SCI. MAR., 59 (Supl. 1): 31-39 SCIENTIA MARINA 1995

TOPICS IN MARINE BENTHOS ECOLOGY. *R. SARDÀ and J.D. ROS (eds.).*

Chemical defense in the seaweed *Laurencia obtusa* (Hudson) Lamouroux*

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SUMMARY: The marine red alga *Laurencia obtusa* is a low preference food for the herbivorous gastropods *Littorina striata* and *Osilinus atratus*, when offered in the presence of *Enteromorpha ramulosa* or *Ulva rigida*, in laboratory assays using the agar suspension method. Neither nitrogen content nor total and available caloric contents could explain this low preference. *L. obtusa* produces secondary metabolites that can lower its palatability to herbivores. The low susceptibility to grazing by snails in these species of algae seems to be associated with the presence of these secondary metabolites. Feeding by *Osilinus* was significantly inhibited by crude extract, non-polar and polar fractions of *Laurencia*. The polar fraction did not deter feeding by *Littorina*. Some of the pure metabolites (elatol, isoobtusol, and obtusol) isolated from the non-polar fraction of *L. obtusa* significantly reduced feeding by both snails. In contrast, obtusane significantly diminished grazing by *Osilinus* but not by *Littorina*. To test whether extracts and individual terpenes were toxic on non-grazer species, their effects on survival of fish larvae (*Sparus aurata*) were analyzed. Elatol was the most effective in producing the death of larvae, while obtusane was not active at the used concentrations.

Key words: gastropods, feeding deterrents, *Laurencia*, fish larvae, ichthyotoxicity, terpenes.

RESUMEN: DEFENSAS QUÍMICAS DEL ALGA *LAURENCIA OBTUSA (HUDSON)* LAMOUROUX. – El alga roja *Laurencia obtusa* es un alimento de baja preferencia para los gasterópodos herbívoros *Littorina striata* y *Osilinus atratus*, respecto a otras algas (*Enteromorpha ramulosa* o *Ulva rigida*), en experimentos de laboratorio usando el método de suspensión en agar. Ni el contenido en nitrógeno ni el contenido calórico de las algas pueden explicar esta baja preferencia. *Laurencia obtusa* produce metabolitos secundarios que pueden actuar como defensas químicas. La baja susceptibilidad del alga al pasto de estos gasterópodos parece estar asociada con la presencia de metabolitos secundarios. Cuando las algas verdes preferidas eran recubiertas por el extracto bruto o la fracción polar o no polar de *Laurencia*, la alimentación de *Osilinus* se veía significativamente inhibida. Sin embargo, la fracción polar no tenía efecto sobre *Littorina*. Los metabolitos puros (elatol, isoobtusol y obtusol) aislados de la fracción apolar de *L. obtusa* reducían significativamente el pasto de los dos gasterópodos. El obtusano disminuía significativamente el pasto de *Osilinus* mientras que su presencia no afectaba a la actividad de *Littorina*. Para examinar si los extractos y los terpenos aislados eran activos sobre especies no herbívoras, ensayamos su efecto sobre la supervivencia de larvas de peces (*Sparus aurata*). El elatol fue el compuesto más efectivo en producir la muerte de las larvas, mientras que el obtusano no mostró actividad a las concentraciones utilizadas.

Palabras claves: gasterópodos, inhibidores de la alimentación, *Laurencia*, larvas de peces, ictiotoxicidad, terpenos.

INTRODUCTION

The importance of plant secondary metabolites in reducing herbivory in terrestrial communities is well-documented and generally accepted as one of the most effective means of defense against herbivores (Rosenthal and Janzen, 1979; Coley *et al.*, 1985). The chemical ecology of marine plants has been studied less extensively than that of terrestrial plants, but many secondary compounds that have been isolated from marine algae exhibit strong biological activities (Bakus *et al.*, 1986; Faulkner, 1984, 1986, 1987, 1988, 1990, 1991, 1992, 1993; Hay *et al.*, 1987; Hay and Fenical, 1988).

*Received February 25, 1994. Accepted November 7, 1994.

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Several recent papers show that some of the hundreds of bioactive secondary metabolites isolated from seaweeds (Norris and Fenical, 1982) function as effective chemical defenses against a variety of herbivores (Targett *et al.*, 1986; Hay *et al.*, 1987, 1988b, d; Paul, 1987; Paul *et al.*, 1987, 1988. Wylie and Paul, 1988; Van Alstyne and Paul, 1988, 1990. Duffy and Hay, 1990; Paul *et al.*, 1990; Hay, 1991, 1992).

Marine algae of the genus *Laurencia* are distributed worldwide (Saito, 1967, 1969a, b, 1982; Saito and Womersley, 1974), and contain many different kinds of compounds (Erickson, 1983; Martín *et al.*, 1983). In the Canary Islands the genus is represented by the cosmopolitan species *Laurencia obtusa* (Hudson) Lamoroux, among others (Gil-Rodriguez and Haroun, 1993). *L. obtusa* produces a varying array of vastly different compounds. Sesquiterpenoids and nonterpenoid C acetogenins (González *et al.*, 1978; Martín and Darías, 1978; Caccamese *et al.*, 1982; Erickson, 1983; González *et al.*, 1983; Faulkner, 1984; Norte *et al.*, 1989, 1991; Öztunç *et al.*, 1991) have been isolated from *L. obtusa* collections in different islands of the Canarian Archipelago. The major metabolites obtusane, elatol, isoobtusol, and obtusol (Fig. 1) are present in the Canarian species *Laurencia obtusa*.

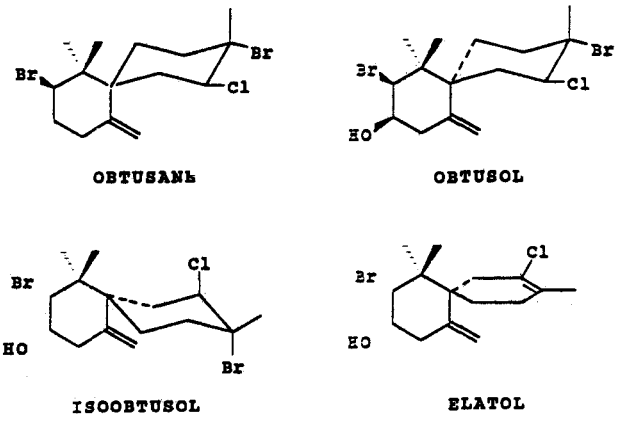

FIG. 1. – Chemical structures of the major halogenated sesquiterpenoids isolated from *Laurencia obtusa*.

One of the best studied terpenes is elatol: this compound, which was originally reported in *L. elata* from Australia (Sims *et al.*, 1974), has been isolated from *L. obtusa* collections in the Canary Islands (González *et al.*, 1976), Belize (Norris and Fenical 1982), and Jamaica (Brennan *et al.*, 1987), among many other sites. Obtusane, isoobtusol, and obtusol have been only isolated from Canarian species. These sesquiterpenoids have a chamigrene skeleton and similar level of halogenation. Obtusane and obtusol differ by only one hydroxyl group. Obtusol and its isomer isoobtusol differ in the enantiomeric relationship of their carbon skeleta (Martín *et al.*, 1986). Both compounds differ in the isomerism of the carbons 2, 3 and 6. In obtusol these are S, R and S, respectively, while in isoobtusol are completely contrary (R, S and R, respectively). This last isomerism is maintained in elatol.

The biological activity of these metabolites led to pharmaceutical studies. Elatol has antibacterial (Martín *et al.*, 1986; Caballero and Melián, 1988), and ichthyotoxic properties, and is exceptionally toxic against fertilized sea urchin eggs, and totally inhibited cell division (Norris and Fenical, 1982). Isoobtusol has shown cytotoxic and antimicrobial activities González *et al.*, 1982; Martín *et al.*, 1986). Obtusol is inactive as an antimicrobial agent (Martín *et al.*, 1986; Caballeroand Melián, 1988).

Several biological studies have shown that *Laurencia obtusa* is a poor food for many herbivorous animals. Ogden (1976) showed that herbivorous fishes and the sea urchin *Diadema antillarum* in Caribbean coral reefs significantly avoided feeding on *L. obtusa*. Hay *et al.* (1988b, d), who have focused their studies on the ecological implications of herbivory, have shown the defensive value of elatol against tropical herbivorous fishes and sea urchins both in field and laboratory assays.

In previous studies on feeding preference in single choice experiments we have seen that *L. obtusa* is one of the algal species least consumed by the herbivorous snail *Littorina striata* (Granado and Caballero, 1991).

In this study we examine the feeding deterrent effects of non-polar and polar extracts and pure metabolites from *L. obtusa* against the herbivorous gastropods *Littorina striata* and *Osilinus atratus*. Other authors have reported that nitrogen content (Mattson, 1980) and caloric content (Himmelmann and Carefoot, 1975) could influence food preference of herbivores. To test this possibility in our algae, we analyzed the nitrogen and caloric content of the seaweed used in feeding trials.

In addition to test whether these extracts could possibly affect species other than grazers, we ran toxicity tests by exposing fish larvae (*Sparus aurata*) to the same extracts.

MATERIAL AND METHODS

Studied species

The marine red alga *Laurencia obtusa* and two greens, *Enteromorpha ramulosa* (Smith) Hooker and *Ulva rigida* Linneaus, were collected by hand at low tide at Dos Roques beach on the northwestern coast of Gran Canaria. A voucher specimen of *L. obtusa* has been deposited at the herbarium of the Departamento de Biología Vegetal at the Universidad de La Laguna (TFC Phyc. 5589).

The algae were transported to the laboratory in a cooler. After epiphytes and animals were removed from their surfaces, the fronds were rinsed with distilled water to remove sediment and salt, and oven dried at 60 °C for 48 hours (or to constant weight). Although this treatment can affect volatile or minor metabolites, it seems no to affect the patterns of major secondary metabolites from *L. obtusa* when compared to dried samples at room temperature and in the dark by thin layer chromatography. Dried plant material was ground through a Wiley mill to a particle size of 0.5 mm in diameter and stored in a desiccator until the start of the trials.

The herbivores used in the chemical feeding deterrent experiments were the gastropods *Littorina striata* King and Broderip, and *Osilinus atratus* Wood. The snails were collected in the upper intertidal zone at San Cristóbal beach, and in the lower intertidal zone at Taliarte, respectively. Once in the laboratory, snails were kept in aquaria, within covered plastic containers. The animals were starved for 24 hours before use.

For the toxicity tests we used the fish larvae, *Sparus aurata* Linnaeus, grown in aquaculture conditions at the Instituto Canario de Ciencias Marinas.

Extraction and isolation of secondary metabolites

Specimens of *L. obtusa* collected in January 1992, were extracted in a Soxhlet apparatus with acetone. The extract was filtered, and the solvent was evaporated under reduced pressure with a rotary evaporator to give a dark-green viscous mass (5.3% of dry weight). The crude extract was repeatedly partitioned (3 times) between hexane and water (1:1).

The hexane layer was concentrated *in vacuo* (4.7% of dry weight, non-polar fraction). To obtain the polar fraction the aqueous layer was extracted (3 times) with diethyl ether and concentrated to dryness (0.5% of dry weight).

The non-polar fraction dissolved in hexane was divided in equal parts, and each was chromatographed on thin layer plates (TLC) using ethyl acetate-hexane (EtOAc:Hex, 3:7) as the developing system. The compounds were detected either by UV lamp (254 nm) or by spraying with H_2SO_4-HOAc-H_2O solution followed by heating at 120°C. The areas that exhibited similar profiles were combined to give different fractions in order of increasing polarity. The less polar fraction was rechromatographed on preparative TLC (EtOAc-Hex, 1:19) to yield obtusane. Elatol was obtained after repeated TLC of the third area from the non-polar fraction. Fraction 5 was purified by successive preparative TLC with EtOAC-Hex (1:9) as solvent to give isoobtusol and obtusol.

The four metabolites were compared to previosly purified and identified standards and their properties were identical in all respects with those reported for authentic materials (González *et al.*, 1979: Sims *et al.*, 1974).

Chemical feeding deterrent assays

Food preference studies (Granado and Caballero, 1991) indicated that *Enteromorpha ramulosa* and *Ulva rigida* were highly preferred by periwinkles while *Laurencia obtusa* was rarely eaten.

A feeding choice experiment in which the snails were presented with a choice of two types of food-agar suspensions per Petri dish was run. We used the green algae *Enteromorpha* and *Ulva*, whole *Laurencia*, and residue of the extracted *Laurencia* as possible foods, in combinations of two items (*Enteromorpha* or *Ulva* *vs.* whole *Laurencia*, *Enteromorpha* *vs.* *Ulva*, *Enteromorpha* or *Ulva* *vs.* extracted *Laurencia*, and whole *Laurencia* *vs.* extracted *Laurencia*).

Crude extract, non-polar and polar fractions, and isolated terpenes from *Laurencia* were tested individually in a second set of feeding experiments to determine relative deterrent effects. Fractions were dissolved in diethyl ether at known concentrations and added to dried ground *Enteromorpha* or *Ulva*. The extract, non-polar and polar fractions were added approximately at natural concentrations (5.3%, 4.7% and 0.5% dry weight of a green alga, respectively), and the pure metabolites at 1% dry weight of a green alga. The solvent was evaporated and the ground alga was suspended in 1.8% agar-seawater. The media was then poured into two opposite compartments of four-way divided Petri dishes. Untreated green algae to be used as a control diet (ether only) were poured into the other two compartments. Rietsma *et al.* (1982) have reported that preference tests among four different foods per Petri dish gave variable results, consequently, tests were conducted using only two foods per dish with the two opposite compartments being filled with the same food.

To each of these dishes, we introduced two *Osilinus* or four *Littorina*, which were allowed to graze for 30 min in the dark. Each of these tests were replicated at least five times.

Feeding was assessed by counting the number of feeding marks left on the surface of the agar suspension as described in Valiela *et al.* (1979) and Rietsma *et al.* (1982). Marks were easily counted under a dissecting microscope. The number of feeding marks in the two compartments with the same food was expressed as a percentage of the total feeding marks in each dish. These percentage data were arcsine-transformed before analysis (Sokal and Rohlf, 1981; Underwood, 1981; Peterson and Renaud, 1989). All analyses were performed using paired *t*-test with SigmaPlot 4.0 package.

Geiselman (1980) developed the agar suspension method to test the palatability of algae and the occurrence of a chemical factor rather than size or toughness as responsible for the feeding deterrent against the herbivore. We suppose that there is a close relationship between the number of bites left on the surface of the agar suspension and the food actually consumed by the animal, independently of the chemoreceptive mechanisms (smell, taste, etc.) used by the herbivores (see Frazier, 1992, for a discussion about how animals perceive secondary metabolites). In any case, if the animal bites the food-agar suspension before recognizing that it is not palatable, the method would be more conservative.

Similar methods coating palatable algae with crude extracts or secondary metabolites of unpalatable seaweeds have been used in evaluating their feeding deterrent effects toward other herbivores (Geiselman, 1980; Paul and Fenical, 1986; Renaud *et al.*, 1990; Irelan and Horn, 1991; Steinberg and Van Altena, 1992).

Fish larval toxicity assays

The extract, fractions and pure metabolites from *Laurencia obtusa* were tested for fish toxicity by using the larvae of gilthead bream (*Sparus aurata*). We used an adaptation of the method developed by Paul (1985). All experiments were carried out on yolk-sac stages with 1 day old larvae. In the laboratory, these larvae were reared in beakers containing 200 ml of aereated seawater at 18°C and salinity 35‰ . Approximately 25 larvae per beaker were used.

Compounds were dissolved in a small amount of ether (100 µl Et_2O) and stirred into seawater (200 ml) at known concentrations. Seawater and solvent controls were run simultaneously. Toxicity was defined as death of 100% larvae within 1h (acute toxicity) and 24 hours (Paul, 1985). Serial dilutions were tested until the lowest ED_{100} (lowest concentration for 100% mortality) was determined.

Each of these tests was assayed 2-3 times with at least four replicates.

Nitrogen and caloric contents

The organic nitrogen content of algae was measured in triplicate with a Perkin-Elmer CHN elemental analyzer (model 2400), using acetanilide as the standard.

Total and available caloric values were determined with an IKA-Calorimeter system (model C700T). Available caloric content was defined according to Tenore (1981), and calculated by determining the caloric loss after weak acid hydrolysis (in 1 N HCl for 8h at room temperature). Initial and final weights and absolute caloric content were determined and available calories calculated by difference.

Ash-free dry weight (AFDW) was determined by weight loss after ashing at 500°C for 24 h.

RESULTS AND DISCUSSION

Chemical feeding deterrent assays

Littorina striata and *Osilinus atratus* preferred to feed on *Enteromorpha ramulosa* and *Ulva rigida* over *Laurencia obtusa* (Fig. 2). Neither snail showed clear preference for one of the two different green algae assayed ($p=0.67$ for *Littorina* and $p=0.33$ for *Osilinus*, $n=6$ trials, paired *t*-test). When *L. obtusa* was presented together with the green algae, we only observed 0.3-1% of the total bites in the compartments with the red alga in each Petri dish ($p<0.001$, for both snails).

The deterrent role of extracted metabolites is shown by the result in which both snails preferred the residue of extracted *Laurencia* over whole *Laurencia* ($p=0.0012$), when we offered a choice between these two food types (Fig. 2, 5th set of bars). *Osilinus* was apparently sensitive to some material still present in extracted *Laurencia* when given with *Enteromorpha* ($p<0.001$), while *Littorina* fed on two food types ($p=0.334$) (Fig. 2, 4th set of bars). This response was independent of the control used (*Enteromorpha* or *Ulva*).

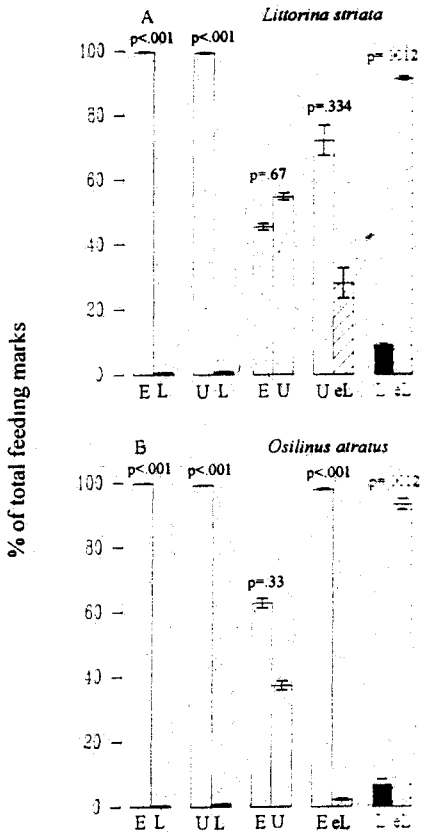

FIG. 2. – Food preferences of (A) *Littorina striata* and (B) *Osilinus atratus*, expressed as a % of total feeding marks. Vertical bars through each histogram show ± 1 SE. Significance values are from the paired-sample *t*-test. E: *Enteromorpha ramulosa*; U: *Ulva rigida*; L: *Laurencia obtusa*; eL: extracted *L. obtusa*.

These results support the hypothesis that the low preference for whole *Laurencia* was due to an extractable factor. However, we think that extracted *Laurencia* had some factor which made it unpalatable for *Osilinus*; this factor may be the structural polysaccharides of the cell wall which contains sulfate (Kloareg and Quatrano, 1988).

Nitrogen content, C/N, and caloric content were not related to feeding preference (Table 1). All three algae have high nitrogen content, although *Laurencia* had the highest, while its C:N ratio was intermediate between *Enteromorpha* and *Ulva*. Total and available caloric contents were lower in the green algae relative to *Laurencia*. These results suggest that for similar high nitrogen content, the food preference is probably determined by another factor.

Crude extract from *L. obtusa* significantly deterred snail feeding. Grazing by *Littorina* was reduced by 91%, p=0.0036, and 99% for *Osilinus*, p<0.001, relative to controls (Fig. 3). The result of the feeding tests using feeding fractions agreed well with the chemical analysis. TLC of extract from *Laurencia*

TABLE 1. – Chemical composition of the algae used in the food preference experiments.

	Algal composition					
Alga	N (%)	C:N	Total KJ/g	Avail. KJ/g	% Avail. KJ	% AFDW
Enteromorpha	3.33	8.94	9.83	3.60	36.6	66.6
Ulva	4.00	8.48	11.61	3.76	32.4	67.5
Laurencia	4.85	8.62	13.86	5.02	36.2	70.9
extracted *L**	3.61	8.04	9.73	4.06	41.7	52.9

*:*L* = *Laurencia*

revealed the presence of obtusane, elatol, isoobtusol, and obtusol with some other minor metabolites. TLC examination showed that extracts from *Enteromorpha* and *Ulva* did not contain secondary metabolites.

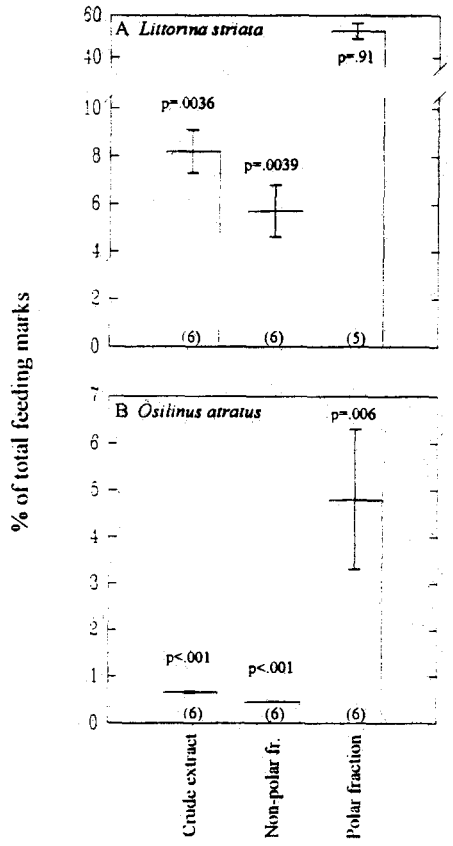

FIG. 3. – The effects of crude extract, non-polar and polar fractions from *Laurencia obtusa* added to food-agar suspension on feeding by (A) *Littorina striata* and (B) *Osilinus atratus*, expressed as a % of total feeding marks, the difference up to 100% is the percentage of the controls. Vertical bars through each histogram show ± 1 SE. Sample size is in parentheses at the base of each histogram. Significance values are from the paired-sample *t*-test.

Non-polar fractions reduced snail feeding by a significant 95-99% (p=0.0039 and p<0.001 for *Littorina* and *Osilinus*, respectively, Fig. 3). Hexane-soluble fractions contained the major compounds above mentioned.

Polar fractions, which amounted to 0.5% of dry weight algae, inhibited feeding preference by *Osilinus* (p=0.006, paired *t*-test); but did not affect *Littorina* feeding (p=0.906, Fig. 3A). The TLC of the polar fraction showed no presence of the major metabolites from *Laurencia*.

These results agree with the hypothesis that the presence of secondary metabolites plays an important role in the unpalatability of the algae. Thus the presence of obtusane, elatol, isoobtusol, and obtusol seemed to be critical for palatability.

Terpenes determined the palatability of the algae, but the snails responded in different ways to the different compounds (Fig. 4). The presence of elatol at 1% of the alga s dry weight reduced feeding more than 90% in *Littorina* and 99% in *Osilinus* (p<0.001, in both cases). Hay *et al.* (1988a) found that elatol also decreased feeding activity in fish. Isoobtusol, the second more effective metabolite, decreased feeding activity by 89% for *Littorina* (p=0.016) and 99% for *Osilinus* (p=0.001). Obtusol was an effective feeding deterrent towards both herbivorous snails (p=0.012, grazing reduced 81% relative to controls, and p<0.001, grazing reduced 98%, respectively). Obtusane did not inhibit feeding by *Littorina* (p=0.287) and showed lower activity than others metabolites for *Osilinus*, although still high (95%, p=0.002).

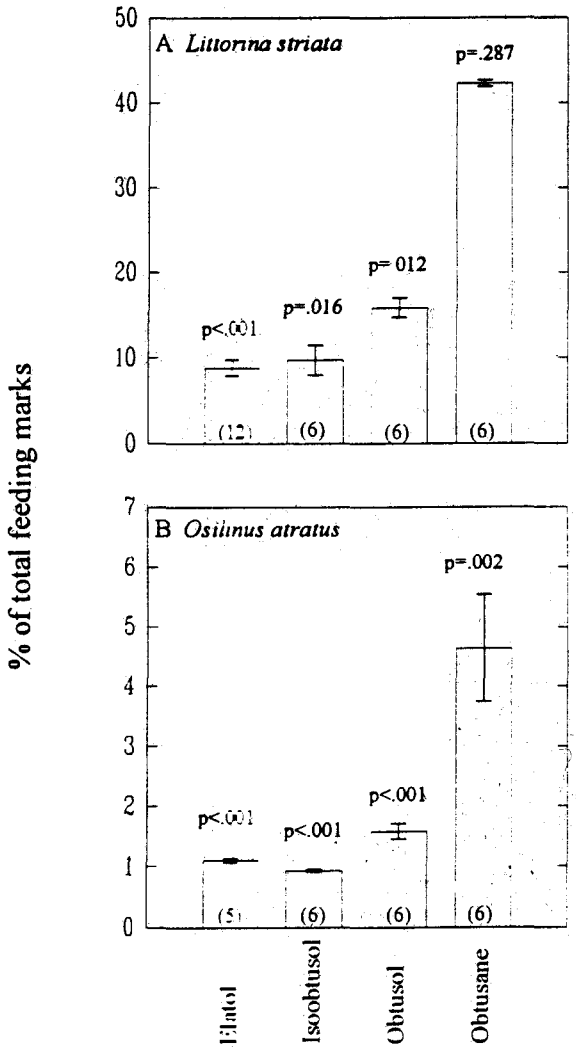

FIG. 4. – The effects of elatol, isoobtusol, obtusol, and obtusane on feeding by (A) *Littorina striata* and (B) *Osilinus atratus*, expressed as in Fig. 3. Symbols and statistical test are as in Fig. 3.

Our results support the hypothesis that the content of secondary metabolites, rather than nutrients and caloric contents of algae, control palatability. The snails avoided *Laurencia*, which contains very active sesquiterpenes, and selected algae low in secondary metabolites. Steinberg (1985) reported similar results with another herbivorous gastropod, *Tegula funebralis*, which also rejected algal species with high levels of secondary metabolites (polyphenolic compounds). In another case, Hay *et al* (1988c) demostrated that the dictyopterenes A and B from the brown seaweed *Dictyopteris delicatula* deterred feeding by fishes. These studies, involving distant geographic areas and taxonomically diverse algae, herbivores, and secondary metabolites, provide a growing body of evidence that support this hypothesis. In this study, there was no negative correspondence between nitrogen content and secondary metabolites. Also in previous studies nitrogen and/or caloric content were unrelated to food preference (Paine and Vadas, 1969; Vadas, 1977; Nicotri, 1980; Duffy and Hay, 1991; Neighbors and Horn, 1991). We think that the metabolites from *L. obtusa* of Gran Canaria may function as chemical defenses against herbivory in Canarian waters.

Fish larval toxicity assays

The results of fish larval toxicity assays for 1 hour and 24 hours are shown in Table 2. All of the metabolites tested, except obtusane, were toxic in 24 hours. Elatol was the most toxic to fish larvae. As little as 1.5 μgml^{-1} produced a 100% mortality of larvae after 24 h. Obtusol had the same effect at 2.5 μgml^{-1}, and the non-polar fraction at 0.75 μgml^{-1}. These results suggest the possibility of additive or synergystic toxic effects when these two compounds are together in the non-polar fraction. Solvent controls showed no toxic effects for the larvae after 1 h; in the 24 h experiments, solvent controls reduced less than 10% the larvae survivorship with respect to the seawater controls.

Polar fraction at 0.5 μgml^{-1} produced 100% mortality within 48 hours, while ten times this concentration produced the same effect within 8 h. Obtusane did not show acute toxicity at the greater concentration tested (20 μgml^{-1}). At this concentration, approximately 38% of larvae died within 24 h. Isoobtusol produced 100% mortality at 20 μgml^{-1} within 10 h, whereas its toxic effect became apparent during the first hour with 10% mortality.

TABLE 2. – Minimum concentration for producing 100 % mortality in 1 h. and 24 h. (ED_{100} in μgml^{-1})

	Bioassay ED	
Compounds tested	1 h	24 h
Non-polar traction	30	0.75
Obtusane	—	—
Elatol	7.5	1.5
Isoobtusol	—	10
Obtusol	20	2.5
Polar fraction	—	—

—: not detected toxicity at tested concentrations.

Compounds with minute structural differences differ in their biological activities. Martín *et al.* (1986) have proposed that the difference observed in bioactivity among obtusol, isoobtusol and elatol must be related with their enantiomeric relation. They found that isoobtusol presented a remarkable antimicrobial activity on both Gram + and Gram - bacteria, but this activity was not observed in the isomer, obtusol. Antimicrobial activity was maintained in elatol. In contrast, in our study, while elatol and obtusol were the most ichthyotoxic compounds, isoobtusol was the least toxic, and obtusane had no toxic effect on fish larvae. Other studies have shown similar results: Hay *et al.* (1988a) have reported that compounds isolated from different *Laurencia* species, which have very similar carbon skeletons and levels of halogenation, differed in their deterrent effects against tropical fishes. In contrast, Paul and Fenical (1986) did not find different activity among major terpenoid metabolites produced by Caulerpalean algae in antimicrobial, toxicity and feeding deterrent assays. Consequently, the structure of the compounds do not seem to be useful in predicting antifeeding and toxic properties.

ACKNOWLEDGEMENTS

This research was supported by Grant 117/080390 awarded by Consejería de Educación y Cultura from Gobierno Autónomo de Canarias. I. Granado was funded by a grant "Formación del Personal Investigador" from the Ministerio de Educación y Ciencia. The authors wish to thank the Grupo de Investigación en Acuicultura from Instituto Canario de Ciencias Marinas for allowing us to use their installations, and particularly D. Hipólito Fernández-Palacios Barber for his generous supply of fish larvae. The authors are also grateful to Drs. Manuel Norte and J. Javier Fernández (Centro de Productos Naturales Orgánicos "Antonio González", Universidad de La Laguna) who kindly supplied the purified obtusane which was used as reference in isolating our product. The authors acknowledge the valuable discussions and encouragement from Dr. Ivan Valiela. The authors would like to thank two anonymous reviewers who provided useful criticism and advice.

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