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The joint action in the bioactivity studies of Antarctic lichen *Umbilicaria antarctica*: Synergic-biodirected isolation in a preliminary holistic ecological study

Jose L.G. Galindo[a], Benito F. García[a], Ascensión Torres[a], Juan C.G. Galindo[a], Joanne G. Romagni[b], Francisco A. Macías[a,*]

[a] Allelopathy Group, Department of Organic Chemistry, INBIO, School of Sciences, Institute of Biomolecules (INBIO), University of Cadiz, C/Repu'blica Saharaui, 7, 11510, Puerto Real, Cádiz, Spain
[b] Department of Biology, DePaul University, 1 E. Jackson Blvd., Chicago, IL 60604, United States

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ABSTRACT

Antarctica is one of the world's most inaccessible regions. This area is also unique in that it has a terrestrial biota dominated by non-vascular plants, of which lichens and mosses are typically the dominant life-forms. A phytochemical study of Antarctic lichen (*Umbilicaria antarctica*) collected from maritime Antarctica has been carried out. The hexane, acetone and butanol extracts have been subjected to a preliminary general bioactivity test using wheat etiolated coleoptiles. A chromatographic study of the acetone extract was performed and seven known compounds were isolated. The general bioactivity of the compounds on etiolated wheat coleoptile has been assessed and joint action studies on mixtures of the compounds were carried out – a methodology that may be the way to a holistic approach in the ecological studies of lichens. The results corroborated the activity exhibited by the original fractions, which in turn support the use of this bioassay to determine joint interactions responsible for the bioactivity shown by *U. antarctica*.

1. Introduction

The Antarctic continent is the coldest, highest, driest, windiest and the most isolated landmass on Earth. Growth conditions are therefore extreme and species exist at the physiological limits of survival. Endurance strategies will be more important when fewer resources become available. Far harsher weather conditions or other intense abiotic stress factor –intense cold, high UV radiation or long periods of darkness, will also push to the limit life forms strategies of subsistence.

It is estimated that about 18,500 different lichen species have been described all over the world and 427 of them are in Antarctica (Øvstedal and Smith, 2001; Øvstedal and Smith, 2011). More or less, they can be found in any part of Antarctica, including in the most environmentally adverse niches. Furthermore, over 100 species of mosses and hepatics have been described but only two flowering plants are present (Sancho and Pintado, 2011). One of the main questions arising is whether Antarctic lichens and mosses possess some type of adaptation mechanism that allows them to survive better than higher vascular plants under such extreme conditions.

Lichens compete mainly for space but the reasons why some lichen species predominate over others are not well understood and they have not been widely studied. One of the reasons for this is the difficulty in growing lichens under controlled laboratory conditions. Another important factor is that the chemical content of these vegetable organisms has been poorly studied from an ecological point of view.

Although the mechanisms by which lichens tolerate extreme stress have not been investigated in detail, it is generally assumed that they rely on the physiological integration of the symbionts (Selbmann et al., 2010). As a successful life strategy for survival under extreme or unfavorable conditions, lichen symbiosis may partially explain the wide range of substrates they can grow in and their wide distribution, including extreme environments such as polar and alpine areas or deserts, and their ability to colonize a wide range of substrates within these habitats (Kappen, 2000).

Lichen secondary products can comprise up to 20% of the thallus dry weight (Fahselt, 1994), but in most lichens the amount varies from 5 to 10%. Such a high carbon allocation represents a

* Corresponding author.
E-mail address: famacias@uca.es (F.A. Macías).

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huge biosynthetic effort and a high investment of carbon and energy resources by the organism. Consequently, the question arises as to what are the advantages that lichen achieves by synthesizing these compounds.

The ecological function of these compounds is important and requires an in-depth study. It would be of great interest to identify the bioactivity and to determine the exact mechanism of action of these energetically expensive bioactive compounds. This is especially interesting in an environment like Antarctica, where the growth season is very limited and therefore the energetic output to make these compounds is proportionally higher.

Umbilicaria antarctica is an endemic lichen from Antarctica (Sung et al., 2008) and has been studied by different research groups. Changon et al. found that the MeOH extract or its components (i.e. gyrophoric acid, lecanoric acid, methyl orsellinate, and methyl gyrophorate) exhibit significant inhibitory effect of PTP1B, which has been widely recognized as a potential drug target for the treatment of type 2 diabetes and obesity (Changon et al., 2009). Other studies revealed the strong antioxidant role of some of their extracts or its components such as lecanoric acid (Luo et al., 2009).

Allelopathy has been widely studied in terrestrial ecosystems and there are many cases where allelopathy can help to explain the structure of a community (Chou, 2006; Macías et al., 2007). However, allelopathic studies have not yet been carried out in the terrestrial ecosystems of Antarctica. Although secondary metabolites are not absolutely essential for the survival and growth of lichens (Bentley, 1999), their study has revealed many possible advantages. However, the functions of these compounds in lichen symbioses are still poorly understood (Hager et al., 2008) and very few evaluations of the possible role of allelopathy in the establishment of lichen communities have been carried out (Romagni et al., 2004). These compounds can function as allelopathic agents and they may affect the development and growth of neighboring lichens, mosses and vascular plants as well as microorganisms (Kershaw, 1985; Lawrey, 1986, 1995; Macías et al., 2007; Romagni et al., 2004; Rundel, 1978). The main roles of lichens secondary metabolites include protection against a large spectrum of microbes, animal predators and plant competitors, defense against environmental stress like UV radiation and desiccation, and physiological regulation of metabolism. (Huneck and Yoshimura, 1996). The studies about allelopathy can provide new information considering the biotic factors in understanding the evolution of lichens in their ecosystem through the phytotoxic study of the major secondary metabolites, by first conducting a study of chemical interactions between these compounds (endointeractions, which occur between components within a plant species) and developing a preliminary holistic study could be detected and determined if lichens have complex chemical defense systems (Lila and Raskin, 2005).

A common approach for the study of plant extracts is bioactivity-guided fractionation. With this technique, extracts are screened in an effort to identify those that contain biologically active compounds. The active extracts are then partitioned and purified, with the ultimate goal of identifying single active compounds. A modification of bioactivity-guided fractionation is Synergic Biodirected Isolation (SBI) (Fernández et al., 2016), which allows the kind of interactions that exist between the major secondary metabolites to be identified and their influence on the activity of the extracts to be assessed by relating the activity profiles of the pure compounds with those of the original extracts.

The aim of the study described here was to identify the compounds responsible for the bioactivity of lichen *U. antarctica* and to determine the type of joint interaction between them. A preliminary bioassay was carried out to select the appropriate extract and fractions. The major metabolites were subsequently isolated and identified (Fig. 1), (Table 1). A bioassay was then carried out on major compounds and on different binary mixtures with different proportions of the compounds and the type of interaction responsible for the observed activity in the original fraction was assigned.

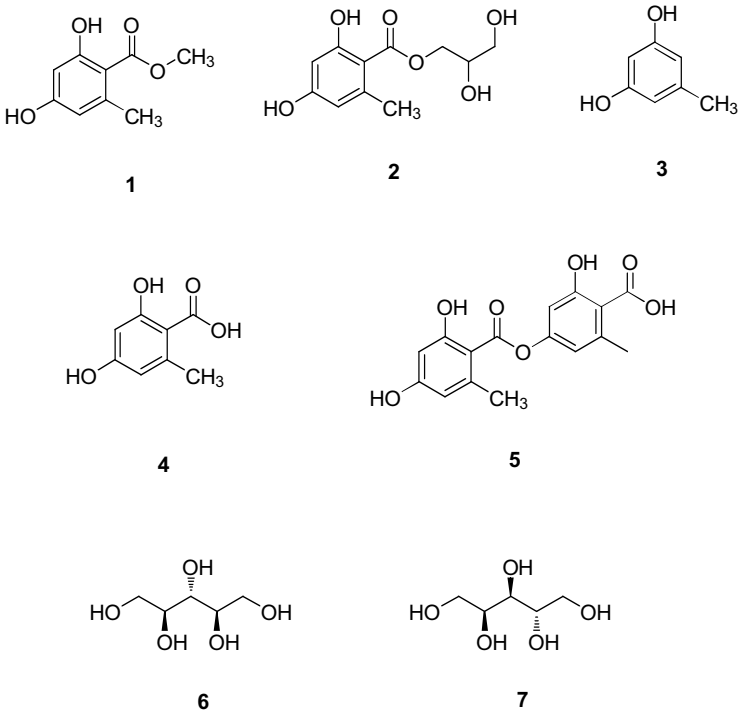

Fig. 1. Compounds Isolated from Umbilicaria antarctica.

Table 1
Percentages of fatty acids from the hexane extract in *Umbilicaria antarctica*.

Fatty acids	%
Myristic acid	0.28
Palmitic acid	10.63
Palmitoleic acid	0.84
Stearic acid	1.32
Oleic acid	18.62
Linoleic acid	42.80
Linolenic acid	3.57

An important technique used in this research is the coleoptile bioassay, which was employed as a tool to determine joint interactions with the aid of an appropriate statistic for discrimination or determination of the relationship between the level of activity, dose and proportions of components in binary or more complex mixtures, as reported for other recent studies (García et al., 2015; Rial et al., 2016).

2. Results and discussion

2.1. Lichen composition

Four samples from Primavera Base in Caleta Cierva (20 g), Byers Peninsula (15.8 g), Juan Carlos I Base in Livingston Island (12.3 g), and Gabriel de Castilla Base in Deception Island (11.6 g) were powdered with liquid nitrogen. The samples were extracted separately using a Sohxlet system with solvents of increasing polarity. The combined extracts of *n*-hexane (360 mg), acetone (5.9 g), and *n*-butanol (10.5 g) were concentrated under vacuum at 40 °C using a rotary evaporator and stored in a freezer at −20 °C prior to study. The extracts, at concentrations of 0.8, 0.4 and 0.2 mg mL^{-1}, were subjected to an etiolated wheat coleoptile bioassay. This test is widely used to evaluate the sensitivity of wheat to a wide range of bioactive substances (Hancock et al., 1961). This bioassay showed the highest inhibition for the hexane extract, with values above 90% at the three concentrations tested, and this extract also showed the most consistent activity profile. This activity was even higher than observed for commercial herbicide Logran® (around −85%) (Fig. 2).

Given the bioassay results and the rich composition in low polarity compounds, gas chromatography (GC) was used to determine the hexane extract composition and to identify the fatty acids: myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic (Table 1). This lichen is very rich on oleic and linoleic acids (18.62% and 42.80%, respectively). These compounds were previously found from *U. antarctica*, but as triglycerides of fatty acids. (Huneck et al., 1984).

The acetone extract (5.9 g) showed moderate activity, with 50% inhibition at the highest concentration (0.8 mg mL^{-1}). This fraction was chromatographed under appropriate conditions to give three fractions: Acetone-1 (649 mg), Acetone-2 (3.8 g) and Acetone-3 (1.4 g). Acetone-2 and Acetone-3 had a similar composition according to the TLC results.

The Acetone-1, Acetone-2 and Acetone-3 fractions were bioassayed with etiolated wheat coleoptiles. The Acetone-2 fraction was the most active and this was submitted to a successive chromatographic fractionation and a final purification by HPLC to give seven compounds, which were identified by comparison of their physical and spectroscopic data (IR, MS, ^{1}H NMR and ^{13}C NMR) with those previously reported in the literature: methyl orsellinate (**1**) as colorless crystals, orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**) (Barros et al., 2008), ribitol (**6**) and arabitol (**7**) (Chapman et al., 1994) (Fig. 1).

The Acetone-3 fraction had a similar TLC profile to Acetone-2 and, as a consequence, this was studied in the same way, with the following compounds identified: methyl orsellinate (**1**), 2,3-dihydroxypropyl orsellinate (**2**) (Yan et al., 2010), orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**), ribitol (**6**) and arabitol (**7**) in different proportions to those found in the Acetone-2 fraction (Fig. 1).

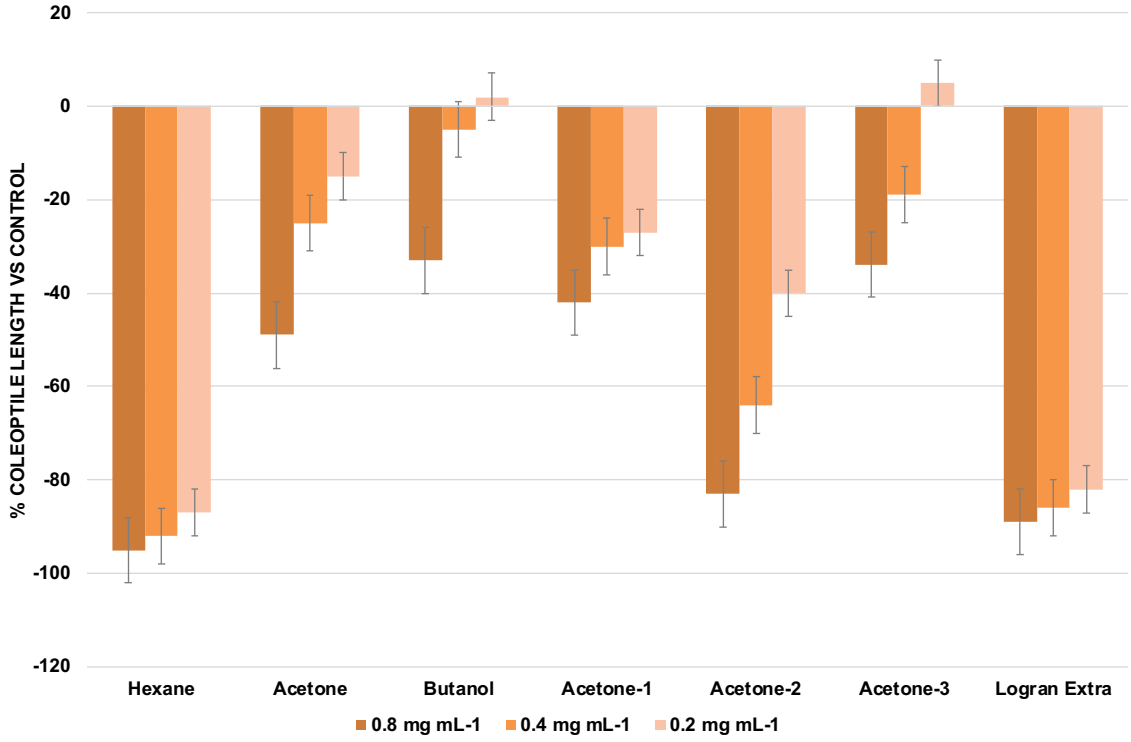

Fig. 2. Coleoptile bioassay for extracts and fractions from *Umbilicaria antarctica*.

Table 2
Amount and proportions of compounds from Acetone-2, Acetone-3 fractions.

Compounds	Acetone-2 fraction		Acetone-3 fraction	
	Isolated mass (mg)	%[a]	Isolated Mass (mg)	%[a]
Methyl orsellinate (**1**)	382	12.48	282	22.69
2,3-Dihydroxypropyl orsellinate (**2**)	–	–	1.5	0.12
Orcinol (**3**)	191	6.24	44	3.54
Orsellinic acid (**4**)	957	31.21	1	0.08
Lecanoric acid (**5**)	1531	49.94	1	0.08
Ribitol (**6**)	1.9	0.06	713	57.35
Arabitol (**7**)	1.9	0.06	202	16.26

[a] Weight percentage of each isolated compound relative to the total weight of the mass fraction.

Compounds **1**, **5**, **6** and **7** were previously isolated from *U. antartica*. (Changon et al., 2009; Luo, et al., 2009; Chapman et al., 1994). However, compounds **2**, **3** and **4** are isolated for the first time from this lichen.

2.2. Preliminary coleoptile assay

The bioactivity results for the extracts, fractions and compounds on wheat coleoptiles are shown in Figs. 2 and 3. The hexane extract showed the highest activity, with an average of 90% inhibition at all concentrations tested. The acetone extract showed an inhibitory activity on coleoptile elongation close to 50% at 0.8 mg mL^{-1} and also a good activity profile with dilution. However, the Acetone-2 fraction showed a high inhibitory activity at 0.8 mg mL^{-1} (>80%) when compared to the activity of the acetone extract. This fraction showed the highest inhibition and its activity profile decreased uniformly with dilution to give a reasonable value (40% inhibition) at 0.2 mg mL^{-1} (Fig. 2). The Acetone-2 and Acetone-3 fractions should have a similar composition according the TLC study but they do differ in the proportions of compounds in each fraction (Table 2) and activity (–7% versus –86%, respectively, at 0.8 mg mL^{-1}) (Fig. 2). This difference could be due to joint action between compounds and this possibility could guide the study on the isolation of compounds as well as the assessment of the potential presence of synergistic, antagonistic or additive effects.

The active compounds isolated from acetone extract were methyl orsellinate (**1**) (1 mM, –81%) and lecanoric acid (**5**) (–41% at 1 mM), while orcinol (**3**), orsellinic acid (**4**), ribitol (**6**) and arabitol (**7**) had activity values below ±10% at this concentration and can be assigned as inactive (Fig. 3) A small quantity of 2,3-dihydroxyprolyl orsellinate (**2**) was isolated from the Acetone-3 fraction but it was not found in the Acetone-2 fraction, so this compound could not be tested. The most active compound (**1**) is characterized by a rapid loss of activity, whereas the activity of **5** decreased more steadily. The logIC50 values obtained for the compounds with the best profile activity (**1**) and (**5**) by the dose-response sigmoidal model are shown in Table 3.

2.3. Isobolographic analysis of the chemical interactions in the coleoptile bioassay

Studies on binary mixtures are often conducted with the aim of elucidating the effect that one specific chemical has on the biological action of another. The results can be interpreted in relation to reference models by the use of isobolograms. However, the amount of data needed for these analyses is large and such experiments are therefore rarely repeated. The joint effect of the majority of chemical mixtures can be predicted using the reference model named 'Independent Similar Action by Bliss' (Tallarida, 2001). This approach becomes challenging, however, when the mixtures include chemicals that synergize or antagonize the effects of other components (Cedergreen et al., 2007).

The isobole method is independent of the mechanism of action and applies under most conditions. In this approach, no assumptions are made as to the behavior of each agent and it is therefore applicable to multi-component mixtures. An isobole is

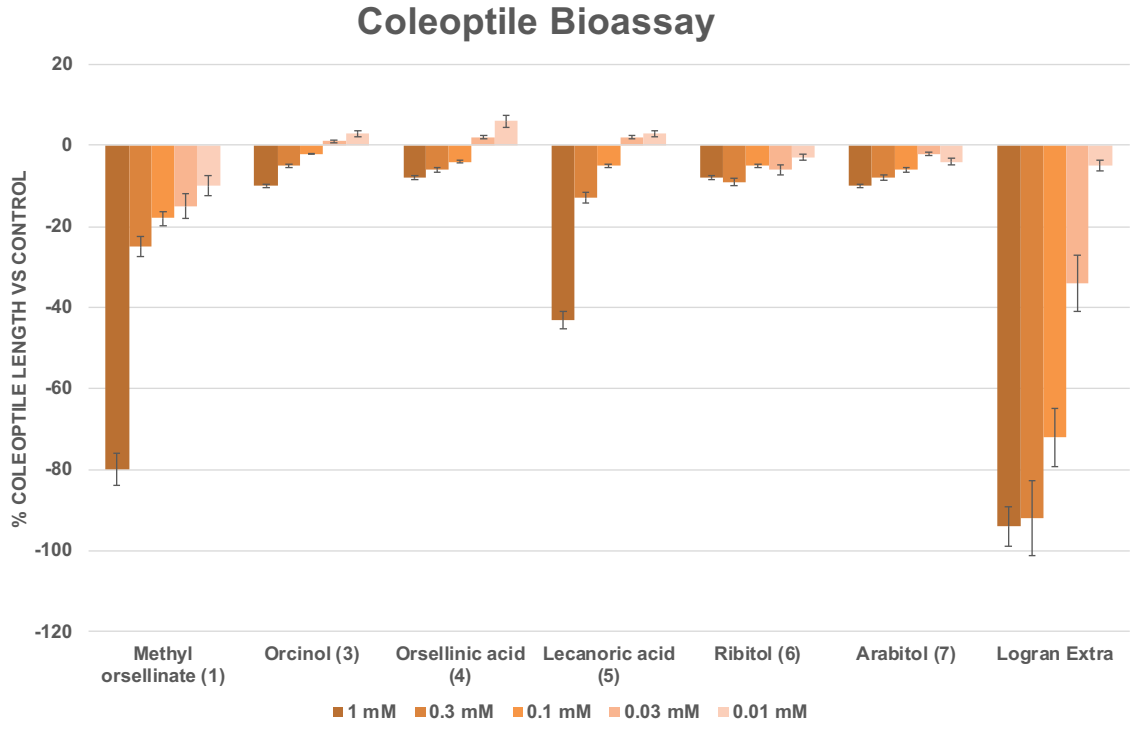

Fig. 3. Coleoptile bioassay for compounds isolated from *Umbilicaria antarctica*.

Table 3
Log IC50 values for active compounds obtained from the sigmoidal dose-respose model.

Active compounds	$y = -y_{min} + \frac{(y_{max}-y_{min})}{1+10^{(logIC50-x)}}$		
	y_{max}	y_{min}	log IC50
Methyl orsellinate (**1**)	−100	−6.97	−3.242
Lecanoric acid (**5**)	−100	$-1{\cdot}10^{-7}$	−2.778

Table 4
Types of interactions with Student's *t*-test ($\alpha = 0.1$) for the ED25 of isolated compounds from *Umbilicaria antarctica* in the etiolated wheat coleoptile bioassay.

	Binary Mixture				
Combination Ratio	1/3	1/4	1/5	1/6	1/7
9:1	additive	additive	additive	additive	additive
3:1	[a]	additive	additive	[a]	[a]
3:2	[a]	additive	additive	additive	additive

[a] the slope is not significantly different from 0 'ANOVA in linear regression'.

an 'iso-effect' curve in which a combination of constituents (da, db) is represented on a graph, the axes of which are the dose-axes of the individual agents (Da and Db). If the agents do not interact, the isobole (the line joining the points that represent the individual doses with the same effect as the combination) will be a straight line (line of additivity). If synergy occurs, i.e., the effect of the combination is greater than expected from their individual dose-response curves, the dose of the combination needed to produce the same effect will be less than the sum of the individual components and this point is located below the line of additivity. The opposite applies for antagonism, where the dose of the combination is greater than expected and the point is located above the line of additivity. Student's *t*-tests were carried out in the isobolographic analysis in order to evaluate the interactions between the compounds (Berenbaum, 1989).

The study described here involved an investigation of binary mixtures in the elongation of the etiolated wheat coleoptile in terms of deviations from the reference model, dose-level dependence, combinations of different proportions and isobole asymmetry.

The interactions of 15 binary mixtures with methyl orsellinate (**1**), orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**), ribitol (**6**) and arabitol (**7**) isolated from *U. antarctica* were evaluated in the etiolated wheat coleoptile bioassay. Student's *t*-test was applied with a confidence level of 90% and all interactions were additive, as shown by the results in Table 4 and the isobolographs (Fig. 4), where all ratios are adjacent to the line of additivity. In the normalized isobologram from binary mixtures with methyl orsellinate (**1**) and lecanoric acid (**5**), the axes represent the dose of each compound for the measured effect in values in per unit.

2.4. Combination analysis: comparison of the additive and experimental regression lines

The isobologram involves the use of sets of equally effective dose combinations for a single effect and it is therefore limited to that specific effect level. In contrast, a more general isobolar analysis that examines combinations of compounds over a range of effects would provide more complete information. Thus, a classification of synergism, antagonism or additivity depends not only on the compound and the effects measured, but also on the fixed ratio combination and the total dose in the combination.

In order to achieve the goal outlined above, a comparison between the theoretical additive curve, constructed from curves of individual compounds for each fixed ratio, and the experimental curve can be performed. The additive regression line and the line obtained from the experimental results must be compared in order to assess whether a given interaction type (synergism, additivity or antagonism) is found at some mid-range effect and whether this extends to other dose levels. The F-distribution test with 95% confidence limits provides a convenient statistic to distinguish whether the two lines differ.

In this way, it is possible to establish whether the experimental and theoretical dose-effect curves are significantly different or, if this is not the case, the additive interaction detected in the statistical analysis of isobologram ED25 remains constant for the different levels of activity. The results shown in Table 5 indicate that in all cases both linear regressions are not different and therefore the additive interaction remains constant at all levels of activity.

The results obtained in the study of the chemical interactions show additive interactions between all binary mixtures and different levels of activity between the tested compounds.

A range of different activities have been reported for secondary metabolites from lichens, i.e., photoprotection, antimicrobial or anti-feedant, and antifreezing amongst others (de Vera et al., 2008; Fahselt, 1993; Lawrey, 1995), and the mechanisms of action of these compounds are unclear. It is possible that during the evolution of lichens under the extreme conditions of the Antarctic ecosystem in which they develop, it has not been necessary to develop the complex defense mechanisms that lead to the appearance of synergistic or antagonistic effects to alleviate various adverse events and allow the emergence of resistance to adverse biotic factors (insects and microorganisms).

2.5. Analysis of the activity profile of an active complex mixture: synergic biodirected isolation (SBI)

The importance of context in the development of ecological theory about plant chemical defensive strategies generally seems to be ignored in the literature. It is still routine practice for scientists to investigate an extract from plants with a view to finding the single chemical entity responsible for the effect, and this may lead to inconclusive findings. If a combination of substances is needed to achieve the effect, the biodirected isolation method is required to trace the activity without losing it.

It is possible that a small number of such compounds could exert the effect observed for the extract. The activity of compounds found in fractions makes it conceivable that they are involved in some type of interaction.

Activity caused by synergistic, antagonistic or additive interactions is usually not comparable to the activity of a single active compound, unless such a compound already participates in the combination.

The specific structural factors that determine the activity of a particular combination of compounds remains unclear. The same holds true for the combined effect because the nature of such an effect cannot be predicted on the basis of an individual compound acting in isolation. In some cases, a non-inhibitory concentration of a specific compound inhibits growth when this compound acts additively, antagonistically or synergistically with other compounds that are present and such joint action is the most common situation. One of the aims of the study presented here was to clarify these interaction effects by carrying out etiolated wheat coleoptile bioassays on binary mixtures of compounds.

Analysis of the activity profiles obtained in the bioassay-guided isolation showed that the Acetone-2 fraction had the best activity profile (Fig. 2). However, this activity profile was not shown by the major active compounds isolated from this fraction. For example, methyl orsellinate (**1**) and lecanoric acid (**5**) both showed marked

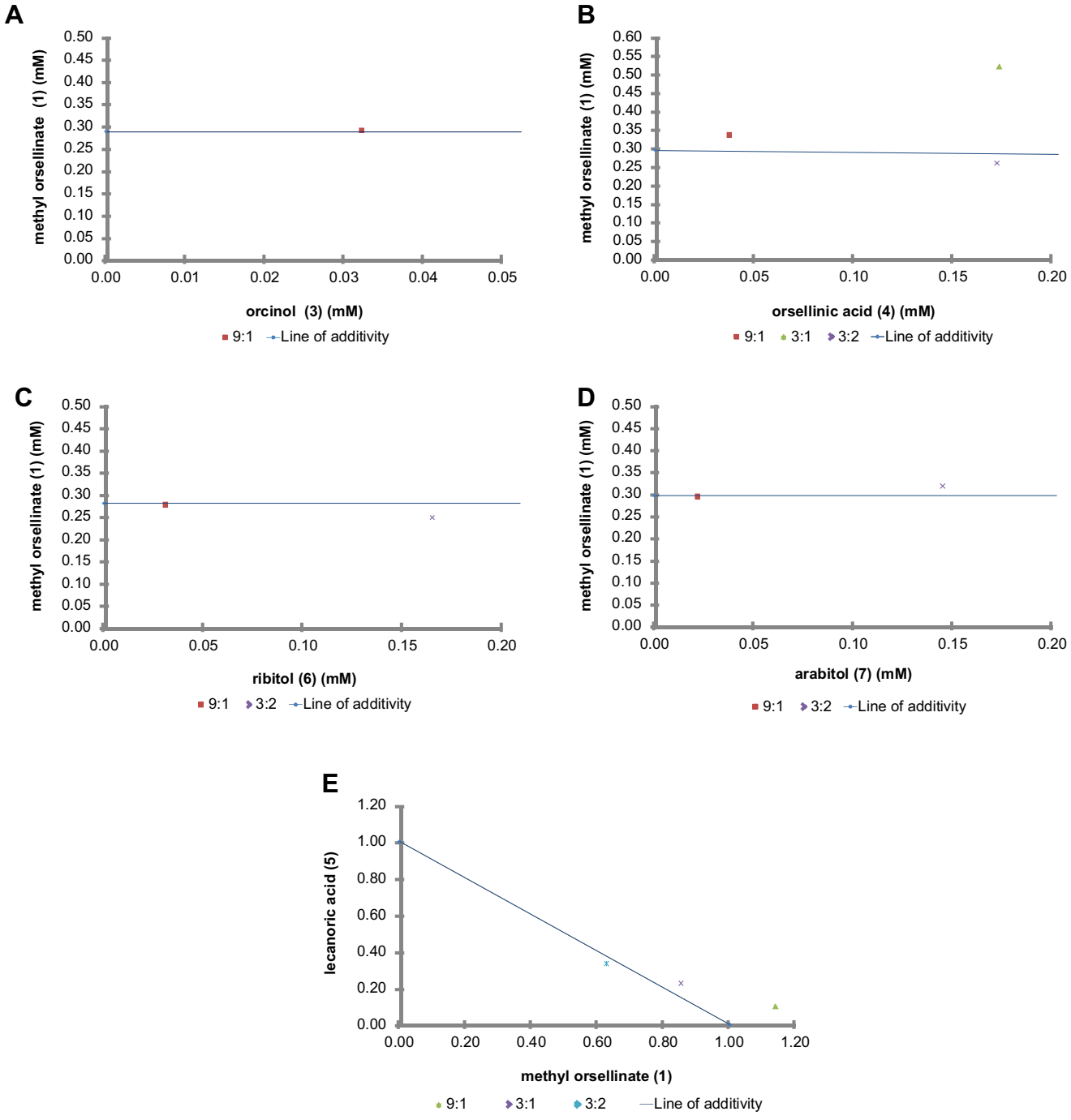

Fig. 4. ED25 isobolograms for the binary mixtures with methyl orsellinate (**1**) and orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**), ribitol (**6**) and arabitol (**7**) in the etiolated wheat coleoptile bioassay: (A) binary mixture **1**/**3**; (B) binary mixture **1**/**4**; (C) binary mixture **1**/**6**; (D) binary mixture **1**/**7**; (E) Normalized isobologram of binary mixture **1**/**5** (in this graph the axes represent the dose of each compound for the measured effect in values in per unit).

Table 5
Comparison of the additive and experimental regression lines for binary mixtures of compounds with F-distribution ($\alpha = 0.05$) (if $F_{cal} > F$ there is a significant difference).

	Binary mixture of compounds									
	1/3		1/4		1/5		1/6		1/7	
Combination ratio	F_{cal}	F	F_{cal}	F	F_{cal}	F	F_{cal}	F	F_{cal}	F
9:1	0.70	3.98	0.01	3.98	3.15	3.98	0.00	3.98	0.03	3.98
3:1	–	–	1.87	9.01	3.53	3.98	–	–	–	–
3:2	–	–	0.87	3.98	2.08	3.98	0.89	3.98	0.76	3.98

decreases in activity upon dilution, whereas the other compounds **3**, **4**, **6** and **7** had activity values below ±10 at 0.2 mM (Fig. 3).

On applying Synergic Biodirected Isolation (SBI) it is possible to propose that the activity profiles shown by the fractions Acetone-2 and Acetone-3 are the result of the sum of the activities of the major compounds isolated. A study was developed about the activity of the major secondary metabolites and their interactions were evaluated jointly, which may be the way to a holistic approach.

The Bliss Independence criterion is more appropriate than the Loewe additivity model (Goldoni and Johansson, 2007). The main assumption of the Bliss independence criterion is that two or more

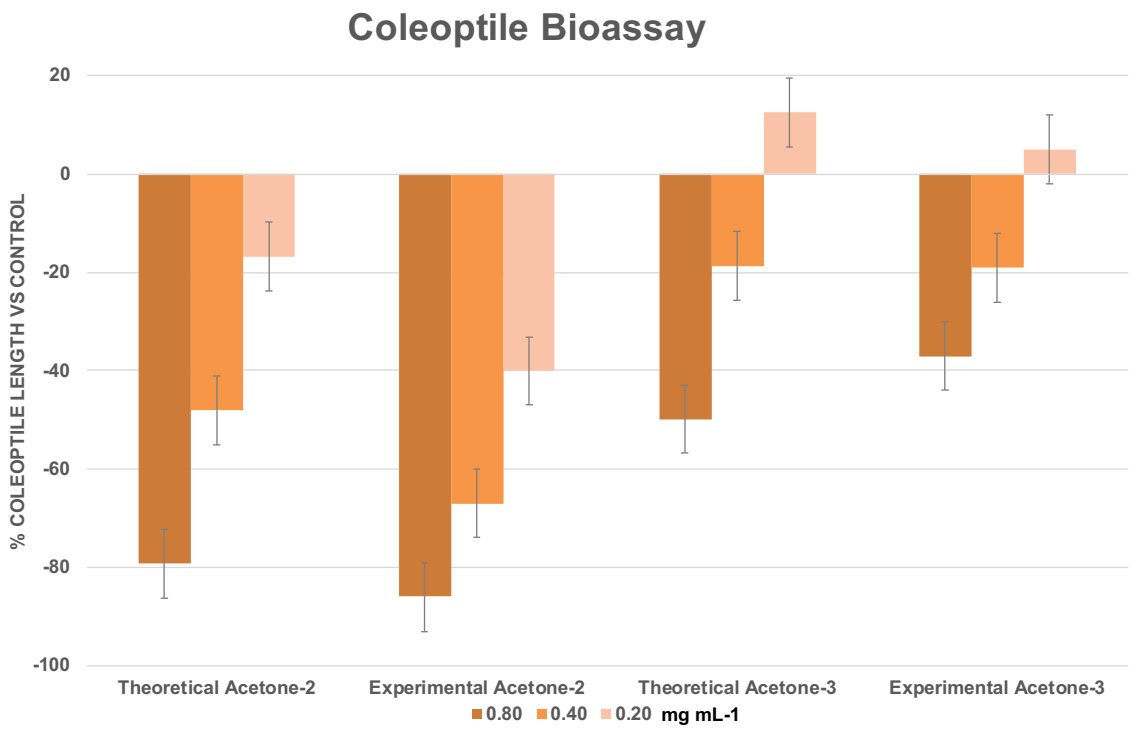

Fig. 5. Comparative activity results (theoretical vs experimental) for Acetone-2 and Acetone-3 fractions.

compounds act independently from one another, i.e., if they fulfill the criterion, the mode, and possibly also the site of action of the compounds in the mixture, always differs.

Analysis of all the factors (compounds and interactions) responsible for the activity profile of a mixture of chemical compounds can be a very laborious task, since it would involve establishing the dose-effects of all compounds present in the mixture as well as the study of all possible interactions that can occur between all compounds (combinations of binary mixtures and tertiary mixtures or higher).

As in a classical analysis of the activity of a chemical mixture, on considering only the dose-effect of active compounds the identities of the active compounds that cause the activity of the extract can be established in some cases. This classical study can be completed by considering the chemical interactions between the compounds detected in the mixture. Therefore, if any antagonistic or synergistic effects are detected, these can have an influence to a greater or lesser extent and even cancel each other, with these interactions reflected in activity profile of extract.

Moreover, in various studies on the interactions of a mixture of compounds, the observed effects are additive in the majority of cases, and synergistic and antagonistic effects are observed less commonly, albeit to different extents (García et al., 2015; Rial et al., 2016).

Table 6
Comparison of theoretical and experimental activity profile of Acetone-2 and Acetone-3 fractions with F-distribution ($\alpha = 0.05$) (if $F_{cal} > F$ there is a significant difference).

	Acetone-2	Acetone-3
F-distribution test	*Theoretical vs Experimental*	*Theoretical vs Experimental*
Fcal	0.99	0.99
F	9.28	9.28

The Bliss Independence criterion was considered to calculate the theoretical activity profile of the Acetone-2 and Acetone-3 fractions. In this process, all compounds isolated in both fractions were taken into account and their profiles were compared with the experimental profiles. In the qualitative mode shown in Fig. 5 the theoretical profile resembles the experimental profile in both cases.

However, bearing in mind that there are only independent additive interactions, it was necessary to confirm that there is no difference between the theoretically calculated profiles and the experimental ones. This was achieved by applying a linear model to the profiles followed by the F-distribution test. This analysis showed that there is no difference between the theoretical and experimental profiles. The results obtained are shown in Table 6 and Fig. 6.

In summary, the etiolated wheat coleoptile bioassay can be used as a preliminary test to detect active compounds and the most influential interactions that cause the activity profiles of complex mixtures without the need for laborious studies involving, in most cases, a large investment of money and time.

In contrast to the above, if the theoretical and experimental profiles are significantly different, it could indicate the existence of an unknown compound or some kind of interaction that has not been evaluated. In such cases further study of the mixture would be required.

Several authors have developed more complex methods to study interactions in extracts (Junio et al., 2011), such as Synergy-Directed Fractionation with *Hydrastis canadensis*, where the synergistic effect of the antimicrobial activity of extracts of roots (containing the alkaloid berberine) was enhanced by adding non-active flavonoids that were isolated from the leaves.

The results presented above allow several conclusions to be drawn:

The habitat of lichens in Antarctica is quite inhospitable and the dryness, cold and exposure to high levels of UV, are the main

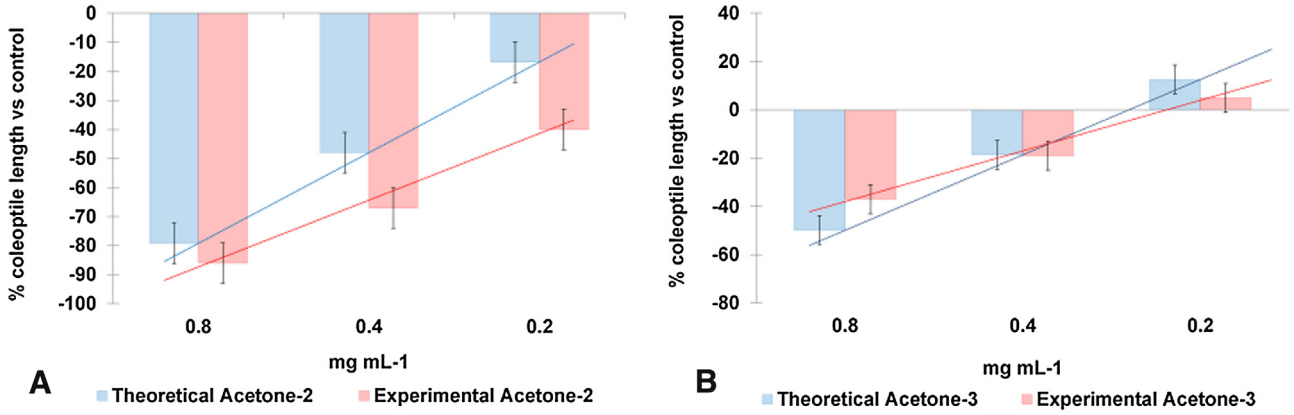

Fig. 6. Comparison of theoretical and experimental activity profile with F-distribution ($\alpha = 0.05$): (A) Acetone-2 fraction, (B) Acetone-3 fraction.

detrimental factors, although there is no strong competition with other species of flora and they are not attacked by insects or herbivores. From an ecological and evolutionary viewpoint, it is striking that many plant defenses are involved in limiting the acquisition of nutrients and/or in the toxication of the insect. This teleological nomenclature for evolutionary strategies is useful at a holisitc level to describe the ecological outcome. In plant defenses against insects, authors speak of the interactions between chemicals and organisms (Duffey and Scout, 1996), distinguishing between complex defenses (nonlinear relationships/multiplicative) and multiple defenses (linear relationships/additive). It could be argued that due to the lack of competition for survival against the flora and fauna, it is possible that the lichen has not evolved to "complex defenses", thus simplifying their survival to "multiple defenses" and therefore to an additive behavior, as identified in this joint action study on compounds obtained from lichens. Although it is necessary to apply this methodology in more specific bioassays (insecticide, cytotoxicity and others), as well as to carry out exointeraction studies (interactions between compounds present in the medium, but released by different species), to complete a holistic study and to understand better the factors that may influence the processes occurring in ecosystems.

The chemistry simplicity found during the chromatographic isolation study on this lichen, with only three families of compounds identified [fatty acids (7), polyols (2) and phenolic compounds (5)], led us identify the adaptation mode to extreme ecosystems. The energetic and photoprotective function of fatty acids is known, and about to membrane protection in freezing situations. The polyols are also known antifreeze properties, but the complete function in the lichens of phenolic compounds remains unclear. There are studies that have shown that orcinol, lecanoric acid and orsellinic acid have UV protective action due their radical scavenging levels (Barros et al., 2008; Luo et al., 2009) and this could be the main reason for adaptation to an extreme ecosystem with high levels of UV during the Antarctic summer.

Considering the phenolic compounds identified in this lichen, methyl orsellinate (**1**) was the most active on wheat coleoptiles and this finding enabled us to design a joint action study in different binary mixtures between this compound and orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**), ribitol (**6**) and arabitol (**7**). Additive behavior was found in binary mixtures of major compounds from *U. antarctica* on wheat coleoptiles and it was possible to determine easily and rapidly the most influential interactions that cause the activity profiles of the original fractions.

This new methodology involving a preliminary analysis of compounds and interactions can be applied to different Antarctic lichen extracts to identify the interactions and the major compounds that have the most influence in the activity.

It would be interesting to carry out this kind of joint action study on Antarctic lichens to identify different and more specific activities on living organisms in their own environment in an effort to explain their coevolution in this inhospitable ecosystem.

3. Experimental

3.1. Biological material

Samples of *Umbilicaria antarctica* were collected in January 2006 during the corresponding government-funded campaigns (Special actions CGL2005-25090-E/ANT) in different locations of the Antarctic Peninsula and the nearest archipelagos: Caleta Cierva (64°10′S, 60°57′W) near Primavera Base and Highlander Peak, Byers Peninsula and Livingston island (62°32′45"S, 61°13′07"W), and Deception Island (62°58′38"S, 60°40′33"W). After identification of the lichens, the samples were stored in boxes under dark conditions and labelled with weight, position of harvest, characteristics of the soil and harvest date.

3.2. Extraction identification and isolation

Four samples from Primavera Base in Caleta Cierva (20 g); from Byers Peninsula (15.8 g); Juan Carlos I Base in Livingston Island (12.3 g), and from Gabriel de Castilla Base in Deception Island (11.6 g) were powdered with liquid nitrogen.

Raw lichen samples were extracted with hexane using ultrasound (10 min) to isolate triglycerides from the cell structure. These extracts were then concentrated and a transesterification was performed to separate the three lipid chains and create volatile compounds for GC–MS analysis. A Varian 220-MS Ion Trap was used to analyse lipid extracts, fatty acids were classified relative to the retention times of known acid chains and library mass spectra. An internal standard (C-17 fatty acid) was added to lipid extracts for quantification by peak integration.

After defatting extracts, the components were extracted using a Sohxlet apparatus and solvents of increasing polarity. The combined resultant extracts of *n*-hexane, acetone, and *n*-butanol were concentrated under vacuum at 40 °C using a rotary evaporator and stored in a freezer at −20 °C prior to study.

The acetone extract was selected due to the TLC results and it was fractionated by column chromatography (CC) on silica gel using mixtures of hexane and ethyl acetate to yield three fractions, Acetone-1, Acetone-2, and Acetone-3. The Acetone-1 fraction

showed a slightly clearer resolution by TLC. The Acetone-2 and Acetone-3 fractions were chromatographed on silica gel eluting with hexane/ethyl acetate in suitable proportions. Purification by HPLC with Hibar Si60 (Merck) and Phenomenex columns using $CHCl_3$/Methanol 19:1 gave methyl orsellinate (**1**) as colorless crystals, 2,3-dihydroxypropyl orsellinate (**2**), orcinol (**3**), orsellinic acid (**4**), lecanoric acid (**5**), ribitol (**6**) and arabitol (**7**) (Fig. 1, Table 2).

3.3. Coleoptile bioassay

Wheat seeds (*Triticum aestivum* L. cv. Catervo) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 25 ± 1 °C for 3 days (Hancock et al., 1961). The roots and caryopses were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were studied under a green safelight (Nitsch and Nitsch, 1956). Extracts, fractions, compounds and binary mixtures were pre-dissolved in DMSO (0.1%) and diluted in phosphate-citrate buffer containing 2% sucrose at pH 5.6 to the final bioassay concentrations [0.8, 0.4 and 0.2 mg mL^{-1} for extracts and fractions; 1.0, 0.3, 0.1, 0.03 and 0.01 mM, for compounds; 1.0, 0.6, 0.3, 0.2, 0.01 mM, and for binary mixtures with different ratios (9:1, 3:1, 3:2)], according to the amounts obtained from the fractions in the bioassay-guided isolation.

Parallel controls were also run. The commercial herbicide Logran®, whose original formulation is a combination N^2-*tert*-butyl-N^4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea (triasulfuron, 0.6%), was used as an internal reference at the same concentrations and under the same conditions as reported previously. In the reproducibility of the dose-response curve (in the bioassay of the binary mixtures), pure compound was used as control. Buffered aqueous solutions with DMSO and without any test compound were as used as a control for all the samples assayed.

Five coleoptiles and 2 mL of solution were placed in each test tube (three tubes per dilution) and the tubes were rotated at 6 rpm in a roller tube apparatus for 24 h at 25 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test and are presented as percentage difference from the control (Martin and Luna del Castillo, 1990). Positive values represent stimulation and negative values represent inhibition.

3.4. Isobolographic data analysis

The dose-effect curves were constructed from data obtained in the coleoptile bioassay and they represent the effect against the logarithm of the dose. The logarithmic transformation results in an S-shaped curve that is approximately linear in the mid-range. ANOVA in linear regression was applied to determine whether the slope was significantly different from 0 with 95% confidence limits [for log(dose)-effect data this is a test to determine whether the effect is dose dependent] (Tallarida, 2000).

An isobolographic analysis was performed to characterize the interaction between the tested compounds. Isobolographs were constructed using ED25 values (doses that produce 25% inhibition with respect to the control) obtained when the compound was administered alone or in combination. The theoretical additive doses (Zadd) with their variance (Vadd) for each combination were computed from the equieffective doses (ED25) of the single compounds according to the method described by Tallarida (Tallarida, 2000). The experimental data (Zexp) and their variance (Vexp) were determined from the respective dose-response curve. A statistical comparison was made between the experimentally determined (Zexp) and the theoretically calculated (Zadd) values by applying Student's *t*-test with 90% confidence limits (Tallarida, 2000).

3.5. Data analysis: comparison of the additive and experimental regression lines

The dose-effect data for the compounds were used to construct a theoretical additive curve, for each fixed ratio, according to the method described by Tallarida (Tallarida, 2001). The linear regressions of the additive and the experimental curves were evaluated by applying a test to distinguish two linear regressions using the F-distribution with a 95% confidence limit (Tallarida, 2000).

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