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Detection of synergistic combinations of *Baccharis* extracts with Terbinafine against *Trichophyton rubrum* with high throughput screening synergy assay (HTSS) followed by 3D graphs. Behavior of some of their components

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ABSTRACT

Forty four extracts from nine *Baccharis* spp. from the *Caulopterae* section were tested in combination with terbinafine against *Trichophyton rubrum* with the HTSS assay at six different ratios with the aim of detecting those mixtures that produced a ≥50% statistically significant enhancement of growth inhibition. Since an enhanced effect of a combination respective of its components, does not necessarily indicate synergism, three-dimensional (3D) dose–response surfaces were constructed for each selected pair of extract/antifungal drug with the aid of CombiTool software. Ten extracts showed synergistic or additive combinations which constitutes a 22% hit rate of the extracts submitted to evaluation. Four flavonoids and three ent-clerodanes were detected in the active *Baccharis* extracts with HPLC/UV/ESI-MS methodology, all of which were tested in combination with terbinafine. Results showed that ent-clerodanes but not flavonoids showed synergistic or additive effects. Among them, bacchotricuneatin A followed by bacrispine showed synergistic effects while hawtriwaic acid showed additive effects.

Introduction

The genus *Baccharis* L., with over 500 species throughout the world, is the most numerous in the Asteraceae family, with geographical distribution restricted to the Americas, from Canada to south Argentina and Chile (Fielding 2001; Abad and Bermejo, 2007). In particular ninety six species were described in Argentina (Giuliano 2001, 2005), of which nine [*B. articulata* (*Ba*), *B. crispa* (*Bc*), *B. gaudichaudiana* (*Bg*), *B. microcephala* (*Bm*), *B. penningtonii* (*Bp*) *B. phyteumoides* (*Bphy*), *B. sagittalis* (*Bs*), *B. triangularis* (*Btria*) and *B. trimera* (*Btrim*)] possess alate stems (Rodriguez et al. 2010). With the exception of *B. triangularis*, all of them belong to *Caulopterae* section.

Most of these alate species have been traditionally used in Argentina, and neighboring countries, for ailments related to fungal infections (Barboza et al. 2009; Di Stasi and Akiko 2002; Sorarú and Bandoni 1978), and this precedent have shown to enhance the probability of detecting antifungal plants mainly against dermatophytes (Svetaz et al. 2010).

In spite of their registered ethnopharmacological uses, previous studies on the antifungal activity of *Baccharis* species were not very promising (Gutkind et al. 1981; Vivot et al. 2009; Svetaz et al. 2010). However, *Baccharis* spp. possess flavonoids (Gonzaga Verdi et al. 2005; Alcaraz et al. 2012) and diterpenoids of clerodane and labdane types (Abad Martínez et al. 2005; Akaike et al. 2003; Alcaraz et al. 2012; Dai et al. 1993; Gianello and Giordano 1989; Gianello et al. 2000; Gonzaga Verdi et al. 2005; Hayashi et al. 2005; Herz et al. 1977; Juan Hikawczuk et al. 2002, 2006; Petenatti et al. 2007; Stapel et al. 1980; Wagner et al. 1978; Zdero et al. 1986) which have showed antifungal properties in earlier reports (Cole et al. 1991; Coll and Tandrón 2005; Murthy et al. 2005).

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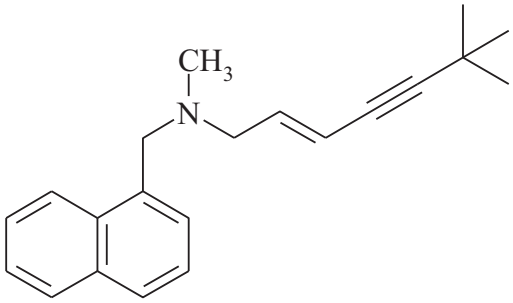

Fig. 1. Chemical structure of terbinafine-HCl.

In order to increase the possibilities of detecting antifungal activity in *Baccharis* extracts, a series of forty four Argentinean alate *Baccharis* extracts (BE) were tested for antifungal properties in both ways, alone and in combination with an antifungal drug.

For the antifungal assays, a dermatophyte was selected as the test organism, due to the previous demonstration that there exists a significantly higher probability of detecting antifungal activity in traditionally used plants, when they are tested against dermatophytes rather than against other type of fungi (Svetaz et al. 2010). Among dermatophytes, *Trichophyton rubrum* was chosen because it is the major cause of chronic or recurrent human superficial infections (Weitzman and Summerbell 1995).

In turn, terbinafine hydrochloride (terb) (Fig. 1), a synthetic allylamine antifungal agent, was used as the combination partner, since it is the preferred antifungal drug to treat mycoses produced by dermatophytes (Gokhale and Kulkarni 2000).

Regarding the method of choice to test BE in combination, the High Throughput Screening Synergy Assay (HTSS) developed by Zhang et al. (2007) was applied at a first instance of screening. This strategy consisted in testing one mixture of both the extracts and the antifungal drug, each one at a sub-optimal dose (producing 10–20% of the maximum activity) with the aim of finding mixtures which, in combination, reached a ≥90% growth inhibition. This simplification, which was used to screen thousands of microbial extracts, led to 0.1% hit rate. However, when a moderate number of extracts are available, this approach runs the risk of not detecting any synergistic extract (Jansen et al. 2009).

So, considering that improvements in the scale and sensitivity of synergy identification techniques would lead to a higher number of synergistic combinations, we herein present the results of application of the HTSS assay to mixtures of BE with terb against *T. rubrum*, by testing them at six different ratios (instead of one) of low doses of each extract and drug. From them, those combinations that produced a statistically significant enhancement of activity ≥50% (instead of ≥90%) inhibition growth were selected.

Since an enhanced effect of a combination respective of its components, does not necessarily indicate synergism (Chou 2010), a three-dimensional (3D) dose–response surfaces were constructed for each selected pair of extract/antifungal drug with the aid of CombiTool software (Dreβler et al. 1999). A 3D model is considered the most complete way to describe synergistic, additive or antagonistic interactions since it elucidates the shape of the dose–response surfaces, identifies the regions of significant synergy and antagonism, and presents a complete map of drug interactions in a way that can be easily interpreted (Prichard and Shipman 1990).

As a result of the application of HTSS assay to six combinations per extract followed by 3D response-surface methodology, several BE with synergistic or additive activity were detected, which represented a much higher percentage of hits that we might have obtained using a single combination, therefore broadening the possibilities of taking advantage of the chemical diversity that *Baccharis* spp. offers.

The analysis of the chemical profiles of selected BE, obtained by HPLC/UV-ESI MS, allowed the detection of flavonoids and entclerodanes which were tested in combination with terb with the aim of finding the components of BE that display the synergistic or additive activity observed in BE.

Materials and methods

Plant material

Ba, *Bc*, *Bg*, *Bm*, *Bp*, *Bphy*, *Bs*, *Btria* and *Btrim* were collected in different locations in Argentina. Areas of 100 m^2 were delimitated, and aerial parts of wild plants of each area were collected constituting each, a different specimen. All samples were botanically identified by one of the authors (MG), and voucher specimens were deposited at the Herbarium of the National University of Rosario (UNR), Argentina (Voucher specimens and locations are detailed in Table S1, Supplementary Material).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2013.06.015

Preparation of extracts

Plant aerial parts were air dried and powdered in a Fristzch Pulverisette 15 mill (Germany). Extraction was performed by maceration with MeOH (Ciccarelli, San Lorenzo, Argentina) (3 × 24 h) mechanically shaken in a Heidolph RZR 50 shaker (Germany). BE were filtered, pooled, and the solvent was concentrated at <40 °C *in vacuo* in a rotary evaporator Buchi R-205 (Flawil, Switzerland).

Chemicals

Terb hydrochloride (≥98%), quercetin **1** (≥99%), luteolin **2** (≥98%) and apigenin **3** (≥97%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); genkwanin **4** (≥98%) was obtained from Chengdu Preferred Biological Technology Co., Ltd. (Chengdu Sichuan, China). Bacchotricuneatin A **6** (≥95%) was a generous gift from Prof. H. Wagner (München, Germany), who isolated it from *B. tricuneata* (Wagner et al. 1978) and bacrispine **5** (≥95%) and hawtriwaic acid **7** (≥95%) were generous donations from Prof. C. Tonn and L. Favier who isolated them from *Bc*, *B. flabellata* and other spp. (Alcaraz et al. 2012; Ceñal et al. 1997; Juan Hikawczuk et al. 2006; Simirgiotis et al. 2000).

Fungal strain and inoculum preparation

For the antifungal evaluation, *T. rubrum* CCC 110 from the Culture Collection of CEREMIC (CCC), Reference Center of Mycology, Faculty of Biochemical and Pharmaceutical Sciences, Suipacha 531-(2000)-Rosario, Argentina, was used.

The strain was grown on Sabouraud-chloramphenicol agar slant for 7 d at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid), and sub-cultured every 15 d to prevent pleomorphic transformations. Inocula of spore suspensions were obtained according to reported procedures and adjusted to 1–5 × 10^3 spores with colony forming units (CFU)/ml (CLSI 2008).

Determination of MIC

Minimum Inhibitory Concentration (MIC_{100}) was determined by using broth microdilution techniques according to the guidelines of the CLSI for filamentous fungi (M38 A2) (CLSI 2008). Microtiter

trays were incubated at 28–30 °C in a moist, dark chamber. MIC was visually recorded at the seventh day. For the assay, stock solution of terb or BE in DMSO (final concentration ≤1%) were diluted with RPMI-1640 (Sigma, St. Louis, MO, USA); 200 μl was poured into the first well and then, 100 μl were transferred to the next well containing 100 μl of RPMI-1640 buffered to pH 7.0 with 4-morpholinepropanesulfonic acid (MOPS, Sigma–Aldrich, St. Louis, MO, USA). The same procedure was performed for all wells of the same file obtaining two-fold dilutions of the drug. A volume of 100 μl of inoculum suspension was added to each well (with the exception of the sterility control where sterile water was added to the well instead) rendering concentrations from 100 to 1.6×10^{-4} μg/ml for terb, from 1000 to 15.6 μg/ml for BE and from 250 to 1.95 μg/ml for pure compounds. Endpoints were defined as the lowest concentration of sample resulting in total inhibition (MIC_{100}) of visual growth compared to the growth in the control wells containing no antifungal. Tests were carried out in duplicate.

Determination of growth inhibition percentage of T. rubrum

After incubation, the trays prepared for MICs, which contains Sample Test Wells (SaTW) (BE or terb in DMSO, culture medium and inoculum); a growth control well (GCW) (culture medium, inoculum, the same amount of DMSO used in SaTW, but sample-free) and a sterility control well (SCW) (sample, medium and sterile water instead of inoculum) were placed on an Epichemi3 darkroom (UVP, Upland, CA). The percentage of *T. rubrum* growth inhibition was obtained by image analysis (Rühl and Kües 2009; Márquez-Rocha et al. 1999; Kelly et al. 2006). Three non-overlapping photographs of each culture were recorded with a high resolution cooled digital monochrome CCD camera (Hamamatsu C8484-51-03G) (Rühl and Kües 2009). Pictures were analyzed using LabWorks version 4.6 (UVP) in the following subsequent steps: (i) Function 'Define AOI (Area of Interest)'. This function was used to isolate an area of interest from the rest of an image; (ii) Function 'Select measurement'. This function defines a set of parameters for pellet detection and description: Area (*A*), Optical Density (OD) and Integrated Optical Density (IOD) being IOD = $A \times$ OD; (iii) Function 'Filter Ranges'. This function sets up a measurement range, in order to restrict the objects to be counted. A 10-pixel area was set as minimum to exclude any remaining smaller object different to pellet from further analysis; (iv) Function 'Measurements Data'. Parameters for all wells were calculated for the 'Exclude setting', and data were saved in separate spreadsheet files, where each row presents the information for one specific object and each column, the specific object parameters, as defined above. These object data were submitted to further analysis to calculate the 'Percentage of growth inhibition' = 1 − (IOD SaTW − IOD SCW)/(IOD GCW − IOD SCW) × 100.

High-throughput synergy screening assay (HTSS)

Six combinations for each of the forty four BE (total 264 combinations) were tested in combination with terb (Fig. 2). Sub-inhibitory concentrations (500 and 250 μg/ml) for BE (MIC_{100} > 1000 μg/ml) and 1/2, 1/5 and 1/10 MIC for terb ($MIC_{100} = 25 \times 10^{-4}$ μg/ml) were used in combination in a microtiter tray by duplicate as explained in the text. A quantified inoculum, similar as the described for MIC determinations, was added to each well. The inhibition percentage of each mixture was determined after 7 d incubation time, as explained above.

Statistical analysis

The effective enhancement of the inhibition growth of each mixture was compared to the inhibition growth of each component alone at the same concentration as it was in mixture, by using the two-way ANOVA test for analyses of variance followed by LSD Least Significant Difference of Multiple Comparisons. A $p < 0.05$ was considered significant.

Response-surface methodology (RSM)

The graphs were generated with the CombiTool program kindly provided by Dr. Jürgen Sühnel (Jena, Germany) (Dreβler et al. 1999). Curve dose–responses from 1000 to 15.6 μg/ml for each BE and from 100 to 1.6×10^{-4} μg/ml for terb were constructed. Parameters *a* and *m* were determined by means of median effect equation at various concentrations of BE and terb with the nonlinear regression module of the Origin software package (www.originlab.de).

$$\text{Median-effect equation}: Ef(d) = \frac{d^m}{a^m + d^m} \quad (1)$$

In Eq. (1), *Ef* = effect observed (percentage of growth inhibition) for each dose; *d* = dose (or concentration of each BE or of terb); *a* = median effect dose that inhibits the system under study by 50%; *m* = slope of the curve.

Analysis with CombiTool software

With these *a* and *m* parameters, the Loewe Additivity response surface (LA) is calculated for each BE and terb. The comparison of the surface obtained with experimental data *vs* the zero response surface allows the direct visualization of spheres which, if they fall into the surface are indicative of additivism, over the surface indicate synergism and below the surface show antagonism.

Analytical profiling of BE by HPLC/UV/ESI-MS

The filtered methanolic *Baccharis* extracts (that have showed synergistic or additive effects with terb in CombiTool) were submitted to a HPLC/UV/ESI-MS Bruker micrOTOF Q II/Agilent Technologies LC 1200 Series (Bruker Daltonics, MA, USA) chromatograph, equipped with an electrospray ionization source operated in positive mode (ESI+) with Nitrogen as nebulizing gas (1.0 bar) and drying gas (4 L min^{-1}, 200 °C); capillary 4500 V and end plate offset at 500 V; the temperature of the heated capillary gas was set to 200 °C, with a spectrometer QTOF with capacity for measuring high resolution exact mass (HRMS). Mass accuracy was verified by infusing Na-formiate (10 mM) (Sigma–Aldrich, St. Louis, MO, USA) dissolved in MeOH:H_2O (50:50). HPLC was performed with an Agilent ZORBAX Eclipse XDB-C18 Rapid Resolution 3.0 mm × 50 mm, 1.8 micron 600 bars column, at 25 °C to obtain extracts profiles and Rt of pure compounds. Sample (5 μl) were injected using an autosampler (Agilent HiP-ALSSL+). The flow rate

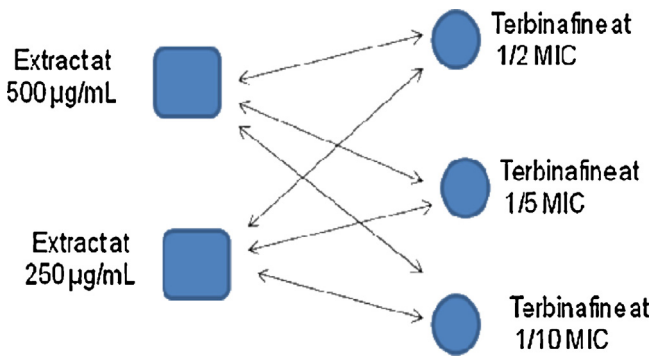

Fig. 2. Scheme showing the six combinations tested. MIC_{100} of each *Baccharis* extract (BE): >1000 μg/ml. MIC_{100} of terbinafine (terb): 25×10^{-4} μg/ml.

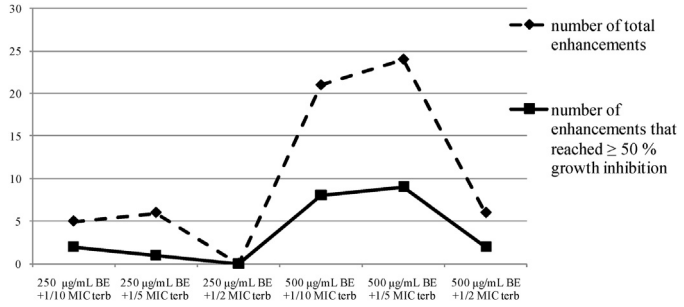

Fig. 3. For each ratio of *Baccharis* extract (BE)/Terbinafine (terb), the amount of total enhancements is showed with the points on dashed line. The amount of the enhancements that reached ≥50% of growth inhibition is showed with points in the solid line.

was set to 0.2 ml/min impulsed by an Agilent 1200 series G1312B SL binary pump) and the gradient (where A = water with 0.1% formic acid (v/v) and B = methanol with 0.1% formic acid (v/v) was as follows: t = 0–1 min, A:B (50:50, v/v); t = 1–15 min, A:B (20:80, v/v), t = 15–30 min A:B (0:100), for a total run time of 30 min. The post-run equilibrium time was 5 min. The compounds were monitored at 240 nm. The HPLC flow was introduced into the mass spectrometer *via* an ESI source. For the analysis of chromatograms and mass spectra, the Data Analysis 4.0 SP1 software (Bruker Daltonik GmgH, Germany) was used. Pure compounds were characterized by direct infusion to ESI using a syringe pump (kd Scientific, Holliston, MA, USA) recording the HRMS in positive mode. To establish the retention times, authentic samples of the flavonoids **1**–**4** and of the ent-clerodanes **5**–**7** were used (see Chemicals, above).

Two-drug combination of terb with the detected flavonoids and ent-clerodanes of Baccharis spp.

In an experiment similar to the detection of MIC (see above), an inoculum of *T. rubrum* was incubated in 96-well plates (Greiner Labortechnik) for 72 h with serial dilutions of terb ($100–1.0 \times 10^{-4}$ μg/ml) in addition to a sub-inhibitory concentration (31.25 μg/ml) of each *Baccharis* component (**1**–**7**). From dose–response curves, calculated with a four parameter logistic curve (SigmaPlot® 11.0), the MIC_{50} value (defined as the concentration of test compounds required to inhibit 50% of the fungal growth) of terb alone and in combination with the fixed amount of the *Baccharis* component, was determined. All assays were carried by triplicate. All data are expressed as mean ± SD.

Analysis of the combination effect

The nature of the interaction (synergism, additivism, or antagonism) between each natural product (NP, namely **1**–**7**) and terb, as a function of MIC_{50}, was assessed by the combination index method (CI) (Chou 2006) which is represented by the following equation:

$$CI = \frac{MIC_{50}, \text{terb, comb.}}{MIC_{50}\text{terb}} + \frac{C\,NP, \text{comb.}}{MIC_{50}\,NP}$$

where 'MIC_{50} terb, comb', is the MIC_{50} value for terb in each two-drug combination and 'C NP, comb' is the fixed concentration of

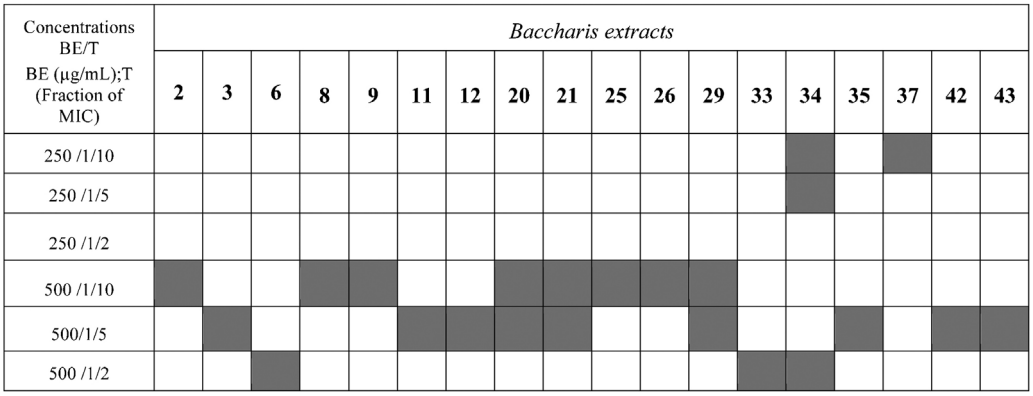

Fig. 4. Extracts (= 18) that showed a significant ($p < 0.05$) growth inhibition enhancement, reaching ≥50% inhibition in at least one combination. In gray, all the combinations that showed such enhancement. MIC_{100} terb = 25×10^{-4} μg/ml.

Table 1
Composition of mixtures at which synergistic (S) or additive (Ad) effects between BE and terb, were detected.

Extract No.		250 μg/ml BE with 1/10 MIC terb CT[a]	250 μg/ml BE with 1/5 MIC terb CT[a]	500 μg/ml BE with 1/10 MIC terb CT[a]	500 μg/ml BE with 1/5 MIC terb CT[a]
Ba	**2**	Ad		S	
	8			S	Ad
	9		S	S	
Bc	**11**		Ad		S
	12	S	S	Ad	S
Bphy	**25**		S	Ad	
	26	Ad		S	
Bt	**34**	S	S	Ad	
	35	S		Ad	
	43	S	Ad		Ad

[a] Columns highlighted in gray show the synergistic (S) and additive (Ad) mixtures obtained with CombiTool program (CT). *Ba*: *Baccharis articulata*; *Bc*: *Baccharis crispa*; *Bphy*: *Baccharis phyteumoides*; *Bt*: *Baccharis trimera*. MIC_{100} terb = 25×10^{-4} μg/ml.

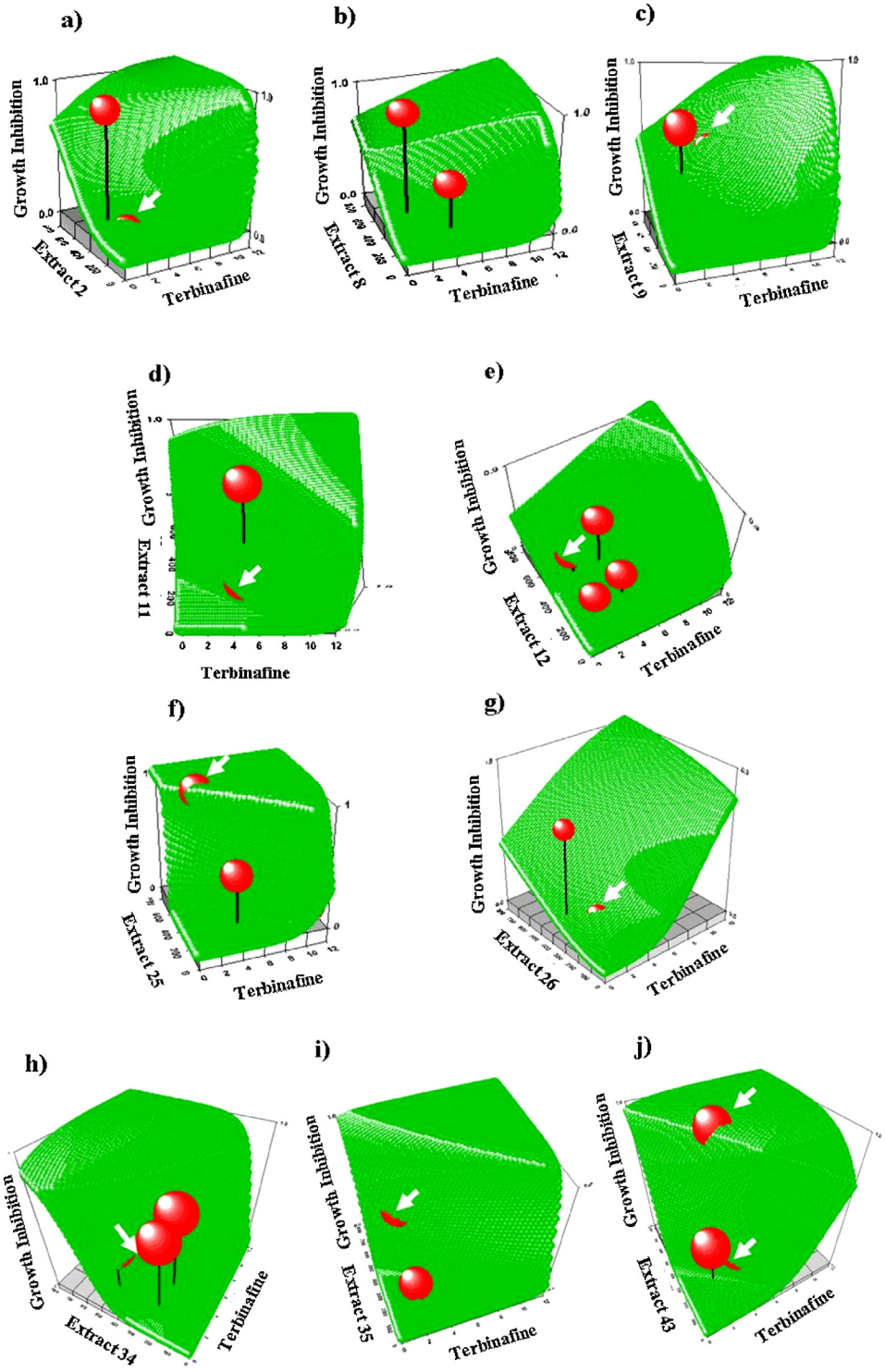

Fig. 5. Surfaces of experimental combination effects for the antifungal effect of *Baccharis* extracts and Terbinafine obtained with CombiTool software. Large spheres correspond to experimental combination effects. Filled spheres that fall within this surface indicate additivism (showed by white arrows) and spheres over the surface, indicate synergism. (a) Extract 2: *Baccharis articulata*, *Ba*2; (b) Extract 8: *Baccharis articulata*, *Ba*8; (c) Extract 9: *Baccharis articulata*, *Ba*9; (d) Extract 11: *Baccharis crispa*, *Bc11*; (e) *Baccharis crispa*, *Bc12*; (f) *Baccharis phyteumoides*, *Bphy*25; (g) *Baccharis phyteumoides*, *Bphy*26; (h) *Baccharis trimera*, *Btrim*34; (i) *Baccharis trimera*, *Btrim*35; (j) *Baccharis trimera*, *Btrim*43.

each individual NP in the combination with terb. 'MIC_{50} terb' and 'MIC_{50} NP' represent the MIC_{50} for each, terb or NP, alone. The Combination Index (CI) identify synergistic (CI < 1), additive (CI = 1), and antagonistic interactions (CI > 1) (Chou 2010).

The dose reduction index (DRI), which is a measure of how many-fold the dose may be reduced as compared with the doses of each drug alone (Chou 2006), is calculated as follows: DRI = 'MIC_{50} terb alone'/'MIC_{50} terb, comb' with each combination partner. A DRI value >1 is indicative of the same effectiveness with lower doses, which could have beneficial clinical implications such as lower toxicity or lower chance of occur resistance.

Checkerboard and isobolograms

The uppermost row (A) of a 96-well microtiter plate was filled with compounds **5** or **6** at a concentration of about two times the expected MIC_{50}. Each following row (B–H) contained half the concentration of the previous one. The same procedure was carried out along the columns (**1**–**12**) with terb. So, each well contained a unique combination (100 μl) of the two substances (one ent-clerodane and terb). To this solution, 100 μl of RPMI-1640 broth containing a *T. rubrum* inoculum = 10^3 CFU/ml was added to the wells. The plate was then incubated at 30 °C for 7 d. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth, were used to construct the isobolograms (Berembaum 1981; Wagner and Ulrich-Merzenich 2009).

Results and discussion

Determination of the enhancement of fungal growth inhibition by BE

The effect on *T. rubrum* growth inhibition of forty four BE and of terb, each one acting alone, was determined with the CLSI microbroth dilution method (CLSI 2008). Results showed that not any BE produce 100% inhibition at 1000 μg/ml (MIC_{100} > 1000 μg/ml) while terb showed a $MIC_{100} = 25 \times 10^{-4}$ μg/ml.

Six combinations for each of the forty four BE with terb (total 264 combinations) were prepared and tested against *T. rubrum*: two sub-inhibitory concentrations (500 and 250 μg/ml) of each BE (which consistently generated less than 20% of inhibition of the fungal growth) with three sub-inhibitory concentrations (1/2, 1/5 and 1/10 MIC) of terb (Fig. 2).

Each mixture was incubated with the fungal inoculum in a 96-microtiter plate, by duplicate, according to Materials and Methods.

The inhibition percentage displayed by each combination was compared to the ones produced by each component alone at the same concentration at which it was in the mixture and results were statistically analyzed with the bi-factorial ANOVA followed by the Least Significant Difference (LSD) tests (Montgomery 1999). Table S2 (Supplementary Material) summarizes the whole information obtained for the 44 extracts. Fig. 3 shows, for each combination ratio, the number of BE that produced a significant enhancement (points in the dashed line) of the fungal inhibition growth, no matter which was the achieved percentage. It can be observed that the number of enhancements significantly vary at different ratios. So, in the ratio 250 μg/ml BE-1/10 MIC terb only 5 of the 44 BE produced a significant enhancement ($p < 0.05$) of antifungal activity. Instead, in the ratio 500 μg/ml BE-1/5 MIC terb, 24 of the 44 BE showed a significant enhancement. Another interesting observation is that if only the combination 250 μg/ml BE-1/2 MIC terb have been used, no BE with enhanced activities would have been detected.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2013.06.015

Of the BE that showed capacity of significant enhancement, we selected those that reached ≥50% inhibition in at least one combination (full line in Fig. 3). So, only eighteen extracts (**2**, **3**, **6**, **8**, **9**, **11**, **12**, **20**, **21**, **25**, **26**, **29**, **33**, **34**, **35**, **37**, **42** and **43**), (Fig. 4) were ranked for submitting to 3D analysis.

Analysis of the combinations of BE with terb in 3D, with CombiTool software

A response-surface generated with the CombiTool program (Dreβler et al. 1999) was constructed for each of the eighteen extracts. By using the experimental effect of each dose, we constructed a dose–response curve for each BE alone, and for terb alone. These data were fit by nonlinear regression to median-effect equation (see section Materials and Methods) in order to obtain the parameters *a* and *m* necessary to build additivity surfaces as determined by Loewe.

Of the eighteen extracts, only ten (*Ba*2, *Ba*8, *Ba*9, *Bc*11, *Bc*12, *Bphy*25, *Bphy*26, *Bt*34, *Bt*35, *Bt*43) showed at least one combination with synergism or additivism in the response-surfaces (Fig. 5).

Fig. 5(a, c, d, f, g and i) shows two spheres for extracts *Ba*2, *Ba*9, *Bc*11, *Bphy*25, *Bphy*26 and *Bt*35 respectively; one falls within the zero surface or slightly above, and thus, it is considered additive. The other one falls over the surface and therefore shows a clear synergistic effect. Fig. 5b shows two synergistic combinations for extract *Ba*8. Fig. 5e shows three synergistic combinations and one additive mixture for extract *Bc*12. Fig. 5h shows two synergistic and one additive combination for extract *Bt*34, and Fig. 5j shows two additive and one synergistic mixture for extract *Bt*43. The compositions of mixtures at which each BE shows synergistic or additive effects are shown in Table 1.

The ten BE that showed synergistic or additive combinations with the CombiTool software constitutes a 22% hit rate (10/44) of the extracts submitted to evaluation.

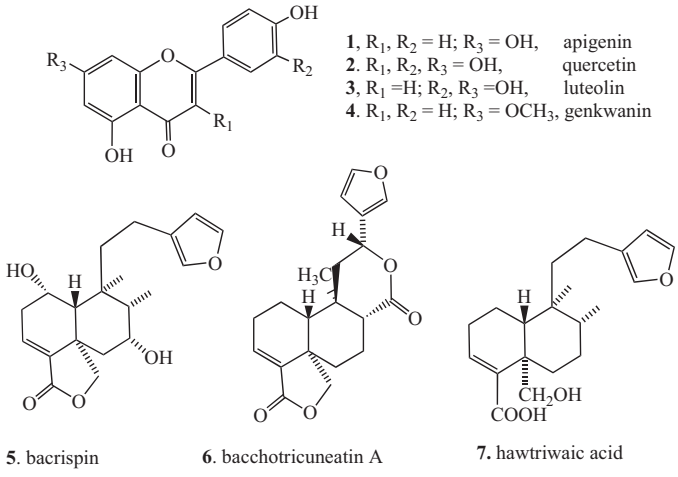

Fig. 6. Chemical structures of reference compounds: flavonoids **1**–**4** and ent-clerodanes **5**–**7** detected in methanol extracts of *Baccharis articulata*, *B. crispa* and *B. trimera*.

Table 2
Flavonoids and ent-clerodanes detected in ethanolic *Baccharis* extracts. Common names, molecular formula, HRMS and Rt obtained in a HPLC/UV/ESI-MS chromatograph.

	Common name	Mol. formula	MW	HRMS ($M+H^+$)	Rt (min)	Ref.
1	Quercetin	$C_{15}H_{10}O_7$	302.04	303.0522	5.7	Lommen et al. (2000), Tsimogiannis et al. (2007)
2	Luteolin	$C_{15}H_{10}O_6$	286.05	287.0567	6.2	Zhang et al. (2005), Tsimogiannis et al. (2007)
3	Apigenin	$C_{15}H_{10}O_5$	270.05	271.0621	8.2	Zhang et al. (2005), Tsimogiannis et al. (2007)
4	Genkwanin	$C_{16}H_{12}O_5$	284.07	285.0761	13.0	Xie et al. (2011)
5	Bacrispine	$C_{20}H_{26}O_5$	346.18	347.1855	7.9	Ceñal et al. (1997)
6	Bacchotricu neatin A	$C_{20}H_{22}O_5$	342.15	343.1545	8.0	Wagner et al. (1978), Simirgiotis et al. (2000)
7	Hawtriwaic acid	$C_{20}H_{28}O_4$	332.20	333.2086	16.8	Hsü et al. (1971)

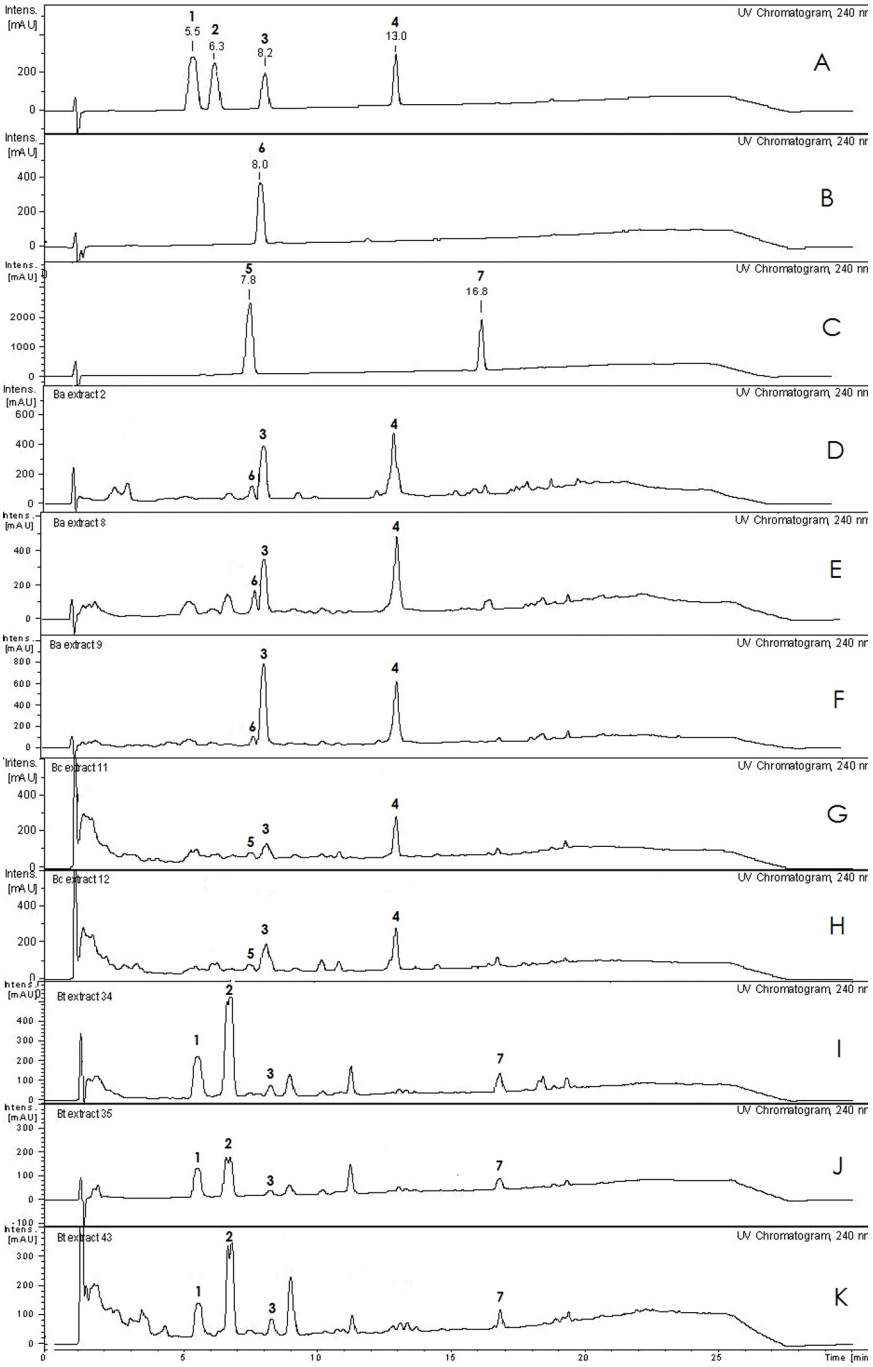

Fig. 7. HPLC chromatograms of selected extracts and reference compounds. Detection: UV 240 nm. (A, B, C) Reference compounds: (A) flavonoids, **1**: quercetin (Rt: 5.5 min); **2**: luteolin (Rt: 6.3 min); **3**: apigenin (Rt: 8.2 min); **4**: genkwanin (Rt: 13 min). (B) and (C) ent-clerodanes. (B) **6**: bacchotricuneatin A (Rt: 8.0 min); (C) **5**: bacrispine (Rt: 7.8 min); **7**: hawtriwaic acid (Rt: 16.8 min). D, E, F: *B. articulata* extracts *Ba*2, *Ba*8, *Ba*9 respectively; G, H: *B. crispa* extracts *Bc*11, *Bc*12 respectively; I, J, K: *B. trimera* extracts *Bt*34, *Bt*35, *Bt*43 respectively.

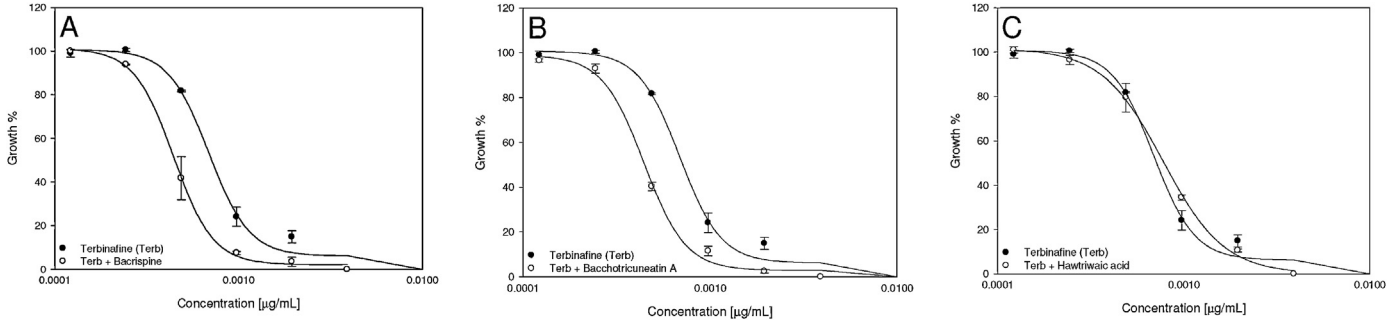

Fig. 8. Dose–response curves of the antifungal drug terbinafine in combination with a fixed amount (31.25 μg/ml) of each ent-clerodane bacrispine **5** (A), bacchotricuneatin A **6** (B) and hawtriwaic acid **7** (C), detected in *Baccharis* extracts.

Analytical profiling and detection of metabolites in selected BE by HPLC/UV/ESI-MS

The analytical profiles of *Ba*2, *Ba*8, *Ba*9, *Bc*11, *Bc*12, *Btrim*34, *Btrim*36 and *Btrim*42 extracts that showed synergistic or additive mixtures in CombiTool was achieved with HPLC/UV/ESI-MS. A three step gradient of solvent mixtures with a constant flow of 0.2 ml/min with 240 nm as the detection wavelength, resulted in the most suitable conditions. The flavonoids quercetin **1**, luteolin **2**, apigenin **3** and genkwanin **4** along with the ent-clerodanes bacrispine **5**, bacchotricuneatin A **6** and hawtriwaic acid **7** (Fig. 6) were detected in the selected BE, based on the coincidence of Rt and HRMS of authentic samples (Table 2). Reference flavonoids were obtained from commercial sources as detailed in section Materials and Methods. Instead, bacchotricuneatin A was a generous gift from Prof. H. Wagner (München, Germany) and both, bacrispine and hawtriwaic acid were a donation from Prof. C. Tonn and L. Favier (San Luis, Argentina) as stated above.

The HPLC-UV (240 nm) chromatograms shows that flavonoids **1** and **2** were present in all *Btrim* extracts (Fig. 7I, J and K); **3** was found in all *Ba*, *Bc* and *Btrim* extracts (Fig. 7D–K) and **4** was detected in all *Ba* and *Bt* (D–F and I–K). Regarding ent-clerodanes, **5** was detected in *Bc* (G and H); **6** in *Ba* (D–F) and **7** in *Btrim* extracts (I–J). These compounds have been previously described in these *Baccharis* spp. (Alcaraz et al. 2012; Gonzaga Verdi et al. 2005).

In each two-drug combination assays (including terb and one of the natural compounds **1**–**7**), ent-clerodanes **5**–**7** but not flavonoids **1**–**4** produced shifts of the dose–response curves of terb toward lower concentrations (Fig. 8A–C).

MIC_{50} values of terb alone and in dual combinations with **5**–**7**, along with the Combination Index (CI) and the Dose Reduction Index (DRI) (Chou 2006, 2010) are showed in Table 3. MIC_{50} terb values decreased from 6.90 to 4.40×10^{-4} μg/ml (DRI = 1.55) when mixed with **5**; from 6.90 to 4.60×10^{-4} μg/ml (DRI = 1.50) when mixed with **6** and from 6.90 to 5.24×10^{-4} μg/ml (DRI = 1.32) when mixed with **7**. In turn, CI values were 0.89, 0.79 and 1.01 respectively, indicating synergism for **5** and **6**, and additivism for **7**.

Isobolograms of bacrispine **5** or bacchotricuneatin A **6** with terb, performed at different concentrations of both partners (as detailed in section Materials and Methods) are depicted in Fig. 9. The *x* axis represents terb and the *y* axis, either compound **5** (Fig. 9 A) or **6** (Fig. 9 B). The line connecting MIC_{50} values of both

Table 3
MIC_{50} values (μg/ml) of terbinafine and ent-clerodanes (detected in selected *Baccharis* spp.) either alone or in two-drug combination. The Dose Reduction Index (DRI) of terbinafine in combination with its partners and the Combination Index (CI) were calculated for *Trichopyton rubrum* cells. Data are represented as mean ± SD (triplicate tests).

		MIC_{50} (μg/ml) of the NP alone	MIC_{50} (μg/ml × 10^{-4}) of terb alone	MIC_{50} terb (μg/ml x 10^{-4}) in combination with NP[a]	DRI_{terb}	CI
Terbinafine	6.90 ± 1.92					
NP at a fixed concentration (31.25 μg/ml)	**5** Bacrispine	125.23 ± 12.30		4.44 ± 1.65	1.56	0.88
	6 Baccho A	250,00 ± 08.23		4.60 ± 1.54	1.50	0.79
	7 Hawtri A	125.35 ± 17.45		5.24 ± 1.98	1.31	1.01

[a] Mixture of Terbinafine (terb) + 31.25 μg/ml of each NP (natural product). Baccho A: Baccotricuneatin A; Hawtri A: Hawtriwaic acid; CI: Combination Index; DRI: Dose Reduction Index.

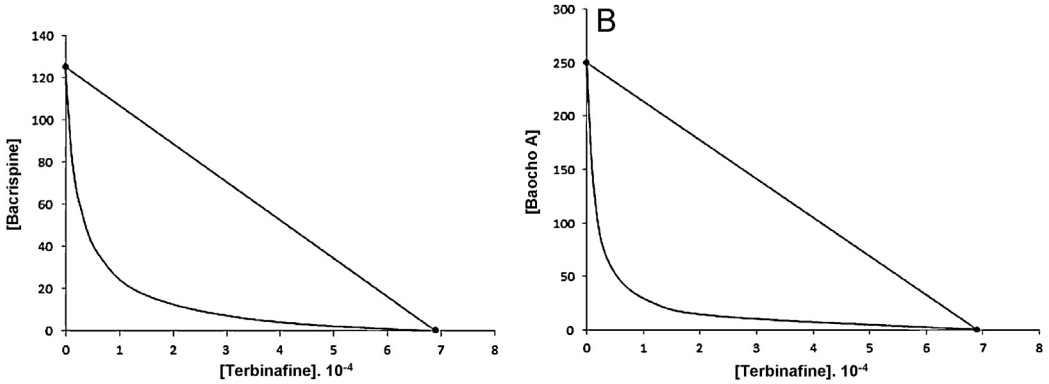

Fig. 9. Isobologram analyses: MIC_{50} concentrations of terbinafine are plotted on *x*-axis and MIC_{50} values of either bacrispine **5** (Fig. 9A) or bacchotricuneatin A **6** (Fig. 9B) on *y*-axis. The line connecting these two points is the line of additivity. Concave curves located below the additive line indicate synergism.

partners represents the line of no interaction (line of indifference). Data below the line of indifference resulting in a concave curve, indicate synergism (CI < 1). Data above the line of indifference that form a convex curve, indicate antagonism (CI > 1) (Wagner and Ulrich-Merzenich 2009).

From the analysis of isobolograms, it is clear that both, bacrispine **5** and bacchotricuneatin A **6** showed synergistic effects with terb. Of them, **6** appears to exert a higher synergistic effect since a more concave curve than with **5**, was obtained.

Conclusions

The application of HTSS assay to mixtures of 44 extracts of *Baccharis* spp. of the Caulopterae section with terb at six different ratios against *T. rubrum* followed by three-dimensional (3D) dose–response surfaces, allowed to detect 10 BE (22%) that showed synergistic or additive combinations. Four flavonoids and three ent-clerodanes were detected in the active *Baccharis* extracts by HPLC/UV/ESI-MS and these pure compounds were tested in combination with terb. Results showed that ent-clerodanes but not flavonoids showed synergistic or additive effects. Among them, bacchotricuneatin A **6** followed by bacrispine **5** showed synergistic effects while hawtriwaic acid **7** showed additive effects.

These results clearly show that the study of non-antifungal *Baccharis* extracts in combination with terb by using an HTSS assay with less stringent conditions that the previously reported (Zhang et al. 2007), followed by 3D CombiTool analysis (Dreβler et al. 1999), allow the detection of 10 antifungal BE-terb combinations. In addition, in these BE, flavonoids and ent-clerodanes were detected, of which the last ones showed synergistic or additive activities. Instead, if tested alone, no BE would have been submitted to further analysis, taking away the possibility of finding antifungal components for the developing of new antifungal drugs.

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