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PII: S0731-7085(18)32295-7
DOI: https://doi.org/10.1016/j.jpba.2019.01.026
Reference: PBA 12443

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 10 October 2018
Revised date: 10 January 2019
Accepted date: 13 January 2019

Please cite this article as: Espino M, Solari M, de los Ángeles Fernández M, Boiteux J, Gómez MR, Silva MF, NADES-MEDIATED FOLK PLANT EXTRACTS AS NOVEL ANTIFUNGAL AGENTS AGAINST *Candida albicans*, *Journal of Pharmaceutical and Biomedical Analysis* (2019), https://doi.org/10.1016/j.jpba.2019.01.026

NADES-MEDIATED FOLK PLANT EXTRACTS AS NOVEL ANTIFUNGAL AGENTS AGAINST Candida albicans

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Highlights

- Natural solvents showed outstanding performance as plant biocompounds vehicle.
- Antifungal activity of *Larrea* extracts were higher than their individual compounds.
- Topical formulations prepared with Larrea extracts showed satisfactory performance.

Abstract

Candida albicans is an opportunistic pathogenic yeast commonly found in mouth, gastrointestinal tract and vagina. Under certain conditions, it causes skin, mucosal and systemic infections. With growing concern over the emergence of resistant strains to conventional antifungals, the development of novel antifungal agents for the management of this pathogen is an urgent need. In the present work, novel bioextracts from folk medicinal plants were directly used as active ingredient in a topical formulation for dermal candidiasis. With the aim to replace hazardous traditional reagents, a natural solvent composed by lactic acid: glucose: water (LGH) was used as vehicle for bioactive compound extraction. Furthermore, phenolic and alkaloid composition were determined by HPLC and their individual antifungal effect was evaluated. LGH extracts of *Larrea* spices demonstrate a significant antimicrobial activity against *C. albicans* being higher than their individual bioactive constituents. Notably, the mixture of *Larrea cuneifolia* and *L divaricata* extracts in topical formulations reveal a synergistic antifungal effect highlighting their potential for candidiasis treatment.

Keywords: *Candida albicans,* NADES, pharmaceutical formulations, medicinal plants, phenolic compounds, alkaloids

1. Introduction

Candida albicans is the most prevalent fungal pathogen in humans causing candidiasis; giving rise to severe morbidity in millions of individuals world-wide [1]. This fungal infection affects predominantly superficial skin and mucosa (oral and vaginal). Also, it can lead to life-threatening systemic infections, known as invasive candidiasis [1-4]. The conventional therapeutic treatments for candidiasis are associated with several side effects that limits the dose and dosing frequency. Moreover, the extensive use of a limited number of antifungal agents, particularly azoles, has conducted to the emergence of resistant strains, a problem of growing concern. For dermal candidiasis, the relapse is highly frequent [4, 5]. In this context, the development of novel antifungal agents for the management of this pathogen represents not only a greatest challenge but also an urgent need for medicine [4-7].

Throughout history, medicinal plants have been successfully explored for their antimicrobial properties being an interesting alternative to synthetic drugs. Bioactive compounds of plants are mainly secondary metabolites, among them phenolic compounds and alkaloids are the most relevant group [8] .Interestingly, these compounds have been reported for their therapeutic activities such as cardio-protective, anti-inflammatory and antimicrobial [9].Traditionally the extraction of plant metabolites are carried out with solvents such as chloroform, methanol, ethanol, hexane, diethyl ether and water [10, 11]. Although these extracts are obtained from natural sources, their extraction procedure presents many disadvantages for human health and for the environment [12]. Therefore, the development of natural solvents turns to be a major priority to achieve safer extracts.

Natural deep eutectic solvents (NADES) have been introduced in the last decade as promising green media. They are eutectic mixtures consisting of natural metabolites bound together by inter-molecular interactions, particularly hydrogen bonding [13].Their common components are naturally present in all types of cells and organisms such as sugars (glucose, fructose, sacarose, etc.); organic acids (lactic, malic, citric acids, etc.); urea and choline chloride [14]. NADES have gained attention in chemistry for the extraction and separation of analytes from natural sources due to their outstanding advantages including biodegradability, low toxicity, solute stabilization, sustainability and low cost [14-16]. Recently, plant extract mediated by NADES have been evaluated for their antimicrobial properties [17], this opens interesting possibilities for their application in phytomedicine. To best of our knowledge, NADES bio extracts have not been used in dermal formulations.

In the present work, NADES extracts of *Thymus vulgaris, Origanum vulgare*, *Larrea divaricata* and *L. cuneifolia* were evaluated against *Candida albicans*. In order to clarify what compound in folk plant extracts shows antifungal properties, phenolic and alkaloid composition were determined and their individual antimicrobial effect were compared with the extracts. Furthermore, extracts cream formulations were prepared and challenged in terms of physical parameters as well as biological activity.

2. Material and methods

2.1. Chemicals and standard solutions

Compounds for NADES preparation including glucose anhydrous (≥ 99 %) and L (+) lactic acid (85-90 %) were purchased from Biopack (Bs. As., Argentina). Analytical standards, apigenin 95 % (Api), naringenin ≥95 % (Nar), caffeic acid ≥99 % (Caf), nordihydroguaiaretic acid ≥97 % (NDGA) and rosmarinic acid ≥99 % (Ros) were purchased from Sigma Aldrich (St. Louis, MO,USA). Quercetin dihydrate ≥97 % (Quer) were obtained from Alfa Aesar (Haverhill, MA, USA); tyrosol >99,5% (Tyr) from Fluka

Analytical (St. Louis, MO,USA); and trans-ferulic acid ≥ 99% (Fer) from SAFC (St. Louis,MO,USA). Alkaloids theophylline (Theop), theobromine (Theob), caffeine (Caffe), piperine (Pip) and Harmaline (Har), were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) of chromatographic grade was purchased from J. T. Baker (USA). Formic acid (85 %) (FA) was obtained from Sintorgan (Bs.As., Argentina). Stock solutions were prepared by dissolving each standard at a concentration of 1000 μg mL^{-1} in NADES. Standard working solutions at concentrations of 50, 25, 15, 5 μg mL^{-1} were obtained from stock solutions. All these solutions were stored in dark-glass bottles at 4 °C. Mueller Hinton agar, Sabouraud dextrose agar (Laboratorios Britania S.A., Bs.As. Argentina) and trypto-caseine soy broth (Biokar, France) were used for antimicrobial assays. Cream base for hydroquinone composed by cetyl alcohol 15 %, propylene glycol 10 %, beeswax 1 %, sodium laureth sulphate 2 %, aqua q.s. 100 %. Silicone 344, silicone 1000 were purchased from Parafarm (Saporiti, Bs.As.).

2.2. NADES preparation

The NADES was prepared using a method previously described by Dai and co-workers [15]. The components mixture (lactic acid and dextrose; 5:1) with 15 % of water (H_2O, v/v), named as LGH, was placed in a 20 mL amber glass vial. After, the mixture was heated in a magnetic stirrer with temperature control (Fisatom model 752A, Brasil) at 40 °C for 60 min.

2.3. Plant material and extracts preparation

Larrea cuneifolia, Larrea divaricata, Thymus vulgaris and *Origanum vulgare* plants were cultivated at a greenhouse under natural radiation and identified by means of morphological, anatomical, and histochemical analyses. Leaves were harvested during flowering period and immediately frozen in liquid nitrogen, then lyophilized in darkness.

Before the extraction, lyophilized material was grounded up to a fine powder with liquid nitrogen.

Extraction was performed according to Espino et al. [18].Lyophilized plant material (375 mg) and extraction solvent (LGH, 5 mL) were placed in a 15 mL centrifuge tube (ratio plant- solvent of 75 mg mL^{-1}), homogenized by a vortex during 15 s. The suspensions were processed by ultrasound (Cleanson, Argentina, 200 W output power, 20 kHz frequency) during 42 min at 40 ˚C (± 2 ˚C). Then, the system was centrifuged for 30 min (Presvac DCS-16-RV), and the supernatant was filtered (0.45 µm). The extraction was performed in triplicate.

2.4. Antimicrobial activity

2.4.1. Microorganisms

Candida albicans strain was provided by the Dermatology area of Hospital Lagomaggiore (Mendoza, Argentina). The pathogen was grown on Sabouraud dextrose agar for 24 hs at 35 °C. Then, a suspension was prepared in sterile saline solution (sodium chloride 0.9 %) and adjusted to 0.5 Mc Farland, immediately before its use.

2.4.2. Antimicrobial screening of bio-extracts and individual constituents

Disk diffusion method standardized according to the recommendations of the CLSI documents M44-A2 was used [19]. Mueller Hinton agar (MHA) supplemented with 10 % methylene blue solution (5 mg mL^{-1}) and 2 % of glucose was added to petri plates (15 cm). Culture medium was inoculated with *C. albicans* suspension. Sterile filter paper discs (9 mm of diameter) were impregnated with 15 µL of plant extract (filtered through 0.2 µm) and fluconazole disc (25 µg, Neo-sensitabs^{TM}) was included as positive control. All discs were placed in inoculated MHA and incubated at 35 °C for 48 h. The experiment was performed

in triplicate and the antimicrobial activity of extracts and individual constituents was determined by measuring the inhibition area of microbial growth.

2.4.3. Minimum inhibitory concentration assay

Broth macrodilution method was performed for the determination of the minimum inhibitory concentration (MIC) following the recommendations of the CLSI documents M27- A3 [19]. Six serial dilutions of the extracts were prepared in Trypto-caseine Soy Broth (TSB): 75, 37.5, 30, 25, 20, 18.75, 7.5, 3.75, 1.87 mg mL^{-1}. *C. albicans* suspension was diluted 1:2000 in TSB. The assay was performed in tubes as follows; 500 µL of each extract dilution was added to 4.5 mL of the pathogen dilution and incubated 48 h at 35 °C. Positive controls were performed using fluconazole as antifungal agent at 0.5 and 400 µg mL^{-1}. A solvent control (LGH) for the six extract dilutions was included to confirm that the solvent did not present any antimicrobial effect. For this purpose, adequate aliquots of LGH were diluted with TSB to achieve the same concentrations tested for the bioextracts. Also, a culture and a sterilization control were included. The test was performed in triplicate. MIC results were interpreted as the lowest concentration of the extract that exhibit total microbial growth inhibition (first clear tube). Finally, in order to evaluate the fungistatic and fungicidal effect of extracts, the suspension from this clear tube and from the two consecutives tubes (one higher and one lower concentrations) were inoculated in Sabouraud dextrose agar plates (24 h at 35 °C).

2.5. Phenolic and alkaloids composition

Phenolic (Tyr, Nar, NDGA, Caf, Fer, Ros, Api, Quer) and alkaloids compounds (Theop, Theob, Pip, Har, Caffe) were determined using a HPLC-DAD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The HPLC instrument was a Dionex Ultimate 3000 consisting of vacuum degasser unit, autosampler, quaternary pump

and chromatographic oven. The detector was a Dionex DAD-3000 (RS) model. Chromeleon 7.1 software was used to control all the acquisition parameters of the HPLC-DAD system and also to process the obtained data. Ultrapure water with 0.1 % FA (A) and ACN (B) were used as mobile phase. The column temperature was held at 30 °C and the injection volume was 5 µL.

HPLC phenolic separation was carried out in a Zorbax SB-Aq column (4.6mm × 150 mm, 5 m) Agilent Technologies, using the following gradient: 0–3 min, 5 % B; 3–11 min, 15% B; 11–15 min, 32 % B; 15–15.5 min, 40 % B; 15.5–16 min, 50 % B: 16–16.5 min 50 % B; 16.5–17 min 30% B; 17–17.5 min 15% B; 17.5–18.5 min 5% B; 18.5–20 min 5 % B. The mobile phase flow was 1.2 mL min^{-1}.

Alkaloids separation was carried out in a Accucore polar premium column c18 (3 mm x 100 mm, 2,6 µm) using the following gradient: 0–1.5 min, 25 % B; 1.5- 3.75 min, 42% B; 3.75–6.5 min, 50 % B; 6.5–9 min, 50 % B; 9–9.75 min, 25 % B; 9.75–10 min 10 % B; 10–12.5 min 10% B. The mobile phase flow was 1 mL min^{-1}.

The identification and quantification of phenolic and alkaloids compounds were based on the comparison of the retention times (tR) and absorbance values of detected peaks in solvents with those obtained by injection of pure standards of each analyte. Chromatograms were recorded at 280 (Tyr, Nar, NDGA, Theob, Caffe, Theop), 320 (Caf, Fer, Ros, Api, Pip) and 370 nm (Quer, Har).

2.6. Preparation of topical formulations

Cream formulations were developed mixing cream base with different extract concentrations and silicones. The mixture was kept under constant agitation until complete homogenization at room temperature. The formulations were characterized in terms of colour, homogeneity, odour, consistency and extensibility. Extensibility test was performed following the method proposed by De Paula et al [20]. A determined amount of cream (25

mg) was submitted to compression under four plates of known weight (5; 2; 2; 5 g). The plates were subsequently displayed over the sample at 1-min intervals and the spreading radio reached by the sample was measured in the vertical and horizontal axis. The results were expressed as the spreading area as a function of the applied mass accordingly to Eq. 1:

$$S = \frac{d^2\pi}{4} \qquad (1)$$

were, *S:* spreading area (mm^2) resultant from the applied mass; and *d*: is the mean diameter reached by the sample (mm).

Antimicrobial activity of formulations was also evaluated using disk diffusion method described in Section 2.4.2. with modifications. Culture medium was inoculated with *C. albicans* suspension. Holes of 6 mm diameter were made on the agar plate and filled with each topical formulations. The experiment was performed in triplicate and the cream efficacies were determined by measuring the inhibition area of microbial growth.

3. Results and discussion

NADES have been introduced as sustainable solvents for bioactive compounds extraction from medicinal plants [21]. In this sense, a NADES composed by lactic acid: dextrose: water (LGH) was used for *L. divaricata*, *L. cuneifolia*, *T. vulgaris* and *O. vulgare* extract preparation.

In order to illustrate the potential of NADES in the extraction of phenolic and alkaloids compounds, comparisons of the extraction efficiencies with the traditional solvents methanol and water were performed (Supplementary Figure 1 and 2). The results demonstrated that LGH shows outstanding extractability for both polar and weak polar compounds compared to conventional solvents.

3.1. Antimicrobial screening of LGH plant-extracts

In the present study, the antimicrobial screening of extracts against *Candida albicans* was performed by disc diffusion method. The results showed differences among the four plant extracts tested (Figure 1). The highest inhibition was achieved with *L. divaricata* and *L. cuneifolia* extracts, whereas *T. vulgaris* and *O. vulgare* showed the lowest inhibitions. Vast scientific knowledge supports the applications of the genus *Larrea* in antimicrobial assays. Alcoholic extracts of *L. divaricata* and *L. cuneifolia* showed considerably activity against filamentous fungi (*Lenzites elegans, Schizophyllum commune, Pycnoporus sanguineus, Ganoderma applanatum, Fusarium oxysporum, Penicillium notatum, Aspergillus niger* and *Trichoderma* spp) [22]. Zampini et al. demonstrated the activity of *Larrea* ethanolic extracts against antibiotic-resistant bacteria [23].Taking into account the aforementioned, *L. divaricata* and *L.cuneifolia* extracts were selected for following assays.

3.2. Phenolic and alkaloid composition

Considering the complexity of plant matrices it is difficult to attribute the antimicrobial activity of an extract to a specific compound. Nevertheless, researchers have reported the relationship between the phenolic and alkaloids composition of the extracts and their antimicrobial activity [8, 10, 24, 25].

In this work, phenolic and alkaloids compounds in *Larrea* extracts were determined by HPLC-DAD following the procedure described in section 2.5. Results are shown in Table 1. NDGA was the phenol found at highest concentration in *Larrea* extracts. Caf was detected in both species while Nar was only detected in *L. divaricata* extract. Interestingly, *L. cuneifolia* also presented Fer; and high concentrations of Quer and Ros. Furthermore, the alkaloids detected in both extracts were Theop and Pip.

Previous studies performed in *Larrea* species have identified the presence of caffeic and ferulic acids as well as methyl ethers of quercetin and kaempferol. [26, 27]. Our group also reported naringenin, luteolin and cinnamic, vanillic and caffeic acids in *L. divaricata* water extracts [8]. To our knowledge there are no reports concerning alkaloid composition in *Larrea* spieces.

3.3. *Antimicrobial activity of bioactive compounds*

With the aim to determine which compounds in *Larrea* extracts show antifungal activity against *Candida albicans*; the individual alkaloids and phenols detected (Section 3.2.) were evaluated. For this purpose, disc diffusion method was used and each biocompound was tested at the concentration found in the extracts. As can be seen in Figure 2 all the biocompounds studied present antimicrobial activity against *C. albicans.* Among the bioactive metabolites present in *L. divaricata* extract, the results demonstrated that NDGA and Caf showed the highest antifungal inhibition (Figure 2A) while for *L. cuneifolia* NDGA, Caf, Fer, Pip and Theop presented a marked antimicrobial activity (Figure 2B). It has to be pointed out that any of the biocompounds tested achieved the inhibition obtained with the extracts.

3.4. Minimum inhibitory concentration assay

With the aim to quantify the minimum inhibitory concentration (MIC) of *L. divaricata* and *L.cuneifolia* bio-extracts against *C. albicans*, broth macrodilution method was performed. Serial dilutions of the bio-extracts were tested between: 1.875 - 75 mg mL^{-1}. The lowest concentration with no visible growth was defined as the MIC (first clear tube). The results highlight that *C. albican*s is more susceptible to *L. divaricata* than *L. cuneifolia* extract, being the MIC of 20 and 25 mg mL^{-1}, respectively. Taking into account the MIC values,

selected dilutions were sub-cultured in a solid media to verify the fungistatic or fungicidal effects of the extracts. As can be seen in Figure 3, fungicidal activity was achieved at the same concentrations that MIC values. Fluconazole, the positive control, showed fungistatic effect at the lowest concentration tested (0.5 µg mL^{-1}), whereas at the highest (400 µg mL^{-1}) it was fungicidal. It has to be pointed out that, at the concentrations tested, LGH allows the microorganism growth, without interfering with the extract antimicrobial activity (Figure 3).

3.5. Preparation of topical formulation

Taking into account the MIC values of *L. cuneifolia* and *L. divaricata* extracts, topical formulations for *C. albicans* control were prepared. In a preliminary study, two silicones (S_{1000} and S_{344}) were tested at concentrations between 12.5 and 25 %. The results indicated no significant difference for silicone type and concentration; thus S_{344} at 25 % was selected. Four cream formulations were prepared using *L. cuneifolia* (C) and *L. divaricata* (D) extracts at 0.5x and x ; being x the MIC value (C 0.5x, C x, D 0.5x and D x). Also, two combinations of both extracts were tested using 0.25x and 0.5x of each one (D 0.25x-C 0.25x and D 0.5x-C 0.5x).

The evaluation of cream's organoleptic characters (colour, odour, consistency and homogeneity) was found to be satisfactory and no signs of separation and deterioration were observed over a period of 60 days. The results of extensibility test showed no significant differences between topical formulations prepared with different plant extracts and concentrations for at least 60 days (Figure 4). When comparing the *Larrea* topical creams with a commercial antifungal, similar spreading areas were achieved. Extensibility is an important characteristic of creams designed for skin application, being responsible of correct dosage, ease of application, extrudability from the package, and consumer preference [20].

In order to evaluate the antimicrobial activity of the topical formulations prepared, disk diffusion method was performed. As can be seen in Figure 5, *L. divaricata* creams at 0.5x and x presented an inhibition area of 2 and 3 mm^2 respectively. A similar performance was observed for *L. cuneifolia* creams. Interestingly, the combination of *Larrea* species (D 0.5x-C 0.5x) showed the highest *C. albicans* inhibition (8 mm^2). These results reveal that the extracts of both *Larrea* species present a synergistic effect improving the antimicrobial activity against *C.albicans.*

Previous studies have demonstrated the potential of creams based on plant extracts for the treatment of *C. albicans* infections. Creams prepared with dried alcoholic extracts of *Juglans nigra* showed satisfactory inhibition for vaginal *C. albicans* infections [28]. El-Gied et al. reported the in vitro activity of creams and ointments prepared with *Mangifera indica* extracts [29]. Moreover, Oridupa and co-workers found that the herbal cream and ointment of *Phyllanthus amarus* extract showed acceptable physicochemical and significant in vitro antimicrobial activity against *C. albicans* [30].

4. Conclusions

The present work represents an advancement in the knowledge of chemical composition and antifungal properties of *Larrea* medicinal plants. NADES bio extracts were used for the first time in dermal formulations. The extraction ability of NADES opens interesting possibilities for their use as vehicles of phenolic and alkaloid bioactive compounds for pharmaceutical applications. LGH extracts of *Larrea* spices demonstrate a significant antimicrobial activity against *C. albicans* being higher than their individual bioactive constituents. Notably, the mixture of *L. cuneifolia* and *L. divaricata* extracts in topical formulations reveal a synergistic antifungal effect highlighting their potential for candidiasis treatment. Further researches, including *in vitro* and *in vivo* assays, are needed for the applicability of this topical formulation.

Acknowledgments

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo (Mendoza, Argentina). The authors are particularly grateful to Dra. Viviana Parra from Dermatology area of Hospital Lagomaggiore (Mendoza, Argentina) for providing *Candida albicans* strain.

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Figure Captions

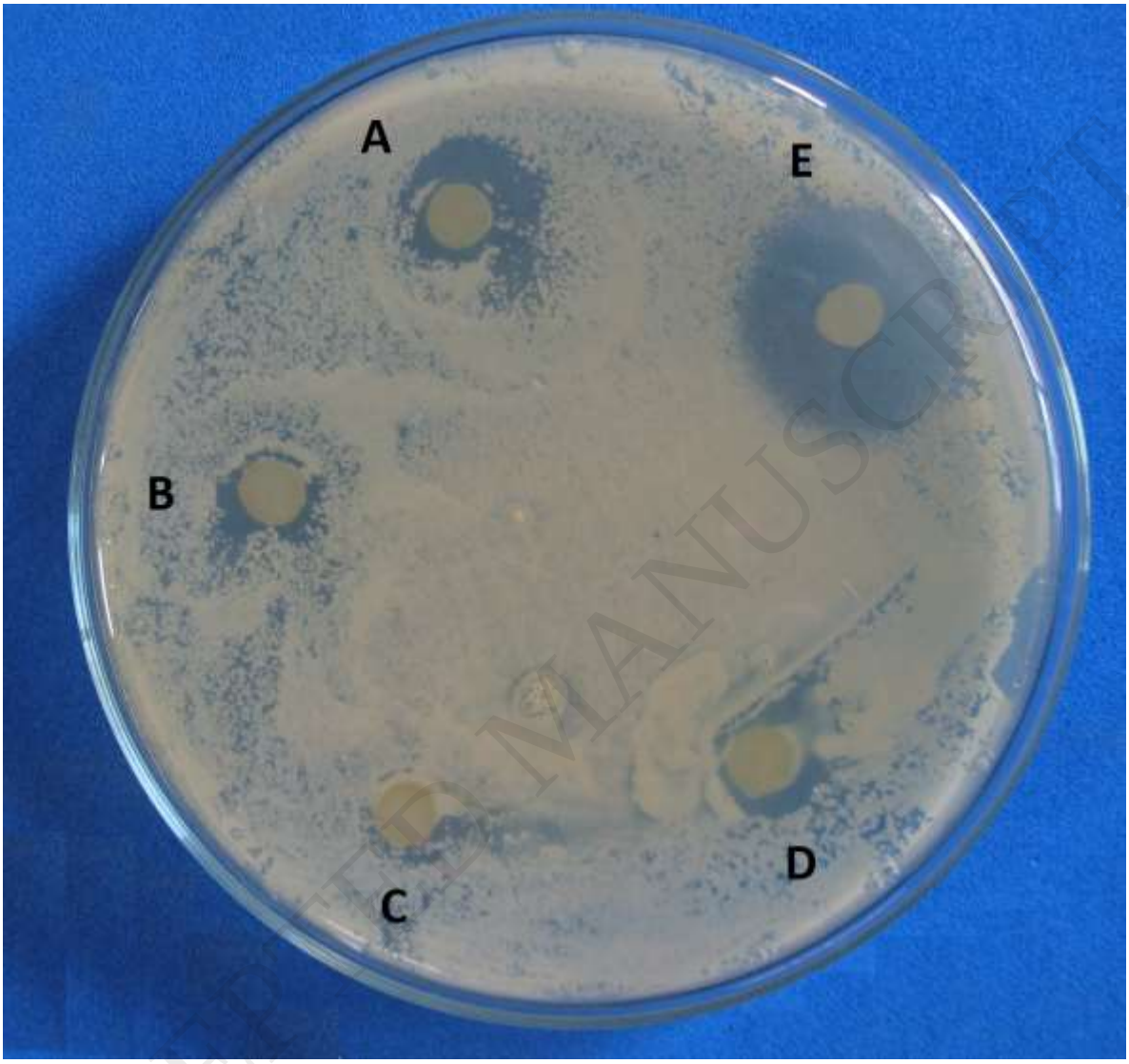

Figure 1: Antimicrobial activity of LGH-plant extracts by disc diffusion method: A: *Larrea divaricate*, B: *Thymus vulgaris,* C: *Origanum vulgare*, D: *Larrea cuneifolia*, E: Fluconazole disc.

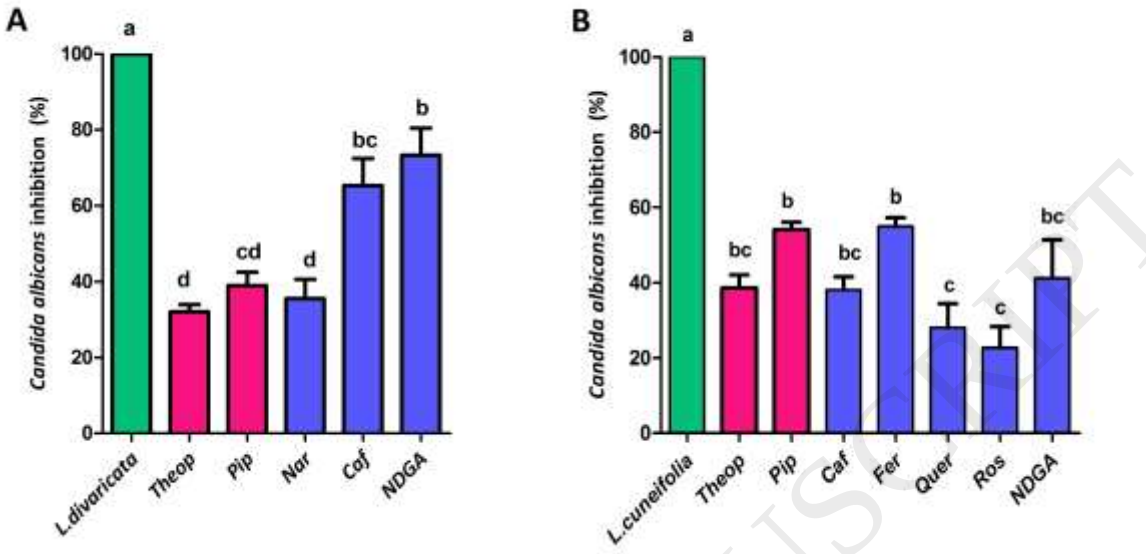

Figure 2: *Candida albicans* inhibition of *Larrea* LGH-extracts and its individual biocompounds, A: *Larrea divaricata* extract, B: *Larrea cuneifolia* extract. Different letters represent significant differences.

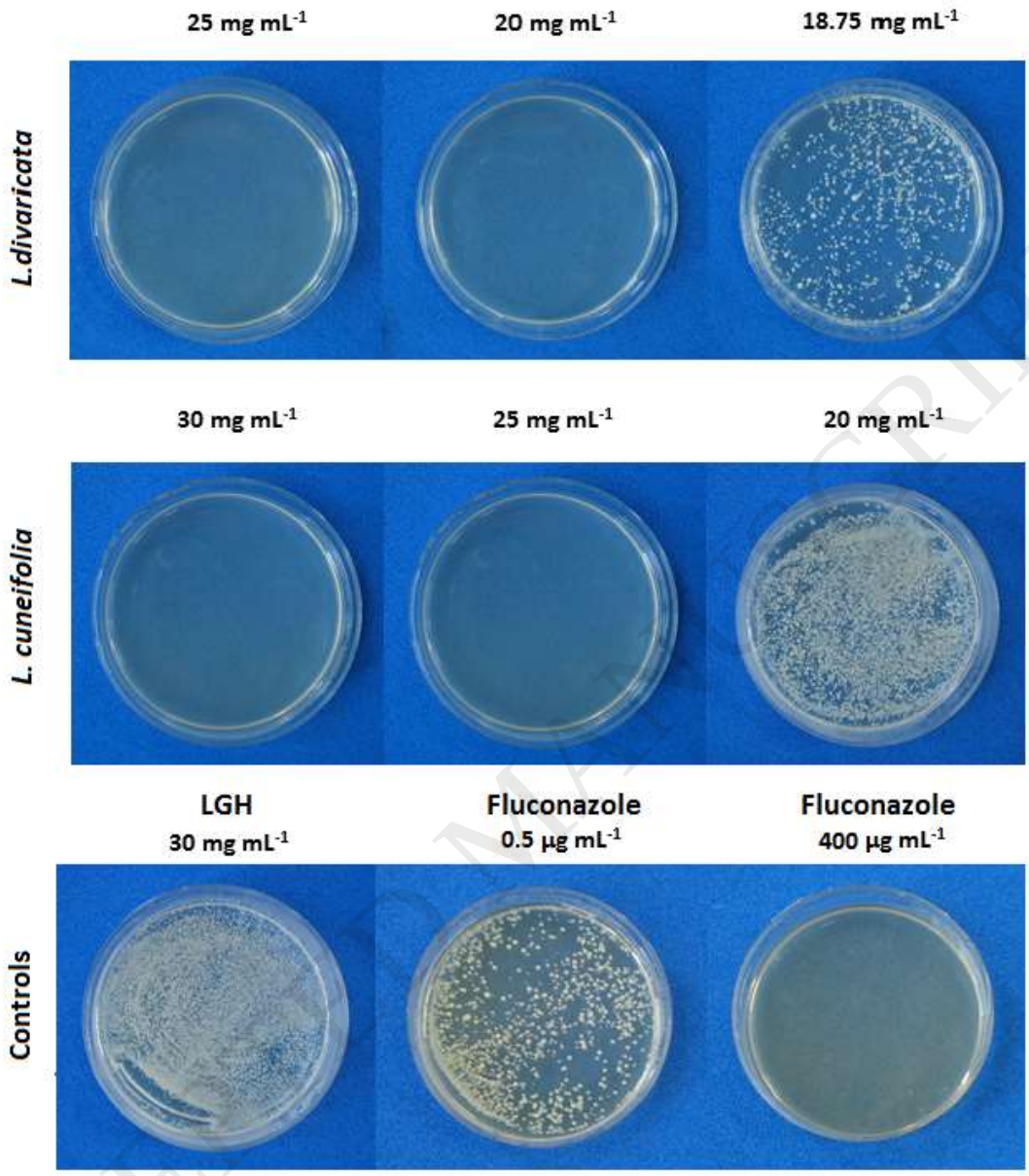

Figure 3: Fungitoxic effect of *Larrea divaricata* and *Larrea cuneifolia* LGH-extracts at different concentrations.

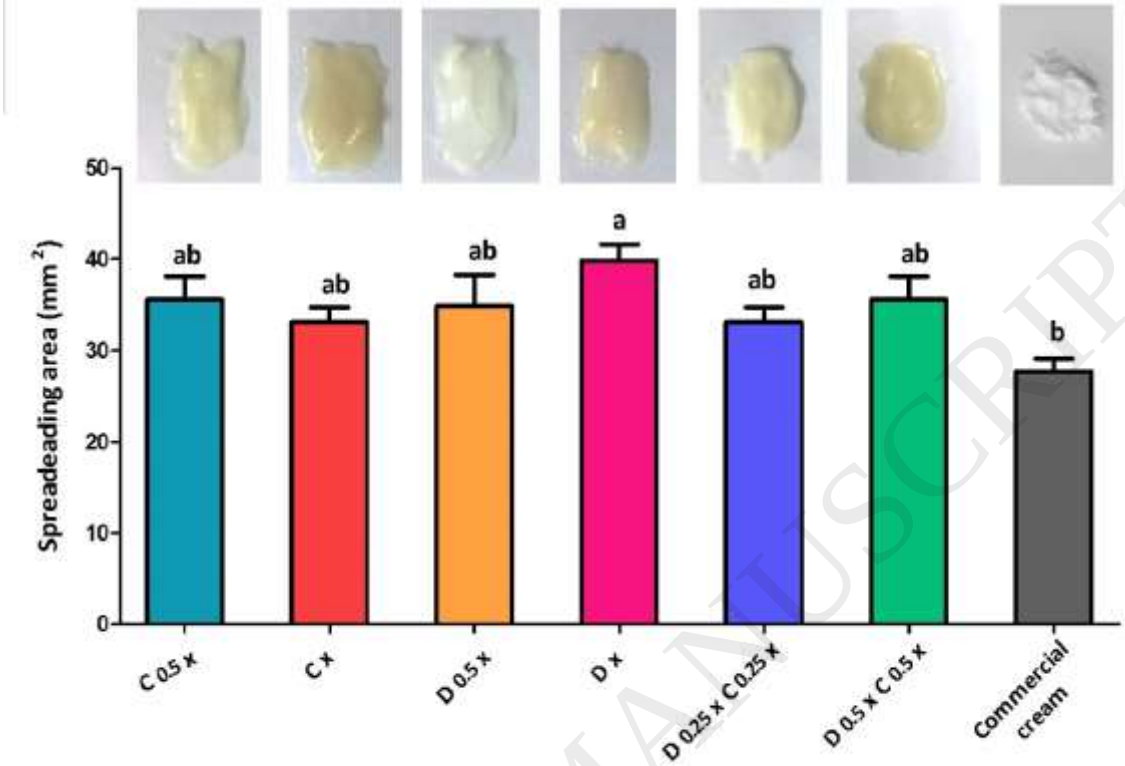

Figure 4: Spreading areas (mm^2) of topical formulations. *Larrea divaricata* (D) and *Larrea cuneifolia* (C) bioextracts at different concentrations, where x is the MIC value. Different letters represent significant differences.

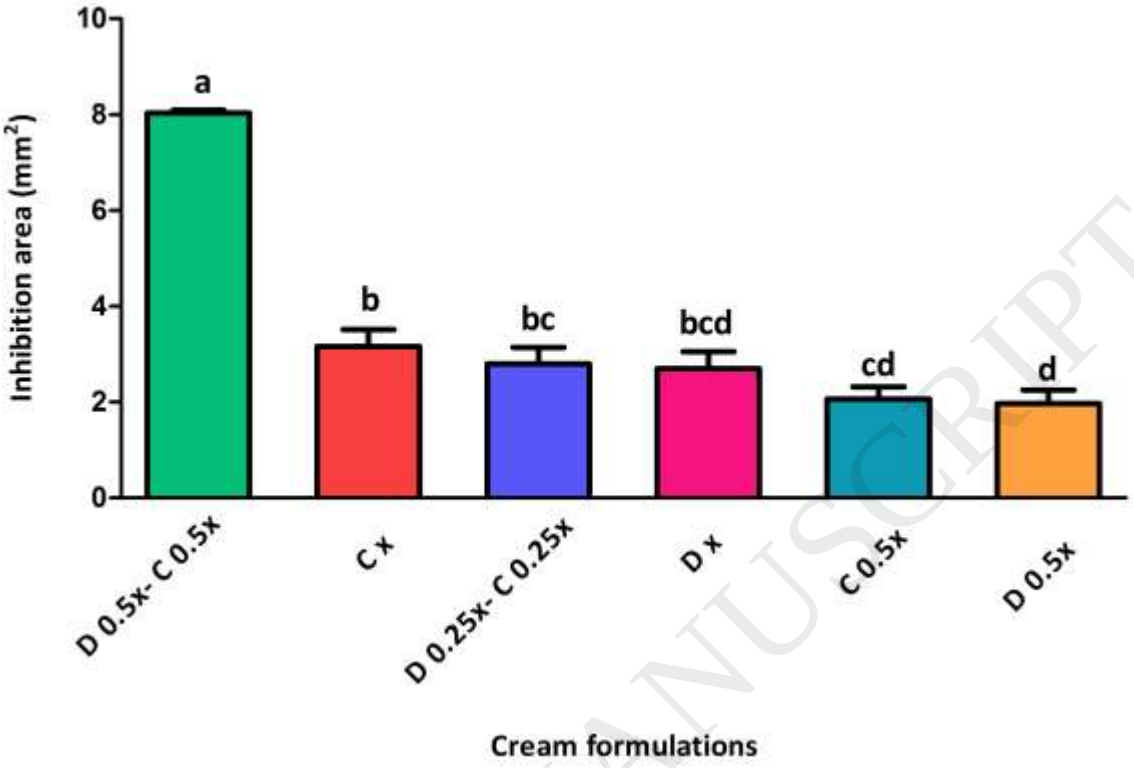

Figure 5: Antimicrobial activity of *Larrea divaricata* and *Larrea cuneifolia* cream formulations by disc diffusion method. Different letters represent significant differences.

Supplementary Figure 1: Phenolics extraction efficiency (%) of different solvents (LGH, MeOH and H_2O) from A: *L. divaricata*, B: *L. cuneifolia*, C: *T. vulgaris* y D: *O. vulgare*. Results are expressed as percentual extraction efficiency.

Supplementary Figure 2: Alkaloids extraction efficiency (%), of different solvents (LGH, MeOH and H_2O) from A: *L. divaricata*, B: *L. cuneifolia*, C: *T. vulgaris*. Results are expressed as percentual extraction efficiency.

Table 1: Concentration of bioactive compounds detected in plant extracts (μg mL^{-1}).

		Extracts[a]	
		L. cuneifolia	*L. divaricata*
Phenolics	Quercetin	170.98 ± 4.97	n.d
	Caffeic acid	7.65 ± 0.07	2.94 ± 0.26
	Ferulic acid	12.13 ± 0.07	n.d
	Rosmarinic acid	111.43 ± 0.06	n.d
	Naringenin	n.d	1.83 ± 0.04
	Nordihydroguaiaretic acid	5073.88 ± 61.61	6881.27 ± 48.22
	Tyrosol	n.d	n.d
	Apigenin	n.d	n.d
Alkaloids	Theophylline	66.00 ± 0.12	24.73 ± 0.34
	Theobromine	n.d	n.d
	Harmaline	n.d	n.d
	Piperine	33.13 ± 0.39	44.84 ± 0.46
	Caffeine	n.d	n.d

nd: non detected

[a] mean ± standard error (n = 3)