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Ultrasound-Assisted Extraction of an Extract with Dermatological and Nosocomial Activity from *Agave nuusaviorum*, a Mexican Endemic Plant

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Featured Application: *A. nuusaviorum* leaves have a potential application for the development of new antimicrobial drugs to treat onychomycosis and dermatophytosis.

Abstract: The revolutionary development of green technology, such as ultrasound-assisted extraction, has had a significant impact on the extraction methods of natural compounds with pharmacological potential. These methods are considered green because they are environmentally friendly, minimizing the use of toxic solvents, energy consumption, and CO_2 generation. Antimicrobial resistance is a global public health problem. Pathogens of dermatological and nosocomial relevance are more frequent because they are resistant to antimicrobials. *Agave nuusaviorum* leaves are used in traditional medicine to treat onychomycosis and inflammatory processes. Here, the antimicrobial activity of hydroethanolic extract of *A. nuusaviorum* leaves obtained by ultrasound-assisted extraction was evaluated. The antimicrobial activity was tested against 14 pathogens with dermatological and nosocomial relevance by the agar diffusion technique. The extract exhibited an antimicrobial effect on all microorganisms tested, showing inhibition zones of 9–16 mm for *Staphylococcus aureus*, *Candida albicans*, *Candida parapsilosis*, and eleven isolates of *Trichophyton*. The extract's minimum inhibitory concentration ranged between 0.5 mg/mL for *Staphylococcus aureus*, 0.25–1 mg/mL for yeast, and 0.4–1.25 mg/mL for molds. The phytochemical screening revealed the presence of terpenes, phenolic acids, tannins, carbohydrates, and steroids in the extract. The results showed that *A. nuusaviorum* leaves have broad-spectrum antimicrobial potential.

Keywords: green extraction method; *Trichophyton rubrum*; *Trichophyton mentagrophytes*; *Candida albicans*; *Staphylococcus aureus*; plant extract; minimum inhibitory concentration

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1. Introduction

The revolutionary development of green technology has made a significant impact on the extraction of natural compounds with use potential for the pharmaceutical industry, due to the development of green extraction methods [1] such as ultrasound extraction. Ultrasound-assisted extraction (UAE) is environmentally friendly because it avoids or reduces the use of organic solvents in the obtention of bioactive compounds, reduces processing time, intensifies heat and mass transfer transport, increases extraction yield, preserves a better extract quality, and reduces energy consumption and CO_2 generation [2–4]. Green extraction techniques, such as supercritical fluid extraction (SFE), ultrasound-assisted

extraction (UAE), and microwave-assisted extraction (MAE), among others, are efficient methods of extracting phytochemicals. This is one of the reasons why many extracts are more bioactive than those extracted by conventional methods [5–7].

Some of the main causal agents of dermatophytosis and onychomycosis are the molds *Trichophyton mentagrophytes* and *T. rubrum*, and the yeast *Candida albicans* [8–11], while the bacteria *Staphylococcus aureus* is one of the main causal agents of nosocomial infections [12–14]. The persistence of these infections is associated with the resistance acquired by microbial strains to commercially available antimicrobials. This situation has become a global public health problem. Infections associated with resistant microbial strains cause about 700,000 deaths per year in the world and, by 2050, they could reach 10 million deaths [15]. Consequently, the search for therapeutic alternatives to treat the causal agents of infectious diseases is a necessity [16,17], as well as research to identify and elucidate new molecules with antimicrobial potential [18]. Some plant species used in traditional medicine may be a source of molecules with this type of activity.

Agave nuusaviorum A. García-Mend., a species previously confused with *A. potatorum* Zucc., is a Mexican endemism that grows in the mountains of the high Mixteca of Oaxaca, where it is locally known as "papalometl", "papalomé" and "yavi-ticushi" (Mixtec). In this region, the species is used for artisanal mezcal production, food, and medicinal purposes [19,20]. An infusion of *A. nuusaviorum* leaves is consumed orally for the treatment of onychomycosis and some problems related to inflammatory processes (documented by the authors). In addition, the cooked or roasted leaves of this plant are applied in the form of a poultice to alleviate the inflammation caused by bumps and falls [19–21].

Although *A. nuusaviorum* is a plant used in traditional medicine, the species lacks phytochemical or biological studies that validate its medicinal use. Therefore, it constitutes a valuable and promising natural resource to obtain novel and effective secondary metabolites for the treatment of pathogenic microorganisms that affect humans. Due to the use of *A. nuusaviorum* in the traditional medicine for diseases of fungal and bacterial etiology, evaluating its antimicrobial potential will be interesting. This work aimed to investigate the antimicrobial activity of the hydroethanolic extract obtained by UAE from leaves of *A. nuusaviorum* against pathogenic species with worldwide clinical relevance for humans, such as *Staphylococcus aureus*, and species of *Candida* and *Trichophyton*.

2. Materials and Methods

2.1. Plant Material

Agave nuusaviorum leaves were collected in the municipality of San Pedro and San Pablo Teposcolula, Oaxaca, Mexico, in April 2013, with the permission of the municipal authorities. For determination of the species, García-Mendoza (2010)'s method was followed [19]. A voucher specimen was prepared and deposited in OAX herbarium, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Oaxaca, Instituto Politécnico Nacional.

2.2. Plant Extract

The leaves were oven-dried (Riossa Model. H-48) at 50 °C for six days. The dried material was then ground and sifted to a particle size of 0.25 mm, following the method described by Ghafoor et al. (2009) with some modifications [22]. A sample of 1 g of dried leaves was kept in a glass flask with 50 mL of 70% ethanol solution. UAE was performed in an ultrasound bath (Cole-Parmer, Vernon Hills, IL, USA, Mod. 08890-21), and parameters were fixed at 42 kHz, 70 W, 120 min, and 28 °C. Temperature and time of extraction were controlled from the ultrasound bath panel. After the extraction process, the sample was filtered with Whatman No. 2 filter paper. The supernatant was concentrated in a rotary evaporator (Büchi, Flavil, Switzerland, Mod. R-210) under reduced pressure at 50 °C. The extract was stored at 4 °C until use. Preliminary extraction trials were carried out with water, 50% ethanol and different extraction times and solvent/sample ratios (Supplementary Material Table S1).

2.3. Microorganisms

Staphylococcus aureus ATCC 6538, *Candida albicans* ATCC 10231, *Candida parapsilosis* CFQ-L-08, *Trichophyton mentagrophytes* CFQ-H-92, and *T. rubrum* CFQ-H-93 were provided by the Faculty of Chemistry of the Universidad Nacional Autonoma de Mexico (UNAM). In addition, nine *Trichophyton* strains were isolated from patients with onychomycosis (*T. rubrum* FM309, *T. mentagrophytes* FM796, *T. rubrum* FM943, *T. rubrum* FM941, *T. rubrum* FM932, *T. rubrum* FM774, *T. rubrum* FM626, *T. rubrum* FM794, and *T. rubrum* FM934) and were provided by the Faculty of Medicine of UNAM. The microorganisms' determination was confirmed by biochemical tests [23]. *Staphylococcus aureus* was maintained on Mueller–Hinton agar (MHA) and fungal cultures on potato–dextrose agar (PDA, Difco). *Trichophyton* antifungal assays are performed with mycelium or spores; spores were used for this study, so *Trichophyton* isolates were grown on PDA for seven days until sporulation. Spores were washed with 3 mL of sterile saline (0.8%), the plate was shaken to release the spores, and filtered with Sartorius 1238 filter paper. Spore counts were obtained using a Neubauer chamber, and 2×10^4 spores/mL were used for the assays.

2.4. Antimicrobial Assay

The analysis of antimicrobial activity was determined by the agar disk diffusion method for bacteria [24] and yeast [25]. Yeast and bacterial suspensions (1×10^4 CFU/mL) were inoculated on MHA, while fungal spore suspensions (2×10^4 spores/mL) were inoculated on PDA. Filter paper discs (6 mm) were dipped in the plant extract solution (5 mg/mL), and then the discs were placed on agar plates and incubated at 30 °C for 24 h for *S. aureus*, *C. albicans*, and *C. parapsilosis*, while for molds the incubation time was 5 days. Roswell Park Memorial Institute (RPMI) 1640 synthetic culture medium was used as a negative control, while gentamicin (16 g/mL) and amphotericin B (64 g/mL) were used as positive controls for bacteria and fungi, respectively. Antimicrobial activity was evaluated as the absence of microbial growth around the disc impregnated with plant extract.

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC)

The minimum inhibitory concentration (MIC) of the extract was performed by the broth dilution method for bacteria, yeasts, and molds using methods M07-A9, M27-A3, and M38-A2, respectively [26–28].

MIC was performed by successive dilutions of the extract from an initial concentration of 1.25 mg/mL in RPMI 1640 culture medium, with glutamine and without bicarbonate, buffered to pH 7.0 with MOPS (3-[N-morpholino]propanesulfonic acid) for fungi, and Mueller–Hinton broth for bacteria. An aliquot of 100 µL of microbial suspension (1×10^4 CFU/mL) or fungal spores (2×10^4 spores/mL) and 100 µL of the extract was added to each well of a 96-well microtiter plate. Serial dilutions of the extract were prepared in 96-well plates, starting from a stock solution at 1.25 mg/mL. The microplates were incubated according to the microorganism: yeasts (24 h at 30 °C), *S. aureus* (18 h at 30 °C), and molds (7 days at 27 °C). After the incubation period, turbidity was measured (λ = 600 nm) in a microplate reader (BioTek, PowerWave XS2). RPMI 1640 culture medium was used as a negative control. The MIC was defined as the lowest concentration of extract that inhibited the growth of microorganisms by the broth dilution method. The minimum microbicidal concentration (MMC) was performed by adding 50 µL of the mixture corresponding to the tested MIC (plant extract + microorganism) on agar plates and incubated under the conditions indicated above for each microorganism. The MMC was defined as the lowest concentration that inhibited the growth of microorganisms on agar plates. Three replicates of all assays were performed for each microorganism.

2.6. Phytochemical Screening

The leaf extract of *A. nuusaviorum* was evaluated by qualitative phytochemical reactions for secondary metabolites present in plants. This test was performed for saponins,

phenolic acids, tannins, carbohydrates, and terpenes/steroids. The color intensity and/or precipitate formation was considered as a positive response to the tests.

Test for phenols and tannins. Two milliliters of 5% ferric chloride solution was added to 1 mL of *A. nuusaviorum* leaf extract. A black or blue-green color indicated the presence of tannins and phenols [29].

Test for Terpenes/Steroids. In a thin layer of chromatography with plant extract, the vanillin-orthophosphoric acid reagent was sprayed. A colorimetric reaction indicated the presence of terpene-type compounds [29].

Test for carbohydrates. A 6% orcinol-sulphuric acid solution was sprayed onto the chromatographic plates containing the plant extract, and then heated to 110 °C for 10 min. The presence of color was considered a positive reaction [30].

Test for saponins. A total of 0.1 g of the extract was added to 10 mL of distilled water and subsequently boiled for 10 min and leaked. Two milliliters of distilled water was added to the filtrated solution and shaken vigorously. The permanence of foam was considered as a positive sample [31].

2.7. Data Analysis

Microbial growth was determined by turbidity at 600 nm. Areas under the curves (AUCs) were calculated using GraphPad Prism v. 5 software (GraphPad Software Inc., La Jolla, CA, USA). The inhibitory effect on microbial growth was obtained by comparing the AUCs of each treated microorganism with the negative control, considered as maximum microbial growth. The data were analyzed by a one-way ANOVA using the Dunnett's multiple comparison test.

3. Results

3.1. Antimicrobial Assay

The extract of *A. nuusaviorum* showed an antimicrobial effect against all the microorganisms tested. The extract produced inhibition halos between 9 and 16 mm in ten (70%) of the fourteen isolates studied (Table 1).

3.2. Minimum Inhibitory Concentration and Minimum Microbicidal Concentration

The extract showed a MIC of 0.5 mg/mL against *S. aureus*; for fungal microorganisms it was 0.25–1.25 mg/mL, while for all the microorganisms evaluated, the MMC was observed in the range of 0.4 to 1.25 mg/mL (Table 1). The growth curves showed that the microbial strains significantly reduced absorbance with extract; all assays showed an extract-concentration-dependent decrease in absorbance. The anti-*Staphylococcus aureus* effect of extracts is shown in Figure 1. Interestingly, the extract had a lower MIC value against *C. parapsilosis* than in *C. albicans* (Figure 1). A significant antifungal effect was observed in the reduction in absorbance for *Trichophyton* isolates (Figure 1). The results showed that the extract exhibited antimicrobial potential against the microorganism tested. The AUCs for microorganisms tested were significantly lower compared to control growth (100%) and showed decreases of 75% for *S. aureus*, 96% for *C. parapsilosis*, 40.2% for *C. albicans*, and about 50% for the molds, except for the FM796 and CFQ-H-92 whose inhibition was 82% and 66%, respectively (Figure 1B(a,b)).

Table 1. Antimicrobial activity of *Agave nuusaviorum* leaf extract. Agar diffusion method (ADM), minimum inhibitory concentration (MIC), and minimum microbicidal concentration (MMC).

Microorganism	ADM	MIC (mg/mL)	MMC (mg/mL)
Bacterium			
Staphylococcus aureus	++	0.5	1
Yeast			
Candida albicans	++	1	1
Candida parapsilosis	+	0.25	1
Molds			
Trichophyton mentagrophytes CFQ-H-92	+++	0.75	1
Trichophyton rubrum CFQ-H-93	+++	1	1
Trichophyton rubrum FM309	++	0.75	0.75
Trichophyton rubrum FM626	++	0.75	0.75
Trichophyton rubrum FM774	+++	0.4	0.4
Trichophyton rubrum FM794	+	1	1
Trichophyton mentagrophytes FM796	+++	1	1
Trichophyton rubrum FM932	+++	1.25	1.25
Trichophyton rubrum FM934	++	0.5	1
Trichophyton rubrum FM941	++	1	1.25
Trichophyton rubrum FM943	++	1	1

+: 6 to 9 mm inhibition halo; ++: >9 to 13 mm inhibition halo; +++: >13 to 16 mm inhibition halo.

3.3. Phytochemical Screening

Phytochemical analysis of *A. nuusaviorum* extract showed a strong positive test for carbohydrates, terpenes, and steroids, while the saponins were absent (Table 2).

Table 2. Phytochemical screening of *Agave nuusaviorum* leaf extract.

Compounds	Extract
Saponins	—
Phenolic acids	+
Tannins	+
Carbohydrates	++
Terpenes	++
Steroids	++

++: strong positive test, +: weak positive test, —: negative test

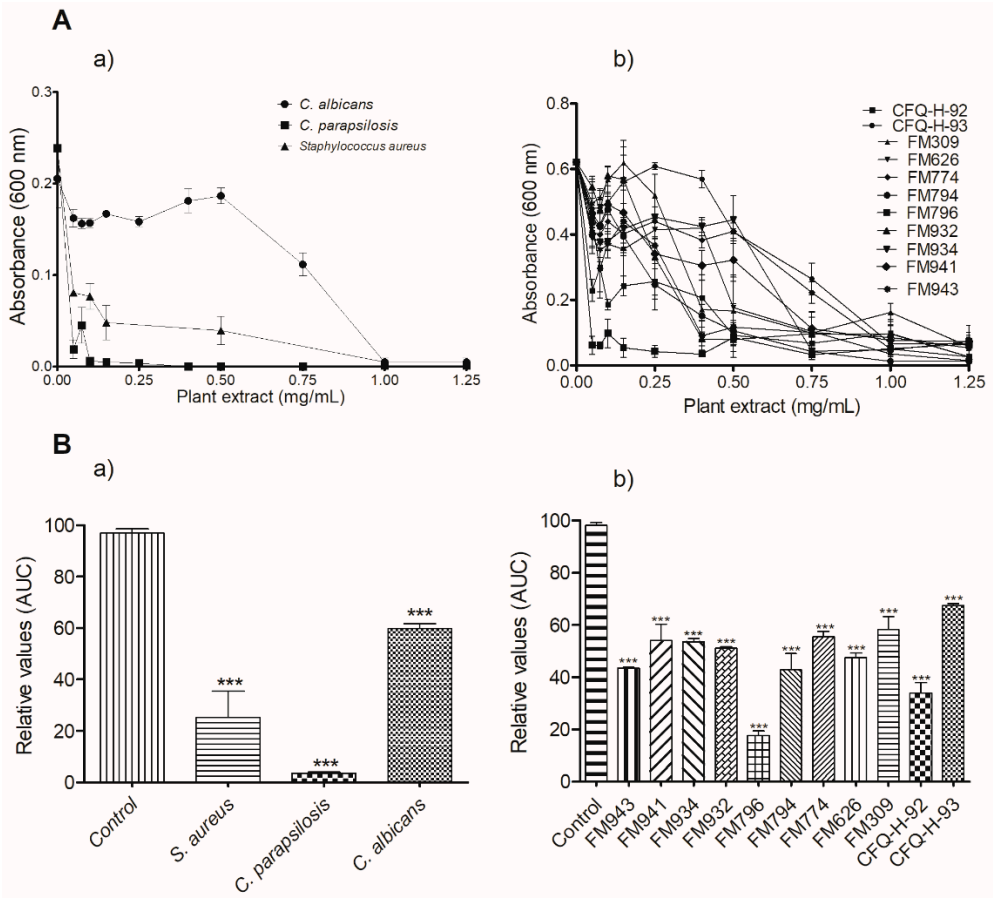

Figure 1. Antimicrobial effect of *Agave nuusaviorum* leaf extract. (**A**) Turbidity curves of bacterium and yeasts (**a**) and mold microorganisms (**b**). Values represent means ± SE for three replicates. (**B**) Growth percentages were obtained from a comparison of the areas under the curves (AUC) of microorganisms compared to the control (AUC established as 100%). AUC of bacterium and yeasts (**a**) and mold microorganism (**b**). Significant differences *** $p < 0.001$, in comparison with the control group, were detected by one-way analysis of variance (ANOVA)-Dunnett´s multiple comparison test.

4. Discussion

The extract of *A. nuusaviorum* obtained by UAE was active against *Staphylococcus aureus*, yeasts, and molds tested. It suggests that *A. nuusaviorum* leaf extract has a broad-spectrum antimicrobial effect. The green extraction methods employed here, such as ultrasound extraction, minimize the use of solvents and energy [1–3]. We chose ethanol as a solvent because it is one of the most environmentally friendly and biodegradable compared to other organic solvents [32]. Chemat et al. (2017) compared the energy consumption and CO_2 generation of three extraction methods: UAE requires the lowest energy consumption (0.25 kW·h vs. 6.0 kW·h for maceration, and 8.0 kW·h for Soxhlet) and generates the lowest amount of CO_2 per 100 g of solid extract (200 g vs. 3600 g for maceration, and 6400 g for Soxhlet). So, the UAE method represents an eco-friendly alternative for obtaining bioactive plant extracts. Also revealed is the fact that the UAE process may involve single or combined mechanisms. The possible mechanisms are fragmentation, erosion, capillarity, detexturation, and sonoporation leading to cell disruption and increased mass transfer [4]. Additionally, ultrasonic waves improve concentration and synthesis processes using so-called sonochemical methods [33–35].

The extract presented MIC values ≤1.25 mg/mL for all the microorganisms tested. These data suggest that the extract contains promising antimicrobial molecules according to the criteria established by Ríos and Recio (2005) [36], and is important for further identification or isolation of bioactive molecules. Remarkably, the antibacterial activity

of *A. nuusaviorum* against *S. aureus* (MIC 0.5 mg/mL) was higher than those previously reported for *Agave* species, e.g., *A. picta* and *A. sisalana* showed MIC values for *S. aureus* of 7.0 mg/mL and 10 mg/mL, respectively [37,38]. Likewise, the antifungal activity against *Candida* species (MIC 0.25–1 mg/mL) was higher than those reported in the literature, e.g., *A. sisalana* exhibited MIC values > 10 mg/mL [38,39]. In addition, the antifungal activity against *Trichophyton* species (0.4–1 mg/mL) was higher than the data reported in the literature in other *Agave* species that showed MIC values > 3.5 mg/mL [37]. Interestingly, *C. parapsilosis* was more sensitive to the extract evaluated here than *C. albicans*, which suggests that the extracts tested could be a more effective therapeutic alternative for species other than *C. albicans*. A similar effect was observed on *T. mentagrophytes* (CFQ-H-92) and *T. rubrum* (CFQ-H-93). Furthermore, the extract inhibited the growth of all clinical isolates of *Trichophyton* (Figure 1B(a,b)). However, a positive correlation between in vitro and in vivo studies is not always observed [40], so it is important to continue in vivo evaluations of *A. nuusaviorum* extract to validate in vitro findings.

A possible explanation for the higher antimicrobial activity observed by the extract of *A. nuusaviorum* obtained by UAE may be that these bioactive metabolites are found in higher amounts in the evaluated extract due to the green extraction method used. UAE generates a higher heat and mass transfer, increases the extraction yield, and improves the quality of the extract [2–4]. In addition, UAE has been reported to increase the bioactivity of plant extracts due to a higher extraction of phenolic compounds, mainly flavonoids, which enhance the bioactivity of these extracts against different microorganisms [41].

Phytochemical screening indicates that the extract contains carbohydrates, phenolic acids, steroids, tannins, and terpenes. The data show that carbohydrates, steroids, and terpenes are the most abundant in the extract studied (Table 2). These compounds are biologically active and they are present in other *Agave* species [42]. Terpene-type compounds have been reported as potent inhibitors for *S. aureus* [43–45] and *Candida* species [46]. Other authors report antibacterial [47] and antifungal [48] effects for phenolic compounds by different mechanisms; for example, the tannin compounds react with proline-rich protein to form irreversible complexes, resulting in the inhibition of cellular protein synthesis. These types of compounds may be present in the extracts evaluated here, so they could be responsible for the observed antimicrobial effect.

In addition, this study reveals an added value for the leaves of *A. nuusaviorum*, since its leaves are an agro-industrial waste in the artisanal production of mezcal, so this plant material could be used for ethnopharmacological purposes. Finally, this research could be continued with the chemical characterization of the metabolites present in the extracts to generate a broad-spectrum antimicrobial herbal preparation from the leaves of *A. nuusaviorum* and evaluation of the antimicrobial activity of the extract in a murine model to validate in vitro results.

5. Conclusions

The extract obtained by a green method, UAE, from *Agave nuusaviorum* leaves, has promising antimicrobial activity against pathogens of dermatological and nosocomial relevance. This finding validates the use of *A. nuusaviorum* in traditional medicine for the treatment of onychomycosis. It should be noted that the antimicrobial activity of *A. nuusaviorum* was higher than previously reported for other *Agave* species. The results suggest that *A. nuusaviorum* leaves are an important source for the development of new antimicrobial drugs to treat onychomycosis and dermatophytosis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122010446/s1, Table S1. Ultrasound-assisted extraction conditions of *A. nuusaviorum* leaf extract.

Author Contributions: L.L.-R., P.C.-R. and R.H.-G. jointly conducted the research, compiled data, and conducted the laboratory analysis. E.G.-S. performed the data analysis, statistical analysis and wrote the paper; L.L.-R. and R.S.-G. contributed to the draft and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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