Comparative Phytochemical Profiling and Evaluation of the Antibacterial and Antifungal Efficacy of *Stevia rebaudiana* Bertoni and *Marrubium Vulgare* L. Hydroethanolic Extracts

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Abstract. This study evaluates and compares the phytochemical profiles and antimicrobial efficacy of *Marrubium vulgare* L. and *Stevia rebaudiana* Bertoni hydroethanolic extracts. Using HPLC-ESI-MS/MS, we identified including marrubiin, verbascoside, and steviol glycosides. We tested these extracts against four bacterial strains and *Candida albicans*. Gram-positive strains exhibited significant susceptibility to *M. vulgare* (MIC 0.78–1.56 mg/mL). In contrast, *S. rebaudiana* showed superior antifungal activity against *C. albicans* (MIC 1.56 mg/mL). These distinct profiles highlight the specific potential of *M. vulgare* as an antibacterial and *S. rebaudiana* as an antifungal agent in natural medicine.

1 Introduction

The rise of multidrug-resistant (MDR) pathogens, specifically MRSA and resistant Candida species, poses a severe threat to global health [1]. As conventional treatments lose efficacy, medicinal plants offer a crucial reservoir of bioactive metabolites capable of bypassing resistance mechanisms [2].

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In Morocco, *Marrubium vulgare* L. is a staple of traditional medicine, widely used to treat respiratory and digestive ailments [3]. Its biological activity is largely attributed to diterpenes, particularly marrubiin [4]. Conversely, *Stevia rebaudiana* Bertoni, although native to South America, has been successfully acclimatized in North Africa [5]. While primarily valorized for its sweetening properties, Stevia leaves contain a rich matrix of polyphenols and terpenoids with overlooked antimicrobial potential [6].

This work characterizes the inhibitory effects of hydroethanolic extracts against selected bacterial and fungal strains from Moroccan-grown *M. vulgare* and *S. rebaudiana* using a diverse set of microbial isolates, including fungi and bacteria of differing Gram status. Likewise, we characterize their chemical profiles using HPLC-ESI-MS/MS to associate specific phytochemical markers with the observed antimicrobial activity.

2 Materials and Methods

2.1 Plant Material and Preparation

Field sampling of *M. vulgare L.* aerial biomass was conducted within the Fez-Meknes region during the flowering stage, while *Stevia rebaudiana* Bertoni leaves were sourced from the botanical grounds of the Faculty of Science and Techniques (FST), Fez. Voucher specimens were deposited at the FST Fez Herbarium under accession numbers FSTH-MV-0125/2023 and FSTH-SR-0089/2023. Processing involved cleaning the material with distilled water prior to a 14-day drying period away from direct sunlight (25 ± 2°C). Subsequently, the samples were milled to a fine consistency and refrigerated at 4°C.

2.2 Bacterial and Fungal Strains

We obtained the microbial panel from the University Hospital Center (CHU) of Fez. This included Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853), and the fungal pathogen *Candida albicans* (ATCC 10231). Prior to assays, strains were maintained on nutrient agar at 4°C and activated in Mueller-Hinton Broth (bacteria) or Sabouraud Dextrose Broth (fungi) at 37°C for 24 hours.

2.3 Preparation of Hydroethanolic Extracts

We macerated 50 g of powdered leaves in 500 mL of a hydroethanolic solution (v/v 70:30). The suspension was kept under orbital shaking (150 rpm) for 48 hours at ambient temperature, protected from light. Post-filtration (Whatman No. 1), we removed the ethanol in vacuo at 40°C utilizing a rotary evaporator (Heidolph, Germany). We subjected the aqueous residue to lyophilization to produce the crude extract, which was subsequently weighed to determine yield and stored at -20°C.

2.4 Phytochemical Profiling by HPLC-ESI-MS/MS

We profiled the extracts using a High-Performance Liquid Chromatography system coupled with a Triple Quadrupole Mass Spectrometer. Separation occurred on a C18 reverse-phase column (250 × 4.6 mm, 5 µm) using a mobile phase of 0.1% aqueous formic acid (A) and acetonitrile (B). The gradient increased from 5% B (0–5 min) to 100% B (5–30 min), held until 35 min, at a flow rate of 0.8 mL/min (10 µL injection). Mass spectral data were collected using dual-polarity electrospray ionization. (capillary voltage: 3.5 kV; source temp: 350°C).

Bioactive compounds were tentatively identified (MSI Level 2) by cross-referencing precursor ions ($[M\text{-}H]^-$ or $[M\text{+}H]^+$) and fragmentation patterns with MassBank, GNPS databases, and literature data.

2.5 Antimicrobial Activity Assays

2.5.1 Agar Well Diffusion Method

We screened for antibacterial and antifungal activity via the agar well diffusion technique. Microbial suspensions were adjusted to 0.5 McFarland (1.5 × 108 CFU/mL) and swabbed onto Mueller-Hinton Agar (bacteria) or Sabouraud Dextrose Agar (C. albicans). We dissolved the crude extracts in 10% DMSO (v/v) to 50 mg/mL and introduced 50 µL into 6 mm wells punched into the agar. Gentamicin (10 µg/disc) and Fluconazole (25 µg/disc) served as positive controls, with 10% DMSO as the vehicle control. Following a 24-h incubation period at 37°C, we measured the inhibition zone diameters (mm) in triplicate.

2.5.2 Quantifying the Inhibitory Thresholds (MIC)

We calculated the MIC using broth microdilution in 96-well plates, following CLSI protocols. Extracts were serially diluted (two-fold) in Mueller-Hinton Broth to achieve concentrations ranging from 0.048 to 25 mg/mL. Each well received 10 µL of microbial suspension (5 ×105 CFU/mL) and 20 µL of resazurin sodium salt (0.015%) as a metabolic indicator. Following incubation at 37°C for 24 hours, we recorded the MIC as the lowest concentration that prevents the color shift from blue (oxidized) to pink (reduced).

2.6 Statistical Analysis

We performed all assays in triplicate (n=3) and presented quantitative data as mean ± (SD). After confirming data normality via the Shapiro-Wilk test, we analyzed group differences employing one-way ANOVA followed by Tukey’s HSD test. All statistical computations were executed in GraphPad Prism (v9.0), with significance thresholds set at $p < 0.05$.

3 Results and Discussions

3.1 Chemical Composition

Figure 1 shows the effectiveness of the 70% hydroethanolic maceration. We attained yields of 14.2% (w/w) for *M. vulgare* and 11.5% (w/w) for *S. rebaudiana* leaves.

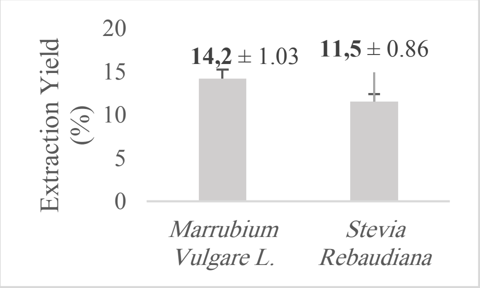

Fig. 1. Extraction yields of the hydroethanolic maceration of *M. Vulgare* and *S. rebaudiana* leaves

These recovery rates align with or exceed standard ranges (8–12%) reported for Mediterranean Lamiaceae and Asteraceae species, which confirms the protocol's efficacy [7]. We selected 70% ethanol to optimize the co-extraction of diverse metabolites, ranging from polar glycosides to lipophilic diterpenes. This combination outperforms pure solvents, it maximizes bioactive recovery as demonstrated in *Mentha* and halophyte matrices [8]. It also yields higher phenolic concentration and antimicrobial power compared to aqueous extraction [9], which enables the simultaneous recovery of sugars and wax esters without complex fractionation [8, 10]. Furthermore, the approach aligns with green extraction principles by reducing solvent usage [11].

3.2 Phytochemical Profiling by HPLC-ESI-MS/MS

Figure 2 below reveals the Total Ion Chromatograms (TICs).

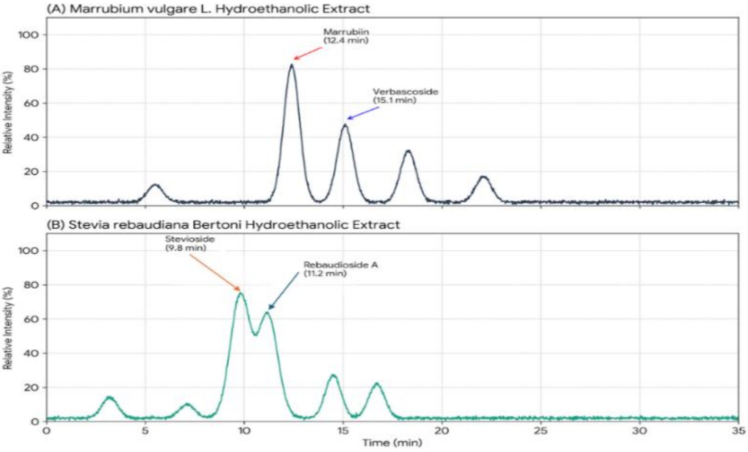

Fig. 2. Total Ion Chromatograms of *M. vulgare* (A) and *S. rebaudiana* (B) hydroethanolic extracts.

Chromatographic analysis resolved distinct peaks. Mass spectral fragmentation patterns with database comparisons allowed us to tentatively identify major constituents listed in Table 1.

Table 1. Major bioactive constituents identified in *M. vulgare* and *S. rebaudiana* hydroethanolic extracts via HPLC-ESI-MS/MS.

Plant Species	Rt (min)	Compound Name	Mode	Precursor Ion (m/z)	Fragment Ions (m/z)
M. Vulgare	12.4	Marrubiin	Pos.	333.2 [M+H]+	315, 297, 135
	15.1	Verbascoside	Neg.	623.5 [M-H]-	461, 161
	18.3	Apigenin-7-O-glucoside	Neg.	431.1 [M-H]-	269, 117
S. rebaudiana	9.8	Stevioside	Neg.	803.3 [M-H]-	641, 317
	11.2	Rebaudioside A	Neg.	965.4 [M-H]-	803, 641
	14.5	Chlorogenic acid	Neg.	353.1 [M-H]-	191, 179

	16.7	Quercetin-3-O-rhamnoside	Neg.	447.1 [M-H]-	301, 151

The analysis emphasized distinct metabolic signatures. *M. vulgare* extracts were dominated by diterpenes (marrubiin) and phenylpropanoids (verbascoside), while *S. rebaudiana* extracts showed high concentrations of steviol glycosides and phenolic acids. Particularly, the Moroccan *M. vulgare* profile is anchored by marrubiin, a furanoid diterpenoid lactone acting as the genus's chemotaxonomic marker [12]. Detected alongside flavonoids like apigenin [13]. this diterpene is synthesized via the MvCPS1 and MvELS pathway and subsequent CYP71AU87 modification [14]. While central to the plant's pharmacology, its P450-mediated activation involves a complex bioactivity profile including potential hepatotoxicity [15]. Remarkably, although polyphenol content varies across Moroccan regions, marrubiin stability keeps its status as a reliable bioactive index [16]. In addition, the significant detection of verbascoside suggests potential synergistic antimicrobial mechanisms [17]. In *S. rebaudiana* cultivated in Fez, the abundance of stevioside and rebaudioside A signals successful acclimatization that mirrors the native South American phenotypes profile. These glycosides drive the plant's intense sweetness, with rebaudioside A offering superior potency [18]. The high concentration of these markers highlights the suitability of local pedoclimatic conditions [19]. a stability confirmed by HPLC and near-infrared quantification studies [20]. Furthermore, enhancing biotic factors, such as introducing arbuscular mycorrhizal fungi, could further expand these yields [21].

3.3 Antimicrobial Activity

We measured preliminary antimicrobial efficacy via the agar well diffusion method. Table 2 details the resulting mean inhibition zone diameters.

Table 2. Antimicrobial efficacy valued via halo diameters (mm) at an extract dosage of 50 mg/mL

Microorganism	***M. Vulgare***	***S. rebaudiana***	**Positive Control**
Gram-Positive Bacteria			Gentamicin (10 µg)
S. aureus (ATCC 25923)	18.5 ± 0.5b	10.2 ± 0.3c	24.0 ± 0.2a
B. subtilis (ATCC 6633)	20.1 ± 0.8b	11.5 ± 0.4c	26.5 ± 0.5a
Gram-Negative Bacteria			Gentamicin (10 µg)
E. coli (ATCC 25922)	12.4 ± 0.6b	09.0 ± 0.2c	22.0 ± 0.4a
P. aeruginosa (ATCC 27853)	08.5 ± 0.3b	NA	19.5 ± 0.3a
Fungi			Fluconazole (25 µg)
C. albicans (ATCC 10231)	11.2 ± 0.4c	16.8 ± 0.6b	21.0 ± 0.5a

Figure 3 provides visual validation of these quantitative results.

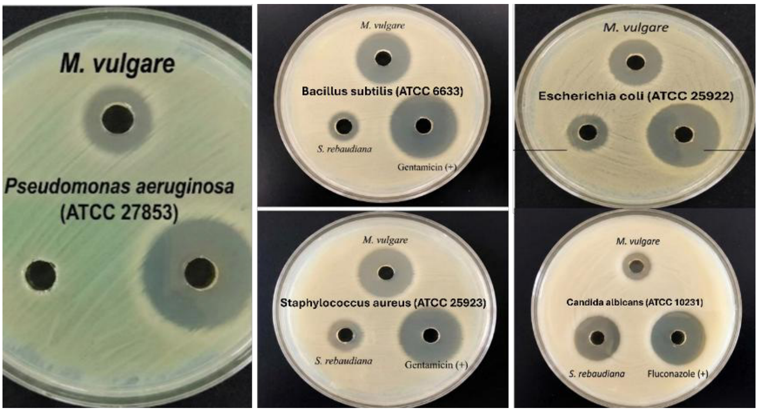

Fig. 3. Composite inhibition profiles.

Top wells (*M. vulgare*) show major efficacy against Gram-positive targets, notably *B. subtilis* (20.1 mm) and *S. aureus* (18.5 mm), surpassing the *S. rebaudiana* extracts (bottom-left). While *M. vulgare* retained moderate activity against Gram-negative *E. coli* (12.4 mm) and *P. aeruginosa* (8.5 mm), the latter resisted *S. rebaudiana* entirely. In contrast, *S. rebaudiana* proved superior against *C. albicans* (16.8 mm). Gentamicin and Fluconazole (bottom-right) confirm assay validity.

To provide a clearer graphical comparison of these distinct antimicrobial profiles, the inhibition zone data are represented in Figure 4.

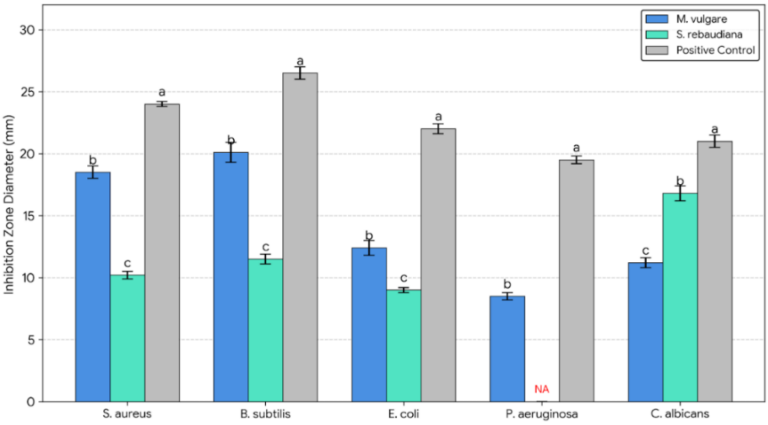

Fig. 4. Inhibition efficacy of *M. vulgare* and *S. rebaudiana* extracts (50 mg/mL). Bars show mean zone diameters ± SD (n=3). Distinct letters indicate significant differences among treatments ($p < 0.05$). NA: No activity. Controls: Gentamicin (bacteria), Fluconazole (fungi).

The inhibition profiles in Fig. 3 validate M. vulgare as a potent anti-Gram-positive agent, with efficacy approaching the antibiotic control. In contrast, S. rebaudiana showed specific antifungal utility, significantly surpassing M. vulgare in suppressing C. albicans growth.

3.3.1 Antibacterial Potential and Selectivity

Our tests confirm a distinct Gram-positive selectivity for *M. vulgare*. The susceptibility of *S. aureus* and *B. subtilis* likely arises from their cell wall architecture; the absence of an outer membrane enables lipophilic diterpenes, such as marrubiin, to penetrate the peptidoglycan mesh and destabilize the cytoplasmic membrane [22]. Conversely, Gram-negative resilience stems from the lipopolysaccharide (LPS) envelope, which creates a hydrophilic blockade against non-polar phytochemicals [23]. *P. aeruginosa* exemplifies this recalcitrance, leveraging both the LPS barrier and efflux systems. Literature confirms that compromising this LPS integrity reverses resistance, which features its critical protective function [24].

We measured inhibitory thresholds by broth microdilution. MIC values are shown in Table 3

Table 3. MIC values of the extracts in mg/mL against the tested pathogens.

Microorganism	***M. Vulgare* (mg/ml)**	***S. Rebaudiana* (mg/ml)**
Gram-Positive		
S. aureus (ATCC 25923)	0.78	6.25
B. subtilis (ATCC 6633)	0.78	3.12
Gram-Negative		
E. coli (ATCC 25922)	3.12	12.5
P. aeruginosa (ATCC 27853)	12.5	>25
Fungi		
C. albicans (ATCC 10231)	6.25	1.56

Quantitative metrics aligned with diffusion patterns. *M. vulgare* registered a notable MIC of 0.78 mg/mL against Gram-positive targets. Conversely, *S. rebaudiana* demonstrated selective antifungal utility at 1.56 mg/mL. Crucially, the *M. vulgare* result satisfies the stringent bioactivity threshold (<1 mg/mL) proposed by Berdgaleeva et al., a standard defining significant plant-derived antimicrobials efficient against various phenotypes [25].

3.3.2 Antifungal Properties of Stevia rebaudiana

The superior activity of *S. rebaudiana* against *C. albicans* (MIC = 1.56 mg/mL) highlights an underappreciated therapeutic dimension beyond its established caloric value [26]. Mechanistically, ent-kaurene glycosides likely disrupt ergosterol biosynthesis or cell wall integrity, paralleling azole kinetics [27]. This membrane-targeting potential aligns with prior observations of suppressed mycelial growth in lipophilic fractions [28]. Furthermore, constituent phenolics may act synergistically to induce oxidative stress, thereby inhibiting the hyphal transition essential for *Candida* virulence [29].

3.3.3 Comparative Therapeutic Value

The divergent profiles involve complementary therapeutic niches. *M. vulgare*'s anti-Gram-positive potency validates its ethnomedicinal application in respiratory and cutaneous staphylococcal infections. On the other hand, *S. rebaudiana* extends its utility beyond caloric reduction, emerging as a viable candidate for functional formulations targeting candidiasis.

4 Conclusion

We characterized the phytochemical and antimicrobial divergence between Moroccan *M. vulgare* and *S. rebaudiana* hydroethanolic extracts. HPLC-ESI-MS/MS analysis confirmed distinct metabolic fingerprints, anchoring *M. vulgare*'s profile in marrubiin and verbascoside, while validating the dominance of stevioside and rebaudioside A in *S. rebaudiana*. Biological assays emphasized a clear functional separation: *M. vulgare* proved highly effective against Gram-positive targets (MIC 0.78 mg/mL), reinforcing its ethnomedicinal utility in respiratory and cutaneous therapies. Conversely, *S. rebaudiana* emerged as a selective antifungal agent against C. albicans, indicating therapeutic potential and exceeding its established dietary role. Ultimately, these distinct profiles suggest complementary applications. Future investigations should prioritize fraction isolation and synergistic modeling with conventional antibiotics to address multidrug-resistant phenotypes.

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