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Phytochemistry, Antimicrobial, and Antibiofilm Properties of Malagasy *Helichrysum manopappoides* Essential Oil

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Abstract

Helichrysum Mill. is one of the largest genera in the Asteraceae family, and in Madagascar, a plant paradise with ca 14,000 species, 111 *Helichrysum* species are known, 110 of which are endemic. In this work, the essential oil of endemic *Helichrysum manopappoides* Humbert, obtained by steam distillation, was investigated chemically and biologically. The spectrometric data obtained by GC-MS (Gas Chromatography–Mass Spectrometry) analysis highlighted the presence of three major compounds, such as eucalyptol (7.38 ± 0.36%), *α*-humulene (14.75 ± 0.79%), and *β*-caryophyllene (19.78 ± 0.89%), which were also structurally confirmed by NMR (Nuclear Magnetic Resonance) spectroscopic investigation. Biological results showed potential antimicrobial, antioxidant, and antibiofilm effects of both the *H manopappoides* essential oil and the main components identified by GC-MS, enhancing an interesting approach for intestinal infections, being active against *Escherichia coli*, *Listeria monocytogenes*, *Shigella sonnei*, and *Salmonella enterica* ser. *typhimurium* strains.

Keywords: asteraceae; antimicrobial properties; antibiofilm activities; *α*-humulene; *β*-caryophyllene; Madagascar

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1. Introduction

Helichrysum Mill., a term derived from the Greek words *helios* (sun) and *chrysos* (gold), both because the bright yellow color of the flower head resembles the sun and for the reason that the genus' plants grow in sunny locations, from the coast to the high hills, is a heterogeny genus of plant species in the Asteraceae family. To date, a total of 564 accepted species has been estimated [1], making it one of the largest and most diverse genera, and one that presents important pharmacological implications among studied angiosperms. Although the African continent hosts the highest concentration of *Helichrysum* species, the genus is also well represented in Australia, Asia, and Europe [1,2]. Plants of this genus may be annuals, herbaceous perennials or shrubs, growing to a height of 60–90 cm (24–35 inches).

The genus was a wastebasket taxon, and many of its members have been reclassified in smaller genera, most notably the *everlastings*, now in the genus *Xerochrysum* Tzvelev.

Among all, the Malagasy endemic *H. manopappoides* (Figure 1) object of this study is a suffrutescent, branched plant with stems covered by a white, cobweb-like tomentum. Leaves are simple, alternate, small, membranous, sessile, and narrowly linear-revolute (10–15 × 0.5–1). They spread at the base but become erect and arched, covered with the same tomentum on both surfaces, and are borne very close together on sterile branches. Inflorescences are homogamous capitula, which are campanulate (bell-shaped) and shortly pedunculate (stalked). They are arranged in dense terminal corymbs and consist of approximately 20 hermaphroditic yellow flowers. The corollas are regular, tubular, slightly widened towards the apex, and 5-toothed. The fruit is a glabrous achene bearing 1–7 short, filiform bristles that taper at the tip [3].

Figure 1. *Helichrysum manopappoides* Humbert plants collected in Madagascar. Photo by Ramaminirina Henintsoa J.B.

Helichrysum plants have a long history of use in traditional and popular medicine, where they have been employed to address ailments ranging from respiratory and urinary tract infections to skin inflammations, wound healing, and digestive issues. In Madagascar, it is the second genus after Croton, which is the richest in medicinal species, with 46 among the 111 species encountered [4]. Numerous scientific studies have validated the use of *Helichrysum* plant species in traditional medicine. Indeed, their antibacterial [5–7], antifungal [8], antioxidant [9,10], antiviral [11–13], anticancer [9,10], and anti-inflammatory [14] properties have been demonstrated through both in vitro and in vivo studies. For example, *Helichrysum odoratissimum* (L.) Sweet, used since ancient times for its healing properties and to soothe burns, is widely employed as an excipient in ointments and salves for acne and pimples, showed potential activity against *Cutibacterium acnes* [15,16]. In Turkey, the healing effects of *Helichrysum graveolens* (M.Bieb.) Sweet are recognized, through in vitro and in vivo studies by Süntar et al. [16], confirming

its antioxidant and anti-inflammatory potential. In Italy, *Helichrysum italicum* (Roth) G.Don, used as herbal tea or infusion, is known for treating digestive disorders, including gastric and intestinal problems; Prof. Rigano et al. [17] demonstrated its antispasmodic activity in vitro on mouse ileum and in vivo on gastrointestinal transit.

These pharmacological effects are linked to diverse bioactive compounds in polar and non-polar extracts [18,19]. Furthermore, the concentration of these secondary metabolites is influenced by environmental factors [20,21], seasons [22], and geographical differences [23]. Metabolomic analysis revealed *Helichrysum* produces chlorogenic acids and phenolic derivatives, particularly flavonoids such as apigenin, quercetin, and kaempferol, contributing to antioxidant effects and reducing inflammation and oxidative stress [24–27]. Studies on mono-, di-, and trichlorogenic acids showed their role in antiviral activity [8,23,24,28].

There is also a lot of research coming from chemical and biological studies on the essential oils of this genus. Essential oils of *H. italicum*, *Helichrysum arenarium* (L.) Moench, *Helichrysum basalticum* Hilliard, *Helichrysum saxatile* Moris, and *Helichrysum incarnatum* DC., rich in terpenoids (α-pinene, β-pinene, limonene, nerol, neryl acetate, γ-terpinene, linalool, geraniol), exhibited strong antibacterial and anti-inflammatory properties [29–33]. The chemical composition of the leaves of different plants studied varies depending on the species analyzed and on the extraction method used. For example, *Helichrysum petiolare* Hilliard & B.L.Burtt leaves essential oil, extracted by steam distillation, was characterized by compounds such as α-pinene, 1,8-cineole, *p*-cymene, and β-caryophyllene [34], while oils obtained by hydrodistillation from the leaves of *Helichrysum cymosum* (L.) D.Don ex G.Don and *Helichrysum stoechas* (L.) Moench were mainly composed of (*Z*)-β- ocimene and α-pinene, respectively [35,36]. Interest in *H. manopappoides* is supported by evidence from other *Helichrysum* essential oils, which showed antimicrobial, anti-inflammatory, and antioxidant activities [37]. Samples from South African species, including *H. odoratissimum*, *Helichrysum petiolare* Hilliard & B.L.Burtt, *H. Helichrysum cymosum* (L.) D.Don ex G.Don, *Helichrysum nudifolium* (L.) Less., and *Helichrysum kraussii* Sch.Bip., exhibited significant in vitro biological activity [37,38], and α-humulene, one common sesquiterpene compound of *Helichrysum* essential oils, demonstrated antibacterial and antibiofilm effects against several bacterial strains [39,40].

In this context, the lack of chemical and biological data on *Helichrysum manopappoides* Humbert essential oil led us to investigate this endemic species. The *H. manopappoides* leaves essential oil was chemically characterized by gas chromatography (GC-MS) and NMR (Nuclear Magnetic Resonance) spectroscopy, and was tested and evaluated, together with its majority compounds, for its biological potential, investigating antimicrobial and antibiofilm properties against intestinal microorganisms such as *Escherichia coli*, *Listeria monocytogenes*, *Shigella sonnei*, *Salmonella enterica* ser. *typhimurium*, *Staphylococcus aureus*, and *Candida albicans*.

2. Results and Discussion

2.1. Chemical Composition of Essential Oil by GC-MS and 1D/2D-NMR

The steam-distilled essential oil, obtained from the leaves of *H. manopappoides*, was an orange-yellow oil. In total, fifty-three metabolites were identified by GC and GC-MS analysis and tabulated in Table 1 based on linear retention indices (LRIs) and retention times on an apolar DB-5 MS column and clearly divided, due to the chemical structure, into five different classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and other compounds.

Table 1. Chemical composition (relative %) of *Helichrysum manopappoides* Humbert essential oil collected wild in Madagascar.

No.	Compound [a]	LRI [b]	LRI [c]	Area (%) [d]
1	1-Methylbutyl hydroperoxide	857	855	0.07 ± 0.00
2	α-Pinene	933	835	2.09 ± 0.09
3	Camphene	949	851	0.25 ± 0.00
4	β-Pinene	977	876	0.58 ± 0.01
5	β-Myrcene	995	996	0.55 ± 0.02
6	α-Terpinene	1017	1020	0.26 ± 0.00
7	*p*-Cymene	1027	1024	0.48 ± 0.01
8	Limonene	1033	1029	0.49 ± 0.02
9	Eucalyptol	1037	1031	7.38 ± 0.36
10	*cis*-Ocimene	1042	1037	0.13 ± 0.00
11	*trans*-Ocimene	1053	1050	0.71 ± 0.01
12	γ-Terpinene	1062	1059	0.66 ± 0.01
13	Terpinolene	1089	1088	0.44 ± 0.02
14	Linalool	1107	1004	2.31 ± 0.11
15	*endo*-Fenchol	1124	1119	0.16 ± 0.00
16	Borneol	1177	1173	0.24 ± 0.01
17	4-Terpineol	1186	1183	0.27 ± 0.00
18	α-Terpineol	1202	1197	0.59 ± 0.02
19	*endo*-Fenchyl acetate	1224	1220	0.08 ± 0.00
20	Myrtenyl acetate	1331	1326	0.18 ± 0.00
21	δ-Elemene	1338	1338	0.27 ± 0.00
22	*trans*-Carvyl acetate	1343	1342	0.63 ± 0.02
23	α-Ylangene	1374	1373	0.19 ± 0.00
24	α-Copaene	1381	1377	0.91 ± 0.02
25	β-Caryophyllene	1424	1419	19.78 ± 0.89
26	α-Guaiene	1445	1440	1.98 ± 0.05
27	Myltayl-4(12)-ene	1454	1447	0.91 ± 0.02
28	α-Humulene	1469	1466	14.75 ± 0.79
29	*trans*-Cadina-1(6),4-diene	1481	1476	0.94 ± 0.03
30	Germacrene D	1484	1481	1.00 ± 0.04
31	β-Himachalene	1489	1486	2.20 ± 0.10
32	α-Curcumene	1492	1486	0.52 ± 0.01
33	β-Selinene	1493	1490	0.77 ± 0.02
34	Germacrene A	1504	1509	1.26 ± 0.03
35	δ-Amorphene	1508	1511	1.99 ± 0.07
36	γ-Cadinene	1510	1512	1.41 ± 0.04
37	α-Bulnesene	1512	1509	0.90 ± 0.03
38	7-*epi*-α-Selinene	1521	1522	1.19 ± 0.03
39	δ-Cadinene	1525	1523	3.29 ± 0.13
40	γ-Cuprenene	1532	1534	3.68 ± 0.12
41	*trans*-Cadina-1,4-diene	1543	1439	0.43 ± 0.01
42	α-Cadinene	1547	1544	0.90 ± 0.03
43	*trans*-Nerolidol	1571	1569	0.43 ± 0.02
44	Caryophyllenyl alcohol	1576	1573	2.03 ± 0.04
45	Caryophyllene oxide	1592	1589	1.48 ± 0.05
46	Humulol	1618	1618	2.21 ± 0.08
47	1-*epi*-Cubenol	1627	1628	0.59 ± 0.01
48	β-Eudesmol	1635	1638	0.36 ± 0.01
49	β-Acorenol	1639	1637	0.77 ± 0.03
50	Valerianol	1656	1658	3.93 ± 0.18
51	Bulnesol	1669	1671	2.95 ± 0.11
52	Khusinol	1682	1680	0.92 ± 0.03
53	Epoxy-pseudoisoeugenyl isobutyrate	1796	1793	0.15 ± 0.00
	Monoterpene Hydrocarbons			6.64 ± 0.19
	Oxygenated Monoterpenes			11.84 ± 0.52
	Sesquiterpene Hydrocarbons			59.27 ± 2.46
	Oxygenated Sesquiterpenes			15.82 ± 0.56
	Other			0.07 ± 0.00
	Total			93.64 ± 3.73

[a] Components listed in order of elution on a DB-5MS apolar column; [b] LRIs based on the literature (https://webbook.nist.gov/ accessed on 20 November 2025); [c] experimental LRIs on a DB-5MS apolar column; [d] content is the relative area percentage of a single compound in the essential oil sample.

The phytochemical identification of the essential oil compounds, carried out by chromatographic analysis, was also confirmed by means of the NMR spectroscopic technique, in particular by exploiting the HMBC (Heteronuclear Multiple Bond Correlation) tech-

nique, which is a powerful 2D-NMR method that maps long-range connections (typically 2–3 bonds) between different types of nuclei, most commonly protons (^{1}H) and carbon (^{13}C), to identify, in this case, characteristic functional groups that are present in the structures of the majority of organic compounds, such as *β*-caryophyllene and *α*-humulene. *α*-Humulene and *β*-caryophyllene, two cyclic sesquiterpenes, have a very similar structure (Figure 2) but are characterized by specific spectroscopic signatures.

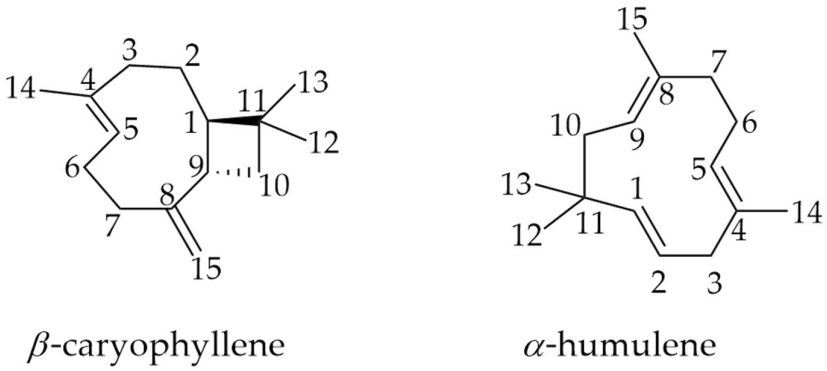

Figure 2. Chemical structure of *β*-caryophyllene and *α*-humulene.

In fact, *α*-humulene presented two characteristic methyl groups, resonating at 18.27 and 15.01 ppm, respectively, for C-14 and C-15, which indicated the double bond and which presented $^{3}J_{CH}$ correlations with the H-9 and H-5 protons at 4.98 and 4.88 ppm, respectively (Figure 3). Finally, the carbon C-1 (140.83 ppm) of the double bond showed clear $^{3}J_{CH}$ correlation with the signal at 1.08 ppm, attributable to six methyl protons H-12 and H-13 (Figure 3).

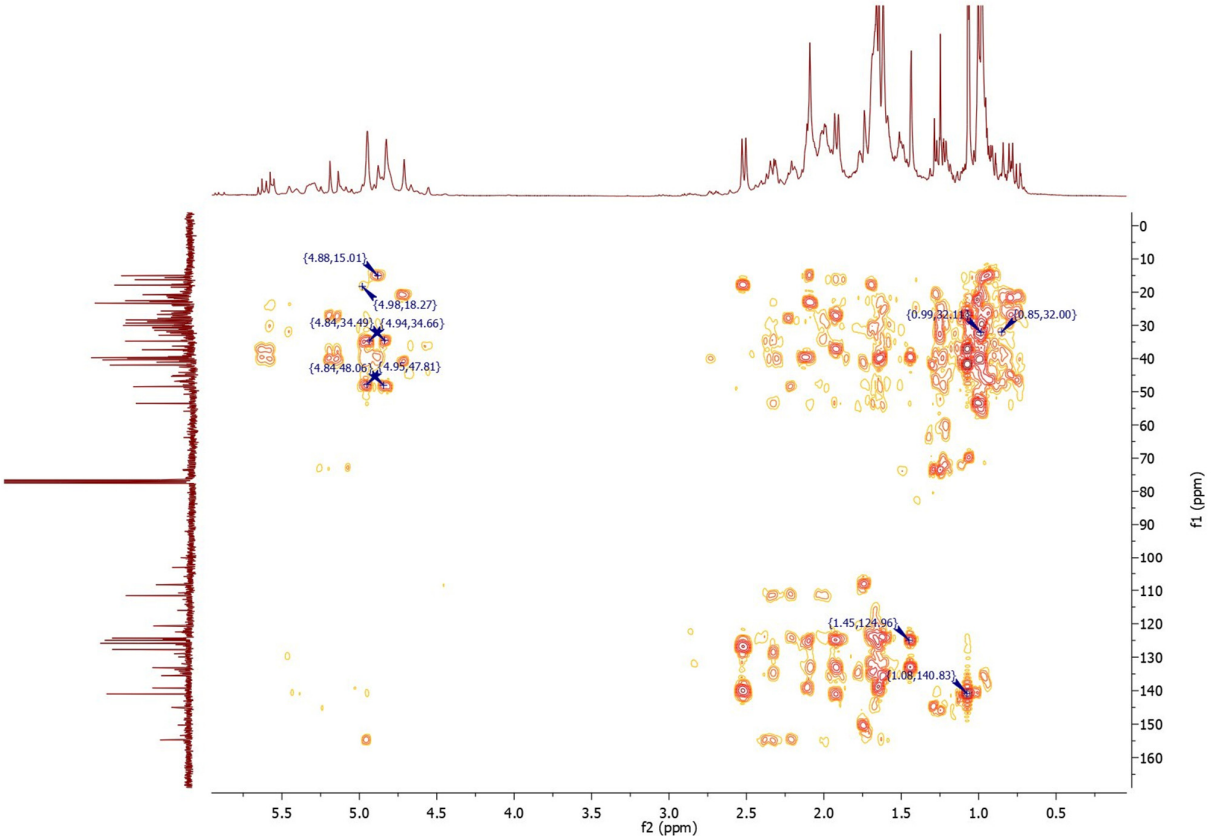

Figure 3. HMBC spectrum of *Helichrysum manopappoides* Humbert essential oil.

In turn, the HMBC spectrum (Figure 3) showed a correlation of the vinyl protons H-15a and H-15b (4.84 and 4.95 ppm) with the sp^3 secondary carbon C-7 (34.49 ppm) and the sp^3 tertiary carbon C-9 (48.06 ppm) for β-caryophyllene. Similarly to α-humulene, the presence of the double bond at the C-4/-5 position is confirmed by the correlation between C-5 (124.96 ppm) and the protons CH_3-14 at 1.45 ppm. These values are in agreement with what is reported in the literature [37,38].

From a compositional standpoint, to date, nothing has been reported in the literature regarding the essential oil of *H. manopappoides*. However, several essential oils from various Malagasy *Helichrysum* species have been investigated. Eucalyptol was found as a major compound, for example, in samples obtained by hydrodistillation from *H. gymnocephalum* (59.7%) [41], *H. bracteiferum* (17.7–27.3%) [41–43], and *H. hypnoides* (51.5%) [42]; α-humulene and β-caryophyllene, found in all Malagasy specimens examined, instead, were the majority metabolites of *H. bracteiferum* [43], *H. cordifolium*, *H. faradifani*, and *H. hypnoides* [38,42] essential oils. It would therefore appear that these compounds are common for this genus. Another study also reported the chemical composition of essential oils from six *Helichrysum* species endemic to Madagascar. *H. benthamii* and *H. dubardii* had their oil compositions dominated by α-pinene (23.1–50.8%) and eucalyptol (26.9–35.7%), respectively. β-Caryophyllene was the major compound of the oils from *H. indutum* (33.1%), *H. bojerianum* (16.1%) and *H. diotoides* (15.0%), whereas 7-epi-silphiperfol-5-*en*-13-oic acid (18.2–40.0%) and 7-*epi*-subergorgiol (7.6–14.8%) were identified in that from *H. hirtum* [44].

2.2. Antimicrobial Properties of *H. manopappoides* Essential Oil

The antimicrobial activity of *H. manopappoides* essential oil was evaluated by determining the minimum inhibitory concentration (MIC) against a panel of microorganisms representative of intestinal infections, including both Gram-positive and Gram-negative bacteria, as well as a pathogenic yeast. In addition to the model strains *Escherichia coli* and *Staphylococcus aureus*, the microbial panel included *Listeria monocytogenes*, *Shigella sonnei*, *Salmonella typhimurium*, and *Candida albicans*.

As reported in Table 2, the sample exhibited a broad-spectrum antimicrobial activity, with MIC values ranging from 12 to 25 mg/mL. The lowest MIC values were observed against *C. albicans* (12 mg/mL), *E. coli* and *L. monocytogenes* (13 mg/mL), indicating a pronounced inhibitory effect against these microorganisms. Moderate activity was detected against *S. aureus* (15 mg/mL) and *S. typhimurium* (20 mg/mL), whereas *S. sonnei* appeared to be the least susceptible strain (25 mg/mL).

Table 2. Determination of the minimum inhibitory concentration (MIC, expressed in mg/mL) of *H. manopappoides* essential oil against Gram-negative and Gram-positive bacterial strains. MIC values are the mean ± SD of three independent biological replicates.

Strain	MIC [mg/mL] + SD
E. coli	13 ± 0.3
S. sonnei	25 ± 0.5
S. tiphymorium	20 ± 0.3
S. aureus	15 ± 0.2
L. monocytogenes	13 ± 0.9
C. albicans	12 ± 0.1

The antimicrobial activity of the main essential oil's constituents was subsequently evaluated individually against the model strains *E. coli* and *S. aureus* (Table 3), revealing distinct activity profiles. Notably, eucalyptol and α-humulene displayed the lowest MIC values against *E. coli* (8 mg/mL), while a reduced efficacy was observed against *S. aureus* (MIC = 16 mg/mL for both compounds). In contrast, β-caryophyllene did not

show significant antimicrobial activity against either strain at the tested concentrations (MIC > 16 mg/mL).

Table 3. Determination of the minimum inhibitory concentrations (MIC, expressed in mg/mL) of eucalyptol, α-humulene, and β-caryophyllene against *E. coli* and *S. aureus*. MIC values are the mean ± SD of three independent biological replicates.

Strain	MIC [mg/mL] ± SD		
	Eucalyptol	**α-Humulene**	**β-Caryophyllene**
E. coli	8 ± 0.1	8 ± 0.3	>16
S. aureus	16 ± 0.5	16 ± 0.6	>16

Among the individual components, α-humulene emerges as the most interesting compound, combining a relevant antimicrobial activity with a relatively high abundance within the essential oil (14.75 ± 0.79%). Although β-caryophyllene represents the most abundant constituent (19.78 ± 0.89%), its limited individual activity suggests a minor contribution to the direct antimicrobial effect. Eucalyptol, despite its good antimicrobial performance, is present at a lower percentage (7.38 ± 0.36%).

Overall, these findings suggest that the antimicrobial potential of *H. manopappoides* essential oil is mainly attributable to the combined action of its bioactive constituents, with α-humulene playing a key role and possible interactions among the essential oil's components contributing to the observed broad-spectrum activity.

These data fall within the range commonly reported for essential oils of the genus *Helichrysum*, which are generally characterized by a moderate but broad-spectrum antimicrobial activity. In fact, previous studies on *Helichrysum* spp. have highlighted a marked variability in antimicrobial potency depending on chemical composition, plant origin, and microbial target, with MIC values often reported in the low-to-mid mg/mL range against both Gram-positive and Gram-negative bacteria, as well as yeasts [45,46]. In this context, the MIC range of 12–25 mg/mL observed here is fully consistent with the antimicrobial profiles described for other *Helichrysum* essential oils.

However, the comparable susceptibility observed among Gram-positive and Gram-negative bacteria suggests that *H. manopappoides* essential oil is not strongly selective toward a specific bacterial group, despite the structural differences between their cell envelopes. Similar non-selective antimicrobial patterns have been reported for complex essential oils, where the combined presence of monoterpenes and sesquiterpenes can overcome, at least partially, the permeability barrier associated with the outer membrane of Gram-negative bacteria [47]. Instead, the pronounced activity against *C. albicans* is also in agreement with previous reports describing antifungal properties for *Helichrysum* essential oils and related sesquiterpene-rich mixtures [45].

But the evaluation of individual sample constituents revealed distinct antimicrobial profiles, with eucalyptol and α-humulene showing higher activity against *E. coli* than against *S. aureus*, while β-caryophyllene was inactive within the tested concentration range. This differential susceptibility is in line with literature data indicating that α-humulene may exert a stronger antimicrobial effect against Gram-negative bacteria, including intestinal pathogens, compared with Gram-positive species [39]. α-Humulene has been described as a bioactive sesquiterpene with antibacterial properties, supporting its relevance as an antimicrobial constituent within essential oils [48].

β-Caryophyllene, despite being the most abundant component of the essential oil, showed limited antimicrobial activity when tested alone. This observation is consistent with previous reports indicating that β-caryophyllene often displays weak or strain-dependent MIC values, while contributing more effectively to antimicrobial activity when

present within complex mixtures [47,49]. Such findings support the hypothesis that β-caryophyllene may play a modulatory role, enhancing or stabilizing the activity of other bioactive constituents rather than acting as a potent antimicrobial agent per se.

From a biological perspective, the observed activity profile suggests that membrane interaction represents a primary determinant of antimicrobial efficacy, particularly for sesquiterpene-rich fractions. The higher susceptibility of *E. coli* compared with *S. aureus* to α-humulene may reflect differences in membrane lipid composition and permeability. Moreover, the discrepancy between compound abundance and individual activity reinforces the concept that the antimicrobial effect of the essential oil cannot be predicted solely based on quantitative composition, but rather on functional interactions among constituents.

2.3. Mechanistic Evaluation of H. manopappoides Essential Oil and of Its Major Compounds Against Bacterial Target

To gain insight into the mechanism underlying the antimicrobial activity of *H. manopappoides* essential oil and of its main constituents, their effects on bacterial membrane integrity were investigated using complementary approaches. In *E. coli*, the NPN uptake assay revealed a pronounced alteration of the outer membrane upon treatment with the essential oil at its MIC (Figure 4).

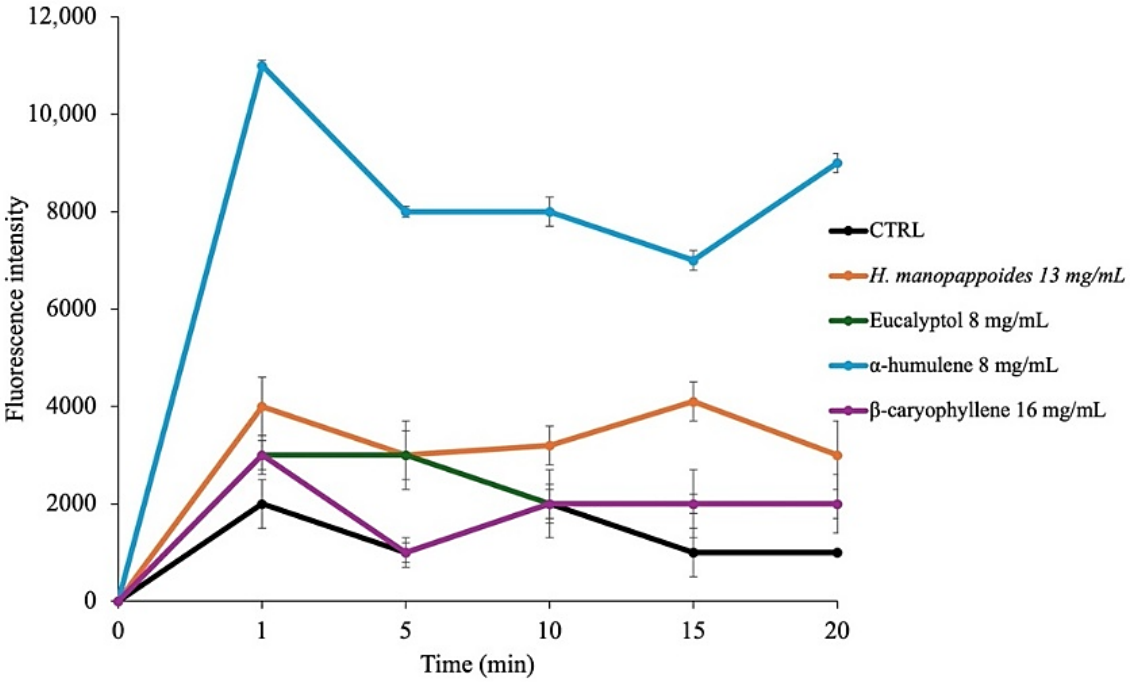

Figure 4. Outer Membrane Damage Assessed by NPN Assay. NPN fluorescence was used to evaluate outer membrane permeability in *E. coli* after treatment with MICs of *H. manopappoides* essential oil, eucalyptol, α-humulene, and β-caryophyllene. Data represent the means of three independent experiments.

Among all treatments, α-humulene induced the most pronounced increase in NPN fluorescence, reaching markedly higher intensity values compared with the essential oil and the other constituents throughout the time course. This result indicates a strong ability of α-humulene to permeabilize the outer membrane of *E. coli*, in line with its higher antimicrobial efficacy against this strain observed in MIC. In contrast, treatment with *H. manopappoides* essential oil resulted in a moderate but consistent increase in fluorescence, suggesting a measurable alteration of the outer membrane, although less intense than that induced by α-humulene alone. Eucalyptol produced a comparable or slightly lower fluorescence increase than the essential oil, indicating a limited but detectable effect on

outer membrane permeability. Conversely, *β*-caryophyllene caused only minimal changes in NPN fluorescence, with values remaining close to the baseline over time, suggesting a weak interaction with the outer membrane under the tested conditions, trends similar to those obtained by Li et al. [50].

To observe if alterations in outer membrane permeability were translated into actual cell damage, time-dependent bacterial lysis assays were subsequently performed. As shown in Figure 5, *H. manopappoides* essential oil induced a significant and progressive increase in bacterial lysis in both *E. coli* (panel A) and *S. aureus* (panel B) compared to the negative control (DMSO).

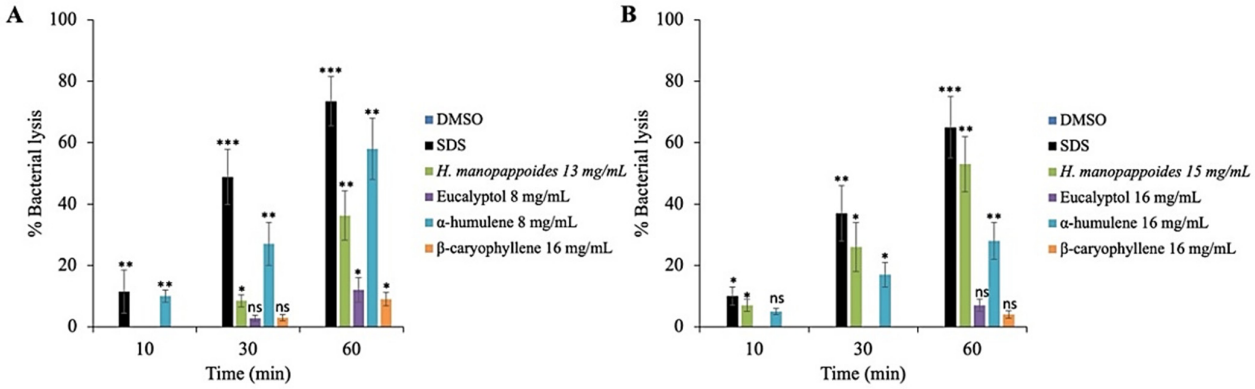

Figure 5. Bacterial lysis activity of *H. manopappoides* essential oil, eucalyptol, *α*-humulene, and *β*-caryophyllene. Panel (**A**) against *E. coli* and panel (**B**) against *S. aureus*. *H. manopappoides* essential oil, eucalyptol, *α*-humulene, and *β*-caryophyllene were tested at their respective MICs. DMSO served as a negative control, while SDS served as a positive control. Bacterial lysis was expressed as a percentage and evaluated over time (10, 30, and 60 min). Data represent the means of three independent experiments. Statistical analysis was performed against the control using a two-tailed paired *t*-test. ns: not significant; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Notably, the lytic effect of the essential oil appeared to develop more rapidly in the Gram-positive strain *S. aureus*, where a marked increase in lysis was already evident at earlier time points (10 min), whereas in *E. coli*, the effect became more pronounced at longer incubation times. This difference suggests a faster interaction of the essential oil with the more accessible cytoplasmic membrane of Gram-positive bacteria. Among the individual constituents, *α*-humulene displayed a distinct kinetic profile, promoting a more rapid and pronounced lysis in *E. coli* than in *S. aureus*, in agreement with its higher antimicrobial activity against the Gram-negative strain observed in MIC assays. Eucalyptol induced a moderate increase in bacterial lysis in both strains, with a generally weaker effect compared to the essential oil and *α*-humulene. In contrast, *β*-caryophyllene showed little or no lytic activity throughout the time course in either *E. coli* or *S. aureus*, with values remaining close to those of the negative control. Overall, these results indicate that bacterial lysis is strongly dependent on both the chemical nature of the treatment and the bacterial species, and further support membrane disruption as a key event underlying the antimicrobial activity of *H. manopappoides* essential oil, consistent with the membrane-targeting mechanism widely reported for essential oils [51].

These findings indicate that outer membrane permeabilization and subsequent cytoplasmic membrane disruption occur in a coordinated manner, leading to progressive loss of cellular integrity. The faster lytic response observed in *S. aureus* compared to *E. coli* is consistent with the absence of an outer membrane barrier in Gram-positive bacteria, allowing more immediate access of lipophilic compounds to the cytoplasmic mem-

brane. Overall, the data support a primarily membrane-targeted mechanism rather than intracellular-specific targets.

Fluorescence microscopy analyses (Figure 6) provided clear confirmation of these effects. Untreated cells of both *E. coli* and *S. aureus* (Panels 1, A-1 and Panel 2, A-1) exhibited predominantly blue DAPI staining, consistent with intact membranes and preserved cellular integrity. In contrast, treatment with *H. manopappoides* essential oil resulted in a marked increase in propidium iodide (PI) uptake in both bacterial species (B-2), as evidenced by intense red fluorescence, indicating extensive membrane permeabilization and loss of membrane integrity [52].

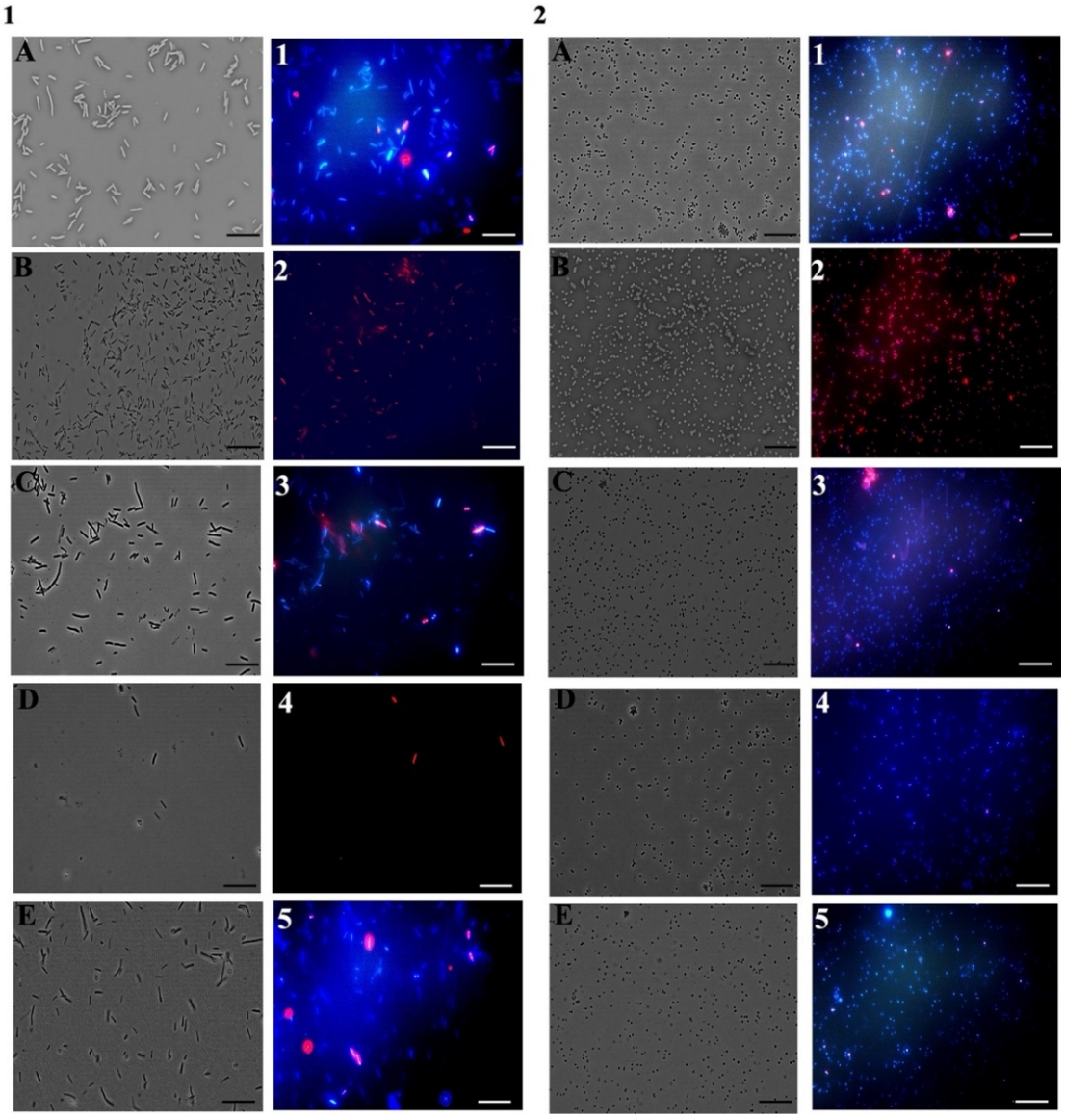

Figure 6. Fluorescence microscopy of *E. coli* (panel (**1**)) and *S. aureus* (panel (**2**)) cells after 1 h of treatment with *H. manopappoides* essential oil (**B-2**), eucalyptol (**C-3**), *α*-humulene (**D-4**), and *β*-caryophyllene (**E-5**) stained with DAPI (blue) and propidium iodide (red). Untreated control cells are shown in (**A-1**). Scale bars: 5 μm.

Distinct staining patterns were observed for the individual constituents, in agreement with the results of the NPN and lysis assays. Eucalyptol-treated *E. coli* cells (Panel 1, C-3) showed limited PI uptake, while *S. aureus* cells (Panel 2, C-3) remained largely DAPI-positive, suggesting a relatively mild membrane effect. In contrast, *α*-humulene treatment induced pronounced PI staining in *E. coli* (Panel 1, D-4), consis-

tent with severe membrane damage and the rapid lytic response observed in this strain, whereas *S. aureus* cells (Panel 2, D-4) appeared mostly blue, reflecting a weaker effect. Finally, *β*-caryophyllene-treated cells (E-5) exhibited predominantly blue staining in both strains, with only sporadic PI-positive *E. coli* cells, further confirming its limited impact on membrane integrity.

Taken together, the concordant evidence from outer membrane permeabilization, bacterial lysis kinetics, and fluorescence microscopy demonstrates that membrane disruption represents a central event in the antimicrobial action of *H. manopappoides* essential oil. Based on these mechanistic findings, the antimicrobial activity observed at the MIC level can be interpreted considering specific interactions between essential oil constituents and bacterial membranes. The more pronounced membrane-targeting effects of the essential oil compared with the isolated compounds support the involvement of possible favorable interactions within the phyto-complex [53]. In this context, the stronger activity of *α*-humulene against *E. coli* relative to *S. aureus* is consistent with literature reports describing a preferential action of this sesquiterpene toward Gram-negative and gut-associated bacteria, through interference with membrane-associated and biofilm-related processes [39,48]. This behavior provides a mechanistic explanation for the lower MIC values and the more pronounced membrane damage observed in *E. coli*.

Although *β*-caryophyllene was the most abundant essential oil constituent, its weak activity as a single compound suggests an indirect role. Previous studies have shown that *β*-caryophyllene can modulate membrane permeability and potentiate the activity of other antimicrobials despite limited intrinsic antibacterial potency [47,49]. Accordingly, within the essential oil matrix, *β*-caryophyllene may function as a membrane-modulating component that enhances the overall antimicrobial efficacy of the phyto-complex.

Eucalyptol also contributed to membrane perturbation, particularly in *E. coli*, in line with its reported ability to interact with lipid bilayers and weaken bacterial envelopes. Overall, these results indicate that the antimicrobial efficacy of *H. manopappoides* essential oil is primarily linked to its capacity to compromise bacterial membrane integrity through the combined and complementary actions of its major constituents, thereby supporting the relevance of the whole phyto-complex rather than individual components alone.

The concordance among MIC data, permeability assays, lysis kinetics, and fluorescence microscopy strengthens the causal link between membrane destabilization and antimicrobial outcome. This mechanistic coherence increases the biological relevance of the findings and supports the interpretation that the whole essential oil exerts a more balanced and sustained membrane-disrupting effect compared with isolated constituents.

2.4. Antibiofilm Activity of H. manopappoides Essential Oil

After characterizing the antimicrobial activity and membrane-targeting effects of *H. manopappoides* essential oil, its ability to interfere with biofilm formation was further investigated. The antibiofilm assay was performed on *Mycobacterium smegmatis*, an established model organism for studying biofilm development and maturation, widely used to evaluate compounds capable of influencing structured microbial communities and persistence [54]. To specifically assess antibiofilm activity independent of growth inhibition, the MIC was first calculated at 15 mg/mL, and sub-MICs of *H. manopappoides* essential oil ranging from 0 to 1 mg/mL were subsequently tested.

As shown in Figure 7, the essential oil showed a clear dose-dependent inhibition of biofilm formation compared to the untreated control (CTRL+). At the lowest concentration tested (0.25 mg/mL), a marked reduction in biofilm biomass was already observed, indicating early interference with biofilm development. Increasing the essential oil concentration to 0.5 and 1 mg/mL resulted in a further reduction in biofilm formation, reaching

approximately 80–85% inhibition at 1 mg/mL, comparable to the effect observed in the antibiotic-treated control (CTRL−).

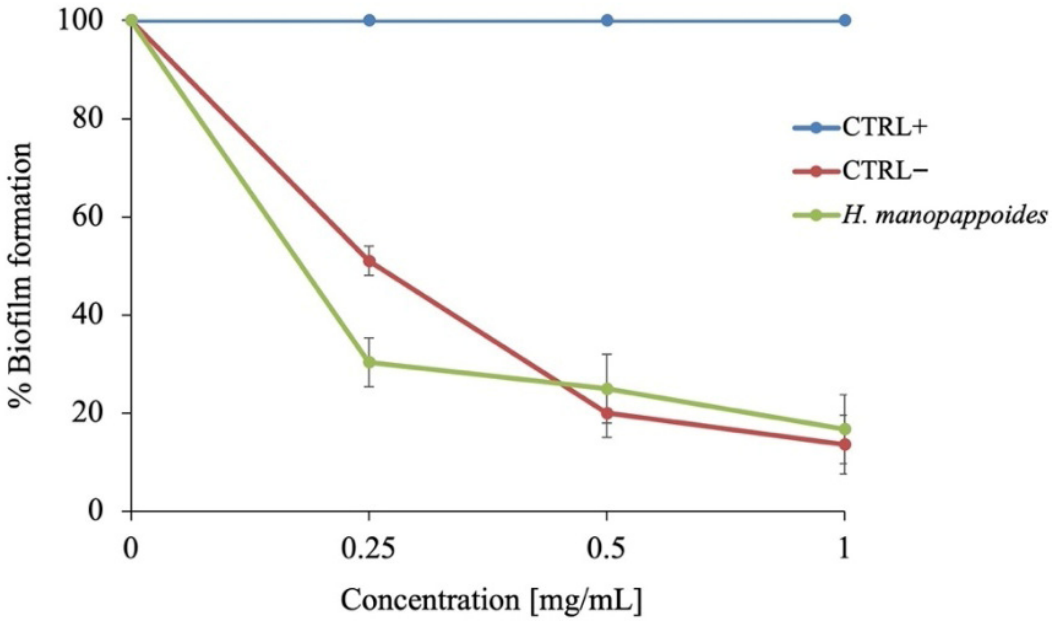

Figure 7. Inhibition of biofilm formation by *H. manopappoides* essential oil on *M. smegmatis*. Biofilm biomass was quantified after treatment with sub-MICs (0–1 mg/mL) of essential oils. CTRL+ cells were untreated; CTRL− cells were treated with kanamycin. Data represent the average of three independent experiments.

In particular, the strong antibiofilm effect observed at concentrations well below the MIC highlights the ability of *H. manopappoides* essential oil to impair biofilm formation without exerting a bactericidal effect. This suggests that essential oil primarily interferes with the early stages of biofilm stabilization and matrix development, processes crucial for bacterial adhesion, maturation, and long-term persistence [55]. This activity is particularly relevant in the context of chronic and device-associated infections, where biofilm formation plays a key role in antimicrobial tolerance and treatment failure [56].

Alterations in membrane integrity are known to affect cell adhesion, quorum sensing, and the production of extracellular polymeric substances, which are critical for biofilm initiation and maturation [57]. In this context, the strong inhibition observed at sub-MICs suggests that the essential oil can modulate biofilm-related pathways independently of direct bactericidal effects.

In addition, α-humulene, identified as one of the most active constituents of *H. manopappoides* essential oil, has been reported to possess antibiofilm properties, particularly against Gram-negative and gut-associated bacteria. Previous studies have shown that α-humulene can interfere with biofilm formation and maturation by affecting membrane-associated processes and regulatory pathways involved in microbial aggregation and persistence [39,48]. This evidence supports a possible contribution of α-humulene to the antibiofilm activity observed for the essential oil, even at concentrations that do not inhibit planktonic growth.

The inhibition of biofilm formation at sub-MICs further suggests that membrane perturbation may interfere with early adhesion processes and surface-associated signaling events. Since biofilm development is tightly linked to membrane-associated regulatory pathways, the ability of the essential oil to modulate these processes without affecting planktonic growth highlights a specific anti-virulence potential rather than a purely bactericidal effect.

2.5. Antioxidant Properties of *H. manopappoides* Essential Oil and Its Main Components

It is well established that essential oils, in addition to exhibiting antimicrobial properties, may also display relevant antioxidant activity [58]. In this context, the antioxidant potential of *H. manopappoides* essential oil and its major constituents, eucalyptol, α-humulene, and β-caryophyllene, was evaluated using two complementary assays: the DPPH radical scavenging assay and the hydrogen peroxide (H_2O_2) scavenging assay (Figure 8).

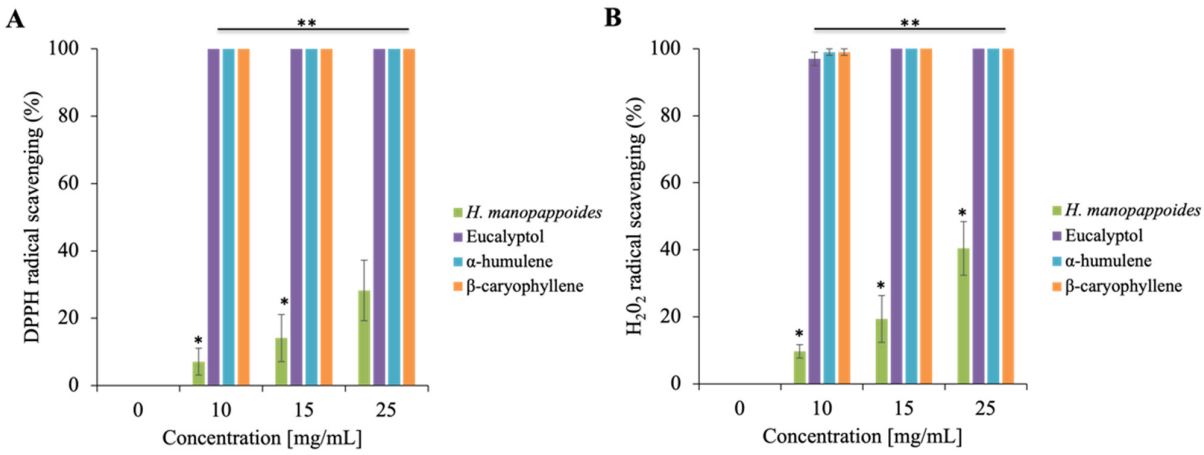

Figure 8. Antioxidant activity of *H. manopappoides* essential oil, eucalyptol, α-humulene, and β-caryophyllene. Panel (**A**) DPPH assay; Panel (**B**) H_2O_2 scavenging assay. *H. manopappoides* essential oil, eucalyptol, α-humulene, and β-caryophyllene were tested at concentrations ranging from 0 to 25 mg/mL. Data represent the average of three independent experiments. Statistical analysis was performed relative to the standard by a two-tailed paired *t*-test. *p*-value (* $p \leq 0.05$; ** $p < 0.01$).

As shown in Figure 8A, all samples exhibited a clear, concentration-dependent increase in DPPH radical scavenging activity. The individual constituents showed markedly stronger antioxidant effects than the whole essential oil across the tested concentration range. Eucalyptol, α-humulene, and β-caryophyllene reached high levels of radical scavenging at relatively low concentrations (10 mg/mL), whereas *H. manopappoides* essential oil displayed a weaker response. A similar trend was observed in the H_2O_2 scavenging assay (Figure 8B). All samples were able to neutralize hydrogen peroxide in a dose-dependent manner, with α-humulene and β-caryophyllene showing the most pronounced scavenging activity, followed closely by eucalyptol. In contrast, the essential oil again exhibited lower overall efficacy compared to its individual constituents, although a progressive increase in activity was evident with increasing concentration.

To allow a quantitative comparison of antioxidant potency, IC_{50} values were calculated and are reported in Table 4.

Table 4. IC_{50} values for the antioxidant activity of *H. manopappoides* sample, eucalyptol, α-humulene, β-caryophyllene, and ascorbic acid (control) in the DPPH and H_2O_2 assays. Values represent the means of three independent experiments and are expressed in mg/mL and ±standard deviation SD.

	IC_{50} [mg/mL] ± SD	
Sample	**DPPH**	**H_2O_2**
H. manopappoides	60 ± 2	30 ± 5
eucalyptol	4 ± 0.5	5.1 ± 0.3
α-humulene	5 ± 0.2	4 ± 0.9
β-caryophyllene	5.2 ± 0.7	4.3 ± 0.7
Ascorbic acid	0.03 ± 0.03	0.04 ± 0.05

The results confirm the trends observed in Figure 8. *H. manopappoides* essential oil showed the highest IC_{50} values in both assays (60 mg/mL for DPPH and 30 mg/mL for H_2O_2), indicating lower antioxidant potency. In contrast, the individual constituents displayed significantly lower IC_{50} values, ranging from 4 to 5.2 mg/mL in the DPPH assay and from 4 to 5.1 mg/mL in the H_2O_2 assay, highlighting their stronger radical scavenging capacity. Ascorbic acid consistently exhibited the lowest IC_{50} values, confirming its high antioxidant efficacy [59]. The reduced activity of the essential oil compared to its components suggests that interactions within the phytocomplex may modulate the overall antioxidant response [60].

2.6. Evaluation of Cytotoxic Effects on CaCo-2 Cells

To evaluate the potential cytotoxicity associated with *H. manopappoides* essential oil and its major constituents on eukaryotic cells, experiments have been carried out on human CaCo-2 intestinal epithelial cells, and cell viability was assessed 24 h after transfection using MTT assay. Cells were treated for 24 h with the highest concentrations corresponding to the MIC values previously determined for each compound, and cell viability was expressed as a percentage relative to untreated control cells (CTRL) as shown in Figure 9.

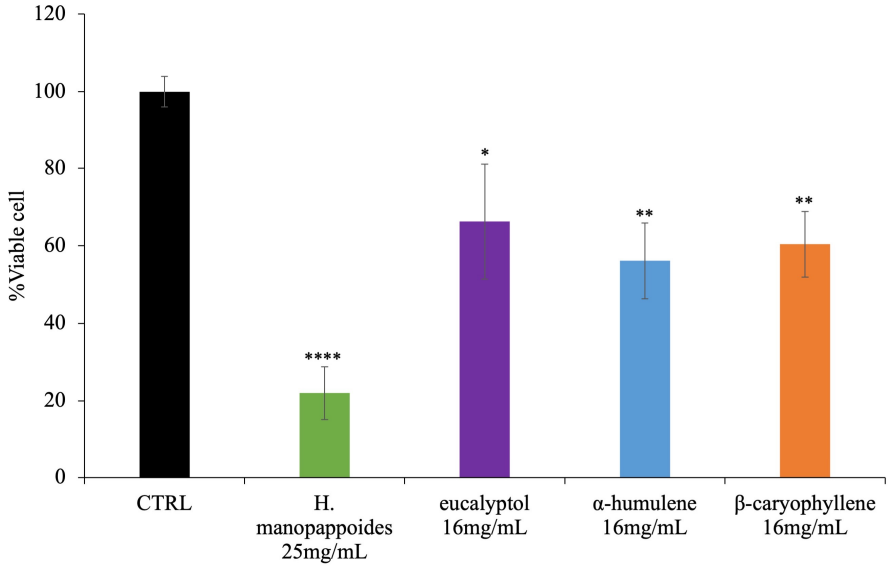

Figure 9. Cytotoxicity of *H. manopappoides*, eucalyptol, *α*-humulene, and *β*-caryophyllene on CaCo-2 cells assessed by MTT assay. Cell viability was evaluated after 24 h of treatment. Cells were treated with the highest concentrations corresponding to the MIC values for each compound. Data are expressed as a percentage of viable cells relative to untreated control (CTRL) and are presented as the means of three independent experiments. Statistical analysis was performed using a two-tailed paired *t*-test relative to untreated cells: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Treatment with *H. manopappoides* essential oil resulted in a marked reduction in cell viability, which dropped to approximately 20–25% compared to the control, indicating a strong cytotoxic effect at the tested concentration. In contrast, treatment with the individual essential oil constituents led to markedly less severe, yet significant, reductions in cell viability.

Specifically, eucalyptol induced a moderate reduction in cell viability, with values around 65–70%, indicating a partial cytotoxic effect. Similarly, *α*-humulene reduced cell viability to approximately 55–60%, showing a slightly more pronounced effect compared to eucalyptol. *β*-Caryophyllene also caused a mild decrease in cell viability, with values around 60–65% relative to the control. These data indicate that, although each major constituent of *H. manopappoides* essential oil exhibits cytotoxic activity when tested individually,

none of them reproduces the strong cytotoxic effect observed with the whole essential oil at equivalent MIC-based concentrations [61].

Overall, these results demonstrate a differential cytotoxicity of the *H. manopappoides* sample and its major constituents on CaCo-2 cells after 24 h of exposure. Notably, the whole oil exerted a substantially greater cytotoxic effect than any of the individual compounds tested at their respective MICs, suggesting that the combined presence of multiple constituents may enhance cytotoxicity compared to single components.

2.7. Effect on Intracellular ROS Production

To further investigate whether the cytotoxic effects observed in CaCo-2 cells were associated with oxidative stress, intracellular reactive oxygen species (ROS) production was evaluated using the CellROX™ fluorescent probe. CaCo-2 cells were treated for 24 h with *H. manopappoides* essential oil and its principal constituents at the highest concentrations corresponding to their respective MIC values, and ROS levels were expressed as relative fluorescence intensity (Figure 10).

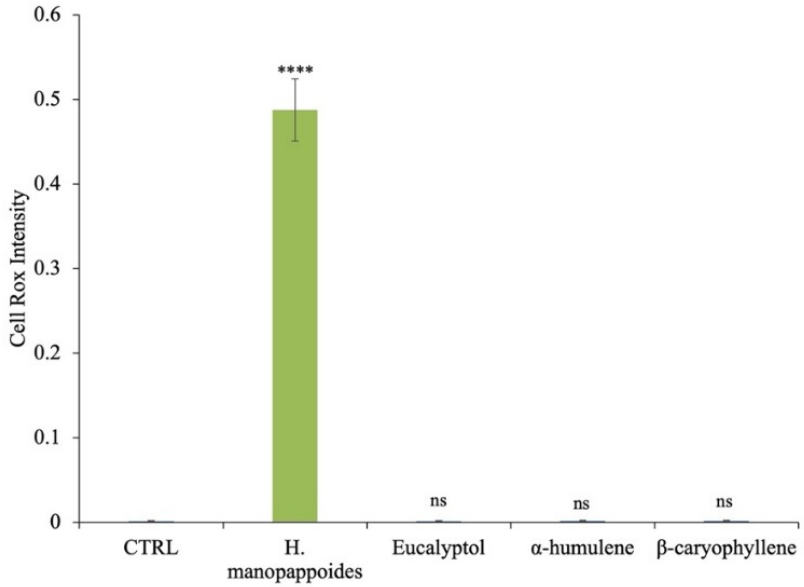

Figure 10. Effect of *H. manopappoides*, eucalyptol, *α*-humulene, and *β*-caryophyllene on intracellular ROS generation in CaCo-2 cells. Oxidative stress was evaluated using the CellROX™ fluorescent probe. CaCo-2 cells were treated with the highest concentrations corresponding to the MIC values for each compound for 24 h before ROS detection. Data are expressed as relative fluorescence intensity and are presented as the means of three independent experiments. Statistical analysis was performed using a two-tailed paired *t*-test relative to the untreated control (CTRL): ns not significant; **** $p < 0.0001$.

Treatment with *H. manopappoides* oil induced a marked increase in intracellular ROS levels compared to untreated control cells, indicating the induction of pronounced oxidative stress. This result is consistent with the strong reduction in cell viability observed in the MTT assay and suggests that the cytotoxicity of the essential oil might be mediated, at least partially, by a ROS-dependent mechanism. In contrast, CaCo-2 cells treated with eucalyptol, *α*-humulene, or *β*-caryophyllene did not show a significant increase in ROS production relative to the control, with fluorescence values remaining close to baseline levels. These findings indicate that, at the biologically active concentrations tested, the individual constituents do not induce oxidative stress in CaCo-2 cells. The selective induction of ROS by the whole essential oil further supports a differential biological activity between the complete phytocomplex and its isolated components.

3. Materials and Methods

3.1. Plant Materials

The leaves of *Helichrysum manopappoides* Humbert were collected by hand from plants growing at Tsaratanàna-Vinaninkarena (19°56′28.3″ S, 47°03′27.8″ E), District of Antsirabe II in the Region of Vakinankaratra, in early February 2025. The plant identification was carried out at the Ethnobotany and Botany Department of the National Center of Pharmaceutical Research (CNARP) in Antananarivo, Madagascar. Voucher specimens have been deposited at the CNARP under the code RHJ 212. The plant material was transported to the laboratory on the same day as the collection to submit immediately to the extraction to avoid an eventual loss of its essential oil content.

3.2. Extraction of Essential Oil

After cutting into small pieces with a knife, the freshly collected leaves (400 g) were subjected to a steam distillation extraction to produce the essential oil, using an appropriate apparatus and water as a source of steam. The plant material was introduced in the upper flask, whereas 1.2 L of distilled water was added to the lower one. The treatment of the plant material lasted 2 h. The essential oil and the condensed water were not miscible, giving two well-separated phases in the separatory funnel of the apparatus. The upper layer was made up of the essential oil, which was collected in a sealed amber vial. The oil was obtained with a yield of 0.14% (*w*/*w*). The obtained essential oil was dried with Na_2SO_4, stored in an appropriate vial under N_2, and placed in the freezer at −20 °C until the time of analysis.

3.3. GC and GC-MS Analyses

GC-MS analysis was performed using a Shimadzu QP 2010. GC-MS analysis was performed using a Shimadzu QP 2010, plus equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) gas chromatograph equipped with a capillary column (DB-5 MS) of 30 m × 0.25 mm i.d., film thickness 0.25 µm, and a data processor. The oven program was as follows: temperature was held at 40 °C for 5 min, then increased at a rate of 2 °C/min up to 260 °C, then isothermal for 20 min. Helium was used as the carrier gas (1 mL min^{-1}). The injector and detector temperatures were set at 250 and 290 °C, respectively. One µL of essential oil solution (3% essential oil/hexane *v*/*v*) was injected in split mode 1:50; MS range 40–600. The settings were as follows: ionization voltage, 70 eV; electron multiplier energy, 2000 V; transfer line temperature, 295 °C; solvent delay, 3 min (Figure S1).

Linear retention indices (LRIs) were calculated on DB-5 MS retention indices using a mixture of pure *n*-alkanes (C_7–C_{30}) (Figure S2), and all the peaks' compounds were identified by comparison with MS and by comparison of their relative linear retention indices with WILEY275, NIST 17, ADAMS, and FFNSC2 libraries. The analyses were performed in triplicate, and the results are expressed as the average of three measurements ± standard deviation.

3.4. NMR Experiments

^{1}H- and ^{13}C-NMR spectra were recorded at 400/100 MHz in $CDCl_3$ on Bruker spectrometers using the residual solvent signal (δ = 7.27 in ^{1}H and δ = 77.00 in ^{13}C for $CDCl_3$) as a reference. HMBC experiment was performed using Bruker microprograms. Deuterated chloroform ($CDCl_3$), *n*-alkanes (C_8–C_{40}), eucalyptol, α-humulene, β-caryophyllene, and hexane were purchased from Sigma-Aldrich (San Louis, MO, USA).

3.5. Bacterial Strain

The antimicrobial activity of the essential oil of *H. manopappoides*, eucalyptol, α-humulene, and β-caryophyllene was evaluated against different Gram-negative strains,

such as *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PAO1 ATCC15692, *Shigella sonnei* ATCC25931, and *Salmonella Typhimurium* ATCC14028, and Gram-positive strains, such as *Staphylococcus aureus* ATCC6538P, *Listeria monocytogenes* ATCC19115 and *Mycobacterium smegmatis* MC^2155 and the yeast *Candida albicans* ATCC14053. *E. coli* and *S. aureus* were used as model strains.

3.6. Determination of Minimum Inhibitory Concentrations (MIC)

The antimicrobial activity of *H. manopappoides* essential oil, eucalyptol, α-humulene, and β-caryophyllene was evaluated by determining the Minimum Inhibitory Concentrations (MICs) against selected bacterial strains using the broth microdilution method, according to CLSI guidelines [62] and according to the procedure reported in Porrello et al. [63].

3.7. N-Phenyl Naphthylamine (NPN) Assay

The outer membrane (OM) permeabilizing activity of *H. manopappoides* essential oil, eucalyptol, α-humulene, and β-caryophyllene was evaluated using the 1-N-phenylnaphthylamine (NPN) uptake assay, adapted from Jia et al. [64] with slight modifications; Porrello et al. [63].

3.8. Bacterial Lysis Assay

Bacterial lysis activity was evaluated according to a previously described method, with slight modifications [65]. *E. coli* and *S. aureus* were used as representative Gram-negative and Gram-positive strains, respectively.

Overnight cultures were diluted to an OD_{600} of 0.1 (5 mL) and incubated at 37 °C with shaking until an OD_{600} of ~1.0 was reached. Cells were harvested by centrifugation (5000× *g*, 5 min), resuspended in PBS (pH 7.4), and adjusted to an OD_{600} of 0.5.

Untreated cells served as controls (Ctrl); DMSO (50% *v*/*v*) and SDS (1% *w*/*v*) were used as negative (Ctrl−) and positive (Ctrl+) controls, respectively. Experimental samples were treated with the tested compounds at MICs previously determined for each strain. Blanks containing PBS and the tested compound were included.

Samples were incubated at 37 °C with shaking, and OD_{600} was measured after 10, 30, and 60 min using a microplate reader. Bacterial lysis was expressed as a percentage according to the following equation:

$$\text{Bacterial lysis } (\%) = 100\% - \left(\frac{OD_{sample}}{OD_{Ctrl}} \times 100 \right)$$

3.9. Fluorescence-Based Viability Assay Using DAPI and PI

Bacterial inner membrane (IM) integrity was examined by fluorescence microscopy using DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) and propidium iodide (PI) as dual-staining probes, based on Di Girolamo et al. [66] and Porrello et al. [63].

3.10. Inhibition of Biofilm Development Assays

The antibiofilm effect of *H. manopappoides* essential oil was investigated against *Mycobacterium smegmatis* using a crystal violet (CV) staining assay adapted from Di Napoli et al. [67].

3.11. DPPH and H_2O_2 Scavenging Capacity Assay

The antioxidant capacity of *H. manopappoides* essential oil and its individual components was evaluated through two complementary assays: DPPH radical scavenging and hydrogen peroxide scavenging, following the methodology described by Napolitano et al. [68].

3.12. Eukaryotic Cell Culture

Human colorectal adenocarcinoma (Caco-2) cells were cultured as reported in Porrello et al. [63].

3.13. Cell Viability Assay

Caco-2 cells were splated in 96-well plates (30,000 cells/well) and allowed to adhere for 24 h. Cells were then treated for 24 h with *Helichrysum manopappoides* sample, eucalyptol, α-caryophyllene, or β-caryophyllene at the concentrations of the corresponding MIC values. Cell viability was subsequently assessed using a standard MTT assay as reported in Porrello et al. [63].

3.14. Measurement of Oxidative Stress

For the evaluation of intracellular oxidative stress, 3000 Caco-2 cells were seeded in 96-well plates and treated 24 h after seeding with *Helichrysum manopappoides* oil, eucalyptol, α-caryophyllene, and β-caryophyllene for 24 h at the indicated concentrations. Oxidative stress levels were analyzed as reported in Porrello et al. [63].

3.15. Statistical Analysis

Statistical analysis was performed using a two-tailed paired Student's *t*-test, suitable for comparing related sample groups. The analysis was conducted in Microsoft Excel (Microsoft Office 365), and results were considered statistically significant when *p*-values were less than or the same as 0.05. Data are expressed as mean $\pm$ standard deviation (SD) from at least three independent experiments.

4. Conclusions

This study provides the first comprehensive chemical and biological characterization of the essential oil obtained from steam distillation of the leaves of *Helichrysum manopappoides* Humbert, an endemic Malagasy species. GC–MS and NMR analyses revealed a sesquiterpene-rich essential oil composition, dominated by β-caryophyllene (19.78%), α-humulene (14.75%), and eucalyptol (7.38%), compounds commonly presented in *Helichrysum* essential oils. This study confirms how spectroscopic and spectrometric analyses, in combination, can ensure the correct identification of complex samples such as essential oils, and, in the future, it would be desirable to understand whether these compounds are markers of leaves only or are present in all parts of the plant. Thanks to its antimicrobial and antibiofilm properties, combined with the in vitro free radical scavenging capacity attributable to its individual constituents, *H. manopappoides* essential oil represents a novel source of bioactive compounds for the management or prevention of intestinal infections. The results obtained during this study may induce further pharmacological research on the oil from *H. manopappoides* and constitute a starting point for its applicative valorization. In addition, they should help to reinforce the conservation of the Malagasy biodiversity in general, and the *Helichrysum* species in particular, by taking into account their importance in local traditional medicine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants15050672/s1. Figure S1. GC-MS chromatograms (A, B, and C) of *Helichrysum manopappoides* Humbert essential oil; Figure S2. GC-MS chromatogram of pure *n*-alkanes (C_7–C_{30}).

Author Contributions: Conceptualization, N.B. and M.V.; methodology, G.C., A.S., M.V., D.D.G., N.B., M.V. and H.J.B.R.; software, G.C., N.B. and M.V.; validation, S.R.R., G.C., A.S., N.B. and M.V.; formal analysis, N.B., M.V. and A.Z.; investigation, S.R.R., G.C., N.B. and M.V.; resources, N.B. and M.B.; data curation, A.Z., N.B. and M.V.; writing—original draft preparation: S.R.R., V.E.R., G.C., A.S., N.B., A.Z. and M.V.; writing—review and editing, V.E.R., A.Z., M.B. and N.B.; visualization, V.E.R., A.Z. and M.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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

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