


Article

Antimicrobial Nanoformulations Based on *Schinus areira* Essential Oil

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Abstract: The goal of this research was to create an antibacterial formulation from *Schinus areira* essential oil (EO) that could spread in water. To achieve this, we developed liposomal formulations of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) or DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) that encapsulated the EO. In addition, we utilized the EO as a reducing and stabilizing agent to synthesize silver nanoparticles (AgNPs). The nanoformulations were characterized by determining their size and zeta potential. In the case of liposomal formulations, chemical composition, and encapsulation efficiency were also determined. Furthermore, antimicrobial activity studies against Gram-positive and Gram-negative model bacteria were carried out for both kinds of formulations. The results obtained showed the successful encapsulation of the *S. areira* EO in multilamellar liposomes of phosphatidylcholine with high efficiency. DPPC liposomes have proven to be a better encapsulation system, retaining more monoterpenes from the EO and therefore presenting antimicrobial activity against *S. aureus* with an minimal inhibitory concentration (MIC) value of 3 mg/mL of EO. On the other hand, it was also possible to obtain AgNPs by using *S. areira* EO, which showed antimicrobial activity against *S. aureus* and *E. coli* at low concentrations of EO, with MIC values of 6.68 µg/mL and 3.4 µg/mL of silver, respectively. The data obtained will contribute to enhancing the biotechnological value of natural products derived from native plant species in Argentina. This will be achieved through the generation of novel formulations with antibacterial activity and potential bioavailability.

Keywords: *Schinus areira*; liposomes; green synthesis of nanoparticles; essential oil; antimicrobials



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1. Introduction

Essential oils (EOs) constitute a complex mixture of chemical compounds derived from the secondary metabolism of plants. They can be synthesized in different parts, such as buds, flowers, leaves, stems, and fruits. The EO chemical composition includes compounds from the family of terpenes, terpenoids, aromatic, and aliphatic constituents, among others, all characterized by having a low molecular weight. The presence, yield, and composition of EO can be influenced by many factors, including climate, plant nutrition, and stress conditions [1,2]. Different techniques are used for its extraction, including steam distillation, solvent extraction, and extraction with supercritical fluids and subcritical water, among others [3]. EOs constitute only a small proportion of the wet weight of the plant

material, usually approximately 1% or less. The antimicrobial activity of EO has been reported against bacteria, yeasts, and fungi. This activity has been studied both in vitro and in vivo and could be related to the presence of different types of compounds, such as aldehydes, phenols, and terpenes, among others [4]. In turn, the EO efficacy as an antimicrobial agent depends on the nature, composition, and orientation of its functional groups [5]. The interest in EOs as antimicrobial agents has increased with the tendency to produce antimicrobials based on natural compounds, making EOs an alternative for the development of new treatments and control strategies for infectious diseases in different areas such as health and food [6,7].

In this regard, Aguaribay or *Schinus areira* L. (synonym: *Schinus molle* L. var. *areira* (L.) DC.), which grows naturally both in the central and northwestern regions of Argentina, showed many traditional uses as a purgative, diuretic, parasitocidal, insecticidal, vulnerary, and topical disinfectant, and for the treatment of rheumatism, stomach upsets, menstrual disorders, bronchitis, conjunctivitis, treatment of colds, and as an expectorant, among others [8–10]. In this sense, it has been used for obtaining EOs from leaves and fruits that showed antimicrobial activity against *Staphylococcus aureus* and clinically isolated methyl-resistant *S. aureus* [11,12]. Due to the probed antimicrobial activity of *S. areira*, in addition to its wide distribution in Argentina, we selected this species to obtain the EO for this work.

An issue that needs to be addressed for further applications of EOs is that they have volatile components that are sensitive to oxygen, light, humidity, and heat [13]. In this sense, different encapsulation systems have been studied for both the whole EO and certain bioactive compounds isolated from it, such as carvacrol, thymol, and eugenol, to improve their stability [14–16]. Different chemical, physical, and mechanical procedures have been described for this purpose [17]. Therefore, the study and development of different EO encapsulating systems offer a great opportunity for applications to several areas, such as food and drug products [18,19]. In particular, liposome encapsulation systems have been widely applied, as they are biodegradable, biocompatible, nontoxic, and nonimmunogenic, so they can carry hydrophobic, hydrophilic, and amphiphilic molecules, improving their pharmacokinetics, biodistribution, selectivity, and low toxicity [20,21]. Liposome-encapsulated EO from *Lavandula angustifolia* has been demonstrated to be active against *Candida auris* [22]. *Zhumeria majdae* EO loaded into nanoliposomes was active against different multidrug-resistant clinical pathogens [6]. Furthermore, multilamellar and unilamellar liposomes were able to carry the EO from *Zanthoxylum tinguassuiba*, improving thermal stability and bioactivity, and liposomes with clove EO showed selective antimicrobial activity against *S. aureus* [23,24].

As another approach, the green synthesis of silver nanoparticles (AgNPs) using different EOs as a reducing and stabilizing agent has been reported [25,26]. In recent years, nanomaterials have been widely studied for their applications in many fields due to their unique physicochemical properties compared to their bulk [27]. Metallic nanoparticles, especially those made of silver, have gained relevance as potential antimicrobial agents, with broad-spectrum activity against Gram-positive and Gram-negative bacteria [28]. Currently, the synthetic approaches to obtaining silver nanostructures could be classified into physical, chemical, and biological or green syntheses [29]. This last synthesis method uses natural reduction components, such as polysaccharides, microorganisms, and plant extraction compounds, among others [30,31].

In this context, the objective of the present work was to develop formulations based on the EO of *S. areira* leaves that are able to disperse in water. With this aim, we obtained liposomes with the EO encapsulated, and silver nanoparticles where the EO acts as a reducing and stabilizing agent. Afterward, we evaluated their antibacterial activity on *S. aureus* and *E. coli*. This study has important implications for expanding the biotechnological potential of the *S. areira* EO, as it represents the first study that describes the formulation and characterization of nanoformulations based on this EO.

2. Results and Discussion

2.1. Obtaining and Characterizing *S. areira* EO

In a previous study, the EO obtained from the leaves of *S. areira* (Aguaribay) located in Santiago del Estero (Argentina) showed antibacterial action against *S. aureus* [12]. In this way, a new stock of EO was obtained from the same trees. The extraction yield of *S. areira* EO was 1.09% (*v/w*). The composition of the obtained EO is shown in Table 1. Among the main compounds identified are 1-*epi*-cadinol (22.67%), α -pinene (9.33%), β -pinene (11.11%), β -caryophyllene (4.56%), alloaromadendrene (6.13%), *cis*- α -bisabolene (4.08%), (*Z,Z*)- α -farnesene (5.45%), and δ -cadinene (11.50%). The composition obtained shows differences from those reported for *S. areira* EOs obtained from other regions in Argentina; for example, α -pinene, limonene, and camphene were reported as the main compounds in samples collected in Córdoba [32]. In addition, the chemical composition of the EO obtained from the leaves of two *S. areira* specimens collected in Jujuy revealed that the predominant compounds were monoterpenes such as myrcene, limonene, β -phellandrene, α -phellandrene, sabinene, and camphene [11]. Moreover, there was a certain difference detected in the main chemical composition of the EO obtained in this work compared with the EO previously reported from the same trees [12]. There was a 33% reduction in the quantity of 1-*epi*-cadinol; a 10% reduction in allomandrene, α -bisabolol, and β -baryophyllene; and the absence of spathulenol. On the other hand, there was an increase of 84% in the amount of (*Z,Z*)- α -farnesene, and the presence of *cis*- α -bisabolene, α -pinene, and β -myrcene, which were otherwise absent in the previous EO extracted [12]. These results agree with the variation reported for other EOs obtained from the same specimen in different periods, as the EO composition depends not only on the geographical localization, but also on other factors, such as the nutritional conditions, climate, and stage [1]. This shows the importance of establishing stocks of known EO chemical compositions to develop rigorous scientific work.

Table 1. Chemical composition of the EO of *S. areira* leaves.

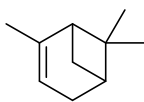
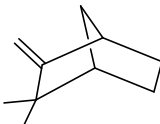
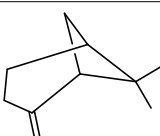
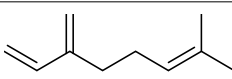
Compound ¹	RI ²	Literature RI ³	Area (%) ⁴
 α -pinene	931	931	9.33 \pm 0.07
 camphene	950	951	0.98 \pm 0.05
 β -pinene	984	984	11.11 \pm 0.16
 β -myrcene	990	990	2.78 \pm 0.03

Table 1. Cont.

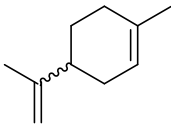
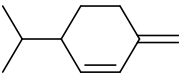
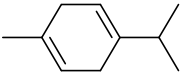
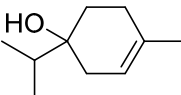
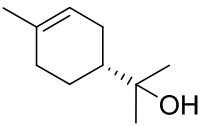
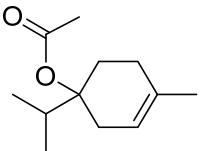
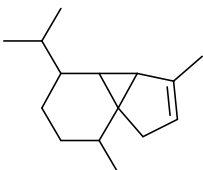
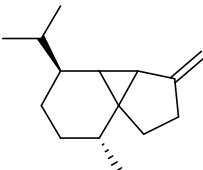
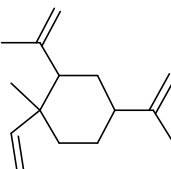
Compound ¹	RI ²	Literature RI ³	Area (%) ⁴
 limonene	1042	1040	0.66 ± 0.02
 β-phellandrene	1045	1045	1.79 ± 0.03
 γ-terpinen	1064	1064	0.15 ± 0.01
 1-terpinen-4-ol	1170	1170	0.13 ± 0.01
 α-terpineol	1198	1198	0.061 ± 0.003
 4-terpinenyl acetate	1330	1335	1.54 ± 0.07
 α-cubebene	1341	1341	0.10 ± 0.02
 β-cubebene	1388	1388	0.55 ± 0.01
 β-elemene	1393	1393	0.98 ± 0.02

Table 1. Cont.

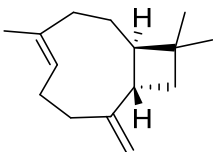
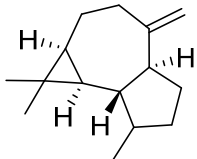
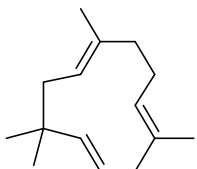
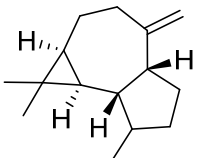
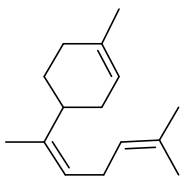
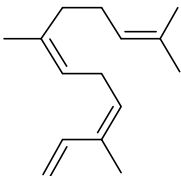
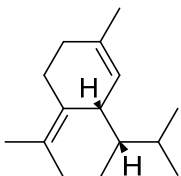
Compound ¹	RI ²	Literature RI ³	Area (%) ⁴
 β -caryophyllene	1437	1437	4.56 \pm 0.13
 aromadendrene	1460	1460	1.04 \pm 0.03
 α -humulene	1474	1474	1.11 \pm 0.21
 alloaromadendrene	1479	1479	6.13 \pm 0.12
 cis- α -bisabolene	1490	1493	4.08 \pm 0.15
 (Z,Z)- α -farnesene	1499	1499	5.45 \pm 0.23
 δ -cadinene	1533	1533	11.50 \pm 0.98

Table 1. Cont.

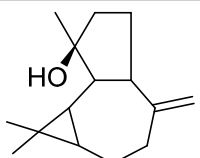
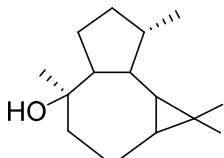
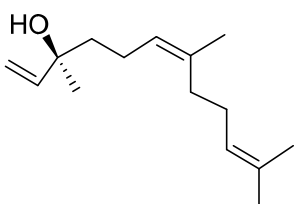
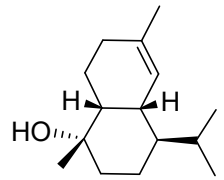
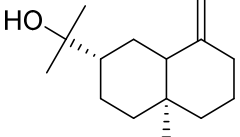
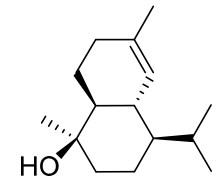
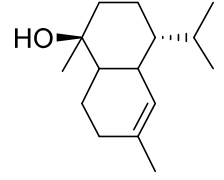
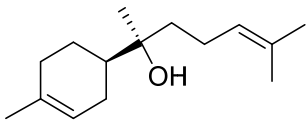
Compound ¹	RI ²	Literature RI ³	Area (%) ⁴
 spathulenol	1570	1570	2.46 ± 0.16
 viridiflorol	1597	1597	0.79 ± 0.02
 nerolidol	1623	1608	1.12 ± 0.02
 δ -cadinol	1634	1634	0.90 ± 0.01
 β-eudesmol	1642	1642	4.17 ± 0.13
 1-epi-cadinol	1653	1645	22.67 ± 0.34
 α-cadinol	1673	1673	0.62 ± 0.01

Table 1. Cont.

Compound ¹	RI ²	Literature RI ³	Area (%) ⁴
 α -bisabolol	1684	1684	3.25 \pm 0.17

¹ Compound identification based on RI, NIST 08 library, and bibliography. ² RI, experimental linear retention indices on a TR5-ms column. ³ Retention index extracted from NIST database. ⁴ Average value of three independent experiments.

2.2. *S. areira* EO Formulations

As was previously stated, despite the potential applications of EOs, their bioavailability is limited because they are not soluble in water [33]. In this context, the possibility of obtaining formulations based on *Schinus areira* EO that can disperse in water represents an significant milestone in the expansion of the potential applications of EOs. In this way, two different EO formulations were developed in this work, one using PC-liposomes and the other using silver nanoparticles with EO components in their capping.

2.2.1. Encapsulation of EO into Liposomes

Liposomes have been extensively studied as carriers of compounds, showing great potential as a delivery system for topical use. Among the methods used, the Bangham method, or thin-film hydration method, is one of the most widely used and simplest techniques for the formulation of liposomes [20,34]. Furthermore, several studies have used the thin film hydration method to prepare liposomes encapsulating EOs [20]. In this sense, MLV liposomes of DPPC/EO (1:1 *w/w*) and DMPC/EO (1:1 *w/w*) were obtained. Both liposome formulations showed high encapsulation efficiency (EE) values, being (90 \pm 8)% and (82 \pm 16)% for the DPPC/EO and DMPC/EO liposomes, respectively. These EE values are in good agreement with previous reports for other EOs using MLV liposomes of phosphatidylcholine [34,35]. Regarding the chemical profile of the encapsulated EO, monoterpenes, such as terpineol, myrcene, α -pinene, and β -pinene, were found in a significantly lower proportion or were absent in liposomal preparations (Figure 1A). As the liposomal formulation involves a step of solvent displacement for the lipid film preparation, this procedure could produce a degradation or volatilization of some of the compounds mentioned above. Similar results were described for nanoformulations based on EO produced from *Lavandula x intermedia*, in which there was a reduction in the relative quantity of pinene and myrcene in the liposomal formulation obtained [36]. In addition, the method used for the EO liposomal formulation requires a hydration step at 50 °C, which could promote the loss of more volatile compounds, as described by Hammoud et al. during the encapsulation of α -pinene in Phospholipon 90 H liposomes [37]. Although both lipid formulations showed a reduction in monoterpenes, DPPC was able to retain significantly more monoterpenes than DMPC (Figure 1B). This could be associated with the fact that compounds such as pinene, being highly lipophilic compounds, are expected to be entrapped in the lipid membrane, so the strength of their interaction influences their incorporation into the vesicles. Furthermore, it was previously reported that monoterpenes such as α -pinene show an affinity for the hydrophobic region of the lipid membrane due to their high hydrophobicity and chemical structure that does not contain polar groups [38]. This interaction would be weaker in the case of DMPC lipids since their hydrocarbon chain length is shorter (14 C) than that of DPPC (16 C), thus offering a smaller hydrophobic interaction surface. On the other hand, compounds such as 1-epi-cadinol that are larger and more polar (have an OH group) could interact better with hydrophobic and hydrophilic phospholipid groups in the liposome bilayers; therefore, the liposome is enriched with these compounds, increasing its relative proportion in comparison with pure EO (Figure 1A).

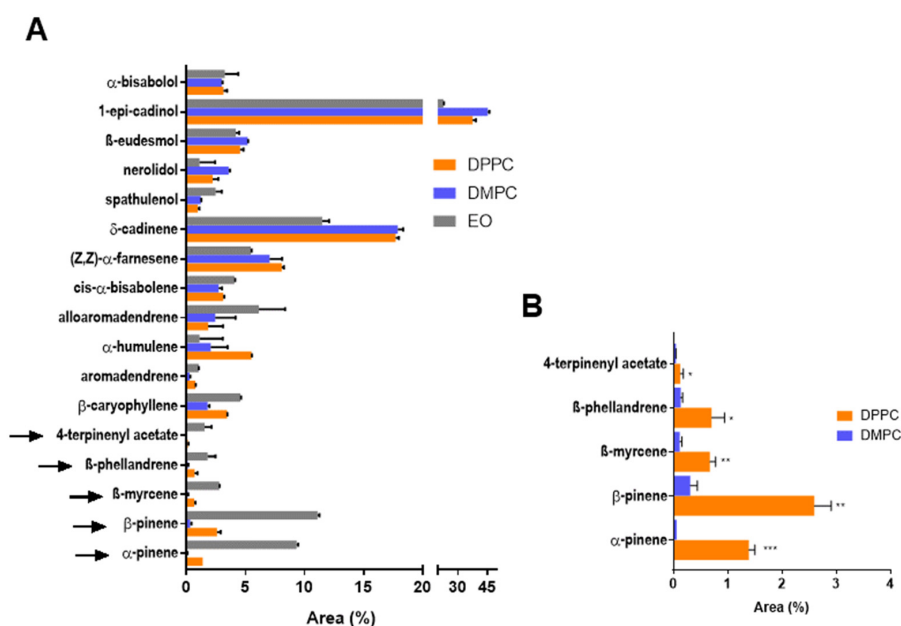


Figure 1. Chemical profile of major components of pure *S. areira* EO and EO liposomal formulations (A). Chemical profile details of monoterpenes (with arrows in Figure 1A) on both liposomal formulations (B). Each determination was made for three EO liposomal formulations. The error bars indicate the standard deviation of the averaged values. *** $p > 0.001$; ** $p > 0.01$; * $p > 0.05$ *t* Student no parametric.

The formulations generated were stored under refrigeration for 15 days. After this period, the EE was determined again for both formulations. There was a relative decrease in EE in comparison with that obtained at the initial time of 16% for DPPC/EO liposomes. On the other hand, almost no difference was observed in DMPC/EO liposomes. In both cases, the chemical composition of the encapsulated EO remained unchanged.

2.2.2. Green Synthesis and Characterization of AgNPs

Another approach for obtaining *S. areira* EO-based nanoformulations was to obtain AgNPs by green synthesis using the EO as a biocomposite. The progression of the synthesis was followed by a color change in the solution from colorless to pale brown (Figure 2A), indicating the formation of AgNPs. The UV-Vis spectrum was registered between 300 and 800 nm (Figure 2B), showing the absorption maximum wavelength at 432 nm, which corresponds to the characteristic surface plasmon resonance absorption of spherical AgNPs. This plasmon absorption band in the visible light region is typical for AgNPs, and the spectral position of the absorption maximum and the bandwidth are determined by the size and shape of the metal nanoparticles [39].

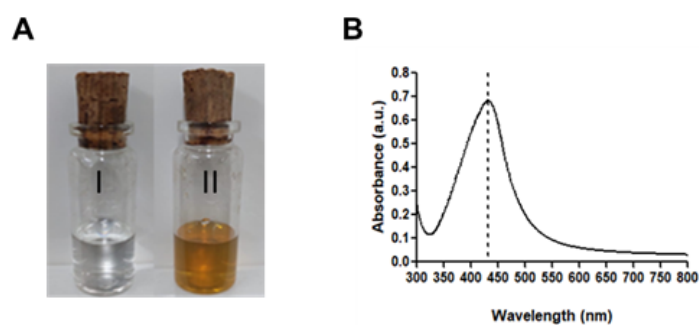


Figure 2. Synthesis of AgNPs with the EO of *S. areira*. (A) Color change of AgNO₃ solution (I) after AgNP formation (II). (B) UV-Vis spectra of AgNPs. The dotted line indicates the wavelength of maximum absorption.

2.2.3. Size and Zeta Potential of Formulations

The obtained liposomes were characterized in terms of their size and zeta potential. In the case of DPPC, DPPC/EO liposomes showed a size ((230 ± 54) nm) significantly lower than the control liposomes ((1267 ± 256) nm). After 15 days of refrigerated storage, the formulation sizes did not change significantly, with a small reduction in the size of both, control and EO-containing liposomes (Figure 3A).

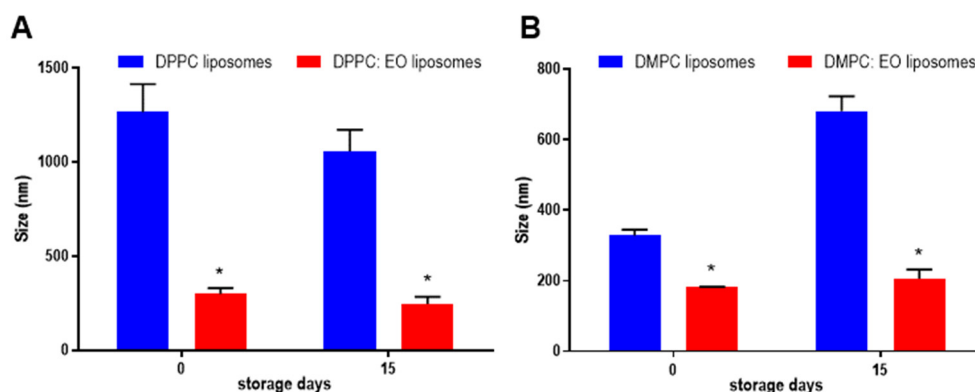


Figure 3. Determination of liposomal formulation size by DLS: (A) DPPC and DPPC/EO liposomes; and (B) DMPC and DMPC/EO liposomes. The data represent an average of three independent measurements, and the error bars indicate the standard deviation of the averaged values. * $p < 0.05$. t Student no parametric.

Liposomes of DMPC/EO also presented a significant size reduction ((182 ± 3) nm) compared with the DMPC liposomes ((329 ± 28) nm). The smaller size found for DMPC in comparison with DPPC liposomes could be attributed to the shorter hydrocarbon chain of DMPC, which implied a lower packing parameter that can favor the formation of smaller particle diameters [40]. After storage, the DMPC/EO liposome size ((206 ± 45) nm) was similar to that obtained at the initial time, and the control DMPC liposomes showed a significant increase in their size ((682 ± 72) nm), which could be produced by the fusion events during storage.

In summary, both types of liposome formulations carrying EOs showed smaller sizes than the controls (Figure 3B). The decrease in the size of liposomes could be due to the presence of hydrophobic compounds of EOs that produce a greater cohesion of Van der Waals forces between phospholipid hydrocarbon chains, favoring a greater packing in lipid membranes and an increase in the surface curvature of liposomes, as was described for monoterpenes encapsulated in soybean phosphatidylcholine [20].

The zeta potential values of the liposomal formulations of DMPC/EO, DPPC/EO, and the corresponding controls were also determined by DLS measurements. The zeta potential of DPPC/EO liposomes ((-14 ± 5) mV) was significantly lower than that obtained for control DPPC liposomes ((-5 ± 3) mV). This behavior would be related to the exposure of negative charges or ionizable molecular groups of EO components, such as spathulenol, nerolidol, viridiflorol, δ -cadinol, β -eudesmol, α -cadinol, 1-epi-cadinol, and α -bisabolol, on the lipid–water interface of the vesicles obtained, as was reported for other EOs [18,41]. After 15 days of storage, DPPC/EO liposome zeta potentials turned into less negative values ((-6 ± 2) mV), showing values closer to those obtained for the control DPPC liposomes (Figure 4A). These facts would be a result of the partial release of EO compounds into the media, as the EE assays showed.

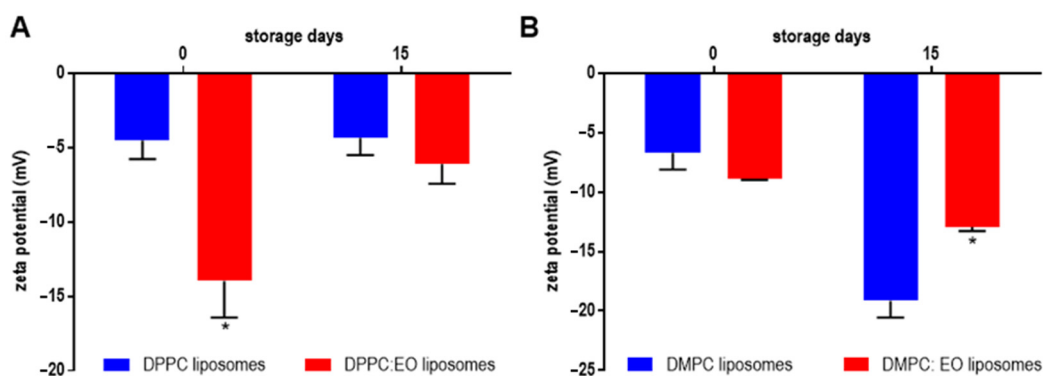


Figure 4. Determination of zeta potential by DLS: (A) DPPC and DPPC/EO liposomes and (B) DMPC and DMPC/EO liposomes. The data represent an average of three independent measurements, and the error bars indicate the standard deviation of the averaged values. * $p < 0.05$. t Student no parametric.

In the case of DMPC/EO and DMPC control liposomes, the zeta potential values were (-8.9 ± 0.1) mV and (-7 ± 2) mV, respectively, at the initial time. On the other hand, there was a decrease in zeta potential values after 15 days of incubation. The DMPC/EO and DMPC liposomes presented zeta potential values of (-12.9 ± 0.6) mV and (-19 ± 2) mV, respectively, which denote a significant difference between both types of liposomes (Figure 4B). These data would indicate a reorganization of the EO components into liposomes, exposing more hydrophilic chemical groups to the lipid–water interface over time. On the other hand, these surface parameter changes do not imply changes in the content of the EO chemical components or the size of the vesicles, taking into account the size values and EE of DMPC/EO liposomes during the storage time.

In the case of AgNPs, TEM microscopy confirms the presence of quasi-spherical nanoparticles with an average diameter of (15 ± 5) nm (Figure 5) and a zeta potential value of (-45 ± 7) mV. The net negative charge exhibited by AgNPs may be due to the exposure at the surface of the nanoparticle of different components present in the EO of *S. areira*, in agreement with results obtained in DPPC liposomes. This negative capping confers colloidal stability to the AgNPs, preventing their agglomeration. To the best of our knowledge, this is the first report of AgNPs obtained using *S. areira* EO as a source of reducing and stabilizing agents.

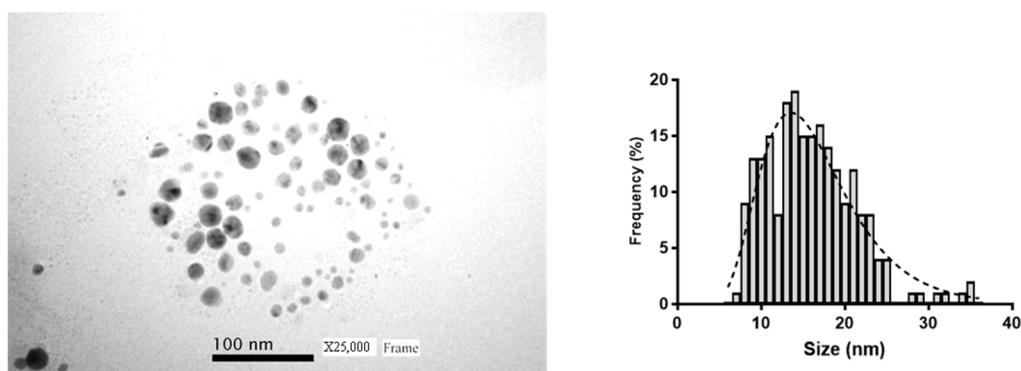


Figure 5. TEM images and frequency size distribution histograms of AgNPs obtained in this work. Histograms were obtained from more than 100 AgNP size determinations of different images. Dashed lines represent the Gaussian fitting of the data.

The compounds present in the EO of *S. areira* were responsible for the reduction in Ag^+ to Ag^0 to obtain AgNPs. Other authors have also shown the ability of the EO of *Myristica fragrans* to synthesize AgNPs using a methodology similar to that of the present

work [25]. It is interesting to note that *M. fragrans* EO is composed, among other substances, of limonene and pinene, also present in the EO of *S. areira*. In addition, AgNP synthesis based on oil–water interface methods has also been reported with the EO of *orange peel*, whose key component is limonene [26]. The data obtained in the present work and those described by other authors indicate limonene as a common component in the EO used for the green synthesis of AgNPs, so it could play a key role in the silver reduction process, although more studies are necessary to corroborate it.

2.3. Antimicrobial Activity of the Formulations

The pure *S. areira* EO showed antibacterial activity against *S. aureus* but not against *E. coli*, in good agreement with a previous report [12]. The MIC and MBC values were 0.3 mg/mL for pure EO. The fact that EO has the same MIC and MBC values implies that this EO exhibits bacteriostatic and bactericidal action against *S. aureus* [42]. This obtained MIC value is greater than that which was previously reported by us [12]. The antimicrobial activity of *S. areira* EO reported previously by other authors against *S. aureus* showed a wide dispersion of results. Regarding the MIC values, for example, the work of Celaya et al. using EO from plants located in Jujuy, Argentina reported significantly low MIC values (6–30 µg/mL) [11], but Onder et al., who tested the EO of *S. areira* isolated from plants located in Turkey, obtained high MIC values in the order of 2 or 4 mg/mL [43]. Martins et al., who obtained the EOs from plants located in Portugal, reported MIC values more similar to that reported in this work, with an MIC value of 125 µg/mL for the same reference strain of *S. aureus* [44]. These differences should be ascribed, as was previously pointed out, to the differences in the profiles of compounds in each EO.

Afterward, the antimicrobial action of liposomes and AgNPs carrying EO was assayed. Liposomal formulations of DPPC/EO showed antibacterial action against *S. aureus* but not against *E. coli*, in agreement with the EO results. However, the MIC value was 3 mg/mL. This value was almost one order of magnitude higher than that for the pure EO. On the other hand, the DMPC/EO liposomes did not show antibacterial activity against either *S. aureus* or *E. coli*, even at the highest tested concentration (3 mg/mL of EO used).

In turn, no antibacterial activity against *E. coli* or *S. aureus* was detected in control experiments with pure DMPC and DPPC liposomes.

Besides the different reports on the antimicrobial activity of *S. areira* EO, as far as our knowledge, there is no report of liposomal encapsulation of this EO with an antimicrobial propose. However, different encapsulation approaches in liposomes were evaluated for other EOs [45]. Although many works reveal that liposomal encapsulation could boost the antimicrobial action of EOs, some studies, such as that by Varona et al., showed particularly for *S. aureus* that the encapsulation of EOs from *Lavandula hybrid* reduced the antimicrobial action. The authors suggest that the peptidoglycan layer of Gram-positive bacteria may hinder the action of the formulations on Gram-positive strains [46]. Additionally, Kherzi et al. showed that the encapsulation of *Mentha pulegium* EO on nanostructured lipid carriers increases the MIC value for *S. aureus* in comparison with the free EO [47].

The lower antibacterial activity found in the present work may also be because the EO components are encapsulated in a multilamellar system that has a strong interaction with phospholipids, as could be inferred from the size values obtained immediately after encapsulation and the EE values. In the particular case of DPPC/EO liposomes, they present a differential interaction with EO compounds that could favor EO availability. However, the EO–phospholipid interaction may affect the release kinetics, which could result in a lower effective EO concentration.

On the other hand, in DMPC/EO liposomes, EO–phospholipid interactions that could be favored by the phase state of lipid membranes seem to be more strong and stable. Moreover, it is necessary to take into account that certain monoterpene compounds, as mentioned above, were reduced to a greater extent during encapsulation in DMPC liposomes in comparison with DPPC ones. This fact could indicate that these compounds would contribute to the antimicrobial activity observed. It is important to note that some

of those compounds, such as α -pinene, β -pinene, and myrcene, have been previously reported to be responsible for antibacterial activity in other EOs [48–51].

Since only DPPC/EO liposomes have shown antibacterial activity, the evaluation of antibacterial activity by the plate diffusion technique of the whole formulation, the pellet, and the supernatant was carried out only in this formulation. The pellet and whole formulation showed antimicrobial action against a lawn culture of *S. aureus*, while the supernatant did not, confirming that the antibacterial activity observed was from the EO encapsulated in liposomes (Figure 6).

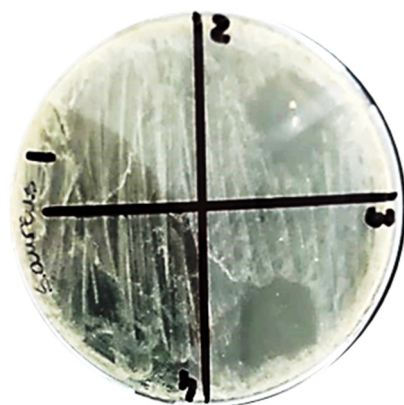


Figure 6. Antibacterial activity of DPPC/EO liposomes evaluated using the agar diffusion technique: (1) PBS buffer; (2) whole formulation; (3) pellet of the formulation; and (4) supernatant of the formulation.

Afterward, the antibacterial activity of AgNPs synthesized with the EO of *S. aureus* was evaluated. The MIC and MBC values are listed in Table 2. For the sake of comparison, chemical AgNPs made with citrate were also included, as this chemical synthesis is probably the most typical among all synthetic strategies [52]. Both kinds of AgNPs (with citrate and with EO) showed high activity with lower MIC and MBC values for both tested bacteria. In agreement, the plate diffusion assay against both bacteria confirmed the antibacterial activity of the AgNPs obtained (Figure 7).

Table 2. MIC and MBC values for EO-based formulations and citrate AgNPs used in this study.

Formulation ¹	<i>E. coli</i>		<i>S. aureus</i>	
	MIC	MBC	MIC	MBC
Pure EO ¹	-	-	0.3 mg/mL	0.3 mg/mL
DPPC/EO ¹	-	-	3 mg/mL	-
DMPC/EO ¹	-	-	-	-
AgNPs with EO ²	6.68 μ g/mL	26.75 μ g/mL	3.4 μ g/mL	6.8 μ g/mL
AgNPs with citrate ²	3.4 μ g/mL	26.75 μ g/mL	3.4 μ g/mL	26.75 μ g/mL

¹ Expressed as mg of EO/mL; ² expressed as μ g of silver/mL.

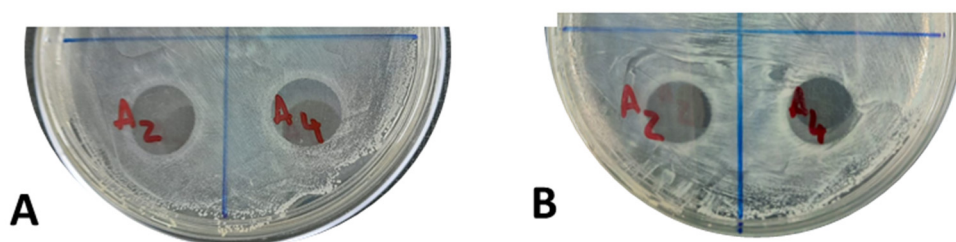


Figure 7. The antibacterial activity of AgNPs using the agar diffusion technique: (A) *E. coli* and (B) *S. aureus*. A2 and A4 represent different batches of AgNPs.

The results obtained are consistent with those published by other researchers that demonstrate the broad-spectrum antibacterial activity of AgNPs obtained by green synthesis [53,54]. Furthermore, previous work has demonstrated that nanoparticles obtained with EOs showed a much higher activity than the EO used for the synthesis, as we found in this work [25,55]. In this way, Vilas (2014) reported a differential activity of AgNPs in Gram-negative and Gram-positive bacteria, with more effectiveness in the *E. coli* strain evaluated, attributing this discrepancy to the bacterial membrane structure [25]. In the present work, with the chemical AgNPs, no differences were observed in the action against *E. coli* and *S. aureus*. On the other hand, for the AgNPs synthesized with the EO, both MIC and especially MBC values were lower against *S. aureus*, indicating that the AgNPs obtained with the EO were more active on this Gram-positive strain. It should be emphasized that, as noted above, the EO used for the AgNP biosynthesis is only active against *S. aureus*. In this sense, an additive effect may occur due to the presence of both silver and EO components of *S. areira* in the nanoparticle. Additionally, it should be pointed out that in previous work [12], the interaction of some EO components of *S. areira* with the *S. aureus* envelope was demonstrated. Therefore, some of the components present in the capping of the AgNPs could more efficiently drive the nanomaterial to the bacterial surface, increasing its antibacterial activity. It was previously reported for other metallic nanoparticles synthesized with EO, a synergic action of both the bioreductant (i.e., EO) and the metal ions, resulted in the excellent antibacterial action of the phytocapped NPs [56]. Overall, the use of EO-derived nanoparticles offers a viable method for reducing the EO dosage, circumventing their limitation, and enhancing their long-term stability [57].

3. Materials and Methods

3.1. Materials

The lipids DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) were both obtained from Avanti Polar Lipids (Alabaster, AL, USA). The medium for all liposomal formulations was PBS buffer (20 mM, pH = 7.40). The water used was ultra-pure, with a conductivity of $0.002 \pm 0.001 \mu\text{S}/\text{cm}$ and a pH = 5.0. The organic solvents used were chloroform, methanol, absolute ethanol, dichloromethane, and acetone obtained from Merck Química (Ciudad De Buenos Aires, Argentina), all of the analytical grade. Silver nitrate and sodium citrate were purchased from Cicarelli (Buenos Aires, Argentina).

3.2. Obtaining Essential Oil and Chemical Characterization

The obtaining and chromatographic analysis of the EO was the same as described by Cutró et al., 2021 [12]. In summary, the EO was obtained by hydrodistillation from leaves of *S. areira* collected from Villa El Zanjón, Santiago del Estero, Argentina (27°52'21.5" S 64°14'33.2" W) [12]. The EO was analyzed in a chromatograph GC Konik 3000 series equipped with a ZB-5 capillary column (30 m \times 0.25 mm \times 0.25 μm) (Phenomenex, Inc., Torrance, CA, USA) and by gas chromatography-mass spectrometry (GC-MS) using a Thermo Scientific Focus GC coupled with a DSQ II electron ionization mass detector. A TR-5MS capillary column (30 m \times 0.25 mm \times 0.25 μm) (Thermo Fisher Scientific) (Waltham, MA, USA) was used. The compounds were identified using the calculated retention indices and their mass spectra analysis. Mass spectra were compared with the NIST 08 library and the spectral data reported in the literature [58].

3.3. Encapsulation of EO into Liposomes

Multilamellar (MLV) liposomes were prepared according to the thin-film hydration method [59]. Briefly, 10 mg of DPPC or DMPC was dissolved in 1 mL of chloroform. Two aliquots of 500 μL were taken, and 5 mg of *S. areira* EO was added to one of them using a Hamilton syringe (controlled by weighing on an analytical balance), ensuring that the mass ratio of EO to lipids was 1:1 *w/w*. After that, the solvent was evaporated under reduced pressure at room temperature for 45 min on both aliquots. The lipid films obtained were

hydrated in a PBS buffer that had previously been filtrated and sterilized; the temperature was maintained at 50 °C for 1 h with vortex agitation.

3.4. Encapsulation Efficiency (EE)

The encapsulation efficiency (EE) of *S. areira* EO was determined by GC-MS spectrometry. The major component of *S. areira* EO (1-epi-cadinol) was chosen as a reference for the determination of encapsulation efficacy. The liposomal suspensions were centrifuged at 13,000 rpm for 30 min at 10 °C. The sediment was then separated and dissolved in 1 mL of absolute ethanol. The loading efficiency is expressed as a percentage of the total amount of *S. areira* EO found in the MLVs at the end of the preparation procedure. For its calculation, the following equation was used:

$$EE = \frac{EO_e}{EO_t} \times 100$$

where EO_e is the total amount of EO in the pellet (determined after rupturing the vesicles with absolute ethanol) and EO_t is the total amount of EO initially added [60].

3.5. Green Synthesis of AgNPs and Characterization

The synthesis was carried out following previous work [25] with modifications. Several synthesis parameters were tested: the concentration of metallic precursor solution ($AgNO_3$), temperature, reaction volume, and the ratio between metallic precursor and reducing and stabilizing agent ($AgNO_3/EO$) for a reproducible synthesis. Briefly, EO diluted in acetone (1:170 *v/v*) was added to a boiling 1 mM $AgNO_3$ solution at a ratio of 1:10 with constant stirring until the color changed from colorless to pale brown. The formation of AgNPs was monitored by UV-Vis spectroscopy using a Unico S2150UV spectrophotometer (NJ, USA). For comparison, a chemical synthesis was also carried out using citrate by the Turkevich method, following the protocol described by Gorup et al. with modifications [61]. Briefly, a volume of 50 mL of an aqueous solution of silver nitrate (1 mM) was heated to 90 °C with agitation. When the solution reached that temperature, 5 mL of dehydrated sodium citrate solution (10 mM) was added drop by drop. The reaction was maintained with constant heating and stirring for 10 min. The synthesis of nanoparticles was evidenced by a color change of the solution to light yellow.

3.6. Size and Zeta Potential Measurements of EO Formulations

The particle size distribution of liposomal formulations was evaluated at 25 °C by dynamic light scattering (DLS) on an SZ-100 nanoparticle analyzer (Horiba Ltd. Japan) equipped with a scattering detection DPPS laser ($\lambda = 532$ nm) at 90° using disposable polystyrene cells. Utilizing the CONTIN technique, normalized intensity autocorrelation functions were examined [62], yielding a distribution of diffusion coefficients (D). Afterward, the hydrodynamic diameter (D_h) was calculated from the obtained D by using the Stokes–Einstein relationship:

$$D_h = \frac{kT}{3\pi\eta D}$$

where k is the Boltzmann constant, T is the absolute temperature, and η the medium viscosity. In light scattering intensity distributions, the peak with the highest scattered light intensity was used to calculate the D_h of the sample. For all calculations, normalization by the polydispersion distribution and scattering light intensity mode were selected from the software's options.

Due to the fact that the vesicles are multilamellar (MLV), the samples exhibit high polydispersion, as can be inferred from the histograms of both samples (Figure S1). In the case of AgNPs, the size and shape were characterized using a JEM-JEOL 1120 EXII transmission electron microscope. Size distribution histograms of AgNPs were obtained from TEM images using the IC measure software v. 2.0.0.286.

The zeta potential value of both liposomes encapsulating EO and AgNPs by green synthesis was also determined by DLS. The measurement volume was 600 μL , and 10 determinations (with 500 runs each) were performed for each sample. The applied voltage and filters were set automatically, and the temperature was 25 $^{\circ}\text{C}$. The zeta potential was calculated from the cell mobility by using the Smoluchowski model. In the case of liposomal formulations, control experiments were carried out with liposomes without EO.

3.7. MIC and MBC Determination

Reference strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were employed. To carry out all the tests, strains from fresh culture were grown in Muller Hinton broth (MH broth, Britania, Argentina) at 37 $^{\circ}\text{C}$. The minimal inhibitory concentration (MIC) was determined by a standard microdilution assay according to CLSI recommendations [63]. After the MIC determination of both liposomal formulations and AgNPs, aliquots of 20 μL from all the wells that showed no visible bacterial growth were seeded on MH agar plates and incubated for 24 h at 37 $^{\circ}\text{C}$. When 99.9% of the bacterial population was killed by the lowest concentration of an antimicrobial agent, it was termed the minimal bactericidal concentration (MBC) endpoint.

3.8. Evaluation of Antibacterial Activity by the Plate Diffusion Technique

The antibacterial activity of both liposomes of DPPC/EO and AgNPs was also tested using the standard plate diffusion technique. A total of 100 μL of bacterial suspension ($\text{DO}_{600} = 0.05$) was uniformly spread on MH agar plates using a sterile Digrafsky loop. Then, a drop with 10 μL of the compound to be evaluated was placed on MH agar plates and incubated at 37 $^{\circ}\text{C}$ for 24 h. In the case of DPPC/EO, the culture plates were divided into four quadrants, and the samples were seeded as follows: 10 μL of sterile PBS buffer; 10 μL of DPPC/EO MLVs suspension; 10 μL of the supernatant after centrifugation at 5000 rpm for 2 min of the initial suspension of DPPC/EO MLVs; and 10 μL of the pellet resuspended in sterile PBS. In another plate, 10 μL of the AgNPs from two different synthesis batches were evaluated.

3.9. Reproducibility and Statistical Treatment of Data

All measurements were carried out in triplicate, and values were statistically analyzed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

4. Conclusions

The results presented in this work show that the EO of *S. areira* leaves could be used to obtain nanoformulations able to be dispersed in water. In the case of lipid formulations, only DPPC/EO liposomes showed antibacterial activity against *S. aureus*. The results obtained suggest that the lipid used for the formulation has an important impact on the profile of the EO encapsulated compounds, with a key effect on the antimicrobial activity, indicating the importance of not only testing the biological activity, but also determining the chemical profiles of the EO-encapsulated formulations in comparison to the pure EO [64].

On the other hand, it was possible to obtain AgNPs by the green synthesis method by using very low quantities of EOs as a source of bioreducing and biostabilizing agents. This formulation has shown stability and antibacterial activity not only against *S. aureus*, but also against *E. coli*.

Overall, two active formulations with antibacterial activity were obtained based on *S. areira* EO, such as DPPC liposomes encapsulating EO and AgNPs obtained by green synthesis. Besides the fact that further experiments should be made to characterize the mode of action of each formulation, these results represent an important milestone for obtaining novel nanomaterials based on natural compounds with high antimicrobial activity that take advantage of EOs and overcome some of the limitations of their free-form.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ddc2020026/s1>, Figure S1. Size distribution of MLV of DPPC or DMPC.

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