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Binary Combinations of Essential Oils: Antibacterial Activity Against *Staphylococcus aureus*, and Antioxidant and Anti-Inflammatory Properties

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Abstract: *Background:* The lack of new antimicrobial drugs and the increased antimicrobial resistance has focused the attention on the employment of essential oils (EOs), particularly in veterinary medicine. *Methods:* EOs from *Origanum vulgare* L., *Juniperus communis* L., *Cistus ladaniferus* L., *Citrus aurantium* L. var. *amara* were tested individually and as binary combinations to study: the *in vitro* antibacterial activity against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), and *Escherichia coli*; the antioxidant capacity by redox-based assays (DPPH, ABTS and FRAP) and the *in vitro* anti-inflammatory activity by bovine serum albumin (BSA) denaturation inhibition assay. *Results:* A good antibacterial activity was observed for *O. vulgare* L. against all strains (MIC = 0.0312%–0.125%, v/v), followed by *C. ladaniferus* L. *O. vulgare* L. also provided the best results in terms of antioxidant and anti-inflammatory activity. Synergistic and additive effects were observed for the EO combinations, *O. vulgare* L./*C. ladaniferus* L. and *O. vulgare* L./*J. communis* L. against *S. aureus* and MRSA, respectively, confirmed also by the reduction of bacterial biofilm, and by antioxidant and anti-inflammatory activities. *Conclusions:* The results suggest that EO combinations may a be promising strategy in veterinary settings for the treatment of infectious diseases caused by *S. aureus*, including drug-resistant and biofilm-forming strains accompanied by oxidative stress and inflammation.

Keywords: essential oils; antibacterial activity; antioxidant activity; anti-inflammatory activity; binary combinations; FIC index; animal infections

1. Introduction

The current lack of new antibacterial drugs, coupled with the continued rise in antimicrobial resistance (AMR), poses a critical problem for global health. To effectively address this challenge requires a One Health approach, an integrated strategy that recognizes the connection of human, animal, and environmental health [1–3].

Combating AMR is critical for several reasons: to safeguard human health, to prevent the emergence and spread of antibiotic-resistant bacteria, to preserve the effectiveness of antimicrobials used in human and veterinary medicine, and to minimize the presence of antibiotic residues in animal-derived food products.

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Given the increasing emergence of drug-resistant bacteria responsible for infections in animal farms, such as mastitis, mammary pustular dermatitis and skin infections, there is an urgent need to explore natural and alternative therapeutic approaches.

In recent years, the attention of many researchers has focused on the study of natural products as a support to the conventional antibiotic therapy. Among various plant-derived secondary metabolites, essential oils (EOs) are widely used in food, cosmetic and pharmaceutical industries (as flavourings, perfumes and fragrances) [4], as well as in the aromatherapy [5].

EOs are complex mixtures of volatile compounds, primarily terpenes, along with aldehydes, alcohols, and esters [6]. These bioactive compounds play a crucial role in plant defense because of their antimicrobial properties but also offer significant therapeutic potential for human and animal health. They have been used in folk medicine for their beneficial properties in treating dermatological disorders, infections, inflammation and pain [7,8]. Numerous studies have documented the antimicrobial, antifungal, antioxidant, and anti-inflammatory properties of various EOs, with particular emphasis on species originating from the Mediterranean region [9]. Among these, EOs from *Cistus ladaniferus* L., *Citrus aurantium* L. var. *amara*, *Juniperus communis* L., *Origanum vulgare* L., are of traditional and new interest due their known and well-documented biological properties such as antibacterial, antioxidant, anti-inflammatory, analgesic, antispasmodic, angiogenic, antiplatelet, antimutagenic and antigenotoxic [10–17].

In more detail, *Cistus ladaniferus* L. (Cistaceae family) shows potential hypoglycemic, hypolipidemic, antihypertensive activities [18–20]. *Citrus aurantium* L. var. *amara* (Rutaceae family), a major crop in Mediterranean regions, is employed also as a sedative, due to its soothing and calming effects [21], and as a natural antiseizure and anticonvulsant agent [22]. *Juniperus communis* L. (Cupressaceae family), widely distributed across Europe, Asia, and North America, is used as a diuretic, and for digestive disorders [23], and exhibits also hypoglycemic, hypolipidemic, and hepatoprotective properties [24]. *Origanum vulgare* L. (Lamiaceae family), one of the most used medicinal plant in infection of the respiratory tract, is also a valid antiparasitic, digestive and antispasmodic agent; in addition, it shows hypoglycemic effects [25].

The study of the pharmacological activities of EOs is of growing interest also in veterinary medicine. In literature there are various *in vitro* studies on the efficacy of EOs against common bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, which are often responsible for mastitis in bovine [26] and sheep [27], mammary pustular dermatitis in ovine [28], exudative epidermitis in pigs [29], bovine respiratory disease [30], cattle endometritis [31]. EOs have shown efficacy also against bacteria isolated from milk samples of mastitic sheep [32] and pathogens isolated from animal-derived products [33]. These data provide a valuable incentive for *in vivo* studies, which are currently too scarce, for potential applications in veterinary medicine [34].

Although the potential therapeutic properties of EOs are well known, recently the attention has been focused on the use of their combinations, in order to enhance their efficacy, reduce the minimum active dose and moderate the possible adverse side effects [35]. The association between EOs can be a valid natural strategy against pathogens responsible of infectious diseases in humans and animals [36] and also against foodborne pathogens [37].

The aim of this study was to evaluate the *in vitro* antibacterial, antioxidant and anti-inflammatory properties of the EOs of *Cistus ladaniferus* L. (Cistus), *Citrus aurantium* L. var. *amara* (Bitter orange), *Juniperus communis* L. (Juniper) and *Origanum vulgare* L. (Oregano), in binary combinations, exploring their potential synergistic or additive effect for possible use against *S. aureus*, a clinically significant Gram-positive pathogen, responsible for common animal infections.

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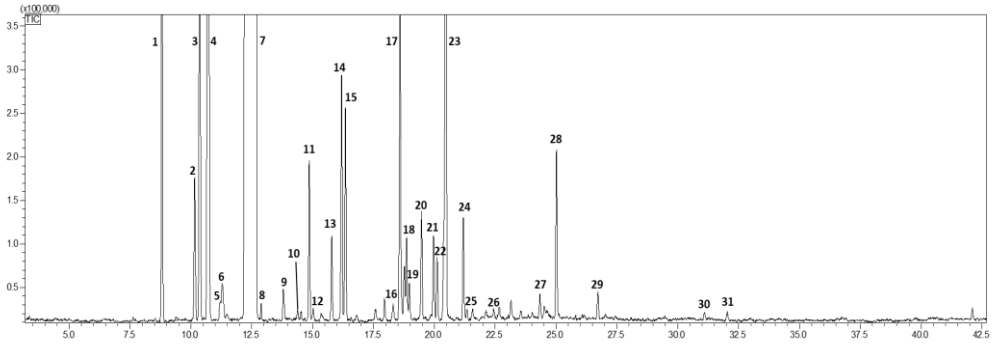
2. Results

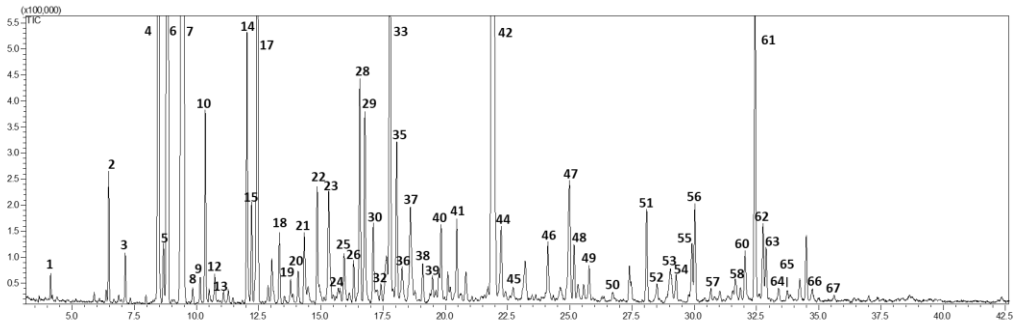
2.1. GC-MS Chromatographic Analysis

The detailed composition of *Citrus aurantium* L. var. amara L., *Cistus ladaniferus* L., *Juniperus communis* L. and *Origanum vulgare* L. EOs obtained through GC-MS are reported in Figure 1 and Table 1.

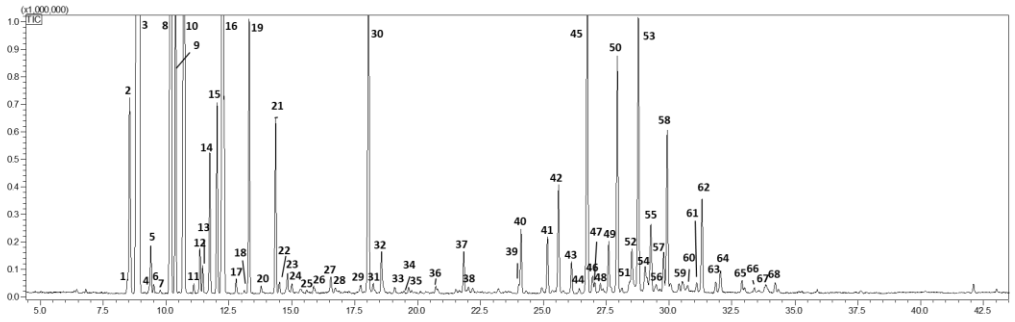
As it's possible to observe, the main components for *Citrus aurantium* L. var. *amara* are limonene (87.87%), followed by myrcene (3.45%) , linalyl acetayte (1,84%) and β-pinene (1.12%); for *Cistus ladaniferus* L. camphene (37.04%), bornyl acetate (21.93%), α-pinene (13.68%), followed by tricyclene (5.08%), 2,2,6-trimethylcyclohexanone (3.95%), borneol (1.65%) and viridiflorol (1.03); for *Juniperus communis* L. α-pinene (42.01%), followed by sabinene (11.66%), myrcene (10.72%), limonene (6.36%), terpinen-4-ol (2.97%) , Germanen D (2.04%), (E)-caryophyllene (1.89%), α -humulene (1.79%), γ – terpinene (1.65%), α –thujene (1.35%), α -cadinene (1.20), terpinolene (1.16%) and p-cymene (1.04); for *Origanum vulgare* L. carvacrol (56.43%), γ-terpinene (13.71%), p-cymene (11.06%), myrcene (1.97%), α-thujene (1.81%) , (E)-caryophyllene (1.65%), sabinene (1.43%), α terpinene (1.23%) and thymol (1.13%).



(a) *Citrus aurantium* L. var. *amara*



(b) Cistus ladaniferus L.



(c) Juniperus communis L.

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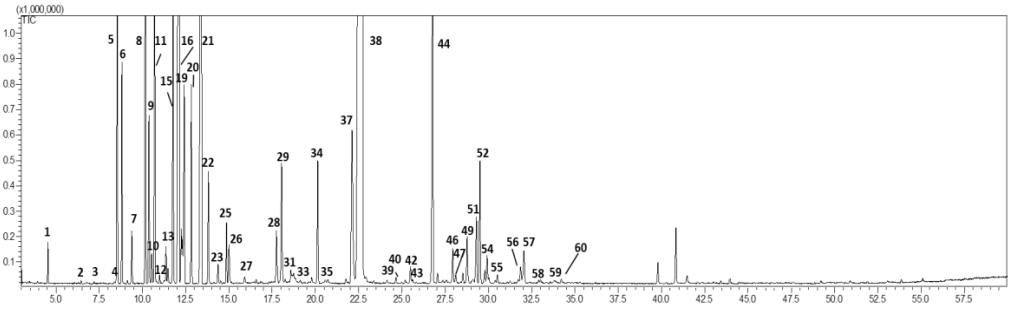

(d) Origanum vulgare L.

Figure 1. Chemical composition of (a) *Citrus aurantium* L. var. *amara* L., (b) *Cistus ladaniferus* L., (c) *Juniperus communis* L. and (d) *Origanum vulgare* L. EOs.

Table 1. Main components of *Citrus aurantium* L. var. *amara* (a), *Cistus ladaniferus* L. (b), *Juniperus communis* L.(c) and *Origanum vulgare* L. (d) EOs identification by GC-MS analysis. (RI exp: Lineare Ritention Index calculated RI pub: Lineare Ritention Indices based on library NIST 11webbook).

(a) Citrus aurantium L. var. amara.

Peak	Compound	RI $_{exp}$	RI $_{pub}$	Area (%) Mean (n = 3)	std.dev
1	a-pinene	930	932	0.88	0.03
2	sabinene	971	972	0.26	0.02
3	b-pinene	973	974	1.12	0.13
4	myrcene	990	991	3.45	0.11
5	octanal	995	998	0.02	0.01
6	p-mentha-1(7),8-diene	1001	1003	0.10	0.02
7	limonene	1027	1030	87.87	0.55
8	(E)-b-ocimene	1042	1044	0.03	0.01
9	octanol	1060	1063	0.06	0.01
10	terpinolene	1084	1086	0.03	0.01
11	linalool	1095	1095	0.30	0.03
12	nonanal	1098	1100	0.03	0.01
13	trans-p-2,8-menthadien-1-ol	1120	1122	0.14	0.02
14	cis-limonene oxide	1149	1152	0.45	0.02
15	trans-limonene oxide	1158	1160	0.34	0.03
16	trans-isocarveol	1187	1189	0.04	0.01
17	α-terpineol	1192	1195	0.59	0.03
18	decanal	1198	1201	0.12	0.02
19	octyl acetate	1207	1211	0.06	0.01
20	trans-carveol	1213	1215	0.20	0.03
21	cis-carveol	1224	1226	0.16	0.01
22	neral	1233	1235	0.11	0.01
23	linalyl acetate	1250	1254	1.84	0.11
24	geranial	1261	1264	0.18	0.02
25	perillaldehyde	1275	1278	0.02	0.00
26	perilla alcohol	1295	1299	0.03	0.00
27	neryl acetate	1356	1359	0.06	0.01
28	geranyl acetate	1378	1379	0.27	0.03
29	(E)-caryophyllene	1416	1417	0.06	0.01
30	(E)-nerolidol	1561	1561	0.02	0.00
31	caryophyllene oxide	1580	1582	0.02	0.01
				98.84	**0.58**

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(b) Cistus ladaniferus L.

Peak	Compound	RI $_{exp}$	RI $_{pub}$	Area (%) Mean (n = 3)	std.dev
1	1,2,3-trimethylcyclopentene	828	822	0.05	0.00
2	1,2,4,4-tetramethylcyclopentene	882	895	0.32	0.02
3	2-methyl-1-propenylcyclopentane	905	915	0.13	0.01
4	tricyclene	920	921	5.08	0.13
5	1,3-dimethylcyclohexanol	926	934	0.17	0.00
6	α-pinene	932	932	13.68	0.31
7	camphene	947	946	37.04	0.15
8	2-methyl-1-hepten-6-one	947	958	0.05	0.01
9	sabinene	970	972	0.08	0.01
10	β-pinene	975	974	0.52	0.03
11	6-methyl-5-hepten-2-one	989	986	0.05	0.01
12	trans-dehydroxylinalool oxide	992	991	0.12	0.02
13	cis-dehydroxylinalool oxide	1008	1006	0.06	0.00
14	p-cymene	1023	1025	0.72	0.05
15	limonene	1029	1030	0.30	0.02
16	1,8-cineole	1031	1031	0.04	0.00
17	2,2,6-trimethylcyclohexanone	1036	1035	3.95	0.10
18	seudenone	1057	1055	0.18	0.04
19	cis-linalool oxide	1072	1069	0.08	0.01
20	2-methylcyclopentanone	1078	1075	0.12	0.01
21	camphenilone	1082	1078	0.29	0.03
22	linalool	1098	1095	0.31	0.05
23	3,4-dimethylcyclohexanol	1108	1105	0.51	0.03
24	exo-fenchol	1121	1118	0.05	0.00
25	α-campholenal	1129	1126	0.23	0.02
26	3-nonen-2-one	1142	1137	0.15	0.01
27	nopinone	1145	1139	0.04	0.01
28	trans-pinocarveol	1147	1141	0.77	0.08
29	camphor	1149	1141	0.73	0.03
30	camphene hydrate	1155	1156	0.31	0.04
31	trans-pinocamphone	1158	1158	0.05	0.00
32	isoborneol	1166	1165	0.08	0.01
33	borneol	1170	1173	1.65	0.16
34	cis-pinocamphone	1178	1176	0.04	0.01
35	terpinen-4-ol	1182	1180	0.60	0.08
36	p-cymen-8-ol	1193	1189	0.18	0.03
37	α-terpineol + myrtenal	1197	1195	0.58	0.09
38	verbenone	1213	1208	0.15	0.01
39	trans-carveol	1228	1223	0.09	0.01
40	isobornyl formate	1235	1235	0.30	0.03
41	linalyl acetate	1256	1254	0.25	0.03
42	bornyl acetate	1290	1287	21.93	0.15
43	isobornyl acetate	1293	1287	0.09	0.01
44	trans-pinocarvyl acetate	1299	1296	0.28	0.02
45	cis-pinocarvyl acetate	1308	1311	0.06	0.01
46	α-cubebene	1345	1349	0.19	0.05
47	cyclosativene	1365	1367	0.57	0.04
48	α-copaene	1374	1374	0.17	0.03
49	sativene	1386	1390	0.14	0.01
50	(E)-caryophyllene	1415	1417	0.03	0.00
51	alloaromadendrene	1462	1458	0.27	0.05
52	γ-muurolene	1477	1478	0.08	0.01
53	viridiflorene	1493	1496	0.22	0.01
54	α-muurolene	1502	1500	0.14	0.02
55	δ-cadinene	1516	1518	0.25	0.02
56	trans-calamenene	1522	1521	0.35	0.03
57	α-calacorene	1542	1544	0.06	0.01
58	palustrol	1569	1567	0.14	0.02
59	spathulenol	1572	1577	0.05	0.02
60	caryophyllene oxide	1581	1582	0.20	0.02
61	viridiflorol	1589	1592	1.03	0.06
62	ledol	1596	1602	0.26	0.03
63	copaborneol	1615	1613	0.27	0.04
64	1-epicubenol	1625	1627	0.06	0.00

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65	α-cadinol	1638	1641	0.05	0.01
66	cadalene	1672	1675	0.05	0.01
67	10-nor-Calamenen-10-one	1699	1702	0.03	0.01
	TOTAL			**97.08**	**0.48**

(c) ***Juniperus communis*** L.

Peak	Compound	RI $_{exp}$	RI $_{pub}$	Area (%) Mean (n = 3)	std.dev
1	tricyclene	920	921	0.07	0.01
2	α-thujene	923	924	1.35	0.08
3	α-pinene	932	932	42.01	0.12
4	α-fenchene	945	948	0.02	0.00
5	camphene	947	946	0.26	0.03
6	thuja-2,4(10)-diene	955	953	0.04	0.01
7	verbenene	960	961	0.02	0.01
8	sabinene	970	972	11.66	0.12
9	β-pinene	973	974	2.38	0.27
10	myrcene	990	991	10.72	0.23
11	δ–2-carene	998	1000	0.05	0.01
12	α–phellandrene	1001	1002	0.26	0.02
13	δ–3-carene	1005	1008	0.17	0.02
14	α–terpinene	1015	1018	0.89	0.08
15	p-cymene	1023	1025	1.04	0.11
16	limonene	1029	1030	6.36	0.17
17	(E)-β-ocimene	1043	1044	0.10	0.02
18	pentyl isobutyrate	1048	1049	0.02	0.01
19	γ-terpinene	1054	1054	1.65	0.12
20	cis-sabinene hydrate	1065	1069	0.04	0.01
21	terpinolene	1083	1086	1.16	0.08
22	p-cymenene	1087	1089	0.09	0.01
23	linalool	1090	1095	0.17	0.01
24	isopentyl isovalerate	1101	1102	0.09	0.01
25	β-thujone	1117	1118	0.02	0.00
26	cis-p-menth-2-en-1-ol	1120	1124	0.07	0.01
27	trans-pinocarveol	1139	1141	0.16	0.02
28	trans-verbenol	1144	1145	0.06	0.01
29	isoborneol	1163	1165	0.08	0.01
30	terpinen-4-ol	1171	1174	2.97	0.14
31	p-cymen-8-ol	1178	1179	0.11	0.01
32	α-terpineol	1183	1186	0.37	0.03
33	verbenone	1201	1204	0.07	0.01
34	trans-carveol	1214	1215	0.03	0.01
35	citronellol	1220	1223	0.02	0.01
36	methyl citronellate	1255	1257	0.07	0.01
37	isobornyl acetate	1279	1283	0.33	0.02
38	2-undecanone	1291	1293	0.05	0.01
39	α-terpinyl acetate	1344	1346	0.05	0.01
40	α-cubebene	1342	1345	0.47	0.04
41	α-copaene	1372	1374	0.38	0.02
42	β-elemene	1388	1389	0.78	0.05
43	sibirene	1398	1400	0.24	0.01
44	longifolene	1406	1407	0.04	0.01
45	(E)-caryophyllene	1415	1417	1.89	0.13
46	γ-elemene	1430	1432	0.14	0.01
47	β-copaene	1428	1430	0.08	0.01
48	cis-thujopsene	1432	1433	0.08	0.01
49	(E)-β-farnesene	1452	1452	0.38	0.03
50	α-humulene	1454	1454	1.79	0.09
51	trans-cadina-1(6),4-diene	1472	1475	0.10	0.02
52	γ-muurolene	1478	1478	0.44	0.04
53	germacrene D	1481	1480	2.04	0.10
54	valencene	1493	1492	0.23	0.03
55	bicyclogermacrene	1501	1500	0.64	0.06
56	β-bisabolene	1507	1505	0.09	0.01
57	γ-cadinene	1515	1512	0.34	0.02
58	δ-cadinene	1520	1518	1.20	0.12
59	selina-4(15),7(11)-diene	1542	1540	0.11	0.02

 doi:10.20944/preprints202411.2294.v1

60	selina-3,7(11)-diene	1546	1545	0.10	0.01
61	(E)-nerolidol	1563	1561	0.09	0.01
62	germacrene B	1565	1559	0.79	0.06
63	spathulenol	1580	1577	0.09	0.01
64	caryophyllene oxide	1583	1582	0.20	0.03
65	humulene epoxide II	1610	1608	0.10	0.01
66	1-epicubenol	1627	1627	0.04	0.01
67	τ-muurolol	1641	1640	0.11	0.02
68	α-cadinol	1652	1652	0.12	0.03
	TOTAL			**98.22**	**1.80**

(d) Origanum vulgare L.

Peak	Compound	RI $_{exp}$	RI $_{pub}$	Area (%) Mean (n = 3)	std.dev
1	methyl 2-methylbutyrate	768	769	0.10	0.02
2	(3Z)-hexenol	850	853	0.01	0.01
3	3-heptanone	886	885	0.01	0.01
4	tricyclene	920	921	0.01	0.01
5	α-thujene	923	924	1.81	0.02
6	α-pinene	932	932	0.74	0.07
7	camphene	947	946	0.18	0.01
8	sabinene	970	972	1.43	0.12
9	1-octen-3-ol	980	978	0.59	0.03
10	3-octanone	986	986	0.11	0.02
11	myrcene	990	991	1.97	0.08
12	3-octanol	997	999	0.02	0.01
13	α–phellandrene	1001	1002	0.16	0.02
14	δ–3-carene	1005	1008	0.05	0.00
15	α–terpinene	1015	1018	1.23	0.12
16	p-cymene	1023	1025	11.06	0.34
17	limonene	1029	1030	0.21	0.04
18	β-phellandrene	1031	1031	0.16	0.03
19	(Z)-β-ocimene	1033	1032	0.87	0.11
20	(E)-β-ocimene	1043	1044	0.68	0.04
21	γ-terpinene	1054	1054	13.71	0.24
22	cis-sabinene hydrate	1065	1069	0.46	0.05
23	terpinolene	1083	1086	0.08	0.01
24	p-cymenene	1087	1089	0.02	0.00
25	linalool	1090	1095	0.26	0.03
26	trans-sabinene hydrate	1097	1098	0.18	0.02
27	cis-p-menth-2-en-1-ol	1115	1118	0.02	0.00
28	borneol	1170	1173	0.28	0.01
29	terpinen-4-ol	1171	1174	0.57	0.07
30	p-cymen-8-ol	1178	1179	0.01	0.00
31	α-terpineol	1183	1186	0.18	0.01
32	(Z)-dihydrocarvone	1210	1207	0.03	0.01
33	(E)-dihydrocarvone	1217	1215	0.02	0.00
34	carvacryl methyl ether	1243	1239	0.52	0.07
35	pulegone	1245	1241	0.02	0.01
36	carvone	1250	1246	0.03	0.01
37	thymol	1290	1289	1.13	0.14
38	carvacrol	1315	1317	56.43	0.57
39	α-cubebene	1342	1345	0.02	0.01
40	carvacrol acetate	1369	1370	0.03	0.01
41	α-copaene	1372	1374	0.03	0.01
42	β-bourbonene	1384	1382	0.08	0.01
43	β-elemene	1388	1389	0.03	0.01
44	(E)-caryophyllene	1415	1417	1.65	0.29
45	β-copaene	1428	1430	0.05	0.01
46	α-humulene	1454	1454	0.17	0.02
47	ε-muurolene	1455	1453	0.05	0.01
48	γ-muurolene	1481	1478	0.05	0.01
49	germacrene D	1481	1480	0.28	0.04
50	γ-amorphene	1496	1495	0.04	0.01
51	(E,E)-α-farnesene	1505	1505	0.39	0.04
52	β-bisabolene	1507	1505	0.60	0.04
53	γ-cadinene	1515	1512	0.07	0.01

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54	δ-cadinene	1520	1518	0.17	0.03
55	(E)-α-bisabolene	1542	1540	0.04	0.01
56	spathulenol	1580	1577	0.12	0.04
57	caryophyllene oxide	1583	1582	0.20	0.03
58	humulene epoxide II	1610	1608	0.01	0.01
59	τ-muurolol	1641	1640	0.02	0.01
60	α-cadinol	1652	1652	0.03	0.01
	TOTAL			**99.34**	**0.30**

2.2. Antibacterial Activity

MIC and MBC values of the EOs tested in our study are reported in Table 2. Among the EOs, *O. vulgare* L. revealed the best inhibitory activity against *S. aureus* and *E. coli*, with MIC values of 0.0312% - 0.125% v/v and MBC values of 0.0625% - 0.25% v/v, respectively, followed by *C. ladaniferus* L. which exhibited a moderate antibacterial activity with MIC values between 0.25% and 0.5% v/v.

Table 2. Antibacterial activity of the EOs tested.

Strains	*Cistus Ladaniferus* L.		*Citrus auratium* L. var. *amara*		*Juniperus communis* L.		*Origanum vulgare* L.	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	(%, v/v)							
S. aureus ATCC 6538	0.25	0.5	1	>1	1	>1	0.0312	0.0625
S. aureus (MRSA) ATCC 43300	0.5	1	1	>1	1	>1	0.125	0.25
E. coli ATCC 10536	0.5	1	1	>1	1	>1	0.0625	0.125

2.2.1. Checkerboard Assay

To study whether *O. vulgare* L. in combination with the other EOs produce a higher bacterial inhibition, a checkerboard assay was performed. The results of the antibacterial activity of EO combinations are given in Table 3.

Table 3. Antibacterial activities of *O. vulgare* L. in combination with the other EOs, determined by the checkerboard test and calculation of the fractional inhibitory concentration (FIC) and fractional inhibitory concentration index **(**FICI).

Strains	**Checkerboard**	**Best combination**[a]		
		FIC	**FICI**	**Effect**
S. aureus ATCC 6538	*O. vulgare* L./*C. ladaniferus* L.	0.250/0.250	0.5	**Synergy**
	O. vulgare L./*C. aurantium* L.var. *amara*	0.250/0.125	0.375	**Synergy**
	O. vulgare L./*J. Communis* L.	0.250/0.062	0.312	**Synergy**
S. aureus (MRSA) ATCC 43300	*O. vulgare* L./*C. ladaniferus* L.	0.5/0.125	0.625	**Additive**
	O. vulgare L./*C. aurantium* L. var. *amara*	0.250/0.5	0.75	**Additive**
	O. vulgare L./*J. Communis* L.	0.5/0.5	1	**Additive**
E. coli ATCC 10536	*O. vulgare* L./*C. ladaniferus* L.	1/0.5	1.5	Indifference
	O. vulgare L./*C. aurantium* L.var. *amara*	1/0.5	1.5	Indifference
	O. vulgare L./*J. Communis* L.	1/0.25	1.25	Indifference

[a] Best combination of sub-MICs of EOs yielding the lowest FICI.

The three tested combinations (*O. vulgare* L./*C. ladaniferus* L., *O. vulgare* L./*C. aurantium* L. var. *amara* and *O. vulgare* L./*J. communis* L.) gave synergistic or additive effects (FICI: 0.312–1) against all tested bacterial strains except *E. coli*, for which they showed indifference (FICI: 1.25–1.5). Specifically, a synergy (FICI from 0.312 to 0.50) with a 4- to 16-fold reduction in the MIC values was recorded for

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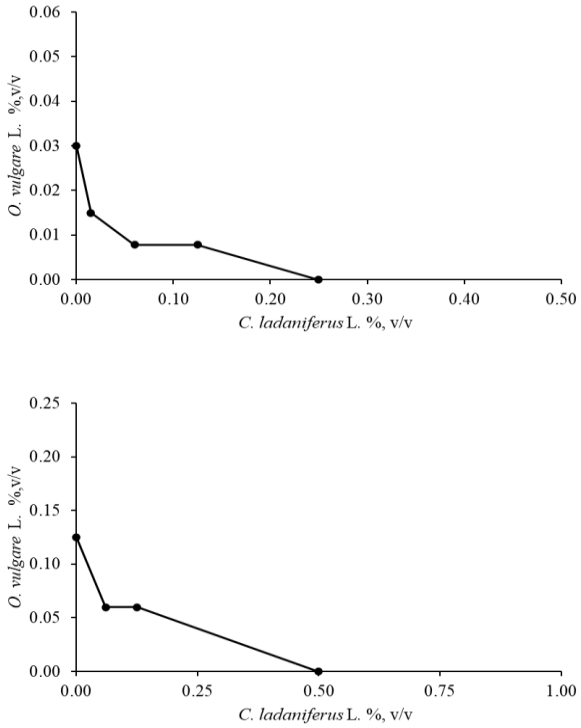
S. aureus ATCC 6538 and an additive effect (FICI from 0.625 to 1) against the MRSA strain. No antagonistic effect was observed.

In terms of concentrations, the combinations that displayed synergistic effect against *S. aureus* ATCC 6538 were: *O. vulgare* L./*C. ladaniferus* L. 1/4+1/4 of sub-MICs corresponding to 0.0078/0.0625% v/v; *O. vulgare* L./*C. aurantium* L. var. *amara* 1/4+1/8 of sub-MICs corresponding to 0.0078/0.125% v/v; and *O. vulgare* L./*J. communis* L. 1/4+1/16 of sub-MICs corresponding to 0.0078/0.0625% v/v. The combinations with additive effects against the MRSA strain were: *O. vulgare* L./*C. ladaniferus* L. 1/2+1/8 of sub-MICs corresponding to 0.0625/0.0625% v/v; *O. vulgare* L./*C. aurantium* L. var. *amara* 1/4+1/2 of sub-MICs corresponding to 0.0312/0.5% v/v; and *O. vulgare* L./*J. communis* L. 1/2+1/2 of sub-MICs corresponding to 0.0625/0.5% v/v of *J. communis* L.

Figure 2 shows representative images of the isobolograms of *O. vulgare* L. in combination with the other EOs against *S. aureus* ATCC 6538 and MRSA ATCC 43300.

S. aureus ATCC 6538 **MRSA ATCC 43300**



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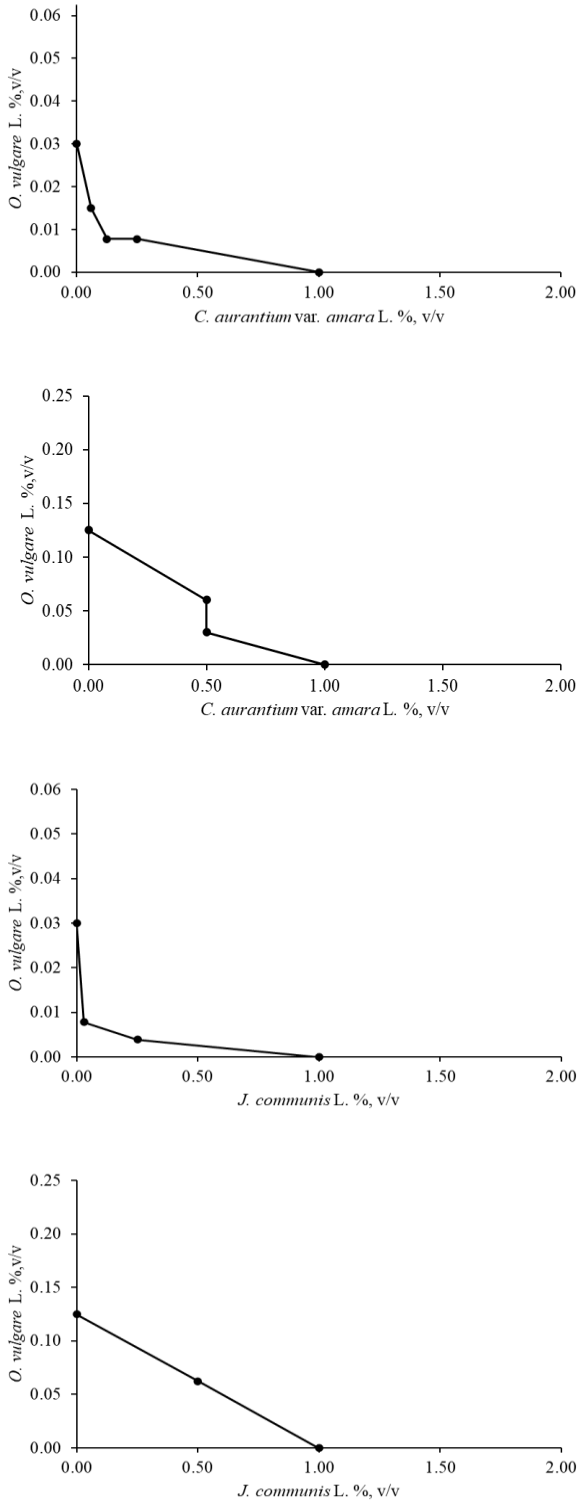

Figure 2. Isobolograms depicting the effect of *O. vulgare* L. in combination with *C. Ladaniferus* L., *C. aurantium* var. *amara* L. and *J. communis* L. against *S. aureus* ATCC 6538 and MRSA ATCC 43300, determined by the checkerboard test and calculation of the fractional inhibitory concentration (FIC).

2.2.2. Effect on Biofilm Formation

Regarding the anti-biofilm effect, the sub-synergistic concentrations of 1/4 + 1/4 EO combinations resulted in a good reduction of *S. aureus* ATCC 6538 biofilm compared with the control (Figure 3).

 doi:10.20944/preprints202411.2294.v1

A good inhibition of biofilm formation with 1/2 synergistic concentration was observed for all combinations. In particular, biofilm reductions of 80% for *O. vulgare* L./*C. ladaniferus* L. (corresponding to 0.0039/0.0312% v/v), 75% for *O. vulgare* L./C. *aurantium* L. var. *amara* (corresponding to 0.0039/0.125% v/v) and 68% for *O. vulgare* L./*J. communis* L. (corresponding to 0.0039/0.125% v/v) were detected. Interestingly, a good inhibitory effect (75% reduction in biofilm formation) was maintained in the presence of the 1/4 synergistic combination of *O. vulgare* L./*C. ladaniferus* L. (corresponding to 0.0019/0.0156%, v/v).

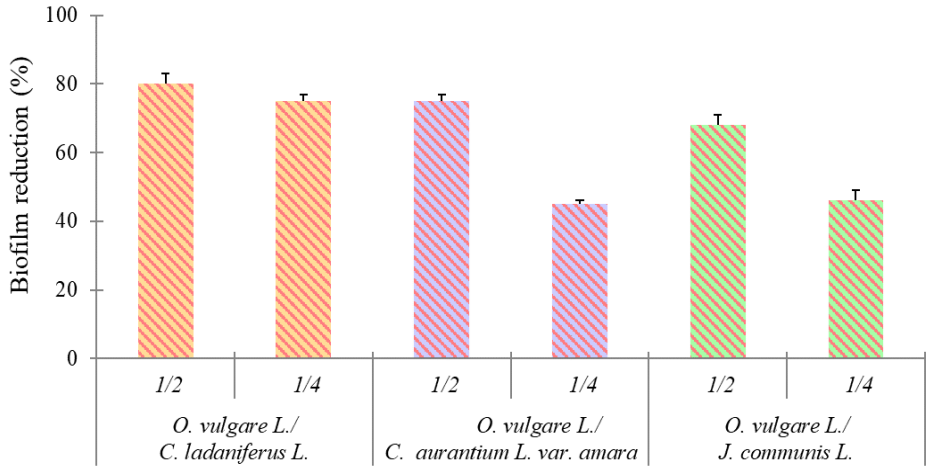

Figure 3. Reduction of biofilm of *S. aureus* ATCC 6538 in the presence of sub-synergistic concentrations of 1/4 + 1/4 EOs combination.

2.3. Antioxidant Activity

In order to characterize the antioxidant properties of the EOs studied, DPPH, ABTS and FRAP test were chosen among the different validated benchmark methods. These redox-based assays measure the reducing capacity of the tested samples under specific conditions. The results of the antioxidant activity of the individual EOs are summarized in Table 4.

Table 4. Antioxidant activity of the tested EOs in three different *in vitro* redox-based assays.

Species	EC_{50} (mg/ml)		
	DPPH	ABTS	FRAP
***C. ladaniferus* L.**	[a] 804.4 ± 49.4	[a] 647.1 ± 85.7	[a] 954.1 ± 50.9
C. aurantium* L. var. *amara	[b] 924.2 ± 25.6	[b] 1077.3 ± 65.3	[b] 1062.0 ± 53.9
***J. communis* L.**	[c] 720.5 ± 89.7	[c] 786.1 ± 18.7	[c] 728.8 ± 50.9
***O. vulgare* L.**	[d] 188.5 ± 37.8	[d] 407.0 ± 72.4	[d] 556.1 ± 63.6
Trolox	149.6 ± 35.7	61.3 ± 8.5	
FeSO₄			66.73 ± 9.9

For each assay, means with the same letter are not significantly different for each other ($p>0.05$).

All EOs showed a medium antioxidant/free radical scavenger activity. The potency order in all assays was higher for *O. vulgare* L., followed by *J. communis* L., *C. ladaniferus* L., and *C. aurantium* L. var. *amara*. For all the assays, the antioxidant activity of the EOs was compared to that of the positive control, Trolox for DPPH and ABTS assay and Fe_2SO_4 for FRAP.

 doi:10.20944/preprints202411.2294.v1

2.4. Anti-Inflammatory Activity

The ability to inhibit the denaturation of bovine serum albumin (BSA) was calculated because the proteic denaturation is recognized as a source of inflammation and the inhibition a key indicator of anti-inflammatory potential [38,39].

Significantly higher activity was also observed in the BSA assay from EO of *O. vulgare* L. compared with the other EOs studied. Results were expressed as IC_{50} and resumed in Figure 4.

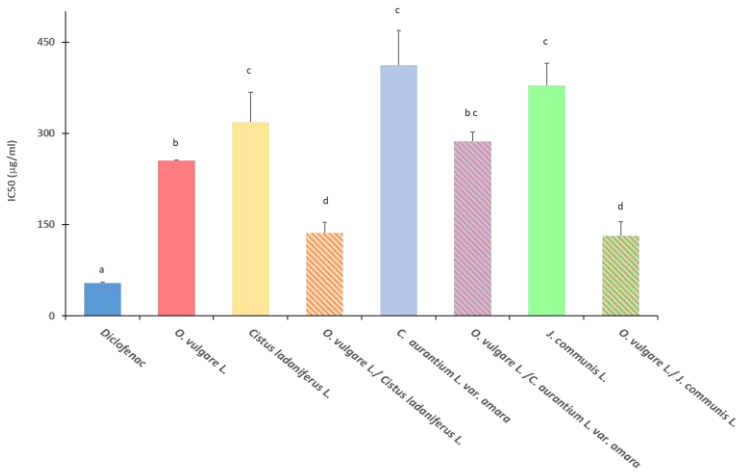

Figure 4. Effect on heat-induced protein denaturation of EOs alone and in combination (1:1), expressed as IC_{50} (inhibitory concentration 50%). Standard anti-inflammatory drug: Diclofenac (sodium salt). Results are expressed as means ± SD of three different experiments. Means with the same letter are not significantly different for each other ($p>0.05$).

2.5. Antioxidant and Anti-Inflammatory Activities of EO Combinations

The EO combinations (*O. vulgare* L./*C. ladaniferus* L., *O. vulgare* L./*C. aurantium* var. *amara* L. and *O. vulgare* L./*J. communis* L. (1:1 v/v) were evaluated for their antioxidant activity by DPPH, ABTS, and FRAP assays and for anti-inflammatory activity by BSA denaturation assay. The results are presented in Table 5 and Figure 4, respectively.

Table 5. Antioxidant activities of the combinations (1:1 v/v) of EOs in the DPPH, ABTS, and FRAP assays.

Combinations	EC_{50} (µg/ml)		
	DPPH	ABTS	FRAP
***O. vulgare* L./*C. ladaniferus*L.**	^{a} 144.3 ± 33.2	$^{a\,b}$ 291.0 ± 68.6	^{a} 338.8 ± 27.4
O. vulgare* L./*C. aurantium* L. var. *amara	^{b} 275.3 ± 24.9	^{b} 366.2 ± 50.5	^{b} 421.1 ± 31.3
***O. vulgare* L./*J. communis* L.**	^{c} 207.7 ± 21.6	^{a} 265.3 ± 30.8	^{a} 298.6 ± 21.7

For each assay, means with the same letter are not significantly different for each other ($p>0.05$).

The combinations analyzed showed for the antioxidant activity (Table 6) an additive effect in the ABTS and FRAP assays with *O. vulgare* L./*J. communis* L., and in DPPH and FRAP assays with *O. vulgare* L./*C. ladaniferus* L.. The other combinations were indifferent. None of the combinations displayed antagonistic effects. This clearly demonstrates that variability exists between the studied methods, and that the employment of different assays, as in this study, provides a better overall assessment of the efficacy.

 doi:10.20944/preprints202411.2294.v1

Table 6. Effect of EOs in combination in DPPH, ABTS, FRAP assay, expressed as sum of fractional inhibitory concentration index (Σ*FIC).*

Combination	DPPH		ABTS		FRAP	
	ΣFIC	**Effect**	**ΣFIC**	**Effect**	**ΣFIC**	**Effect**
***O. vulgare* L./*C. ladaniferus*L.**	**0.945**	**Additive**	1.165	Indifference	**0.964**	**Additive**
O. vulgare* L./*C. aurantium* L. var. *amara	1.758	Indifference	1.240	Indifference	1.154	Indifference
***O. vulgare* L./*J. communis* L.**	1.390	Indifference	**0.989**	**Additive**	**0.947**	**Additive**

Similar kind of interactions have been showed in the BSA assay for the anti-inflammatory activity (Table 7), in fact also in this case the combination of *O. vulgare* L./*J. communis* L. and *O. vulgare* L./*C. ladaniferus* L. showed an additive effect.

Table 7. Effect of EOs assoxiations in BSA denaturation assay, expressed as sum of fractional inhibitory concentration index (ΣFIC).

Combination	BSA	
	ΣFIC	**Interaction**
O. vulgare L./*C. ladaniferus*L.	0.964	**Additive**
O. vulgare L./*C. aurantium* L. var. *amara*	1.774	Indifference
O. vulgare L./*J. communis* L.	0.862	**Additive**

3. Discussion

The EOs from *C. ladaniferus* L., *C. aurantium* L. var. *amara, J. communis* L. and *O. vulgare* L., alone and in binary combinations, have shown antibacterial, antioxidant and anti-inflammatory properties, suggesting that they are natural agents for a potential therapeutic use in the treatment of animal infectious diseases, including those caused by drug-resistant and biofilm-forming bacteria. Furthermore, they may attenuate the oxidative stress and inflammation, that often accompany such infections.

The biological activities of these EOs are related to their chemical composition [40] and, in particular, to major components such as carvacrol for *O. vulgare* L., α-pinene for *J. communis* L., camphene and α-pinene for *C. ladaniferus* L., limonene for *C. aurantium* L. var. *amara* [41–43].

Regarding antibacterial properties of individual EOs, *O. vulgare* L. showed a good inhibitory activity against *S. aureus* and *E. coli*, similar to that demonstrated by *C. ladaniferus*. In contrast, *J. communis* L. and *C. aurantium* L. var. *amara* exhibited lower efficacy against these bacterial strains. The results obtained on *O. vulgare* L. are in line with those of other authors, who highlighted the antibacterial activity of EO against both Gram-positive and Gram-negative strains [13,44]. The antimicrobial activity has also been reported for *C. ladaniferus* and *J. communis* L. EOs [20,45]. About *C. aurantium* L.var. *amara*, the antimicrobial potential and the impact of season's variation on chemical composition and biological activities of its EO have been exhaustively described [46,47].

The results obtained from the DPPH, ABTS and FRAP tests showed good antioxidant/free radical scavenging capacity for all EOs examined individually. The antioxidant effects of EOs derive from their ability to neutralize free radicals by donating hydrogen atoms or electrons, thus protecting biological molecules from oxidative damage. Although the (poly)phenolic constituents are mainly responsible of these properties, other compounds such as cyclic monoterpenes and various functional groups also make an important contribution. Therefore, the combined presence of these diverse components (as in this case; see hereinafter) improves the overall antioxidant activity of EOs [40,48].

About anti-inflammatory activity, the BSA denaturation assay used revealed favorable responses from the tested EOs, with the highest efficacy of *O. vulgare* L. among all samples. This assay is based on the principle that the denaturation, of proteins leads to the loss of their structural integrity and function, resulting in the potential production of auto-antigens. Bioactive compounds present in EOs may protect against this process by preserving the various bonds involved in maintaining protein structure. This protective effect is the basis of the observed anti-inflammatory activity of the EOs studied [38]. The superior biological activities detected in the EO of *O. vulgare* L. are generally attributable to its major component, i.e. carvacrol [49–51]. However, the effectiveness of *O. vulgare* L.

 doi:10.20944/preprints202411.2294.v1

can be influenced by its other minor components, such as *p*-cymene, γ-terpinene, thymol and (E)-caryophyllene [9].

Considering the best response of *O. vulgare* L. in the assays carried out, it was chosen as main EO for the study of binary combinations. Overall, our investigations on the antibacterial, antioxidant, and anti-inflammatory properties of binary combinations (*O. vulgare* L./*J. communis* L., *O. vulgare* L./*C. ladaniferus* L. and *O.vulgare* L./*C. aurantium* L. var. *amara*) revealed noteworthy interactions, quantified using FIC values.

The effects of all tested combinations were synergistic against *S. aureus*, additive against MRSA, while indifferent against *E. coli*. In terms of concentrations, the optimal combination was found to be *O. vulgare* L./*C. ladaniferus* L. against *S. aureus* ATCC 6538 (0.0078% v/v of *O. vulgare* L. and 0 .0625% v/v of *C. ladanifer* L.) and MRSA (0.0625% v/v of *O. vulgare* L. and 0.0625% v/v of *C. ladaniferus* L.). Interestingly, the EO combinations did not reduce the effectiveness of single EOs, as no antagonistic effect was observed. *S. aureus* is a Gram-positive bacterium that can cause a wide range of infections, from minor skin conditions to severe systemic diseases. Its resistance to multiple antibiotics, has led to the emergence of MRSA, a major public health concern. Additionally, *S. aureus* is known for its ability to form biofilms, which enhances its persistence and resistance to treatments. Consequently, addressing *S. aureus* infections, especially those caused by drug-resistant strains, remains a critical area of research. To this regards, some authors have documented the susceptibility of MRSA to tea tree oil [52], others evaluated a potential use of geranium and lavender oils [53].Our data contribute valuable insights into the effectiveness of the EOs analyzed, providing further information on their potential application in combating MRSA.

The subsequent study conducted on *S. aureus* biofilm also evidenced the best inhibitory effect (80% reduction of biofilm formation) by the synergistic association of *O. vulgare* L./*C. ladaniferus* L.. These results highlight the potential of combining EOs against bacterial biofilms which are notoriously difficult to treat. Biofilms are complex communities of microorganisms that adhere to surfaces and are embedded in a self-produced extracellular matrix. They are very difficult to eradicate for their poor susceptibility to conventional antimicrobial agents and host immune defense [54].Therefore, the development of new strategies able to inhibit *S. aureus* biofilm formation is of great interest, considering the ability of this bacterium to cause several diseases.

About antioxidant activity, an additive effect was observed for all binary combinations of EOs under evaluation, whereas in the BSA denaturation assay an additive effect in the anti-inflammatory response was evident only for the combinations *O. vulgare* L./*J. communis* L. and *O. vulgare* L./*C. Ladaniferus* L. Our findings pertaining the antioxidant activity are in line with those of other researchers conducted on different combinations of EOs [55,56]. For example, some authors observed synergistic effects of combinations of EOs, from Laminaceae family, except *O. vulgare* L., such as *Apium graveolens* L., *Thymus vulgaris* L. and *Coriandrum sativum* L. [57], *Thymus fontanesii Boiss. & Reut.*, *Artemisia herba-alba* Asso and *Rosmarinus officinalis* L. [58], *Callistemon lanceolatus* Sweet, *Ocimum gratissimum* L., *Cymbopogon winterianus Jowitt* ex Bor., *Cymbopogon flexuosus* (Nees ex Steud.) Stapf, *Mentha longifolia* (L.) L. and *Vitex negundo* L. [59].

Regarding the anti-inflammatory properties of EO combinations, there are few data in the literature [60]. Instead, some researchers have reported the effect of associations between EOs and common anti-inflammatory drugs [61,62]. However, the study of synergistic anti-inflammatory effects of combined phytochemicals are of growing interest [63].

To give an understanding to the results of the interactions among the binary associations of the EOs in all the assays performed (antibacterial, antioxidant and anti-inflammatory), the observed synergistic effect, defined as the combined effect of the tested compounds greater than the sum of the individual effects [64], could be mainly due to the composition of the EOs, which can affect multiple biochemical processes, enhance the bioavailability of the components, and/or neutralize the adverse effects [59]. On the other hand, an additive effect is considered as the resulting effect of two EOs equal to the sum of the individual effects. For example, some authors have observed for the EO of *O. vulgaris* a synergistic effect in association with EO of *Rosmarinus officinalis* L. and additive effects in association with EOs of *Thymus vulgaris* L., *Ocimum basilicum* L. and *Origanum majorana* L. [65–67].

 doi:10.20944/preprints202411.2294.v1

In general, the synergistic and additive antibacterial effects of the binary associations could be attributed to the main component of *O. vulgare* L. EO, namely carvacrol, and its remarkable effects on the structural and functional properties of the cytoplasmatic membrane [66,68]. However, α-pinene can contribute to the structural damage of cell membrane [69] as well as limonene can alter the cytoplasmic membrane permeability [70]. As for camphene, several studies have documented the antibacterial activity of this terpene and its derivatives [71], particularly as a potential inhibitory agent against *S. aureus* [72,73]. Similarly, for the antioxidant activity, carvacrol can contribute to the resulting additive effect of the binary combinations of EOs assessed in different essays.

Also for the anti-inflammatory activity, the additive effects observed for *O. vulgare* L./*J. communis* L. and *O. vulgare* L./*C. ladaniferus* L. combinations could be due to the main component of *O. vulgare* L. EO [41,74]. In fact, carvacrol is able to reduce the production of inflammatory mediators (IL-1β, IL-4, IL-8 and malondialdehyde and prostanoids) [41] and the induction of IL-10 release [75]; α-pinene reduces the production of inflammatory cytokines (IL-1β, NF-κB, and LTB4) [76,77] and limonene, instead, demonstrated to increase IL-10 levels and reduce TNF-α levels [78,79]. In relation to camphene, it is possible hypothesize its contribution in lipoxygenase inhibition, as documented by other authors for the EO of *Cistus albidus* [80].

The results of this study suggest that the combinations of EOs rich in bioactive components may enhance their overall pharmacological effect, particularly in terms of antibacterial, antioxidant, and anti-inflammatory properties, allowing a reduction in the dose required for activity and the risk of potential side effects. In the specific case, this approach could offer a promising strategy to improve the management of animal infectious diseases, caused by *S. aureus,* including drug-resistant and biofilm-forming strains, whose infections are often accompanied by oxidative stress and inflammation.

4. Materials and Methods

4.1. Essential Oils Sampling

Four commercially available EOs were purchased from two different Italian companies, that provided the following chemical composition: *C. ladaniferus* L. (Cistus) from Laborbio - Collegno (Torino-Italy), *C. auratium* L. var. *amara* (Bitter orange), *J. communis* L. (Juniper) and *O. vulgare* L. (Oregano), from FLORA srl - Lorenzana (Pisa-Italy). The main compounds present in these EOs were: camphene (34%), α -pinene (14%) in Cistus; limonene (85–98%), myrcene (0.8–3%), α-terpineol (0.3–0.9%), α-pinene (0.2–0.9%), linalool (0.2–0.9%) in *C. auratium* L. var. *amara;* α-pinene (40–60%), myrcene (8–18%), sabinene (4–11%), limonene (2–8%), β-pinene (2–6%) in *J. communis* L. and carvacrol (60–80%), *p*-cymene (4–10%), γ-terpinene (3–9%), thymol (0.5–5%), β-caryophyllene (0.5–4%) in *O. vulgare* L. The molecular structure of the main compounds is reported in Figure 3.

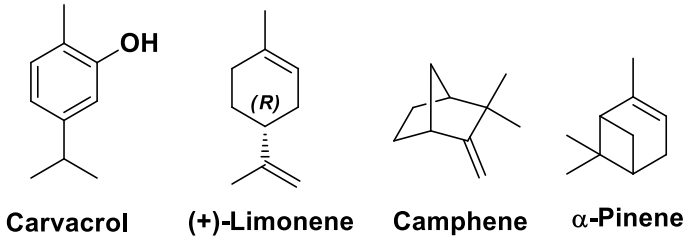

Figure 3. Main compounds present in the studied essential oil samples.

4.2. GC-MS Chromatoghrphic Analysis

The GC-MS analyses were carried out on a GCMS-TQ8030 system (Shimadzu, Milan, Italy) equipped with an AOC-20i auto-sampler. Samples of *Citrus aurantium* L. var. amara EO was injected neat and the injection volume was 0.4μL with a split ratio 1:50at 250°C., instead *Cistus ladaniferus* L., *Juniperus communis* L. and *Origanum vulgare* L. EOs were preliminarily diluted 1:5 v/v in chloroform and the injection volume was 1.0 μL with a split ratio 1:50 at 250°C. The capillary column was anSLB-5ms (Supelco), 30 m × 0.25 mm ID × 0.25 μm film thickness, operated at the following oven program:

 doi:10.20944/preprints202411.2294.v1

50°C (2 min) up to250°C (held 10 min) ≅4°C/min.The mass spectrometric source (EI) was set at 200°C, 0.95 kV; interface: 250°C; acquisition mode was in full scan, range: 40-350 m/z and a scan speed of 1666 amu/sec. Data handling was performed by means of GCMS solution software (Shimadzu). For peak assignment, the following mass spectral libraries were used: FFNSC 2, Adams 4th edition, Wiley 9, NIST11, NIST webbook. n-Paraffins (C7-C40, custom made mixture) were injected apart from real samples in order to measure the Retention Indices. Peak identification was based on library matching of unknowns (similarity index ≥90) and retention index matching of experimental vs. published values (RI filter±10 units) [81]

4.3. Antibacterial Activity

4.3.1. Bacterial Strains and Culture Conditions

The following strains were used: *Staphylococcus aureus* ATCC 6538, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and *Escherichia coli* ATCC 10536. Cultures for antimicrobial tests were grown at 37°C in Mueller-Hinton Broth (MHB, Oxoid, Basingstoke, United Kingdom) for 24 h.

4.3.2. MIC and MBC Determination

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of EOs were determined using a broth dilution micro-method in 96-well round-bottomed polystyrene microtiter plates according to the guidelines of the Clinical and Laboratory Standards Institute [82], with some modifications for EOs. Briefly, *C. ladaniferus* L., *C. aurantium* L. var. *amara*, *J. communis* L. and *O. vulgare* L. EOs were dissolved to 50% using dimethylsulfoxide (DMSO) and then serial twofold dilutions were made in MHB at concentrations ranging from 1% to 0.0039% v/v. DMSO maximum concentration was 0.5% (v/v). Bacterial cultures were inoculated to yield a final concentration of 5×10^5 CFU/mL. Growth control (medium with DMSO and without EO) was included. Plates were incubated at 37 °C for 24 h. The MIC was considered as the lowest concentration of the EO giving the inhibition of visible bacterial growth after incubation for 24 h. To evaluate the inhibition of metabolic bacterial activity, 20 µL of 2,3,5-triphenyl tetrazolium chloride (TTC) 0.125% (w/v) was added in all the wells, followed by 1 hour of incubation. The tetrazolium salt is frequently employed in MIC determinations, when dissolved in water is colorless, but turns red when metabolically active bacteria are present. This red color is directly correlated with the number of living cells. The MBC was determined by seeding 20 µl from all clear MIC wells onto Mueller-Hinton Agar (MHA, Oxoid) plates and was defined as the lowest concentration of EOs that killed 99.9% of the inoculum. The data from at least three replicates were evaluated and modal results were calculated.

4.3.3. Checkerboard Assay

The checkerboard assay was used to determine potential synergistic, additive or even antagonistic effects of combinations of EOs (*O. vulgare* L./*C. ladaniferus* L., *O. vulgare* L./*C. auratium* L. var. *amara* and *O. vulgare* L./*J. communis* L.). Dilutions of two EOs in combinations, from 2 x MIC to serial dilution below, were inoculated in microtiter plates and incubated as described above [83]. The checkerboard test was used to calculate the Fractional Inhibitory Concentration (FIC), according to the formulas: FICA = MICA + B/MICA, FICB = MICB + A/MICB, and FIC Index = FICA + FICB, where MICA + B is the MIC of compound A in presence of compound B, and MICB + A is the opposite.

FIC Index (FICI) values were interpreted as follows: synergistic effect FICI ≤ 0.5; additive effect FICI >0.5 - ≤ 1; indifference FICI >1 - < 2; antagonism FICI ≥ 2 [84]. All experiments were performed in triplicate. The results were also reported as isobolograms, constructed by plotting synergistic concentrations [85].

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4.3.4. Effect on Biofilm Formation

The effect of EO-combinations on biofilm-forming ability of *S. aureus* ATCC 6538 was tested on polystyrene flat-bottomed microtitre plates as previously described [86]. Overnight culture in TSB + 1% glucose (TSBG) of *S. aureus* was adjusted in TSBG to 1 × 10^6 CFU/mL and was dispensed into each well of 96-well polystyrene flat-bottomed microtitre plates containing twofold dilutions of the EO combinations from the 1/4/ + 1/4 combination. After incubation at 37 °C for 24 h, the planktonic phase was removed and each well was washed twice with sterile PBS (pH 7.4), dried, stained for 1 min with 0.1 % safranin and washed with water. The stained biofilms biomass was re-suspended in 30% (v/v) acetic acid and OD_{492} was measured using a spectrophotometer EIA reader. A biofilm control consisting of TSBG medium was included. The reduction percentage of biofilm was calculated using the following equation:

100 – (mean OD_{492} of EO association/mean OD_{492} of control well) × 100 (1)

4.4. Antioxidant Activity

The individual EOs were screened for the antioxidant activity of the tested EOs using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), stable 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and Ferric Reducing/Antioxidant Power (FRAP) assays. Measurements were obtained in triplicate for each sample in each assay. The EC_{50} values were calculated for the control and samples, representing the antioxidant capacity in the sample necessary for 50% of the maximal antioxidant effect.

4.4.1. 2,2-. diphenyl-1-picrylhydrazyl (DPPH) Test

The free radical-scavenging capacity of EOs was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [87], a method based on the reduction of the stable radical DPPH. The reagent mixture consisted of 1.5 ml of 100 mM DPPH in methanol, to which 37.5 µl of solutions containing various concentrations (100–1000 mg/ml) of the EOs to be tested, or of the vehicle alone (DMSO), were added; an equal volume of the solvent employed to dissolve the extracts was added to control tubes. After 20 min of incubation at room temperature, the absorbance was recorded at 517 nm in a UV-Vis spectrophotometer. Trolox reagent was used as blank. Each determination was carried out in triplicate.

4.4.2. 2,2′-. azinobis-(3-ethyl-benzothiazolin-6-sulfonic acid (ABTS) Assay

This method determines the capacity of the EOs to quench the stable 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\cdot+}$). In our experiments, according with Chelly et al. [88], the $ABTS^{\cdot+}$ radical cation was produced by the oxidation of 1.7 mM ABTS with potassium persulfate (4.3 mM final concentration) in water. The mixture was allowed to stand in the dark at room temperature for 12–16 h before use, and then the $ABTS^{\cdot+}$ solution was diluted with phosphate buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7 ± 0.02 at 734 nm. One hundred microliters of a solution containing different concentrations (1000-100 mg/ml) EO samples to be tested or of the vehicle alone (DMSO) was added to 2 ml of the $ABTS^{\cdot+}$ solution, and the absorbance was recorded at 734 nm in a UV-Vis spectrophotometer after allowing the reaction to stand for 6 min in the dark at room temperature. Trolox reagent was used as blank. Each determination was carried out in triplicate.

4.4.3. Ferric Reducing/Antioxidant Power (FRAP) Assay

The ferric reducing ability of the EOs under study was evaluated according to the method described by Chelly et al. [89] with minor modifications. The FRAP reagent contained 10 mM of TPTZ solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). 50 µL of a methanolic solution containing different concentrations (100–1000 mg/ml) of the samples tested

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or of the vehicle (methanol) alone were added to 3 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation at 20 °C for 4 min, using the FRAP reagent as a blank.

4.5. Anti-Inflammatory Activity

The in vitro anti-inflammatory activity of the EOs was carried out according to the method of Belkhodja et al. [90] by monitoring the inhibition of protein denaturation. The method consisted of preparing 0.5 mL of reaction mixture consisting of 0.45 mL BSA (5% aqueous solution) and 0.05 mL of EOs (250 µg/ml). The standard mixture of Diclofenac (0.5 ml) was prepared in the same condition (0.45 mL BSA 5% and 0.05 ml of the standard solution of diclofenac with a concentration of (10-100 µg/ml). pH was calibrated at 6.3 using 1N HCl. After preparation mixtures were incubated at 37 °C for 20 min subsequently heating at 57 °C for 30 min. After cooling the samples, 2.5 mL phosphate buffered saline (pH 6.3) was added to each test tube. Moreover, 0.05 mL distilled water was used in place of essential oil in control test tube whilst product control did not contain bovine serum albumin. The absorbance was measured by the UV-Visible spectrophotometer (Shimadzu UV-1280) at 416 nm and the inhibition percentage of protein denaturation was calculated.

4.6. Antioxidant and Anti-Inflammatory Activities of EOs Combinations

The antioxidant and anti-inflammatory activities were evaluated also on EO binary combinations (O. *vulgare* L./*C. ladaniferus* L., O. *vulgare* L./*J. communis* L. and O. *vulgare* L./*C.auratium* L. var. *amara*). For determining the type of interaction within EOs, different doses of EOs were combined (1:1). Evaluation of different types of interactions (synergism, antagonism or additive effect) between the EOs in binary combinations was carried out by transforming the experimental data studies to fractional inhibitory concentration (FIC) values. The fractional inhibitory concentration fifty percent indexes ($FIC_{50}I$) were determined for each EOS combination according to Sharma et al. [59].

Fractional Inhibitory Concentration (FIC)

The sum of the fractional inhibitory concentration index (ΣFIC) was used to measure interactions from different EOs combinations (1:1) when tested using the DPPH, FRAP, ABTS. The same calculation was used for value the anti-inflammatory capacity of EOs in combination through inhibition of albumin denaturation.

The ΣFICs for each of the combinations were calculated using the following Equation:

$FIC(I)=EC_{50}$ (*a*) in combination with (*b*)/EC_{50} (*a*) independently (2)

$FIC(II)=EC_{50}$ (*b*) in combination with (*a*)/EC_{50} (*b*) independently (3)

where (*a*) is the EC_{50} of one EO in the combination and (*b*) is the EC_{50} of the other EO.

The ∑FICs for each combination were interpreted as synergy where the ∑FICs were less than or equal to 0.5, as additive effects when the ∑FICs were greater than 0.5 but less than or equal to1.0, for indifference, the ∑FICs were greater than 1.0 but less than or equal to 4.0, and for antagonism the ∑FICs were greater than 4.0.

4.7. Statistical Analysis

Results are statistically analyzed by a one-way or a two-way analysis of variance (ANOVA) test, followed by Tukey's honest significant difference, using the statistical software ezANOVA.

5. Conclusions

This study highlighted the antibacterial, antioxidant and anti-inflammatory activities of the EOs of *C. ladaniferus* L., *C. aurantium* L. var. *amara, J. communis* L. and *O. vulgare* L., alone and in binary combinations, suggesting their possible use in the treatment of animal infectious diseases caused by *S. aureus*, including drug-resistant and biofilm-forming strains. Among all EOs, *O. vulgare* L. has been shown to be the most effective in enhancing the antibacterial, anti-biofilm, antioxidant and anti-inflammatory activities of the other EOs when used in combination. Specifically, synergistic and

additive effects were observed for *O. vulgare* L./*C. ladaniferus* L. and *O. vulgare* L./*J. communis* L. against *S. aureus* and MRSA, respectively. The additive effects observed for antioxidant and anti-inflammatory activities are also very important in mitigating infectious diseases associated with oxidative stress and inflammation, as mastitis, and mammary pustular dermatitis compromise animal health and production. In addition, the synergistic effects of these EO combinations could be an important tool in the food industry for the safety and preservation of food of animal origin.

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