

Antibacterial Evaluation of *Matricaria recutita* L., *Achillea millefolium* L. Essential Oil and Tetracycline Combinations in Respect to *in vivo* Toxicity Data[¥]

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[¥]Dedicated to Late Prof. Dr. Nezhun Gören

(Received March 10, 2025; Revised April 11, 2025; Accepted April 17, 2025)

Abstract: *Matricaria recutita* L. and *Achillea millefolium* L. (Asteraceae) aetherolea are among the herbal drug preparations used due to their broad-spectrum antimicrobial effects. The present study aimed to evaluate the *in vitro* antibacterial activity of *M. recutita* and *A. millefolium* oils individually combined with tetracycline. Followed by safety/toxicity evaluation using an *in vivo* animal alternative experimental model, namely *Caenorhabditis elegans*. Chemical verification of Pharmacopoeia quality essential oils was performed both by GC-FID and GC-MS systems, simultaneously. β -Caryophyllene (17%), β -pinene (13.2%), camphor (10%), and sabinene (9.7%) were identified as major components for *A. millefolium* essential oil; whereas, bisabolol oxide A (41.6%), α -bisabolol (19.4%), (*E*)- β -farnesene (17%), α -bisabolol oxide B (5.2%), α -bisabolon oxide A (5%), chamazulene (1.6%), and germacrene D (1.2%) were determined as major components for *M. recutita* essential oil, respectively, in line with the international standards. Antibacterial activities of essential oils and tetracycline were evaluated by microdilution methods against the standard pathogenic strains *Bacillus cereus* NRRL B3711, *Corynebacterium striatum* ATCC BAA-1293, *Streptococcus sanguinis* ATCC 10556, *Staphylococcus aureus* ATCC 700699. Minimal inhibitory concentrations (MIC) were determined followed by the checkerboard combination studies. *A. millefolium* and *M. recutita* essential oils showed *in vitro* inhibitory activity against all tested microorganisms (MIC= 48.7-6250 μ g/mL). The oil combinations with tetracycline showed varying inhibitory antibacterial activity, where *M. recutita* essential oil with tetracycline resulted in synergism against *S. aureus*. *In vivo* toxicity tests on *C. elegans* nematodes resulted in a non-acute toxicity, indicating the relatively safe use of the tetracycline combinations.

Keywords: *Matricaria recutita* L.; *Achillea millefolium* L.; *Caenorhabditis elegans*; synergy; antimicrobial activity. © 2025 ACG Publications. All rights reserved.

1. Introduction

For centuries humans have used plant preparations to treat and protect themselves from diseases caused by external factors including microorganisms, which is documented by ethnobotanical data in Türkiye [1]. In general, the unconscious use of antibiotics, not only causes high treatment costs, but also results in a large variety of microorganisms to develop resistance towards the antibiotics used. In addition, treatments become difficult due to the side effects drugs [2]. As a consequence, a tendency towards utilization of plant products such as essential oils, which have wide effects due to their complex structure and compositions, is employed to test against drug resistance. As matter of fact of their physical, chemical, pharmacological properties, combining essential oils with antimicrobial drugs

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increase the activity, which makes it difficult for microorganisms to develop resistance among other benefits [2,3].

One of the study materials of this work is the genus *Achillea*, an important genus of the Asteraceae family, represented by 42 species, 20 of which are endemic, in the flora of Türkiye (Davis, 1975) [4]. The preparations and essential oil from yarrow - *Achillea millefolium* L., is used due to its antimicrobial effect among other inflammatory skin disorders [5].

The second study material, *Matricaria recutita* L. also from the Asteraceae, known as “chamomile,” is a perennial plant which cultivated in diverse locations including Türkiye. The infusion of its flowers has a sedative and digestive effect. It is used to relieve pain in gum irritation and infections. It is also preferred in the preparation of mouthwashes due to its antimicrobial effect [6]. The essential oil is known to have antipyretic, antiseptic, antibacterial, antifungal, antiviral, insect repellent, anti-inflammatory, antioxidant, antispasmodic, carminative, anti-ulcer, and analgesic properties among many other [7,8].

As it is well known that tetracycline antibiotics are used internally and externally against various bacterial pathogens, however, the development of bacterial resistance limits their use.

In this present study, the *in vitro* antibacterial and combinational activities of pharma grade *M. recutita* and *A. millefolium* essential oil with tetracycline is evaluated. Individual safety profiling using the *in vivo* animal alternative experimental model *Caenorhabditis elegans*, was utilized to the best of our knowledge for the first time.

2. Materials and Methods

2.1. Materials

M. recutita and *A. millefolium* essential oils were obtained from Aromapharm Company, Germany, the standard antibiotics ciprofloxacin and tetracycline were acquired from Sigma-Aldrich (St. Louis, USA) in pharmaceutical grade / highest purity. For *C. elegans* - relevant strains were kindly provided by the Caenorhabditis Genetic Center (Minneapolis, MN, USA). *Bacillus cereus* NRRL B-3711, *Corynebacterium striatum* ATCC BAA-1293, *Streptococcus sanguinis* ATCC 10556, *Staphylococcus aureus* ATCC 700699, were used as test organisms. The microorganisms were stored at -85 °C in glycerol until purity tests just before performing the assays.

2.2. Gas Chromatography-Flame Ionization Detection (GC-FID) And Gas Chromatography-Mass Spectrometry (GC-MS)

The phytochemical analysis of the commercial essential oils was performed by gas chromatography-flame ionization detection and gas chromatography-mass spectrometry system to verify the quality of essential oils [9-14].

2.3. Antibacterial Activity

The *in vitro* antibacterial activity of the *M. recutita* and *A. millefolium* essential oils were evaluated by broth microdilution assay according to a modified Clinical and Laboratory Standards Institute (CLSI) [9,15]. The Minimum Inhibitory Concentrations (MIC), where the lowest concentration of the samples prevented visible growth were calculated. The standard antibiotic ciprofloxacin (CPR) (128-0.25 µg/mL) was used as standard control, where solvent and microbial controls were also added. The assays were repeated at least three times for all the test samples.

2.4. Antibacterial Combinations

Interaction of the test samples were studied using the *in vitro* Checkerboard microdilution assay in 96- well plates. Eight serial dilutions, two-fold dilutions of oils and tetracycline (128-0.25 µg/mL) were prepared, followed by 50 µL aliquots of sample addition to the wells vertically, and 50

Antibacterial activity evaluation of chamomile, yarrow oil and tetracycline combinations

μL aliquots of each antibiotic dilution addition horizontally, so that the plate contained various concentration combination mix of the two test compounds. To ensure the assays, positive growth controls to assess the presence of turbidity were performed in wells without antibacterials. The fractional inhibitory concentration index (ΣFIC), which was defined as the sum of the MIC of each test sample, when used in combination divided by the MIC of the test sample, when used alone. Calculations were as per the equations:

$$\Sigma\text{FIC} = \text{FIC X} + \text{FIC Y}$$

$$\text{FIC X} = (\text{MIC of combined sample and antibiotic}) / (\text{MIC of antibiotic alone})$$

$$\text{FIC Y} = (\text{MIC of combined sample and antibiotic}) / (\text{MIC of essential oil alone})$$

Consequently, the activity types were defined: $\Sigma\text{FIC} \leq 0.5$ = synergism; $0.5 \leq 1$ = additive effect; $>1-4$ = indifferent effect; ≥ 4 = antagonism, as reported [3].

2.5. In Vivo *Caenorhabditis elegans* Toxicity Evaluation

The current *C. elegans* protocols at www.wormbook.org [16] were applied. The wild type N2 was used, where *Escherichia coli* OP50 strain was added into K-agar (Uracil deficient *E. coli*). After inoculation, incubation was allowed for 3 days at 20 °C. At the end of this period, the eggs were collected, and a new K-agar containing *E. coli* was inoculated. 3-day adult worms were collected from Petri dishes and placed then in 12-well tissue culture Petri plate. K-broth containing *E. coli* OP50 was used as the liquid medium. Toxic evaluations were made by adding the essential oil and standard substances to liquid medium. After addition of the worms to the liquid medium, nutritional calculation was carried out by measuring the bacterial optical density immediately at 570 nm. The optical density was measured hourly for 4 hours to determine the acute toxic effect, prolonged to 72 hours. CuCl_2 was used as a toxicity standard. By using non-worm control wells, the effects of *E. coli* lysine or growth on the substances were examined. The net change in nutrition was calculated by the formula:

$$\Delta\text{OD}_{\text{worm}} - \Delta\text{OD}_{\text{without worm}}$$

The results are reported as percentages (%) based on the control values. To determine the lethal effect, 10 ± 1 worms were placed in Petri plates containing 1 mL of the liquid medium. After incubation at 20 °C for 24 hours, wells were examined using a microscope to count living and dead worms. Living worms react when touched by a small wire to distinguish. Lethal concentrations were calculated to determine potential toxic effects [16,17].

3. Results and Discussion

3.1. GC-FID and GC-MS Analysis

In the present study, Pharmacopoeia grade essential oils from commercial sources were evaluated for their safe antibacterial properties. The essential oils were analyzed to confirm their quality by both GC-FID and GC-MS, simultaneously. Overall, 67 components were characterized in both oils representing 99.3 and 96.2% of *A. millefolium* and *M. recutita* essential oils, respectively. β -Caryophyllene (17%), β -pinene (13.2%), camphor (10%), and sabinene (9.7%) were identified as major components for *A. millefolium* essential oil. Bisabolol oxide A (41.6%), α -bisabolol (19.4%), (*E*)- β -farnesene (17%), α -bisabolol oxide B (5.2%), α -bisabolon oxide A (5%), chamazulene (1.6%), and germacrene D (1.2%) were determined as major components for *M. recutita* essential oil, respectively. The components identified both for *A. millefolium* and *M. recutita* essential oils are listed in detail, as in Table 1.

According to previous reports, *M. recutita* essential oil contains the mixture of substances such as alkanes (tricosane, pentacosane); monoterpenes (*E*)- β -ocimene, (*Z*)- β -ocimene, γ -terpinene, *p*-cymene; sesquiterpenes (α -bisabolol, bisabolol oxide A and B, farnesene, germacrene D,

bicyclogermacrene, δ -cadinene, γ -cadinene), sesquiterpene lactone (chamazulene), and fatty acids such as methyl hexadecanoate and methyl oleate in varying amounts [7,18-20]. The most encountered components of *A. millefolium* essential oil are camphor, 1,8-cineole, *cis* and *trans*-sabinene hydrate, borneol, α -thujone, β -thujone, linalool, and α -terpineol [5,21].

The results from the present study are in sound agreement with previous reported work on the chamomile as well as yarrow essential oil compositions [18-21].

Table 1. *Achillea millefolium* and *Matricaria recutita* essential oil components (%*)

RRI ^a	RRI ^b	Component	<i>A. millefolium</i>	<i>M. recutita</i>	Identification method
1008-1039 ^[12]	1032	α -Pinene	6.0	-	<i>t_R</i> , MS
1012-1039 ^[12]	1035	α -Thujene	0.1	-	MS
1044-1084 ^[12]	1072	α -Fenchene	0.3	-	MS
1043-1086 ^[12]	1076	Camphene	1.5	-	<i>t_R</i> , MS
1085-1130 ^[12]	1118	β -Pinene	13.2	-	<i>t_R</i> , MS
1098-1140 ^[12]	1132	Sabinene	9.7	-	<i>t_R</i> , MS
1140-1175 ^[12]	1174	Myrcene	0.3	-	<i>t_R</i> , MS
1148-1186 ^[12]	1176	α -Phellandrene	0.1	-	MS
1154-1195 ^[12]	1188	α -Terpinene	0.5	-	<i>t_R</i> , MS
1178-1219 ^[12]	1203	Limonene	1.0	-	<i>t_R</i> , MS
1186-1231 ^[12]	1213	1,8-Cineole	6.9	-	<i>t_R</i> , MS
1211-1251 ^[12]	1246	(<i>Z</i>)- β -Ocimene	<0.1	<0.1	MS
1222-1266 ^[12]	1255	γ -Terpinene	3.6	0.1	<i>t_R</i> , MS
1232-1267 ^[12]	1266	(<i>E</i>)- β -Ocimene	0.2	0.2	MS
1246-1291 ^[12]	1280	<i>p</i> -Cymene	1.7	0.1	<i>t_R</i> , MS
1261-1300 ^[12]	1298	Terpinolene	0.5	-	<i>t_R</i> , MS
1320-1358 ^[12]	1358	Artemisia ketone	1.5	0.2	MS
1370-1414 ^[12]	1400	Nonanal	-	0.2	MS
1377-1405 ^[12]	1403	Yomogi alcohol	-	<0.1	MS
1385-1441 ^[12]	1437	α -Thujone	1.1	-	MS
1435 ^[13]	1439	γ -Campholene aldehyde	0.1	-	MS
1471-1495 ^[12]	1495	Bicycloelemene	0.1	<0.1	MS
1462-1522 ^[12]	1497	α -Copaene	0.2	<0.1	MS
1476-1523 ^[12]	1510	Artemisia alcohol	-	0.1	MS
1481-1537 ^[12]	1532	Camphor	9.7	-	<i>t_R</i> , MS
1496-1546 ^[12]	1535	β -Bourbonene	0.3	-	MS
1547-1589 ^[12]	1589	β -Ylangene	0.8	-	MS
1549-1597 ^[12]	1590	Bornyl acetate	3.5	-	<i>t_R</i> , MS
1518-1560 ^[12]	1549	β -cubebene	0.4	-	MS
1565-1608 ^[12]	1600	β -Elemene	-	<0.1	MS
1569-1632 ^[12]	1612	β -caryophyllene	17.0	<0.1	<i>t_R</i> , MS
1583-1668 ^[12]	1628	Aromadendrene	-	<0.1	MS
1624-1668 ^[12]	1661	Alloaromadendrene	-	0.1	MS
1647-1689 ^[12]	1659	γ -Gurjunene	0.6	-	MS
1637-1689 ^[12]	1687	α -Humulene	1.5	-	<i>t_R</i> , MS
1643-1684 ^[12]	1695	(<i>E</i>)- β -Farnesene	2.8	17.0	MS
1655-1714 ^[12]	1704	γ -Muurolene	1.6	0.1	MS
1659-1724 ^[12]	1706	α -Terpineol	0.6	-	<i>t_R</i> , MS
1653-1728 ^[12]	1719	Borneol	1.9	-	<i>t_R</i> , MS
1676-1726 ^[12]	1726	Germacrene-D	5.4	-	MS
1696-1743 ^[12]	1726	α -Zingiberene	0.5	-	MS
1686-1753 ^[12]	1740	α -Muurolene	0.5	-	MS
1692-1757 ^[12]	1755	Bicyclogermacrene	-	0.3	MS
1714-1763 ^[12]	1758	(<i>E,E</i>)- α -Farnesene	0.2	0.7	MS
1722-1774 ^[12]	1773	δ -Cadinene	0.9	0.1	MS
1735-1782 ^[12]	1776	γ -Cadinene	1.0	0.1	MS
1748-1783 ^[12]	1783	β -Sesquiphellandrene	0.2	-	MS
1743-1788 ^[12]	1786	<i>ar</i> -Curcumene	0.1	-	MS
1734-1803 ^[12]	1807	α -Cadinene	0.1	-	MS
1893-1941 ^[12]	1941	α -Calacorene	-	0.5	MS
1959-2003 ^[13]	2001	Isocaryophyllene oxide	0.1	-	MS
1936-2023 ^[12]	2008	Caryophyllene oxide	0.6	-	<i>t_R</i> , MS
1995-2055 ^[12]	2050	(<i>E</i>)-Nerolidol	-	0.1	<i>t_R</i> , MS

Antibacterial activity evaluation of chamomile, yarrow oil and tetracycline combinations

Table 1. Continued.

2096-2131 ^[12]	2131	Hexahydrofarnesyl acetone	-	0.3	MS
2074-2150 ^[12]	2144	Spathulenol	0.1	0.4	<i>t_R</i> , MS
	2156	α -Bisabolol oxide B	-	5.2	MS
2136-2198 ^[14]	2187	<i>T</i> -Cadinol	-	0.5	MS
	2200	α -Bisabolol oxide A	-	5.0	MS
	2228	8Z-(2,3)-Dihydromatricaria ester	0.1	-	MS
2178-2234 ^[12]	2232	α -Bisabolol	-	19.4	<i>t_R</i> , MS
2227-2301 ^[12]	2298	Decanoic acid	-	0.5	<i>t_R</i> , MS
2300 ^[13]	2300	Tricosane	-	0.1	<i>t_R</i> , MS
	2423	Azunol	0.2	-	MS
2334-2452 ^[12]	2430	Chamazulene	-	1.6	MS
	2438	α -Bisabolol oxide A	-	41.6	MS
2500 ^[13]	2500	Pentacosane	-	0.3	<i>t_R</i> , MS
2862-2945 ^[12]	2931	Hexadecanoic acid	-	0.4	<i>t_R</i> , MS
Total			99.3	96.2	

^a Relative retention indices reported in the literature [12-14]; ^b Relative retention indices (RRI) experimentally determined against *n*-alkanes; *% calculated from FID data.

3.2. Antibacterial Activity

The potential *in vitro* antibacterial activity of the essential oils and tetracycline combinations were evaluated using the broth microdilution assay. A panel of oral human pathogenic strains *Bacillus cereus* NRRL B3711, *Corynebacterium striatum* ATCC BAA-1293, *Streptococcus sanguinis* ATCC 10556, *Staphylococcus aureus* ATCC 700699 were used, respectively. Minimal Inhibitory concentrations (MIC) of the samples were determined and listed in Table 2, where ciprofloxacin was used as a positive control in the experiments. The moderate to low antibacterial versus *B. cereus* and *S. aureus* are in accordance with literature results and new inhibitory data against *S. sanguinis* and *C. striatum* strains were reported herein for the first time (MIC= 48.7-6250 µg/mL).

In a previous study, inhibitory concentrations of *A. millefolium* essential oil against *S. aureus* and *C. albicans* microorganisms were reported as 5 µg/mL [22]. In another study, *A. millefolium* essential oil showed strong activity with a MIC value of 72 mg/mL against *S. aureus* strain, 4.5 mg/mL against *C. albicans* strain and 18 mg/mL against *C. krusei* strain, suggesting antiyeast activity [23]. In the activity test performed by disc diffusion method, the inhibition zone diameter of *A. millefolium* essential oil against *S. aureus* strain was 15.7 mm with remarkable antibacterial activity [24]. In another study, the inhibition zone diameter was 14 mm where a relatively moderate antibacterial activity was observed [25]. Owlia et al. (2007) tested *M. recutita* essential oil against *S. pyogenes*, *S. mutans*, *S. salivarius*, *S. faecalis* and *S. sanguinis* strains and reported MIC values of 0.1-4 µg/mL [26]. In other study, *M. recutita* essential oil evaluated wide range of microorganism strains and observed high antibacterial activity [27].

To the best of our knowledge, moderate to low antibacterial activity versus *S. sanguinis* and *C. striatum* pathogenic strains were reported herein, for the first time.

3.3. Antibacterial Combinations

The essential oils and standard antibiotic tetracycline were combined varying proportions to determine the synergistic antibacterial potential to tackle future resistance cases. Antibacterial combination results were expressed as the Fractional Inhibitory Concentration Index (FICI). The *M. recutita* essential oil and tetracycline combinations resulted in synergistic effect against *S. aureus*, and additive effects against *S. sanguinis* strains (FICI = 0.46-1.25). The results were listed comparatively in Table 2. The *A. millefolium* essential oil and tetracycline combinations resulted as indifferent effects against *B. cereus* and *C. striatum* (FICI = 1.5-2) strains.

In the literature, only one study was reported for the combination Amphotericin B and *M. recutita* essential oil. The combination was challenged against *C. albicans* strains [28]. To the best of our knowledge, the combination of chamomile oil with yarrow oil using standard antibacterial tetracycline was reported for the first time.

Table 2. Combination results by Fractional Inhibitory Concentration Index (FICI, µg/mL)

Combination	Bacteria	Essential oil			Antibiotic				OUTCOME
		*A	**C	FIC	A	C	FIC	FICI	
ACH + TCY	<i>B. cereus</i>	390	390	1	0.35	0.175	0.5	1.5	Indifferent
MAT + TCY	<i>B. cereus</i>	>6250	>6250	-	<0.087	<0.087	-	-	No activity
ACH + TCY	<i>S. sanguinis</i>	780	390	0.5	0.087	0.35	0.25	0.75	Additive
MAT + TCY	<i>S. sanguinis</i>	48.7	195	0.25	0.087	0.087	1	1.25	Additive
ACH + TCY	<i>S. aureus</i>	>6250	>6250	-	3.18	<0.087	-	-	No activity
MAT + TCY	<i>S. aureus</i>	1562	390	0.24	3.18	0.7	0.22	0.46	SYNERGISTIC
ACH + TCY	<i>C. striatum</i>	195	195	1	0.35	0.35	1	2	Indifferent
MAT + TCY	<i>C. striatum</i>	>6250	>6250	-	<0.087	<0.087	-	-	No activity

ACH: *Achillea millefolium* essential oil; MAT: *Matricaria recutita* essential oil; TCY: Tetracycline;

*A: MIC value individually; **C: MIC value in combination.

3.3. *In vivo* *Caenorhabditis elegans* Toxicity Evaluation

The acute toxicity and lethal concentrations of the tested essential oils were evaluated by using the *in vivo* animal alternative experimental model. As a result of the optical density changes in first four hours, it was determined that essential oils did not show acute toxicity at 5-0.15 mg/mL concentrations. The results of optical density are illustrated in Figures S1-2, within the Supporting information file. Also, % Inhibition concentrations were illustrated in Figure S3 (see supporting information). When examined, both essential oils showed toxicity at relatively high concentrations at 10 mg/mL concentration dependent, the toxicity of both oil decreases at 5-0.15 mg/mL, respectively. In a previous study, Satyal et al. (2015) reported the nematocidal activity of *M. recutita* essential oil on *C. elegans*, where no notable toxicity was observed [29]. The (%) viability in percentage of *C. elegans* versus *M. recutita* and *A. millefolium* essential oils were graphed in Figure 3, where chamomile oil was relatively higher (see Supporting Information). When the results were compared, our findings were consistent and supporting previous findings and data.

As an overall summary, the oils were analyzed to confirm their quality using state of the art chromatographic-spectral analyses. When compared with European Pharmacopoeia 11th Edition [30], the results observed confirmed the quality of essential oils.

According to antibacterial assay results, both essential oils were relatively high in antibacterial activity against the tested pathogenic standard strains. When essential oils were combined with tetracycline, a tendency was observed in the increase of their activities. Especially, *M. recutita* essential oil with the antibiotic tetracycline showed a synergistic effect against *S. aureus* strain.

To evaluate the toxicity exposure *in vivo* *C. elegans* animal alternative experimental model was used. As a result, the data observed in first four hours (see Figure 1), also a 24-hour measurement was performed, where both essential oils were relatively safe at a concentration range of 0.15-5 mg/mL (an seen Figure 2), which was reported herein for the first time, to the best of our knowledge.

In conclusion, the combination of natural substances such as essential oils with antibiotics opens a new insight towards antimicrobial resistance with safer limits. Detailed clinical evaluations towards efficacy in safe aspects still need to be performed as future work. Further activity tests with different oil combinations suggest promising future insights not only for antimicrobial resistance.

Acknowledgements

This study was financially supported by Anadolu University Scientific Research Projects, (BAP No: 1509S622-1605S285) and presented at 48th International Symposium on Essential Oils, 2017, 10-13 September, in Pecs, Hungary.

Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

Author contributions

Contributing authors have accepted all responsibility for the content of this manuscript and approved the content.

Competing Interests

All authors declare no conflict of interest.

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