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# Essential oil and Waste Hydrosol of *Ocimum Tenuiflorum* L.: A Low-Cost Raw Material Source of Eugenol, Botanical Pesticides, and Therapeutic Potentiality

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In this study, essential oils and waste hydrosols of leaves of *Ocimum tenuiflorum* in four different geographical locations were extracted by hydrodistillation method and using gas chromatography/mass spectrometry (GC/MS) for chemical composition analysis. All four essential oil samples contained the main components (*E*)-β-caryophyllene (27.8–49.0%), *trans*-β-elemene (20.3–37.1%) and eugenol (9.0–44.0%). Three of the four hydrosol samples had eugenol in absolute content (94.5–98.6%), while the remaining hydrosol sample had two main components, elemicin (77.8%) and eugenol (14.2%). Essential oils and hydrosols demonstrated larvicidal activities against four important disease-transmitting mosquito species including *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, and *Culex fuscocephala* with 24-h $LC_{50}$ values in the range 15.42–56.01 μg/mL and 53.88–97.80 μg/mL for the essential oils and the hydrosols, respectively. Essential oils and hydrosols strongly inhibited the acetylcholinesterase (AChE) enzyme of electric eels with $IC_{50}$ values in the range of 25.35–107.19 μg/mL. Microemulsion (ME) can be considered as a sustainable pesticide formulation over 300 days and has improved larvicidal activity compared to free essential oil. The *O. tenuiflorum* in Vietnam can be considered a low-cost source of eugenol, botanical pesticides that control disease-transmitting mosquitoes, as well as having therapeutic potential to be further investigated.

## Introduction

*Ocimum tenuiflorum* L. (Lamiaceae; syn. *Ocimum sanctum*), holy basil, is a widely distributed herbal species[1] and has been used for a long time in traditional medicine in many countries.[2] According to Vietnamese traditional medicine, holy basil is used to treat many respiratory diseases, diarrhea, headaches, fever, skin diseases and pneumonia.[3] This plant has been reported to have many biological activities and is safe for humans.[2] The essential oil of this species is considered a good source of natural eugenol,[4] having commercial value in industries such as pharmaceuticals, cosmetics and food as an antiallergic and antibacterial agent.[2] Hydrosols of several essential oils containing eugenol have been reported to exhibit antimicrobial,[5–8] antioxidant,[9] and phytotoxic activities.[10] Eugenol is highly soluble and stable in water at high temperatures,[11] suggesting that large amounts of eugenol may have dissolved in water during the hydrodistillation or steam-distillation process. Hydrosols of *O. tenuiflorum* as well as other eugenol-containing aromatic plants do not seem to have been taken seriously in terms of their value.

Furthermore, if waste hydrosols are not treated, such as removing or recovering essential oil components, they may be hazardous wastes to the environment. A strategy for treating waste sources containing essential oils is to use them as renewable resources to create a basis for the production of biochemicals and other high-value products for the food and feed industry as well as raw materials for industry.[12,13]

The disease burden from mosquito-borne diseases is increasing rapidly every year globally. The expansion of the distribution of disease-transmitting mosquito species has been reported. Synthetic pesticides are showing ineffectiveness in controlling mosquito species, moreover they have disadvantages such as being toxic to human and non-target organisms,

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causing pollution to the environment and water sources, and drug resistance occurs. Essential oils are receiving the attention of many scientists for their potential to control disease-transmitting mosquitoes with advantages such as being safe for human, less toxic to non-target organisms, and difficulty in developing resistance in target species. However, essential oils also have limitations: they are less stable in environmental conditions and insoluble in water.

Alzheimer's disease accounts for 50 to 60% of all dementia cases, thought to be caused by decrease in acetylcholine levels in synaptic clefts. The *Ocimum gratissimum* L. essential oil contains the main ingredient eugenol, which has shown activities on the central nervous system such as increasing sleep time and protecting animals against tonic seizures.[14] Some previous studies have reported the central nervous system protective activity of extracts from leaves of *O. tenuiflorum*, however these studies did not determine the chemical composition.[15,16] The components of essential oils are characterized by their small size and lipophilicity, thus facilitating passage across the blood-brain barrier, and have been shown to have effects on the central nervous system, including the treatment of Alzheimer's disease and Parkinson's disease.

In this study, the yields and chemical composition of essential oils and hydrosols from the leaves of *O. tenuiflorum* in Central Vietnam were investigated. The larvicidal activities of essential oils and hydrosols against four important disease-transmitting mosquito species including *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, and *Culex fuscocephala* have also been evaluated. Furthermore, the acetylcholinesterase (AChE) enzyme inhibitory activities of essential oils and hydrosols were assessed. Additionally, this study presented a method to prepare microemulsion formulations that are stable over a long period of time and have improved larvicidal activity compared to free essential oil. The results of this study aim to provide a scientific basis for the exploitation and use of *O. tenuiflorum* essential oil with high economic efficiency, and environmental safety, in accordance with circular bio-economic principles.

## Results and Discussion

### Chemical Profiles of Essential Oils and Hydrosols

Yields of four essential oil samples (OTDN, Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT, Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1, Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2, Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district) in Vietnam ranged from 0.12 to 0.18% (w/w) (Table 1), these results are consistent with most of the previous studies.

The yields of the HOTH1 and HODN hydrosols were comparable to their essential oils, for HOHT being half that of its essential oil, while HOTH2 was already very low (Table 1). To the best of our knowledge, there have not previously been any complete and detailed reports on the yield and chemical composition of hydrosols of *O. tenuiflorum*.

Agglomerative hierarchical cluster (AHC) analysis for 68 essential oil samples from previous scientific papers and 4 essential oil samples in this study identified 4 chemotypes of *O. tenuiflorum* essential oil (Figure 1).

Cluster 1 has included essential oils that have been characterized by methyl eugenol, cluster 2 has been characterized by essential oils of eugenol and cluster 3 has been characterized by estragol/β-bisabolene/1,8-cineole. Cluster 4 is a group of the main components eugenol/β-caryophyllene/β-elemene, all five samples of essential oils from Vietnam fell into this cluster. Detailed analyses of chemical compositions of 4 samples of *O. tenuiflorum* essential oil from Vietnam are shown in Table 2.

According to our summary, the eugenol content in the leaves of *O. tenuiflorum* is inconsistent and varies greatly from 0% (sample Aust1)[17] to the highest of 84.0% (sample Ind31).[18] The cause of these differences may be due to soil factors, seasons, and geographical location. Previous research by Cung (2018)[19] on leaves collected in May (in Hanoi City) gave a eugenol content of 53.61%. Therefore, eugenol content of *O. tenuiflorum* in Vietnam varied according to the geographical location of the collection site. In some places in Vietnam, *O. tenuiflorum* has shown a content of eugenol above 40.0%,

**Table 1.** Yields of essential oils and hydrosols of *Ocimum tenuiflorum* in Vietnam.

| Collection location | Yield of essential oil (%, w/w) | Yield of hydrosol (%, v/w) |
|---|---|---|
| Da Nang City (DN): 16°02′33″N 108°09′45″E, elevation 15 m. | 0.18 (OTDN) | 0.17 (HODN) |
| Duc Tho district, Ha Tinh Province (HT): 18°30′56″N 105°62′48″E, elevation 5 m | 0.15 (OTHT) | 0.08 (HOHT) |
| Hoang Hoa District, Thanh Hoa Province (TH1): 19° 48′35″N 105°51′40″E, elevation 5 m. | 0.12 (OTTH1) | 0.12 (HOTH1) |
| Thuong Xuan district, Thanh Hoa Province (TH2): 19°54′16″N 105°20′45″E, elevation 31 m. | 0.13 (OTTH2) | 0.03 (HOTH2) |

Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

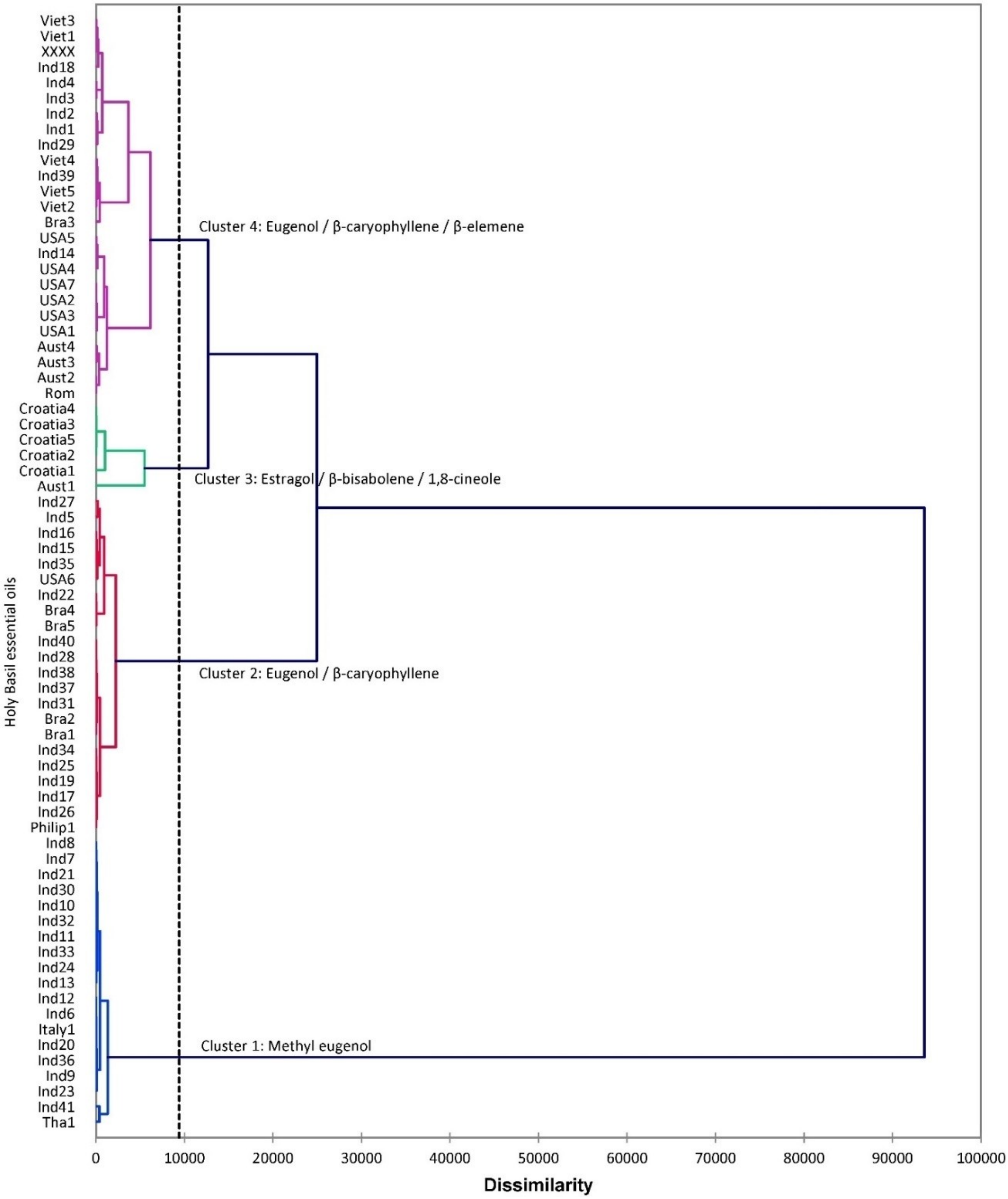


**Figure 1.** Dendrogram obtained from the agglomerative hierarchical cluster analysis of *Ocimum tenuiflorum* essential oil compositions. Viet3 (Vietnam, this study), Viet1 (Vietnam),[19] XXXX (Cuba),[20] Ind18 (India),[21] Ind4 (India),[22] Ind3 (India),[22] Ind2 (India),[22] Ind1 (India),[22] Ind29 (India),[23] Viet4 (Vietnam), Ind39 (India),[24] Viet5 (Vietnam), Viet2 (Vietnam), Bra3 (Brazil),[25] USA5 (USA),[26] Ind14 (India),[27] USA4 (USA),[26] USA7 (USA),[26] USA2 (USA),[26] USA3 (USA),[26] USA1 (USA),[26] Aust4 (Australia),[28] Aust3 (Australia),[28] Aust2 (Australia),[28] Rom (Romania),[29] Croatia4 (Croatia),[30] Croatia3 (Croatia),[30] Croatia5 (Croatia),[31] Croatia2 (Croatia),[30] Croatia1 (Croatia),[30] Aust1 (Australia),[17] Ind27 (India),[32] Ind5 (India),[33] Ind16 (India),[34] Ind15 (India),[34] Ind35 (India),[24] USA6 (USA), Ind22 (India),[1] Bra4 (Brazil),[25] Bra5 (Brazil),[35] Ind40 (India),[24] Ind28 (India),[23] Ind38 (India),[24] Ind37 (India),[24] Ind31 (India),[18] Bra2 (Brazil),[25] Bra1 (Brazil),[25] Ind34 (India),[24] Ind25 (India),[32] Ind19 (India),[36] Ind17 (India),[21] Ind26 (India),[32] Philip1 (Philippines),[37] Ind8 (India),[38] Ind7 (India),[38] Ind21 (India),[39] Ind30 (India),[40] Ind10 (India),[41] Ind32 (India),[18] Ind11 (India),[41] Ind33 (India),[42] Ind24 (India),[32] Ind13 (India),[41] Ind12 (India),[41] Ind6 (India),[43] Italy1 (Italia),[44] Ind20 (India),[39] Ind36 (India),[24] Ind9 (India),[38] Ind23 (India),[1] Ind41 (India),[45] Tha1 (Thailand).[46]

**Table 2.** Leaf essential oil compositions of *Ocimum tenuiflorum* from Vietnam.

| $RI_{calc}$ | $RI_{db}$ | Compound | OTDN | OTHT | OTTH1 | OTTH2 |
|---|---|---|---|---|---|---|
| 897 | 896 | 2,5-Diethyltetrahydrofuran | tr | tr | tr | tr |
| 923 | 923 | Tricyclene | tr | tr | tr | tr |
| 926 | 925 | α-Thujene | tr | tr | tr | tr |
| 933 | 933 | α-Pinene | 0.1 | 0.1 | 0.1 | 0.1 |
| 949 | 950 | Camphene | 0.1 | 0.1 | 0.1 | 0.1 |
| 972 | 971 | Sabinene | tr | tr | tr | tr |
| 977 | 978 | β-Pinene | 0.1 | 0.1 | tr | 0.1 |
| 979 | 978 | 1-Octen-3-ol | – | – | tr | tr |
| 989 | 989 | Myrcene | tr | tr | tr | tr |
| 998 | 999 | 3-Octanol | – | – | tr | tr |
| 1005 | 1006 | Octanal | tr | tr | tr | tr |
| 1006 | 1008 | Hex-(3*Z*)-enyl acetate | tr | tr | – | tr |
| 1017 | 1018 | α-Terpinene | tr | tr | tr | tr |
| 1025 | 1025 | *p*-Cymene | tr | tr | tr | tr |
| 1029 | 1030 | Limonene | tr | 0.1 | 0.1 | 0.1 |
| 1031 | 1031 | β-Phellandrene | tr | tr | tr | tr |
| 1032 | 1032 | 1,8-Cineole | tr | tr | tr | tr |
| 1035 | 1034 | (*Z*)-β-Ocimene | tr | tr | tr | tr |
| 1046 | 1045 | (*E*)-β-Ocimene | 0.1 | 0.1 | 0.1 | tr |
| 1058 | 1057 | γ-Terpinene | tr | tr | tr | tr |
| 1085 | 1086 | Terpinolene | tr | tr | tr | tr |
| 1100 | 1101 | Linalool | 0.1 | tr | tr | 0.1 |
| 1103 | 1104 | 2-Methylbutyl 2-methylbutanoate | tr | tr | – | – |
| 1106 | 1107 | Nonanal | tr | tr | tr | tr |
| 1109 | 1109 | 2-Methylbutyl 3-methylbutanoate | tr | tr | – | – |
| 1113 | 1113 | (*E*)-4,8-Dimethyl-nona-1,3,7-triene | tr | tr | tr | tr |
| 1142 | 1143 | Geijerene | tr | – | – | – |
| 1147 | 1149 | Camphor | tr | tr | – | tr |
| 1172 | 1173 | Borneol | tr | 0.2 | 0.1 | 0.1 |
| 1181 | 1180 | Terpinen-4-ol | tr | tr | tr | tr |
| 1192 | 1192 | Methyl salicylate | tr | – | – | – |
| 1196 | 1195 | α-Terpineol | tr | tr | tr | tr |
| 1198 | 1197 | Methyl chavicol (= Estragole) | – | 0.1 | – | – |
| 1212 | 1211 | Octyl acetate | tr | tr | tr | tr |
| 1245 | 1246 | Carvone | – | 0.1 | – | – |
| 1276 | 1278 | Perilla aldehyde | – | 0.1 | tr | tr |
| 1284 | 1285 | Bornyl acetate | tr | tr | tr | tr |
| 1311 | 1313 | Nonyl acetate | tr | – | – | – |
| **1355** | **1356** | **Eugenol** | **17.2** | **44.0** | **13.2** | **9.0** |
| 1375 | 1375 | α-Copaene | tr | – | tr | tr |
| 1383 | 1383 | *cis*-β-Elemene | 0.8 | 0.7 | 1.3 | 1.0 |
| **1391** | **1390** | ***trans*-β-Elemene** | **24.6** | **20.3** | **37.1** | **28.2** |
| 1396 | 1403 | Methyl eugenol | 0.3 | 0.4 | 0.1 | 0.2 |
| 1396 | 1392 | β-Cubebene | tr | tr | 0.1 | 0.1 |
| 1404 | 1405 | (*Z*)-β-Caryophyllene | – | tr | tr | tr |
| 1414 | 1413 | α-Barbatene | 0.1 | 0.1 | 0.2 | 0.2 |
| **1422** | **1417** | **(*E*)-β-Caryophyllene** | **49.0** | **27.8** | **36.7** | **46.8** |
| 1429 | 1430 | γ-Elemene | tr | – | – | – |

**Table 2.** continued

| $RI_{calc}$ | $RI_{db}$ | Compound | OTDN | OTHT | OTTH1 | OTTH2 |
|---|---|---|---|---|---|---|
| 1432 | 1432 | *trans*-α-Bergamotene | tr | tr | tr | tr |
| 1434 | 1433 | *cis*-Thujopsene | tr | tr | tr | tr |
| 1437 | 1437 | *iso*-Bazzanene | 0.1 | tr | 0.1 | 0.1 |
| 1448 | 1447 | β-Barbatene | 0.2 | 0.1 | 0.2 | 0.2 |
| 1452 | 1452 | (*E*)-β-Farnesene | 0.1 | 0.1 | 0.1 | 0.1 |
| 1455 | 1454 | α-Humulene | 1.9 | 1.2 | 1.6 | 2.0 |
| 1458 | 1458 | *allo*-Aromadendrene | tr | – | tr | tr |
| 1464 | 1463 | *cis*-Muurola-4(14),5-diene | tr | tr | tr | tr |
| 1475 | 1476 | Selina-4,11-diene | 0.2 | 0.2 | 0.2 | 0.2 |
| 1482 | 1488 | Germacrene D | 0.1 | tr | 0.1 | tr |
| 1491 | 1489 | β-Selinene | 0.6 | 0.6 | 0.9 | 1.0 |
| 1493 | 1492 | Valencene | – | – | 0.1 | 0.1 |
| 1498 | 1497 | α-Selinene | 0.6 | 0.6 | 0.9 | 1.0 |
| 1504 | 1504 | α-Cuprenene | tr | – | 0.1 | – |
| 1506 | 1506 | α-Chamigrene | tr | – | 0.1 | 0.1 |
| 1509 | 1511 | Germacrene A | 0.4 | 0.3 | 0.7 | 0.7 |
| 1518 | 1517 | (*E,Z*)-Matricaria ester | tr | 0.1 | 0.1 | – |
| 1519 | 1518 | δ-Cadinene | 0.1 | 0.1 | 0.2 | 0.1 |
| 1522 | 1520 | Myristicin | tr | 0.2 | 0.2 | tr |
| 1522 | 1520 | 1,2-Dihydrocuparene | tr | – | tr | tr |
| 1528 | 1528 | (*E*)-γ-Bisabolene | 0.1 | 0.1 | 0.1 | 0.1 |
| 1531 | 1531 | 10-*epi*-Cubebol | tr | tr | 0.1 | 0.1 |
| 1535 | 1535 | γ-Cuprenene | 0.1 | tr | 0.1 | tr |
| 1550 | 1549 | α-Elemol | 0.4 | 0.3 | 0.9 | 0.5 |
| 1551 | 1551 | (*Z*)-Caryphyllene oxide | tr | – | – | – |
| 1559 | 1557 | Germacrene B | tr | tr | tr | tr |
| 1563 | 1561 | (*E*)-Nerolidol | tr | tr | tr | tr |
| 1584 | 1587 | Caryophyllene oxide | 1.4 | 0.5 | 1.5 | 3.6 |
| 1595 | 1594 | Viridiflorol | tr | tr | tr | 0.3 |
| 1610 | 1613 | Humulene epoxide II | 0.1 | tr | 0.1 | 0.2 |
| 1615 | 1616 | 1,10-di-*epi*-Cubenol | tr | tr | 0.1 | 0.1 |
| 1632 | 1632 | γ-Eudesmol | 0.1 | 0.1 | 0.1 | 0.1 |
| 1634 | 1632 | Caryophylla-4(12),8(13)-dien-5α-ol | tr | tr | tr | 0.1 |
| 1638 | 1635 | Caryophylla-4(12),8(13)-dien-5β-ol | 0.1 | tr | 0.1 | 0.2 |
| 1643 | 1643 | τ-Cadinol | 0.1 | tr | 0.1 | 0.2 |
| 1647 | 1645 | α-Muurolol (=δ-Cadinol) | – | – | tr | tr |
| 1655 | 1656 | β-Eudesmol | 0.1 | 0.1 | 0.1 | 0.1 |
| 1656 | 1655 | α-Eudesmol | 0.1 | 0.1 | 0.2 | 0.3 |
| 1659 | 1658 | *neo*-Intermedeol | 0.6 | 0.6 | 1.0 | 1.1 |
| 1664 | 1665 | Intermedeol | tr | tr | 0.1 | 0.3 |
| 1670 | 1671 | 14-Hydroxy-9-*epi*-(*E*)-caryophyllene | 0.1 | 0.1 | 0.2 | 0.5 |
| 1717 | 1715 | Pentadecanal | – | – | tr | 0.1 |
| 1723 | 1719 | 1-Phenylhepta-1,3,5-triyne | tr | – | – | – |
| 1729 | 1729 | Zerumbone | – | – | – | 0.1 |
| 2490 | 2491 | Dehydrodieugenol | – | tr | tr | tr |
| | | Monoterpene hydrocarbons | 0.3 | 0.6 | 0.3 | 0.4 |
| | | Oxygenated monoterpenoids | 0.1 | 0.3 | 0.1 | 0.1 |
| | | Sesquiterpene hydrocarbons | 78.9 | 52.1 | 80.8 | 81.8 |

**Table 2.** continued

| $RI_{calc}$ | $RI_{db}$ | Compound | OTDN | OTHT | OTTH1 | OTTH2 |
|---|---|---|---|---|---|---|
| | | Oxygenated sesquiterpenoids | 3.1 | 1.8 | 4.5 | 7.6 |
| | | Benzenoid aromatics | 17.5 | 44.7 | 13.5 | 9.2 |
| | | Others | traces | 0.1 | 0.1 | 0.1 |
| | | Total identified | 99.9 | 99.6 | 99.3 | 99.1 |

Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province. Major components are highlighted in **blue bold**.

which is suitable for being a low-cost source of natural eugenol.[4]

The chemical compositions of the three hydrosols HODN, HOHT and HOTH1 were characterized by the absolute dominance of eugenol, with concentrations ranging from 94.5 to 98.6%. Meanwhile, HOTH2 hydrosol was characterized by elemicin with the content of 77.8%, eugenol as the remaining main component with the content of 14.2% (Table 3). The elemicin content in the hydrosols appeared to be inversely proportional to the eugenol content in the essential oil samples, in particular, the elemicin content in the hydrosols increased as the eugenol content in the essential oils decreased. This finding may be a signal to predict eugenol and elemicin hydrosol concentrations of species in the genus *Ocimum* that contain eugenol. According to the results of our AHC analysis (Figure 1), the majority of *O. tenuiflorum* essential oil samples fell into two clusters containing eugenol as the main component.

Eugenol has been identified in high concentrations in essential oils such as *Myristica fragrans* Houtt., *Cinnamomum verum* J. Presl, *Cinnamomum loureirii* Nees. (Saigon cinnamon), *Ocimum gratissimum* Forssk. (basil) and *Ocimum basilicum* L. (sweet basil).[47] However, *Syzygium aromaticum* (L.) Merr. & L.M. Perry essential oil has been considered as the main natural source of eugenol, with a content of 45 to 90% of the total oil.[47] International standards for clove oil have specified the total content of eugenol from 78 to 95%.[48,49] Eugenol content in *S. aromaticum* essential oil in Brazil was up to 89.6%,[50] while in Turkey was 87%.[51] After distillation, clove oil undergoes refining to obtain a product containing almost pure eugenol (i.e., $>$95%).[52] Hydrosol of *S. aromaticum* was reported by Aazza et al. (2011) have a eugenol content of 80.8%.[53] Hydrosol of *C. verum* reported by Didar (2019) was absent of eugenol.[5] The *Ocimum* species also identified as rich in eugenol are considered cost-effective alternatives to clove as a source of eugenol, *O. gratisimum* LR-1 was found to be the richest source of eugenol (53–89%).[54] The *O. basilicum* essential oil with the highest eugenol content of 41.2% was reported by Filho et al. (2006).[55] Studies on hydrosols of *O. basilicum* have reported eugenol contents ranging from 7.0 to 18.9%.[56,57] This finding of this study suggests serious consideration of the hydrosol potential of *O. tenuiflorum* as a source of high purity natural eugenol.

### Preparation and Characterization of Microemulsions

The obtained microemulsions (MEs) (were transparent and homogeneous after 300 days of storage. At time T1 (1 day), the formulas OTHT-ME, OTTH1-ME and OTTH2-ME had a particle size distribution in the range of 15.8–27.2 nm and polydispersity index (PDI) in the range of 0.060–0.085. The OTTH2-ME formula was selected to investigate sustainability at time points T2 (120 days) and T3 (300 days) (Figure 2). The particle size distribution and polydispersity index of OTTH2-ME formula have increased over time. Microemulsions exhibit physical attributes, including low viscosity, optical transparency, thermodynamic stability, and consistent phase sizes ranging from 10 to 200 nm, homogeneous and isotropic. The formula in this study used isopropanol instead of ethanol in the previous study by Hung et al. (2023),[58] and stability of the microemulsion system has been significantly improved.

### Larvicidal Activities

The larvicidal activities against *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, and *Cx. fuscocephala* of the essential oils and hydrosols are presented in Tables 4, 5, 6, and 7, respectively. The larvicidal activities of eugenol, elemicin, (*E*)-β-caryophyllene, and *trans*-β-elemene against the larvae of several mosquito species have been previously reported. Eugenol was effective against mosquito larvae *Ae. aegyptipti* with 24-h $LC_{50}$ of 43.67–93.3 μg/mL,[59–66] *Ae. albopictus* with 24-h $LC_{50}$ of 28.14 μg/mL,[61] *Cx. tritaeniorhynchus* with 24-h $LC_{50}$ of 30.80 μg/mL,[61] and *Cx. quinquefasciatus* with 24-h $LC_{50}$ of 117 μg/mL.[67] In our previous study, (*E*)-β-caryophyllene demonstrated larvicidal activity against *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* with 24-h $LC_{50}$ of 53.08 μg/mL, 49.48 μg/mL, and 139.1 μg/mL, respectively.[68] The compound *trans*-β-elemene demonstrated potent larvicidal activity against *Ae. albopictus* and *Cx. tritaeniorhynchus* with 24-h $LC_{50}$ values of 11.15 μg/mL and 12.05 μg/mL, respectively.[61] There were no significant differences between the larvicidal activities of the four essential oil samples against each mosquito species. Thus, the larvicidal activities of the essential oils may be due to the additional activity of the compounds eugenol, (*E*)-β-caryophyllene and *trans*-β-elemene.

The hydrosols demonstrated weaker toxicity trends toward the larvae than the essential oils. The larvicidal activities of

**Table 3.** Leaf hydrosols composition of *Ocimum tenuiflorum* from Vietnam.

| $RI_{calc}$ | $RI_{db}$ | Compound | HODN | HOHT | HOTH1 | HOTH2 |
|---|---|---|---|---|---|---|
| 896 | 896 | 2,5-Diethyltetrahydrofuran | tr | tr | tr | tr |
| 961 | 960 | Benzaldehyde | tr | tr | tr | 0.1 |
| 979 | 978 | 1-Octen-3-ol | tr | – | tr | 0.3 |
| 997 | 999 | 3-Octanol | tr | tr | tr | 0.1 |
| 1024 | 1025 | *p*-Cymene | – | tr | – | – |
| 1029 | 1030 | Limonene | – | tr | – | tr |
| 1032 | 1032 | 1,8-Cineole | tr | tr | tr | tr |
| 1034 | 1033 | Benzyl alcohol | – | – | – | tr |
| 1070 | 1069 | *cis*-Linalool oxide (furanoid) | tr | tr | tr | tr |
| 1086 | 1086 | *trans*-Linalool oxide (furanoid) | tr | tr | tr | tr |
| 1100 | 1101 | Linalool | 0.2 | tr | 0.1 | 0.5 |
| 1104 | 1104 | Hotrienol | – | – | – | tr |
| 1112 | 1113 | Phenethyl alcohol | – | – | – | 0.1 |
| 1146 | 1145 | Camphor | tr | – | – | tr |
| 1161 | 1162 | Benzoic acid | – | – | – | tr |
| 1171 | 1173 | Borneol | 0.3 | 0.3 | 0.1 | 0.2 |
| 1180 | 1180 | Terpinen-4-ol | tr | tr | tr | tr |
| 1191 | 1192 | Methyl salicylate | tr | – | – | tr |
| 1195 | 1195 | α-Terpineol | tr | tr | tr | 0.1 |
| 1226 | 1229 | Nerol | – | – | – | tr |
| 1245 | 1246 | Carvone | – | 0.1 | – | – |
| 1277 | 1278 | Perilla aldehyde | tr | tr | 0.1 | 1.3 |
| 1300 | 1299 | Perilla alcohol | – | – | – | 0.3 |
| 1310 | 1309 | 4-Vinylguaiacol | tr | – | – | tr |
| 1360 | 1357 | **Eugenol** | **98.4** | **98.6** | **94.5** | **14.2** |
| 1375 | 1375 | α-Copaene | – | – | – | tr |
| 1377 | 1379 | (*E*)-β-Damascenone | – | – | – | tr |
| 1384 | 1383 | *cis*-β-Elemene | – | tr | – | – |
| 1391 | 1390 | *trans*-β-Elemene | tr | 0.1 | tr | tr |
| 1396 | 1394 | Vanillin | tr | tr | tr | – |
| 1402 | 1403 | Methyl eugenol | 0.2 | 0.2 | tr | 0.1 |
| 1419 | 1417 | (*E*)-β-Caryophyllene | 0.1 | 0.2 | 0.1 | 0.8 |
| 1449 | 1449 | (*E*)-Lachnophyllum acid | – | tr | – | – |
| 1452 | 1454 | Vanillal | – | tr | – | – |
| 1455 | 1454 | α-Humulene | tr | tr | tr | 0.1 |
| 1456 | 1445 | *iso*-Eugenol | – | – | tr | – |
| 1476 | 1476 | Selina-4,11-diene | – | tr | – | – |
| 1479 | 1480 | Germacrene D | – | – | – | tr |
| 1492 | 1492 | β-Selinene | tr | tr | tr | - |
| 1492 | 1489 | (*Z,E*)-α-Farnesene | – | – | tr | 0.1 |
| 1496 | 1497 | Bicyclogermacrene | – | – | – | tr |
| 1499 | 1501 | α-Selinene | tr | tr | tr | – |
| 1514 | 1514 | (*Z*)-Lachnophyllum acid | tr | tr | tr | tr |
| 1519 | 1517 | (*E,Z*)-Matricaria ester | tr | 0.1 | 0.1 | 0.1 |
| 1523 | 1520 | Myristicin | tr | tr | 0.2 | 3.2 |
| 1551 | 1548 | **Elemicin** | **0.3** | **tr** | **4.5** | **77.8** |
| 1564 | 1561 | (*E*)-Nerolidol | – | – | – | tr |
| 1584 | 1587 | Caryophyllene oxide | 0.1 | 0.1 | tr | 0.1 |

**Table 3.** continued

| $RI_{calc}$ | $RI_{db}$ | Compound | HODN | HOHT | HOTH1 | HOTH2 |
|---|---|---|---|---|---|---|
| 1599 | 1600 | Methoxyeugenol | tr | – | tr | 0.1 |
| 1611 | 1611 | Humulene epoxide II | tr | tr | tr | – |
| 1616 | 1617 | (*Z*)-Asarone | – | – | tr | 0.2 |
| 1634 | 1632 | γ-Eudesmol | tr | tr | tr | tr |
| 1634 | 1632 | Caryophylla-4(12),8(13)-dien-5α-ol | tr | – | tr | – |
| 1638 | 1635 | Caryophylla-4(12),8(13)-dien-5β-ol | tr | – | tr | – |
| 1643 | 1645 | Agarospirol I (=Hinesol) | – | – | tr | tr |
| 1657 | 1652 | α-Eudesmol | 0.1 | tr | 0.1 | 0.1 |
| 1658 | 1659 | Cadin-4-en-10-ol | – | – | – | tr |
| 1660 | 1658 | *neo*-Intermedeol | 0.1 | 0.1 | 0.2 | tr |
| 1666 | 1668 | Intermedeol | – | – | tr | – |
| 1671 | 1671 | 14-Hydroxy-9-*epi*-(*E*)-caryophyllene | tr | tr | tr | – |
| 1681 | 1678 | Tetradec-9-yn-1-ol | tr | – | tr | – |
| 1729 | 1739 | (*E*)-Coniferyl aldehyde | tr | tr | tr | – |
| 1816 | 1816 | Cryptomeridiol | tr | tr | tr | – |
| 2200 | 2200 | Docosane | – | tr | – | – |
| 2300 | 2300 | Tricosane | – | tr | – | – |
| 2400 | 2400 | Tetracosane | – | tr | – | – |
| 2493 | 2491 | Dehydrodieugenol | 0.1 | 0.2 | 0.1 | tr |
| 2500 | 2500 | Pentacosane | tr | tr | tr | tr |
| 2511 | 2509 | Dehydrodiisoeugenol | tr | 0.1 | tr | – |
| 2600 | 2600 | Hexacosane | tr | tr | tr | tr |
| 2700 | 2700 | Heptacosane | tr | tr | tr | tr |
| 2800 | 2800 | Octacosane | tr | tr | tr | tr |
| | | Monoterpene hydrocarbons | 0.0 | traces | 0.0 | trace |
| | | Oxygenated monoterpenoids | 0.5 | 0.4 | 0.3 | 2.3 |
| | | Sesquiterpene hydrocarbons | traces | 0.1 | traces | 0.2 |
| | | Oxygenated sesquiterpenoids | 0.3 | 0.1 | 0.3 | 0.2 |
| | | Benzenoid aromatics | 99.1 | 99.0 | 99.3 | 95.7 |
| | | Others | 0.1 | 0.3 | 0.1 | 1.2 |
| | | Total identified | 100.0 | 99.9 | 100.0 | 99.7 |

Note: HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province. Major components are highlighted in **blue bold**.

hydrosols HODN, HOHT and HOTH1 were consistent with reports of larvicidal activity of eugenol. Elemicin has shown weak larvicidal activity against mosquito species, with 24-h $LC_{50} > 100$ μg/mL against *Ae. albopictus*.[65] Thus, the larvicidal activities of HOTH2 may have been due to synergistic activity between eugenol and elemicin.

The microemulsions demonstrated stronger toxicity than their free essential oils (Table 8). The small particle size of the MEs may lead to increased contact area with the larvae which improves better penetration into biological tissues and effective distribution of the active substance, enhancing insecticidal activity.[58]

### Moina Macrocopa Toxicity Assay

The hydrosols demonstrated less toxicity to *M. macrocopa* compared to the essential oils (Table 9). The essential oils and hydrosols fall within the category of moderate toxicity (Acute 2: $1 < LC_{50} \leq 10$ μg/mL) in relation to plankton.[69]

### Acetylcholinesterase (AChE) Inhibitory Activity

The AChE inhibitory activities of the hydrosol samples showed a linearly dependent trend on the content of eugenol (Table 10). The AChE inhibitory activity of the hydrosols decreased linearly with their eugenol content, specifically $IC_{50\ HODN}$ (% eugenol =

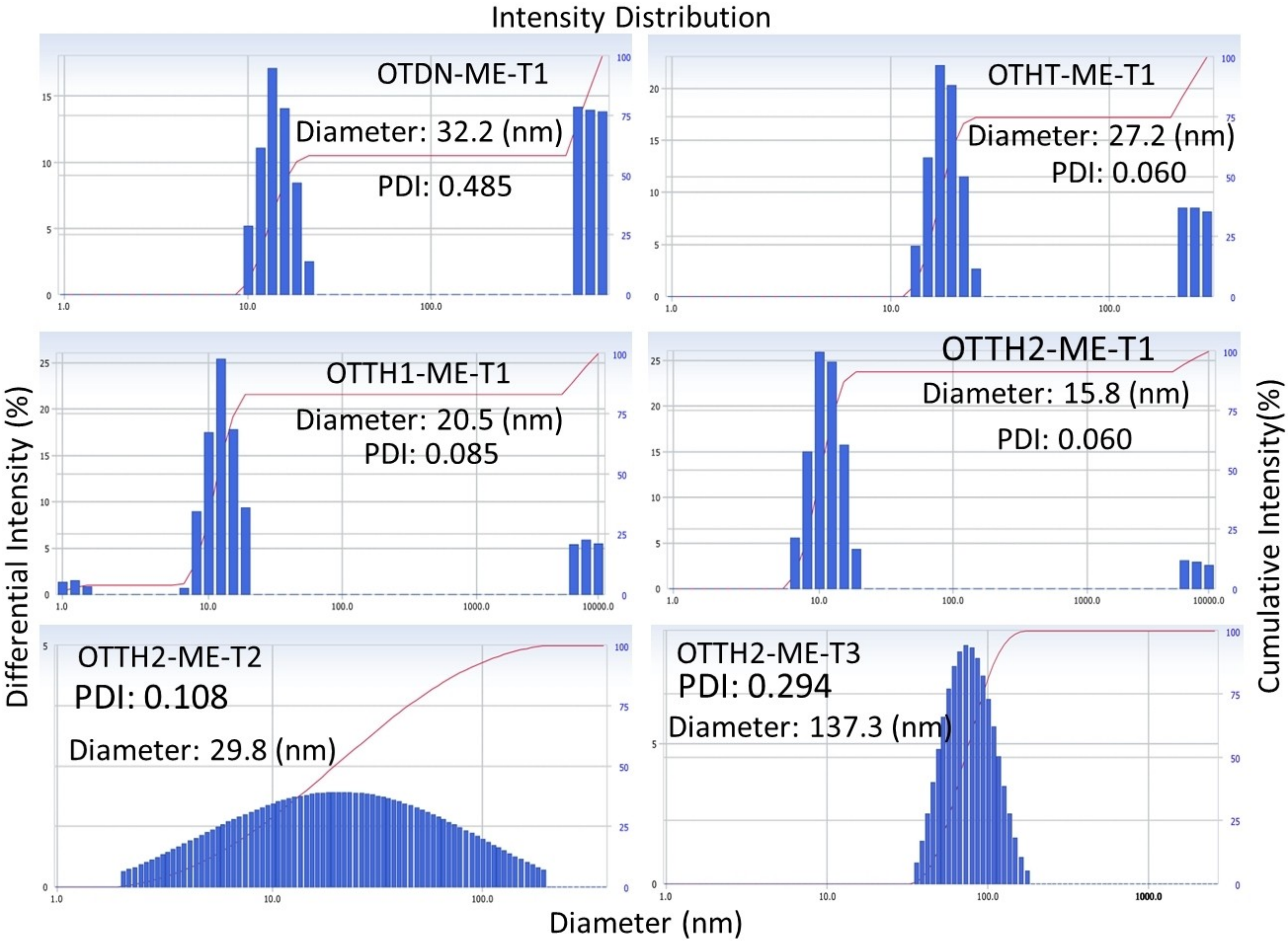


**Figure 2.** Dynamic light scattering (DLS) traces of microemulsions (MEs) at different timepoints. OTDN-ME-T1: Microemulsion of OTDN essential oil at time T1 (1 day); OTHT-ME-T1: Microemulsion of OTHT essential oil at time T1 (1 day); OTTH1-ME-T1: Microemulsion of OTTH1 essential oil at time T1 (1 day); OTTH2-ME-T1: Microemulsion of OTTH2 essential oil at time T1 (1 day); OTTH2-ME-T2: Microemulsion of OTTH2 essential oil at time T2 (120 days); OTTH2-ME-T3: Microemulsion of OTTH2 essential oil at time T3 (300 days).

98.4) $\approx IC_{50\ HOHT}$ (% eugenol = 98.6) $< IC_{50\ HOTH1}$ (% eugenol = 94.5) $< IC_{50\ HOTH2}$ (% eugenol = 14.2). Several previous publications on the AChE inhibitory activity of eugenol have reported $IC_{50}$ values in the range of 29.52 to 62.33 μg/mL.[70,71] *In silico* modeling has shown that *trans*-β-elemene has a stronger AChE inhibitory potential than eugenol.[72] Therefore, elemicin showed weak AChE inhibitory activity, consistent with the previous report of Sathya et al. (2020),[73] Bonesi et al. (2010)[74] reported that at a concentration of 0.06 mM (*E*)-β-caryophyllene inhibited AChE (electric eel) by 32 %; Hung et al. (2021)[68] reported an $IC_{50}$ value of 89.10 μg/mL.

In this study, it was shown that AChE inhibition is not the determining mechanism for the larvicidal activities of essential oils and hydrosols. The AChE inhibitory activity data did not correlate with the larvicidal activity data; HOTH2 had the weakest inhibition of AChE but demonstrated the strongest larvicidal activity when compared with other hydrosols.

Moreover, essential oils are of interest as aromatherapeutic agents for treating Alzheimer's disease, with the evaluation of inhibiting the enzyme acetylcholinesterase (AChE) considered is an important mechanism. The essential oils and hydrosols of *O. tenuiflorum* demonstrated promising AChE inhibitory activities with $IC_{50}$ values between 25.35 and 107.19 μg/mL.[75] The herb *Rhizoma acori graminei* (dry rhizomes of *Acorus gramineus* Soland), used in Asia to treat symptoms reminiscent of Alzheimer's disease (AD), contains eugenol as an active principle.[76] Eugenol has been proposed as a drug to treat neurological diseases such as Alzheimer's, depression, and Parkinson's.[4] Essential oils containing high levels of eugenol also show potential in the management of cognitive diseases such as Alzheimer's disease through their potent AChE inhibitory activity.[77,78]

## Conclusions

We have studied the yield and chemical composition of essential oils and hydrosols extracted from *O. tenuiflorum* leaves in central Vietnam, and proposed that *O. tenuiflorum* grown in Vietnam can be considered as a low-cost source of natural eugenol. Depending on geographical location, the concentration of eugenol in essential oils can be over 40 %, and in

**Table 4.** Larvicidal activity of essential oils and hydrosols against *Aedes aegypti* (µg/mL).

| Material | $LC_{50}$ (95 % limits) | $LC_{90}$ (95 % limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN | 47.82 (43.93–52.11) | 85.39 (75.52–100.97) | 1.5822 | 0.663 |
| OTHT | 41.67 (38.68–44.55) | 59.93 (55.20–67.12) | 0.7325 | 0.866 |
| OTTH1 | 38.62 (35.41–41.42) | 49.33 (46.01–53.72) | 0.0241 | 0.999 |
| OTTH2 | 42.48 (39.31–45.11) | 56.88 (53.16–62.79) | 0.1937 | 0.979 |
| HODN | 97.80 (92.00–103.67) | 136.01 (124.47–158.20) | 1.6512 | 0.800 |
| HOHT | 95.97 (90.51–102.16) | 129.98 (121.13–142.79) | 4.0828 | 0.398 |
| HOTH1 | 93.18 (87.11–99.08) | 132.83 (121.85–151.90) | 1.4693 | 0.832 |
| HOTH2 | 50.09 (45.67–55.11) | 99.10 (86.06–119.99) | 1.7179 | 0.633 |
| Permethrin | 0.00638 (0.00548–0.00744) | 0.0232 (0.0182–0.0318) | 8.868 | 0.031 |
| | | 48 h | | |
| OTDN | 30.41 (28.15–32.92) | 49.15 (44.08–56.99) | 1.3169 | 0.725 |
| OTHT | 37.07 (34.37–39.81) | 50.59 (46.69–56.10) | 0.1147 | 0.990 |
| OTTH1 | 29.07 (27.04–31.37) | 44.97 (40.47–52.14) | 6.9774 | 0.073 |
| OTTH2 | 31.95 (29.55–34.61) | 51.68 (46.40–59.76) | 7.1807 | 0.066 |
| HODN | 78.84 (73.08–84.69) | 116.49 (106.68–131.01) | 0.8873 | 0.926 |
| HOHT | 89.14 (83.97–94.59) | 120.32 (112.89–130.52) | 3.5841 | 0.465 |
| HOTH1 | 72.36 (67.01–78.07) | 110.25 (100.14–125.22) | 1.2009 | 0.878 |
| HOTH2 | 34.11 (31.01–37.57) | 74.09 (64.48–88.43) | 9.8479 | 0.020 |

Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

hydrosols up to 98.6%. Furthermore, essential oils and hydrosols can be considered as sources of botanical pesticides to control four globally-common disease-transmitting mosquito species. In addition, essential oils and hydrosols have shown promise in supporting the treatment of diseases related to the central nervous system through their strong AChE inhibitory activities. Based on the results of this study, we propose that waste hydrosols of eugenol-containing aromatic plants should be investigated for eugenol content and purity, and using these waste sources as raw materials in sustainable production.

## Abbreviations

| | |
|---|---|
| AChE | Acetylcholinesterase |
| AD | Alzheimer's disease |
| AHC | Agglomerative hierarchical cluster |
| DMSO | Dimethyl sulfoxide |
| DN | Da Nang City |
| GC-MS | Gas Chromatographic – Mass Spectral |
| HODN | Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City |
| HOTH1 | Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province |
| HOTH2 | Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province |
| HOHT | Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province |
| HT | Ha Tinh Province |
| $IC_{50}$ | Half maximal inhibitory concentration |
| $LC_{50}$ | Lethal concentration for 50% killing |
| MCT | Coconut oil |
| ME | Microemulsion |
| NC | Negative control |
| Nd | Not determined |
| Nt | Not tested. |
| OTDN | Essential oil of *Ocimum tenuiflorum* collected in Da Nang City |
| OTDN-ME | Microemulsion of OTDN essential oil |
| OTDN-ME-T1 | Microemulsion of OTDN essential oil at time T1 (1 day) |
| OTHT | Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province |
| OTHT-ME-T1 | Microemulsion of OTHT essential oil at time T1 (1 day) |
| OTTH1 | Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province |
| OTTH1-ME-T1 | Microemulsion of OTTH1 essential oil at time T1 (1 day) |
| OTTH2 | Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province |
| OTTH2-ME | Microemulsion of OTTH2 essential oil |

**Table 5.** Larvicidal activity of essential oils and hydrosols against *Aedes albopictus* (μg/mL).

| Material | $LC_{50}$ (95% limits) | $LC_{90}$ (95% limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN | 28.28 (25.99–30.81) | 50.51 (44.82–59.25) | 3.4102 | 0.333 |
| OTHT | 30.05 (27.96–32.42) | 45.78 (41.27–52.93) | 1.3660 | 0.714 |
| OTTH1 | 38.87 (35.89–42.03) | 62.64 (56.53–71.83) | 2.9552 | 0.399 |
| OTTH2 | 31.42 (29.36–33.89) | 44.04 (39.96–50.59) | 0.3551 | 0.949 |
| HODN | 45.14 (41.96–48.39) | 68.42 (62.31–78.11) | 0.5203 | 0.971 |
| HOHT | 50.00 (46.67–53.57) | 75.34 (68.26–87.17) | 6.5150 | 0.164 |
| HOTH1 | 48.62 (45.44–52.35) | 69.32 (63.91–77.08) | 2.3349 | 0.674 |
| HOTH2 | 46.61 (43.35–49.98) | 71.19 (64.65–81.69) | 4.0587 | 0.398 |
| Permethrin | 0.0024 (0.0021–0.0026) | 0.0042 (0.0038–0.0049) | 4.64 | 0.031 |
| | | 48 h | | |
| OTDN | 20.32 (18.60–22.17) | 37.38 (33.15–43.79) | 10.0481 | 0.018 |
| OTHT | 17.93 (16.52–19.45) | 29.90 (26.83–34.51) | 1.8940 | 0.595 |
| OTTH1 | 29.51 (27.03–32.23) | 54.62 (48.26–64.35) | 3.4682 | 0.325 |
| OTTH2 | 26.16 (24.86–27.83) | 34.75 (31.56–42.23) | 0.5281 | 0.913 |
| HODN | 41.48 (38.38–44.70) | 65.24 (59.18–74.42) | 3.3778 | 0.497 |
| HOHT | 30.86 (28.71–33.62) | 41.54 (38.01–46.86) | 5.0920 | 0.278 |
| HOTH1 | 41.97 (39.10–45.15) | 61.29 (56.76–67.43) | 2.5502 | 0.636 |
| HOTH2 | 30.40 (28.52–33.48) | 38.51 (34.66–46.57) | 0.0285 | 1.000 |

Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

OTTH2-ME-T1 Microemulsion of OTTH2 essential oil at time T1 (1 day)
OTTH2-ME-T2 Microemulsion of OTTH2 essential oil at time T2 (120 days)
OTTH2-ME-T3 Microemulsion of OTTH2 essential oil at time T3 (300 days)
PDI Polydispersity index
TH Thanh Hoa Province

## Experimental Section

### Chemicals

Polysorbate 80 (Tween 80) surfactant was purchased from Croda Singapore Pte Ltd (Singapore). Coconut oil (MCT) was purchased from Sternchemie GmbH & Co. KG (Germany). Isopropanol ACS, ISO was purchased from Scharlau, made in Spain. Permethrin and DMSO were purchased from Merck Vietnam (Ho Chi Minh City, Vietnam).

### Plant Material

Fresh leaves of *O. tenuiflorum* (purple type) were collected at the same time (June 2022) in the provinces of Thanh Hoa (TH1, TH2), Ha Tinh (HT), and Da Nang City (DN) (Table 1). Plants in flowering period were used for this study.

### Extraction of Essential Oils and Hydrosols

To determine the yield of essential oils, fresh leaves of holy basil (50 g/each time/three repetitions) were used to extract the essential oil by hydrodistillation using a Clevenger apparatus (Witeg Labortechnik, Wertheim, Germany) for 6 hours. The aqueous solutions obtained after the extraction of the essential oil were extracted with *n*-hexane which was then subjected to solvent recovery using a rotary vacuum evaporator to obtain hydrosols. The essential oils and hydrosols were dried with $Na_2SO_4$ and stored at 4 °C until they were used. The extraction yields of essential oils and hydrosols were calculated from three consecutive extractions.

### Gas Chromatographic – Mass Spectral (GC-MS) Analysis

Gas chromatography–mass spectral analyses (GC–MS) of essential oils and hydrosols were carried out using previously published instrumentation and protocols.[79] A Shimadzu GCMS-QP2010 Ultra (Shimadzu Scientific Instruments, Columbia, MD, USA) with a ZB-5 ms fused silica capillary column (60 m length, 0.25 mm diameter, and 0.25 μm film thickness) (Phenomenex, Torrance, CA, USA), He carrier gas, 2.0 mL/min flow rate, injection and ion source temperatures of 260 °C, and a GC oven program of 50 to 260 °C at 2.0 °C/min was used. A 0.1 μL amount of a 5% (w/v) sample of essential oil in $CH_2Cl_2$ was injected in split mode with a 24.5:1 split ratio. Identification of the essential oil components was carried out with a comparison of MS fragmentation and retention indices (RI) with those available in the databases.[80–83] Quantification was performed using external standards of representative compounds from each compound class.

**Table 6.** Larvicidal activity of essential oils and hydrosols against *Culex quinquefasciatus* (µg/mL).

| Material | $LC_{50}$ (95 % limits) | $LC_{90}$ (95 % limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN | 34.29 (31.38–37.49) | 67.30 (59.40–78.98) | 6.9579 | 0.073 |
| OTHT | 56.01 (52.67–60.93) | 72.70 (66.40–83.94) | 1.9451 | 0.584 |
| OTTH1 | 28.75 (27.11–30.98) | 38.87 (35.14–46.02) | 0.2941 | 0.961 |
| OTTH2 | Nt | Nt | Nd | Nd |
| HODN | 51.92 (48.50–55.68) | 78.07 (70.59–90.64) | 4.2920 | 0.368 |
| HOHT | 59.33 (55.86–65.03) | 75.94 (68.36–92.36) | 0.0510 | 1.00 |
| HOTH1 | 67.20 (62.12–72.77) | 107.89 (97.07–124.23) | 2.6373 | 0.620 |
| HOTH2 | Nt | Nt | Nt | Nt |
| Permethrin | 0.0165 (0.0149–0.0181) | 0.0305 (0.0267–0.0367) | 5.235 | 0.073 |
| | | 48 h | | |
| OTDN | 20.99 (19.41–22.71) | 35.44 (31.81–40.87) | 4.0844 | 0.252 |
| OTHT | 28.05 (25.89–30.44) | 47.55 (42.44–55.49) | 0.9032 | 0.825 |
| OTTH1 | 22.69 (20.95–24.79) | 34.95 (31.80–39.47) | 9.2131 | 0.056 |
| OTTH2 | Nt | Nt | Nd | Nd |
| HODN | 36.59 (37.06–43.55) | 66.97 (60.21–77.11) | 2.3030 | 0.680 |
| HOHT | 49.34 (45.64–53.33) | 81.31 (72.96–94.38) | 6.1209 | 0.106 |
| HOTH1 | 50.32 (46.87–54.04) | 77.40 (69.94–89.61) | 0.9221 | 0.921 |
| HOTH2 | Nt | Nt | Nd | Nd |

Note: Nt: Not tested. Nd: Not determined; OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

### Preparation and Characterization of Microemulsions

Microemulsion formulations of essential oils were prepared according to the method described by Hung et al (2023) with minor modifications.[58] Oil phase includes essential oil, isopropanol and coconut oil (MCT) in a ratio of 3:1:1 (v/v/v, respectively) stirred with a magnetic stirrer H3770-HS (Benchmark Digital Hotplate Stirrer) for 30 minutes. Then Tween 80 (10% v/v) was added to the oil mixture and stirring was continued for an additional 30 minutes. Distilled water (85% v/v) was added to the mixture at a rate of 3 mL/min and stirred until transparent and homogeneous MEs were obtained. The MEs were contained in transparent vials which were stored at 25 °C and 12 h light, 12 h dark cycle. The particle size distributions of the samples were determined on a Zetasizer-Nano ZS instrument (Malvern, UK) by dynamic laser scattering method. The MEs were evaluated for droplet size distribution at two time points of 01 day (T1), 120 days (T2) and 300 days (T3).

### Mosquito Larvicidal Assay

*Aedes* spp. mosquitoes are continuously maintained at Duy Tan University. Egg rafts of *Cx. fuscocephala* were collected and identified according to the method of Pham et al. (2023).[79] Egg rafts of *Cx. quinquefasciatus* were collected in domestic wastewater canals in Da Nang city area. Mosquito eggs were allowed to hatch in tap water overnight. The larvae were fed on a mixture of cat food and yeast (ratio 3:1, w/w). All developmental stages of mosquito species were placed under laboratory conditions of temperature of 25 °C, relative humidity of 75%, 12-hour light/12-hour dark cycle.

Larvicidal activities were performed according to WHO (2005) guidelines with minor modifications.[84] The third instar and early fourth instar larvae were used for larvicidal activity assays. The larvae (25 larvae) were transferred into 250-mL beakers containing 150 mL of distilled water. The essential oils and hydrosols were dissolved with ethanol (Sigma-Aldrich) to obtain 1% stock solutions. Various volumes of the stock solutions were transferred to test beakers containing mosquito larvae to achieve the desired concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. Ethanol was used as negative control, permethrin (Sigma-Aldrich) was used as positive control. Each concentration of agents was repeated 4 times. The number of dead larvae was determined after 24 and 48 h of exposure. Larvicidal activity assays were performed under the same mosquito rearing conditions.

For the microemulsions, different volumes of them were transferred directly into test cups containing larvae to obtain essential oil concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. A mixture of Tween 80, MCT and isopropanol (10/1/1, w/w) that had been stirred for 30 min was used as a negative control (NC).

All experimental procedures involving animals (mice, mosquitoes, and non-target organisms) were carried out in compliance with the "Guideline for the Care and Use of Laboratory Animals," approved by the Medical-Biological Research Ethics Committee of Duy Tan University (DTU/REC2023/NHH07), Vietnam.

### Moina Macrocopa Toxicity Assay

The *M. macrocopa* adults were collected in stagnant water and were reared together with mosquito larvae for 14 days before being

 

**Table 7.** Larvicidal activity of essential oils and hydrosols against *Culex fuscocephala* (μg/mL).

| Material | $LC_{50}$ (95 % limits) | $LC_{90}$ (95 % limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN | 17.29 (15.59–19.14) | 41.27 (35.78–49.41) | 6.4277 | 0.093 |
| OTHT | 15.53 (14.35–16.84) | 25.59 (22.92–29.71) | 4.2632 | 0.234 |
| OTTH1 | 15.42 (14.04–16.92) | 30.60 (26.84–36.37) | 4.0427 | 0.257 |
| OTTH2 | 18.71 (17.21–20.31) | 31.87 (28.54–36.87) | 5.4325 | 0.143 |
| HODN | 79.14 (75.24–83.24) | 116.16 (107.34–129.83) | 1.8972 | 0.868 |
| HOHT | 69.04 (65.51–72.55) | 99.88 (93.07–109.95) | 4.0436 | 0.543 |
| HOTH1 | 74.58 (70.77–78.50) | 110.60 (102.39–123.15) | 4.1320 | 0.531 |
| HOTH2 | 53.88 (52.18–56.51) | 63.04 (59.29–71.15) | 0.0207 | 1.000 |
| Permethrin | 0.0024 (0.0022–0.0026) | 0.0037 (0.0034–0.0043) | 2.1866 | 0.335 |
| | | 48 h | | |
| OTDN | 12.98 (11.77–14.25) | 27.16 (23.87–32.06) | 9.3258 | 0.025 |
| OTHT | 11.92 (10.81–13.12) | 24.30 (21.25–29.11) | 5.5752 | 0.134 |
| OTTH1 | 11.99 (10.76–13.28) | 27.09 (23.40–32.92) | 5.3370 | 0.149 |
| OTTH2 | 14.72 (13.30–16.25) | 32.07 (27.83–38.65) | 6.2535 | 0.100 |
| HODN | 68.24 (64.32–72.22) | 107.72 (99.03–120.71) | 2.0816 | 0.838 |
| HOHT | 66.15 (62.70–69.57) | 95.81 (89.33–105.31) | 2.6202 | 0.758 |
| HOTH1 | 67.61 (64.04–71.16) | 99.40 (92.42–109.69) | 1.4689 | 0.917 |
| HOTH2 | 36.84 (34.14–39.58) | 56.57 (51.98–62.85) | 6.7303 | 0.241 |

Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

**Table 8.** Larvicidal activity of microemulsions of essential oils against *Aedes aegypti* (μg/mL).

| Material | $LC_{50}$ (95 % limits) | $LC_{90}$ (95 % limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN-ME | 28.35 (26.31–30.55) | 45.31 (41.04–51.64) | 8.0425 | 0.090 |
| OTTH2-ME | 21.42 (19.82–23.07) | 33.98 (30.86–38.64) | 4.5639 | 0.335 |
| NC | Inactive | Inactive | Nd | Nd |
| | | 48 h | | |
| OTDN-ME | 21.30 (19.78–22.81) | 31.52 (28.92–35.48) | 1.5645 | 0.815 |
| OTTH2-ME | 17.53 (16.21–18.96) | 27.81 (25.14–31.77) | 2.3822 | 0.666 |
| NC | Inactive | Inactive | Nd | Nd |

Nd: Not determined. NC: Negative control. OTDN-ME: Microemulsion of OTDN essential oil. OTTH2-ME: Microemulsion of OTTH2 essential oil.

used for the assay. Toxicological evaluation of essential oils and hydrosols against *M. macrocopa* was performed under the same protocol and conditions as the larvicidal activity assay.

## Data Analysis

Mortality data were analyzed by log-probit analysis[85] to acquire $LC_{50}$ and $LC_{90}$ values as well as 95 % confidence limits using Minitab® version 19.2020.1 (Minitab, LLC, State College, PA, USA). Analysis of variance was conducted by one-way ANOVA followed by the Tukey test using Minitab® version 19.2020.1 (Minitab, LLC, State College, PA, USA). Differences at $p < 0.05$ were considered to be statistically significant.

Agglomerative hierarchical cluster (AHC) analysis was carried out using XLSTAT v. 2018.1.1.62926 (Addinsoft, Paris, France). The essential oil compositions for the four samples in this work as well as 68 samples reported in the literature were used as operational taxonomic units (OTUs) and the percentages of the most abundant essential oil components (eugenol, methyl eugenol, β-caryophyllene, β-elemene, estragol, 1,8-cineole, β-bisabolene, and *trans*-β-guaiene) were used to define the chemical associations between the essential oils. Dissimilarity was used to determine clusters

**Table 9.** Toxicity of essential oils and hydrosols to *Moina macrocopa* (μg/mL).

| Material | $LC_{50}$ (95 % limits) | $LC_{90}$ (95 % limits) | $\chi^2$ | *p* |
|---|---|---|---|---|
| | | 24 h | | |
| OTDN | 2.58 (2.37–2.83) | 4.95 (4.37–5.82) | 13.125 | 0.041 |
| OTHT | 2.56 (2.37–2.79) | 4.33 (3.88–5.01) | 1.566 | 0.955 |
| OTTH1 | 3.58 (3.32–3.90) | 5.34 (4.91–5.91) | 17.955 | 0.003 |
| OTTH2 | 1.10 (1.06–1.16) | 1.27 (1.20–1.44) | 0.009 | 1.000 |
| HODN | 7.74 (7.02–8.54) | 16.40 (14.26–19.64) | 12.943 | 0.044 |
| HOHT | 6.51 (5.93–7.16) | 13.67 (11.98–16.14) | 20.038 | 0.003 |
| HOTH1 | 8.17 (7.48–8.92) | 14.94 (13.23–17.55) | 3.007 | 0.808 |
| HOTH2 | Nt | Nt | Nd | Nd |
| | | 48 h | | |
| OTDN | 0.87 (0.79–0.95) | 2.04 (1.78–2.41) | 18.939 | 0.004 |
| OTHT | 0.81 (0.74–0.89) | 1.72 (1.52–2.02) | 14.604 | 0.024 |
| OTTH1 | 0.65 (0.59–0.72) | 1.42 (1.25–1.67) | 15.861 | 0.015 |
| OTTH2 | 0.86 (0.79–0.92) | 1.32 (1.23–1.45) | 10.387 | 0.065 |
| HODN | 4.50 (4.12–4.93) | 8.44 (7.47–9.87) | 14.761 | 0.022 |
| HOHT | 3.82 (3.47–4.20) | 8.51 (7.44–10.03) | 20.028 | 0.003 |
| HOTH1 | 4.48 (4.08–4.91) | 8.78 (7.73–10.32) | 5.763 | 0.450 |
| HOTH2 | Nt | Nt | Nd | Nd |

Nt: Not tested. Nd: Not determined. OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected in Duc Tho district, Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

**Table 10.** Inhibition of acetylcholinesterase (AChE) by essential oils and hydrosols (μg/mL).[a]

| Concentration | OTDN | HODN | OTHT | HOHT | OTTH1 | HOTH1 | OTTH2 | HOTH2 |
|---|---|---|---|---|---|---|---|---|
| 500 | 100.76 ± 2.00 | 91.61 ± 2.15 | 93.57 ± 1.69 | 91.94 ± 2.02 | 93.68 ± 1.54 | 91.94 ± 1.92 | 93.68 ± 2.46 | 85.80 ± 1.98 |
| 100 | 98.42 ± 1.24 | 68.63 ± 1.08 | 66.78 ± 1.46 | 62.42 ± 1.87 | 71.02 ± 1.23 | 60.68 ± 2.00 | 68.19 ± 1.31 | 51.60 ± 1.05 |
| 20 | 36.06 ± 1.08 | 32.35 ± 1.47 | 28.32 ± 1.23 | 35.95 ± 1.16 | 37.69 ± 2.46 | 16.56 ± 0.92 | 27.89 ± 0.92 | 18.53 ± 0.72 |
| 4 | 12.72 ± 0.77 | 18.85 ± 1.22 | 6.75 ± 0.46 | 24.07 ± 1.62 | 20.04 ± 0.92 | 12.42 ± 1.13 | 16.45 ± 1.24 | 5.66 ± 0.45 |
| $IC_{50}$ | 25.35 ± 0.42 | 43.94 ± 3.56 | 51.52 ± 3.24 | 50.06 ± 4.08 | 34.21 ± 3.03 | 86.62 ± 5.11 | 51.01 ± 3.41 | 107.19 ± 5.05 |

[a] Galantamine (positive control) had an $IC_{50}$ value of 1.78 ± 0.13 μg/mL. Note: OTDN: Essential oil of *Ocimum tenuiflorum* collected in Da Nang City; OTHT: Essential oil of *Ocimum tenuiflorum* collected Duc Tho district, in Ha Tinh Province; OTTH1: Essential oil of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; OTTH2: Essential oil of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province; HODN: Hydrosol of *Ocimum tenuiflorum* collected in Da Nang City; HOHT: Hydrosol of *Ocimum tenuiflorum* collected in Ha Tinh Province; HOTH1: Hydrosol of *Ocimum tenuiflorum* collected in Hoang Hoa District, Thanh Hoa Province; HOTH2: Hydrosol of *Ocimum tenuiflorum* collected in Thuong Xuan district, Thanh Hoa Province.

considering Euclidean distance and Ward's method was used to define agglomeration.

## *Author Contributions*

Conceptualization: Huy Hung Nguyen, William N. Setzer; Data curation: Huy Hung Nguyen, Prabodh Satyal, William N. Setzer; Formal analysis: Prabodh Satyal, William N. Setzer; Investigation: Duc Giang Le, Hai Giang Nguyen, Thi Uyen Nhi Nguyen, Cam Nhung Nguyen, Thuy Hang Le, Van Huynh Le, Xuan Luong Ngo, Thi Mai Hoa Le, Van Hoa Vo, Thanh Thuong Vo; Methodology: Nguyen Huy Hung, Prabodh Satyal; Project administration: Duc Giang Le; Resources: Duc Giang Le, Nguyen Huy Hung,, Prabodh Satyal; Software: Prabodh Satyal; Supervision: Duc Giang Le; Validation, Huy Hung Nguyen, William N. Setzer; Visualization, Huy Hung Nguyen, William N. Setzer; Roles/Writing – original draft: Huy Hung Nguyen, William N. Setzer; Writing – review & editing: William N. Setzer.

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## Conflict of Interests

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** Acetylcholinesterase · Holy basil · Mosquito larvicidal · *Ocimum sanctum* · Microemulsions

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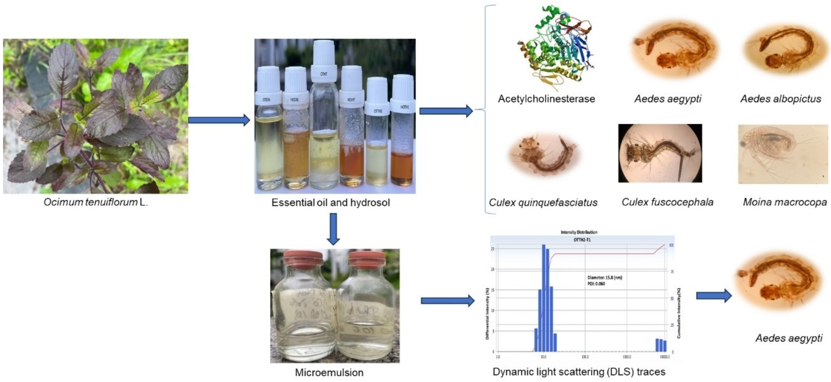



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**Essential oil and Waste Hydrosol of *Ocimum Tenuiflorum* L.: A Low-Cost Raw Material Source of Eugenol, Botanical Pesticides, and Therapeutic Potentiality**

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