Abstract: Volatile plant-derived products were observed to exhibit broad spectrum of biological effects. However, due to their volatility, results of conventional microplate-based bioassays can be significantly affected by the vapors. With aim to demonstrate this phenomenon, antimicrobial, antioxidant, and cytotoxic activities of three essential oils (Alpinia elegans, Cinnamomum iners, and Xanthostemon verdugonianus), one supercritical CO₂ extract (Nigella sativa), and four plant-derived compounds (capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone) were evaluated in series of experiments including both ethylene vinyl acetate (EVA) Capmat sealed and nonsealed microplates. The results clearly illustrate that vapor transition to adjoining wells causes false-positive results of bioassays performed in nonsealed microtiter plates. The microplate layout and a duration of the assay were demonstrated as the key aspects defining level of the results affection by the vapors of volatile agents. Additionally, we reported biological activities and chemical composition of essential oils from A. elegans seeds and X. verdugonianus leaves, which were, according to our best knowledge, analyzed for the first time. Considering our findings, certain modifications of conventional microplate-based assays are necessary (e.g., using EVA Capmat as vapor barrier) to obtain reliable results when biological properties of volatile agents are evaluated.

Keywords: bioassay; broth microdilution; DPPH; essential oil; microtiter plate; MTT; plant compounds; supercritical CO₂ extract; volatilization

1. Introduction

Volatile plant-derived products (VPDPs) are a large group of carbon-based chemicals with low molecular weight and high vapor pressure at ambient temperature including different chemical classes such as hydrocarbons and their derivatives, e.g., benzoquinones, epoxides, methoxyphenols, and quinolines. [1,2]. Volatile products that can be obtained from different plant parts involve essential
oils, extracts, oleoresins, tinctures, distillates, and juice concentrates. They are isolated using an array of techniques such as expression, distillation, concentration, solvent extraction, and supercritical fluid extraction [3]. Essential oils (EOs), the complex mixtures composed mainly of terpenoids, are important representatives of VPDPs with a characteristic aroma and a flavor typical for certain plant families (e.g., Lauraceae, Myrtaceae, and Zingiberaceae) [4–7]. Since ancient times, EOs have been used for their medicinal and organoleptic properties. Nowadays, plant volatiles have various applications in pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries [8]. For example, EO and supercritical CO\textsubscript{2} extract obtained from seeds of \textit{Nigella sativa} L. is used as a medicament for a variety of disorders in the digestive tract, kidney, cardiovascular, respiratory, and immune systems [9,10]. VPDPs were observed to exhibit broad spectrum of biological effects including antimicrobial, anticarcinogenic, antioxidant, and cytotoxic properties [11]. Especially plant species originating from tropical regions are considered valuable sources of biologically active agents due to the stronger pressure of bacterial and fungal pathogens affecting plants in tropical ecosystems [12]. Among the tropical areas, Philippine archipelago belongs to the important centers of biodiversity with a large number of endemic plants. Besides species that has been reported to exhibit medicinal properties [13], less explored medicinal and aromatic plants, such as \textit{Alpinia elegans} (C.Presl) K.Schum., \textit{Cinnamomum iners} Reinw. ex Blume, and \textit{Xanthostemon verdugonianus} Náves ex Fern.-Vill, occur in this region.

In a plant-based drug discovery, the in vitro biological screening using pharmacologically relevant microplate assays is one of the first steps to verify the effectivity and safety of medicinal plants and their constituents [14]. Since the microwell plate was created in 1951 by Hungarian scientist with aim to provide a potentially useful techniques suitable for the high throughput screening, a number of standardized procedures have been established such as methods widely referenced to Clinical and Laboratory Standards Institute (CLSI) [15]. The use of microplate together with a fully automated equipment makes bioassays simple, fast, and reliable, providing reproducible results [16]. In natural product research, they serve to determine biological effects such as antimicrobial [17], antioxidant [18], and cytotoxic activity [19]. Respective, broth microdilution, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and thiazolyl blue tetrazolium bromide (MTT) assays are examples of the most widely used methods for the assessment of plant-derived compounds including volatile agents [20–22]. Although conventional microplate-based bioassays are common in the laboratory practice, in case of VPDPs in vitro testing, they face specific problems due to physicochemical properties of these agents such as high volatility, hydrophobicity, and viscosity [23]. The hydrophobic nature worsens the solubility of VPDPs in water-based media (e.g., agar and broth), that may reduce the dilution capability and unequal distribution of active components through the medium [24]. The volatility causes a risk of active substance losses by evaporation during sample handling, experiment preparation, and incubation depending on its time and temperature conditions [25,26]. Moreover, significant influence of vapors of volatiles on the results of biological tests performed in microtiter plates by spreading of volatiles into adjoining wells has been described [27]. To prevent above mentioned difficulties, some modifications of standardized methods are required. For example, the use of ethylene vinyl acetate (EVA) Capmat was observed to be effective as a vapor barrier in assays for determination of antistaphylococcal activity of thymoquinone in combinations with antibiotics [28] and cytotoxicity of carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, and thymoquinone [29]. However, certain current studies still continue to overlook the significant risk of results affection by vapors of plant volatiles when tested in microplate-based assays [30,31].

With aim to clearly demonstrate the significant results distortion of the standard methods for evaluation of biological properties of VPDPs by their vapors, the series of tests comparing identical experiments performed simultaneously in both sealed and nonsealed microtiter plates were assayed. For this purpose, we tested antimicrobial, antioxidant, and cytotoxic effects of three EOs obtained from Philippine plant species \textit{A. elegans}, \textit{C. iners}, and \textit{X. verdugonianus}, one supercritical CO\textsubscript{2} extract from \textit{N. sativa}, and four plant-derived compounds, i.e., capsaicin, caryophyllene oxide, 8-hydroxyquinoline,
and thymoquinone, as representatives of various classes of biologically effective agents with different levels of volatility. Moreover, chemical composition of EOs and supercritical CO₂ extract tested was analyzed to assess the relationship between their biological activities and chemistry.

2. Results

2.1. Antimicrobial Assay

The results of antimicrobial activity performed using multiplate design when all samples were tested in one replicate in one microtiter plate (Figure 1) were significantly affected by vapors of plant-derived products tested in nonsealed plates. In general, the effectiveness of samples varied ranging from 2 to 1024 µg/mL and from 2 to 512 µg/mL in EVA Capmat sealed and nonsealed plates, respectively. Importantly, 8-Hydroxyquinoline was determined as the most active antimicrobial agent, when its lowest minimum inhibitory concentration (MIC) was found against Staphylococcus aureus with value 2 µg/mL in both EVA Capmat sealed and nonsealed plates. The most affected result of antimicrobial assay was observed for capsain against Candida albicans, although no activity was detected in plates sealed with vapor barrier, MIC 64 µg/mL was found in nonsealed plates. Similarly, A. elegans oil, X. verdugonianus oil, caryophyllene oxide, and thymoquinone did not possessed any growth-inhibitory effect against one of these pathogens C. albicans, Enterococcus faecalis, and S. aureus in EVA Capmat sealed plates; however, certain degree of inhibition with MICs in the range of 256–512 µg/mL was observed in plates without vapor barrier. Except C. iners EO, all samples exhibited some antimicrobial efficacy; however, only 8-hydroxyquinoline was active against all pathogens tested. The detailed results of growth-inhibitory effect of VPDPs against four representatives of both Gram-negative and Gram-positive bacteria and one fungal strain in EVA Capmat sealed and nonsealed plates are summarized in Table 1.

![Figure 1.](Figure 1. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of capsain, 8-hydroxyquinoline, and thymoquinone tested in microtiter plates sealed with vapor barrier ethylene vinyl acetate (EVA) Capmat and nonsealed microplates.)

2.2. Antioxidant Assay

Although the results of antioxidant assay were affected less than those of antimicrobial testing, VPDPs showed different results in series of single-plate designed DPPH tests when one EO (or extract) and one compound were assayed in triplicates together in the same microtiter plate (Figure 2). Among all plant-derived volatiles, only three compounds, namely, capsain, 8-hydroxyquinoline, and thymoquinone, showed some level of antioxidant activity in both EVA Capmat sealed and nonsealed plates with respective half maximal inhibitory concentrations (IC₅₀) in the ranges of 24.22–313.36 and 23.11–199.33 µg/mL, respectively. A summary of all results of DPPH assay is shown in Table 2. The most promising free radical scavenging potential has been observed for capsain (IC₅₀ 24.22 and 23.11 µg/mL in sealed and nonsealed plates, respectively). The result of antioxidant activity of thymoquinone was the most affected by vapors, in contrast to IC₅₀ value 313.36 µg/mL in EVA Capmat sealed plates, lower value 199.33 µg/mL was detected in nonsealed plates. In addition,
certain level of vapors influence is apparent regarding to standard deviations of IC₅₀ values, which are represented by broader range of values in nonsealed plates as seen in Figure 1 showing data of absorbance average of triplicates in one experiment.

![Graphs showing cytotoxic activity of Alpinia elegans, Cinnamomum iners, Xanthostemon verdugonianus essential oils, Nigella sativa supercritical CO₂ extract, capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone to human colon cancer cells Caco-2 tested in microtiter plates sealed with vapor barrier EVA Capmat and nonsealed microplates.](image)

**Figure 2.** Cytotoxic activity of *Alpinia elegans, Cinnamomum iners, Xanthostemon verdugonianus* essential oils, *Nigella sativa* supercritical CO₂ extract, capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone to human colon cancer cells Caco-2 tested in microtiter plates sealed with vapor barrier EVA Capmat and nonsealed microplates.
Table 1. Influence of the vapors of volatile plant-derived products on the results of the antibacterial activity when tested by broth microdilution assay.

| Plant Species/Compound | Candida albicans | Enterococcus faecalis | Escherichia coli | Pseudomonas aeruginosa | Staphylococcus aureus |
|------------------------|------------------|-----------------------|-----------------|------------------------|----------------------|
|                        | Sealed | Nonsealed | Sealed | Nonsealed | Sealed | Nonsealed | Sealed | Nonsealed | Sealed | Nonsealed |
| Essential oil, CO₂ extract |        |           |        |           |        |           |        |           |        |           |
| Alpinia elegans        | >1024   | 512       | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | 256    | 256       |
| Cinnamomum iners       | >1024   | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     |
| Nigella sativa         | >1024   | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     |
| Xanthostemon verdugonianus | 1024   | 256       | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     |
| Capsaicin              | >1024   | 64        | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     |
| Caryophyllene oxide    | >1024   | 256       | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     | >1024  | >1024     |
| 8-Hydroxyquinoline     | 32     | 16        | 512    | 128       | 256    | 64        | 1024   | 512       | 2      | 2         |
| Thymoquinone           | 64     | 32        | >1024  | 512       | 512    | 264       | >1024  | >1024     | 64     | 16        |
| Positive antibiotic control |       |           |        |           |        |           |        |           |        |           |
| Ciprofloxacin          | -      | -         | -      | -         | -      | -         | 0.125  | 1         | -      | -         |
| Fluconazole            | 0.5    | 4         | -      | -         | -      | -         | -      | -         | -      | -         |
| Oxacillin              | -      | -         | 32     | 32        | 1      | 2         | -      | -         | -      | -         |
| Tetracycline           | -      | -         |        |           | 1      | 2         | -      | -         | -      | -         |
Table 2. Influence of the vapors of volatile plant-derived products on the results of antioxidant activity testing using 2,2-diphenyl-1-picrylhydrazyl assay.

| Plant Species/Compound | IC$_{50}$ ± SD $^1$ (µg/mL) | Sealed | Nonsealed |
|------------------------|-----------------------------|--------|-----------|
| **Essential oil, CO$_2$ extract** | | | |
| *Alpinia elegans* | >512 | >512 | |
| *Cinnamomum iners* | >512 | >512 | |
| *Nigella sativa* | >512 | >512 | |
| *Xanthostemon verdugonianus* | >512 | >512 | |
| **Compound** | | | |
| Capsaicin | 24.22 ± 2.57 | 23.11 ± 7.10 | |
| Caryophyllene oxide | >512 | >512 | |
| 8-Hydroxyquinoline | 79.09 ± 24.15 | 61.92 ± 16.53 | |
| Thymoquinone | 313.36 ± 68.71 | 199.33 ± 88.02 | |
| **Positive control** | | | |
| Trolox | 9.94 ± 2.30 | 10.96 ± 1.96 | |

$^1$ IC$_{50}$ ± SD: half maximal inhibitory concentration ± standard deviation.

2.3. Cytotoxicity Assay

Similarly, as in both antimicrobial and antioxidant assays, the results of cytotoxicity were significantly affected when tested in single-plates layouts with four samples in duplicates in one microtiter plate (Figure 2). The values IC$_{50}$ varied in ranges of 0.95–57.40 and 0.18–4.85 µg/mL for EVA Capmat sealed and nonsealed microplates, respectively. The detailed results of the MTT assay performed with human colon cancer cells Caco-2 are listed in Table 3. The lowest cytotoxic effect was observed for caryophyllene oxide (IC$_{50}$ value 57.40 µg/mL) in EVA Capmat sealed plates. Moreover, in case of this compound, the most significant difference in the results was recorded as IC$_{50}$ value determined in plates with vapor barrier was 11 times higher than IC$_{50}$ value in nonsealed plates (IC$_{50}$ = 4.85 µg/mL). The effect of vapors of volatile agents tested on results of cytotoxic assay is obvious when graph curves for each sample tested is compared as shown in Figure 2 displaying data from three independent experiments in duplicates. Moreover, IC$_{50}$ values of *A. elegans* oil, *C. iners* oil, *X. verdugonianus* oil, 8-hydroxyquinoline, and thymoquinone performed in nonsealed plates were not detected, as these values were below the lowest concentration tested.

Table 3. Influence of the vapors of volatile plant derived products on the results of cytotoxicity to human colon cancer cells Caco-2 determined using thiazolyl blue tetrazolium bromide (MTT) assay.

| Plant Species/Compound | IC$_{50}$ ± SD $^1$ (µg/mL) | Sealed | Nonsealed |
|------------------------|-----------------------------|--------|-----------|
| **Essential oil, CO$_2$ extract** | | | |
| *Alpinia elegans* | 23.84 ± 3.29 | n.d.$^2$ | |
| *Cinnamomum iners* | 2.96 ± 0.28 | n.d. | |
| *Nigella sativa* | 21.71 ± 2.79 | 0.18 ± 0.04 | |
| *Xanthostemon verdugonianus* | 12.51 ± 3.62 | n.d. | |
| **Compound** | | | |
| Capsaicin | 11.95 ± 2.72 | 1.71 ± 0.26 | |
| Caryophyllene oxide | 57.40 ± 9.19 | 4.85 ± 1.03 | |
| 8-Hydroxyquinoline | 3.24 ± 1.50 | n.d. | |
| Thymoquinone | 0.95 ± 0.05 | n.d. | |

$^1$ IC$_{50}$ ± SD: half maximal inhibitory concentration of proliferation ± standard deviation, $^2$ n.d.: not detected.
2.4. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

In this study, three EOs hydrodistilled from different parts of Philippines plant species *A. elegans*, *C. iners*, and *X. verdugonianus* were obtained in yields ranging from 0.52% to 2.86% (v/w). Yield of supercritical CO$_2$ extract of *N. sativa* was 5.80% (w/w). Based on the GC/MS analysis equipped with HP-5MS/DB-HeavyWAX columns, a total of 119, 106, 51, and 20 compounds were identified in the samples, representing 93.637/93.186, 95.571/96.676, 94.757/96.114, and 82.349/92.308% of their total contents, respectively. The analysis showed that monoterpenes and sesquiterpenes were the leading chemical classes of the major constituents in the EOs tested, however, *N. sativa* supercritical CO$_2$ extract was composed mainly by fatty acids. The complete chemical composition of all VPDPs analyzed is provided in Tables 4–7.

In *A. elegans* seed EO, D-limonene (16.77/15.39% = 2.39/2.33 mg/kg) was the main compound followed by α-pinene (13.66/12.24% = 1.96/1.86 mg/kg) and caryophyllene oxide (11.37/10.78% = 1.74/1.77 mg/kg). *C. iners* leaf EO was rich in content of caryophyllene (21.00/34.87% = 3.22/6.56 mg/kg), followed by linalool (15.44/13.89% = 3.15/3.02 mg/kg). Pseudolimonene was detected in a significant amount by HP-5MS column (9.50/9.53% = 1.71/1.72 mg/kg), and, conversely, β-phellandrene was found by DB-HeavyWAX column (5.50/5.92% = 1.08 mg/kg). The major component of *X. verdugonianus* leaf oil was α-gurjunene (32.28/19.51% = 3.74/3.64 mg/kg), followed by cyperenone (22.65/52.69% = 2.74/10.96 mg/kg) and caryophyllene (6.38/2.98% = 0.74/0.56 mg/kg). The most abundant component of *N. sativa* supercritical CO$_2$ extract was linoleic acid (71.65/59.24% = 3.01/6.71 mg/kg), followed by ethyl linoleate (5.02/0.26% = 0.14/0.03 mg/kg) and ethyl oleate (2.78/0.07% = 0.07/0.03 mg/kg). Other dominant compounds, oleic acid (19.57/19.52% = 2.20/0.68 mg/kg) and hexadecenoic acid (9.89/1.09% = 1.09/0.10 mg/kg), were detected by DB-HeavyWAX column only.
| R1 | Compound | C² | RF³ | Column 4 | Identification 5 |
|---|---|---|---|---|---|
| Obs. | Lit. | | | HP-5MS | DB-HeavyWAX |
| | | | | (%) | (%) |
| | | | | c | c |
| 1 | 924 | 924 | α-Thujene | MH | 0.765 | 0.117 | 0.016 | - ⁸ | - | RI, GC/MS | - |
| 2 | 932 | 932 | α-Pinene | MH | 0.765 | 13.661 | 1.963 | 12.237 | 1.855 | RI, GC/MS | GC/MS |
| 3 | 945 | 953 | Camphene | MH | 0.765 | 0.245 | 0.035 | 0.225 | 0.032 | RI, GC/MS | Std |
| 4 | 951 | 957 | 2,4(10)-Thujadiene | MH | 0.779 | 0.079 | 0.011 | - | - | RI, GC/MS | - |
| 5 | 971 | 975 | 4(10)-Thujene | MH | 0.765 | 0.282 | 0.040 | - | - | RI, GC/MS | - |
| 6 | 973 | 974 | β-Pinene | MH | 0.765 | 0.521 | 0.073 | 0.455 | 0.066 | RI, GC/MS | Std |
| 7 | 990 | 988 | Myrcene | SH | 0.765 | 0.433 | 0.061 | 0.466 | 0.071 | RI, GC/MS | Std |
| 8 | 1002 | 1004 | Pseudolimonene | MH | 0.765 | 0.090 | 0.013 | - | - | RI, GC/MS | - |
| 9 | 1024 | 1026 | m-Cymene | MH | 0.700 | 1.578 | 0.203 | 1.722 | 0.232 | RI, GC/MS | GC/MS |
| 10 | 1029 | 1029 | β-Phellandrene | MH | 0.765 | 16.770 | 2.390 | 15.394 | 2.333 | RI, GC/MS | GC/MS |
| 11 | 1030 | 1031 | D-Limonene | MO | 0.887 | 0.147 | 0.024 | 0.630 | 0.112 | RI, GC/MS | GC/MS |
| 12 | 1089 | 1098 | α-Campholenal | MO | 0.887 | 0.146 | 0.024 | 0.148 | 0.026 | RI, GC/MS | GC/MS |
| 13 | 1097 | 1095 | α-Pinene oxide | MO | 0.887 | 0.028 | 0.005 | - | - | RI, GC/MS | - |
| 14 | 1116 | 1102 | Thujone | MO | 0.887 | 0.342 | 0.056 | 0.291 | 0.047 | RI, GC/MS | GC/MS |
| 15 | 1121 | 1123 | 1R,4R-p-Mentha-2,8-dien-1-ol | MO | 0.911 | 0.065 | 0.011 | 0.017 | 0.003 | RI, GC/MS | GC/MS |
| 16 | 1131 | 1131 | 4-Acetyl-1-methylcyclohexene | MO | 0.911 | 0.065 | 0.011 | 0.017 | 0.003 | RI, GC/MS | GC/MS |
| 17 | 1134 | 1136 | Limonene epoxide | MO | 0.887 | 0.036 | 0.006 | 0.061 | 0.011 | RI, GC/MS | GC/MS |
| 18 | 1139 | 1137 | L-Pinocarveol | MO | 0.887 | 0.625 | 0.102 | 0.645 | 0.115 | RI, GC/MS | GC/MS |
| 19 | 1145 | 1145 | L-Limonene oxide | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 20 | 1146 | 1146 | Limonene | MO | 0.887 | 0.028 | 0.005 | - | - | RI, GC/MS | - |
| 21 | 1147 | 1147 | Limonene | MO | 0.887 | 0.342 | 0.056 | 0.291 | 0.047 | RI, GC/MS | GC/MS |
| 22 | - | - | Limonene | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 23 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 24 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 25 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 26 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 27 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 28 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 29 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 30 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 31 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |
| 32 | - | - | Camphor | MO | 0.887 | 0.542 | 0.068 | 0.492 | 0.087 | RI, GC/MS | GC/MS |

Table 4. Chemical composition of *Alpinia elegans* seed essential oil.
| Obs. | Lit. | Compound | C<sup>2</sup> | RF<sup>3</sup> | Column 4 | Identification 5 |
|------|------|----------|--------------|-------------|----------|------------------|
| RI   | HP-5MS | DB-HeavyWAX | HP-5MS | DB-HeavyWAX |
|      | (%)   | (%)   | (%)   | (%)   | RI, GC/MS | -    |
| 33   | 1208  | 1204   | Berbenone    | MO          | 9.07     | 0.198  0.033  -  - | RI, GC/MS | - |
| 34   | 1220  | 1229   | Carveol      | MO          | 0.887    | 0.546  0.089  0.103 0.018 | RI, GC/MS | GC/MS |
| 35   | 1229  | 1251   | cis-p-Mentha-1(7),8-dien-2-ol | MO | 0.887 | 0.0451 0.007 0.032 0.006 | RI, GC/MS | GC/MS |
| 36   | -     | 1239   | Isobornyl formate | MO | - | - - 0.357 0.072 | - | GC/MS |
| 37   | 1241  | 1244   | 2-Methyl-3-phenylpropanol | MO | 0.824 | 0.059 0.009 - - | RI, GC/MS | - |
| 38   | 1245  | 1243   | Sclareol      | MO          | 0.907    | 0.697  0.116 0.580 0.106 | RI, GC/MS, Std | GC/MS |
| 39   | 1254  | 1294   | Limonene dioxide | MO | 1.019 | 0.013 0.004 - - | RI, GC/MS | - |
| 40   | 1276  | 1196   | 3-p-Menth-7-en-7-al | MO | 0.887 | 0.131 0.021 - - | RI, GC/MS | - |
| 41   | 1287  | 1287   | Pichtosin     | MO          | 0.957    | 0.042 0.007 - - | RI, GC/MS | - |
| 42   | 1292  | 1228   | D-Verbenone   | MO          | 0.907    | 0.033 0.005 - - | RI, GC/MS | - |
| 43   | 1342  | 1343   | Tricycloexasantalal | A | 0.867 | 0.069 0.011 - - | RI, GC/MS | - |
| 44   | -     | 1345   | α-Cubebene   | SH          | -        | - - 0.115 0.017 | - | GC/MS |
| 45   | -     | 1371   | Cyclosativene | SH          | -        | - - 0.052 0.008 | - | GC/MS |
| 46   | 1380  | 1374   | α-Copaene     | SH          | 0.751    | 0.878 0.122 0.514 0.077 | RI, GC/MS | GC/MS |
| 47   | -     | 1374   | Longicyclene  | SO          | -        | - - 0.279 0.046 | - | GC/MS |
| 48   | -     | 1388   | β-Cubebene    | SH          | -        | - - 0.088 0.013 | - | GC/MS |
| 49   | 1395  | 1389   | β-Elemen      | SH          | 0.751    | 2.001 0.277 2.223 0.342 | RI, GC/MS | GC/MS |
| 50   | 1413  | 1409   | α-Gurjeneene  | SH          | 0.751    | 0.033 0.005 - - | RI, GC/MS | - |
| 51   | 1419  | 1422   | α-Bergamotene | SH          | 0.751    | 0.142 0.020 0.045 0.007 | RI, GC/MS | GC/MS |
| 52   | 1424  | 1415   | α-Santalene   | SH          | 0.715    | 3.154 0.415 1.413 0.213 | RI, GC/MS | GC/MS |
| 53   | 1426  | 1418   | Caryophyllene | SH          | 0.715    | 2.972 0.392 3.576 0.550 | RI, GC/MS | GC/MS |
| 54   | -     | 1436   | γ-Elemene     | SH          | -        | - - 0.035 0.005 | - | GC/MS |
| 55   | 1448  | 1443   | Guai-6,9-diene | SH          | 0.715    | 0.118 0.016 - - | RI, GC/MS | - |
| 56   | 1452  | 1452   | Epi-β-Santalene | MH | 0.751 | 0.425 0.059 0.395 0.060 | RI, GC/MS | GC/MS |
| 57   | -     | 1457   | Altoaromadendrene | SH | - | - - 0.041 0.006 | - | GC/MS |
| 58   | 1460  | 1452   | Humulene      | SH          | 0.751    | 1.198 0.166 0.958 0.144 | RI, GC/MS, Std | GC/MS |
| 59   | -     | 1464   | epi-β-Caryophyllene | SH | - | - - 0.082 0.012 | - | GC/MS |
| 60   | 1467  | 1443   | Aromandendrene | SH          | 0.751    | 0.070 0.010 - - | RI, GC/MS | - |
| 61   | 1481  | 1478   | γ-Muurolene   | SH          | 0.715    | 0.177 0.023 - - | RI, GC/MS | - |
| 62   | 1490  | 1473   | 2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene | SH | 0.745 | 2.972 0.392 3.576 0.550 | RI, GC/MS | - |
Table 4. Cont.

| RI  | Compound                | C<sup>2</sup> | RF<sup>3</sup> | Column<sup>4</sup> | Identification<sup>5</sup> |
|-----|-------------------------|--------------|---------------|-------------------|--------------------------|
|     |                         |              |               | HP-5MS         | DB-HeavyWAX | HP-5MS | DB-HeavyWAX |
|     |                         | (%)         | (%)          | (%)              | (%)         | (%)    | (%)    |
| 63  | 1492 β-Eudesmenene       | SH           | 0.756        | 0.518            | 0.072       | -       | -       |
| 64  | 1498 Eremophilene        | SH           | 0.751        | 1.448            | 0.209       | 1.301   | 0.196  | RI, GC/MS | GC/MS    |
| 65  | 1498 α-Selinene          | SH           | -            | -                | 0.224       | 0.034   | -       | GC/MS     |
| 66  | 1475 α-Himachalene       | SH           | 0.751        | 0.746            | 0.103       | -       | -       | RI, GC/MS | GC/MS     |
| 67  | 1505 β-Bisabolene        | SH           | 0.751        | 4.270            | 0.591       | -       | 4.804  | 0.738    | RI, GC/MS | GC/MS     |
| 68  | 1515 Cubebol             | SO           | -            | -                | 0.525       | 0.086   | -       | -         |
| 69  | 1522 Calamene            | SH           | -            | -                | 4.906       | 0.709   | -       | -         |
| 70  | 1522 α-Maaliene          | SH           | 0.751        | 2.823            | 0.391       | 3.115   | 0.469  | RI, GC/MS | GC/MS     |
| 71  | 1521 Calamene            | SH           | 0.707        | 4.460            | 0.581       | -       | -       | RI, GC/MS | -         |
| 72  | 1632 Ledene oxide-(II)   | O            | 0.830        | 0.195            | 0.030       | -       | -       | RI, GC/MS | -         |
| 73  | 1370 α-Ylangene          | SH           | 0.751        | 0.159            | 0.022       | -       | -       | RI, GC/MS | -         |
| 74  | 1544 α-Calacorene        | SH           | -            | -                | 0.103       | 0.018   | -       | GC/MS     |
| 75  | 1549 Elemol              | SO           | -            | -                | 0.040       | 0.006   | -       | GC/MS     |
| 76  | 1562 Cadala-1(10),3,8-triene | SH          | 0.760       | 0.405            | 0.057      | -       | -       | RI, GC/MS | -         |
| 77  | 1565 α-Calacorene        | SH           | -            | -                | 0.025       | 0.004   | -       | GC/MS     |
| 78  | 1565 Nerolidol           | SO           | 0.819        | 0.035            | 0.055       | -       | -       | GC/MS     |
| 79  | 1576 Spathulenol         | SO           | -            | -                | 0.448       | 0.076   | -       | GC/MS     |
| 80  | 1582 Caryophyllene oxide | SO           | 0.830        | 11.368           | 1.738      | 10.781  | 1.772  | RI, GC/MS | GC/MS     |
| 81  | 1602 Ledol               | SO           | 0.819        | 0.521            | 0.075       | 0.251   | 0.041  | RI, GC/MS | GC/MS     |
| 82  | 1610 Humulene epoxide 2  | SO           | 0.830        | 2.132            | 0.326      | 1.676   | 0.286  | RI, GC/MS | GC/MS     |
| 83  | 1630 α-Acorenol          | SO           | 0.819        | 0.122            | 0.018      | 0.407   | 0.067  | RI, GC/MS | GC/MS     |
| 84  | 1626 Aromadendrene oxide-(2) | SO          | 0.830        | 0.795            | 0.116      | 0.438   | 0.073  | RI, GC/MS | GC/MS     |
| 85  | 1627 Epicubanol          | SO           | 0.819        | 0.856            | 0.027      | 0.626   | 0.103  | RI, GC/MS | GC/MS     |
| 86  | 1640 Caryophylladienol II| SO           | -            | -                | 0.147      | 0.081   | -       | GC/MS     |
| 87  | 1646 α-Muuro dol         | SO           | -            | -                | 0.243      | 0.040   | -       | GC/MS     |
| 88  | 1655 1645 Cubenol        | SO           | 0.819        | 0.071            | 0.011      | -       | -       | RI, GC/MS | -         |
| 89  | 1662 Allilomachalol      | SO           | -            | -                | 1.210      | 0.199   | -       | GC/MS     |
| 90  | 1669 Intermediol         | SO           | 0.819        | 1.729            | 0.261      | 1.053   | 0.176  | RI, GC/MS | GC/MS     |
| 91  | 1675 Ylangenal           | SO           | -            | -                | 0.059      | 0.010   | -       | GC/MS     |
| 92  | 1685 α-Bisabolol         | SO           | 0.819        | 1.383            | 0.209      | 1.137   | 0.196  | RI, GC/MS | GC/MS     |
| 93  | 1612 Isoaromadendrene epoxide | SO      | 0.830        | 3.813            | 0.612      | 0.201   | 0.025  | RI, GC/MS | GC/MS     |
| 94  | 1629 (E)-α-Santalal      | SO           | 0.841        | 1.391            | 0.233      | 1.456   | 0.264  | RI, GC/MS | GC/MS     |
| 95  | 1689 Cedr-8-en-13-ol     | O            | 0.830        | 0.034            | 0.005      | -       | -       | RI, GC/MS | -         |
| 96  | 1740 Isolongifolol       | SO           | 0.819        | 0.814            | 0.123      | 0.806   | 0.132  | RI, GC/MS | GC/MS     |
| RI \(^1\) | Compound | C \(^2\) | RF \(^3\) | Column \(^4\) | Identification \(^5\) |
|---|---|---|---|---|---|
| **Obs. Lit.** | | HP-5MS | DB-HeavyWAX | HP-5MS | DB-HeavyWAX |
| 97 | - | 1766 | Costol | SO | - | - | 0.198 | 0.033 | - | GC/MS |
| 98 | 1814 | 1809 | Ambrial | SO | 0.821 | 0.908 | 0.137 | 0.954 | 0.160 | RI, GC/MS | GC/MS |
| 99 | - | 1899 | Corymbolone | SO | - | - | 0.042 | 0.008 | - | GC/MS |
| 100 | - | - | Menthen-2-ol | MO | - | - | 0.123 | 0.021 | - | GC/MS |
| 101 | - | - | 2-Isopropenyl-4a,8-dimethyl-1,2.3.4.4a.5.6.7-octahydronaphthalene | SH | - | - | 2.146 | 0.323 | - | GC/MS |
| 102 | - | - | Isopiperitenol | MO | - | - | 0.100 | 0.018 | - | GC/MS |
| 103 | - | - | β-(Z)-Curcumen-12-ol | SO | - | - | 0.106 | 0.018 | - | GC/MS |
| 104 | - | - | Germacr-4(15).5.10(14)-trien-1β-ol | SO | - | - | 0.058 | 0.010 | - | GC/MS |
| 105 | - | - | 1-Methyl-8-(1-methylethyl)-tricyclo[4.4.0][2.7]dec-3-one-5-methanol | SO | - | - | 0.333 | 0.055 | - | GC/MS |
| 106 | - | - | Diepicedrene-1-oxide | SO | - | - | 0.175 | 0.029 | - | GC/MS |
| 107 | - | - | 2,5,8-Trimethyltetralin | SH | - | - | 0.273 | 0.039 | - | GC/MS |
| 108 | - | - | Neointermedeol | SO | - | - | 0.360 | 0.059 | - | GC/MS |
| 109 | - | - | Epiglobulol | SO | - | - | 0.709 | 0.117 | - | GC/MS |
| 110 | - | - | 4-(2.4.4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one | MO | - | - | 0.294 | 0.052 | - | GC/MS |
| 111 | - | - | Bicyclo[4.4.0]dec-2-ene-4-ol | SO | - | - | 0.409 | 0.074 | - | GC/MS |
| 112 | - | - | 2-methyl-9-(prop-1-en-3-ol-2-y1)-2(2E)-2-Methyl-4(2.6.6-trimethyl-1-cyclohexen-1-yl)-2-butene-1-ol | MO | - | - | 0.697 | 0.114 | - | GC/MS |
| 113 | - | - | ent-Germacra-4(15).5.10(14)-trien-1β-ol | SO | - | - | 1.662 | 0.284 | - | GC/MS |
| 114 | - | - | 7-Isopropenyl-1,4a-dimethyl-4,6.5.7.8-hexahydro-3H-naphthalen-2-one | SO | - | - | 0.096 | 0.013 | - | GC/MS |
| 115 | - | - | 2,6-Ditet-butyl-4-methylphenyl | MO | - | - | 0.076 | 0.012 | - | GC/MS |
| 116 | - | - | 1-benzyl-2-methyleucilocopropanecarboxylate | MO | - | - | 0.112 | 0.021 | - | GC/MS |
| 117 | - | - | Methyl hexadec-7.10.13-trienoate | E | - | - | 0.070 | 0.012 | - | GC/MS |
| 118 | - | - | 3-Deoxyestradiol | S | - | - | 0.424 | 0.067 | - | GC/MS |
| 119 | - | - | 1-Heptatriacotanol | O | - | - | 0.117 | 0.017 | - | GC/MS |
Table 4. Cont.

| RI  | Compound | C    | RF  | Column | Identification |
|-----|----------|------|-----|--------|----------------|
|     |          |      |     | HP-5MS | DB-HeavyWAX |
| Obs. | Lit.     | (%)  | (%) | (%)    | (%)           |
| 1    |          | 0.069| -   |        |               |
| 2    |          | 0.570| 0.450|        |               |
| 3    |          | 33.768| 31.859|        |               |
| 4    |          | 4.961| 6.545|        |               |
| 5    |          | 28.102| 26.505|        |               |
| 6    |          | 25.938| 27.216|        |               |
| 7    |          | 0.229| 0.424|        |               |
| 8    |          | -    | 0.181|        |               |

Chemical classes
- Aldehydes
- Ketones
- Esters
- Monoterpene hydrocarbons
- Oxygenated monoterpenes
- Sesquiterpene hydrocarbons
- Oxygenated sesquiterpenes
- Sterols
- Others

Total identified (%) 93.637 93.186

1 Retention indices: Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values [32,33]. 2 C = chemical class: A—aldehydes, E—esters, K—ketones, MH—monoterpene hydrocarbons, MO—oxygenated monoterpenes, O—others, S—sterols, SH—sesquiterpene hydrocarbons, SO—oxygenated sesquiterpenes. 3 RF = response factor; 4 column = composition of essential oil detected on HP-5MS and DB-HeavyWAX columns; (%) = relative percentage content; c = content is expressed as concentration in milligram per 1 kg of dry plant material; 5 identification method: GC/MS = mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database; Std = constituent identity confirmed by coinjection of authentic standards; 6 retention indices were not calculated for compounds identified only by DB-HeavyWAX column; 7 literature data not available; 8 not detected.

Table 5. Chemical composition of Cinnamomum iners leaf essential oil.

| RI  | Compound | C    | RF  | Column | Identification |
|-----|----------|------|-----|--------|----------------|
|     |          |      |     | HP-5MS | DB-HeavyWAX |
| Obs. | Lit.     | (%)  | (%) | (%)    | (%)           |
| 1    |          | 0.075| 0.013| 0.043  | 0.008         |
| 2    |          | 0.369| 0.066| 0.210  | 0.049         |
| 3    |          | 0.056| 0.009|        | 8             |
| 4    |          | 1.116| 0.187| 0.679  | 0.164         |
| 5    |          | 1.125| 0.202| 0.810  | 0.144         |
| 6    |          | 0.407| 0.074|        | 9             |
| Obs | RI | Compound          | C   | RF (%) | Column | Identification |
|-----|-----|-------------------|-----|--------|--------|----------------|
|     |     |                   |     |        | HP-5MS | DB-HeavyWAX   | HP-5MS | DB-HeavyWAX   |
| 7   | 1024| 1022 α-Cymene     | MH  | 0.698  | 1.511  | 0.245 | 1.314 | 0.229 | RI, GC/MS | GC/MS |
| 8   | 6   | 1024 D-Limonene   | MH  | 0.69     | -     | -     | 1.479 | 0.326 | -     | GC/MS |
| 9   | 1029| 1029 β-Phellandrene | MH | -       | 5.982  | 1.080 | -     | -     | GC/MS |
| 10  | 1029| 1004 Pseudolimonene | MH | 0.765 | 9.549 | 1.715 | 0.089 | 0.015 | RI, GC/MS | GC/MS |
| 11  | 1048| 1048 β-Ocimene    | MH  | 0.765 | 0.096 | 0.017 | 0.099 | 0.019 | RI, GC/MS | Std, GC/MS |
| 12  | 1058| 1058 γ-Terpinene | MH  | 0.765 | 0.149 | 0.025 | 0.099 | 0.019 | RI, GC/MS | Std, GC/MS |
| 13  | 1088| 1086 Terpinolene  | MH  | 0.765 | 0.118 | 0.020 | 0.350 | 0.073 | RI, GC/MS | Std, GC/MS |
| 14  | 1105| 1095 Linalool     | MO  | 0.869 | 15.466 | 3.153 | 13.899 | 3.023 | RI, GC/MS | Std, GC/MS |
| 15  | 1122| 1121 (Z)-2-Menthenol | MO | 0.869 | 0.177 | 0.031 | -     | -     | RI, GC/MS | - |
| 16  | 1140| 1136 (E)-2-Menthenol | MO | 0.869 | 0.114 | 0.022 | -     | -     | RI, GC/MS | - |
| 17  | 1178| 1174 Terpinen-4-ol | MO  | 0.869 | 0.965 | 0.170 | -     | -     | RI, GC/MS | - |
| 18  | 1186| 1183 Cryptone     | MO  | 0.911 | 0.303 | 0.056 | -     | -     | RI, GC/MS | - |
| 19  | 1191| 1186 α-Terpinol   | MO  | 0.869 | 0.895 | 0.158 | 0.729 | 0.203 | RI, GC/MS | Std, GC/MS |
| 20  | 1196| 1195 (Z)-Piperitol | MO  | 0.869 | 0.031 | 0.005 | 0.082 | 0.019 | RI, GC/MS | Std, GC/MS |
| 21  | 1202| 1143 (Z)-Sabinol  | MO  | 0.887 | 0.033 | 0.006 | -     | -     | RI, GC/MS | - |
| 22  | 1208| 1207 (E)-Piperitol | MO  | 0.869 | 0.052 | 0.010 | -     | -     | RI, GC/MS | - |
| 23  | 1241| 1244 2-Methyl-3-phenylpropanol | MO | 0.824 | 0.039 | 0.007 | -     | -     | RI, GC/MS | - |
| 24  | 1256| 1255 Geraniol     | MO  | 0.869 | 0.472 | 0.102 | 0.580 | 0.127 | RI, GC/MS | Std, GC/MS |
| 25  | 1276| 1273 Phellandral  | MO  | 0.887 | 0.155 | 0.028 | -     | -     | RI, GC/MS | - |
| 26  | 1291| 1285 Safrole      | MO  | 0.969 | 2.028 | 0.402 | 1.983 | 0.486 | RI, GC/MS | Std, GC/MS |
| 27  | 1353| 1345 α-Cubeicene  | SH  | 0.751 | 0.032 | 0.004 | -     | -     | RI, GC/MS | - |
| 28  | 1362| 1356 Eugenol      | SO  | 0.947 | 0.631 | 0.122 | 0.631 | 0.133 | RI, GC/MS | Std, GC/MS |
| 29  | 1369| 1389 Longifolene  | SH  | 0.751 | 0.086 | 0.010 | -     | -     | RI, GC/MS | - |
| 30  | 1380| 1374 α-Copaene    | SH  | 0.751 | 0.452 | 0.069 | 0.414 | 0.082 | RI, GC/MS | Std, GC/MS |
| 31  | 1388| 1387 β-Bourbonene | SH  | 0.751 | 0.069 | 0.007 | -     | -     | RI, GC/MS | - |
| 32  | 1395| 1389 β-Elemicene  | SH  | 0.751 | 0.267 | 0.041 | -     | -     | RI, GC/MS | - |
| 33  | 1403| 1403 Methyleneol  | SO  | 0.879 | 2.028 | 0.402 | 1.983 | 0.486 | RI, GC/MS | Std, GC/MS |
| 34  | 1412| 1571 Sesquisabinene hydrate | SO | 0.819 | 0.260 | 0.042 | 0.184 | 0.041 | RI, GC/MS | Std, GC/MS |
| 35  | 1414| 1414 β-Fumonene   | SH  | -     | -     | -     | 1.223 | 0.215 | -     | GC/MS |
| 36  | 1417| 1417 α-Santalene  | SH  | -     | -     | -     | 0.935 | 0.204 | -     | GC/MS |
| 37  | 1432| 1418 Caryophyllene | SH  | 0.751 | 21.002 | 3.223 | 34.875 | 6.561 | RI, GC/MS, Std, GC/MS |
| 38  | 1435| 1419 β-Ylangene   | SH  | 0.751 | 0.183 | 0.028 | -     | -     | RI, GC/MS | - |
| 39  | 1440| 1439 α-Bergamotene | SH | 0.751 | 0.633 | 0.097 | -     | -     | RI, GC/MS | - |
| 40  | 1446| 1402 Ledol        | SO  | 0.751 | 0.143 | 0.022 | 0.067 | 0.015 | RI, GC/MS | Std, GC/MS |

**Table 5. Cont.**
Table 5. Cont.

| Obs. | Lit. | Compound | C ² | RI ¹ | RF ³ | Column ⁴ | Identification ⁵ |
|------|------|----------|-----|------|------|----------|-----------------|
| 41   | 1453 | 1452     | MH  | 0.751 | 0.112 | 0.017   | 0.054 0.012 | RI, GC/MS       |
| 42   | -    | 1455     | SO  | -     | -     | -       | -     -     | -               |
| 43   | 1456 | 1460     | SO  | 0.751 | 0.302 | 0.046   | -     -     | RI, GC/MS       |
| 44   | 1461 | 1452     | SH  | 0.751 | 4.902 | 0.735   | 2.982 0.536 | RI, GC/MS, Std |
| 45   | 1464 | 1413     | SH  | 0.751 | 0.112 | 0.017   | 8.067 1.449 | RI, GC/MS       |
| 46   | 1470 | 1464     | SH  | 0.751 | 10.216| 1.570   | -     -     | -               |
| 47   | -    | 1477     | SH  | -     | -     | -       | 0.235 0.494 | -               |
| 48   | 1479 | 1472     | SH  | 0.751 | 0.042 | 0.009   | 2.235 0.394 | RI, GC/MS       |
| 49   | -    | 1480     | SH  | -     | -     | -       | 0.162 0.026 | -               |
| 50   | 1482 | 1478     | SH  | 0.751 | 0.755 | 0.045   | -     -     | -               |
| 51   | 1487 | 1484     | SH  | 0.751 | 0.827 | 0.127   | 0.374 0.063 | RI, GC/MS       |
| 52   | 1490 | 1505     | SH  | 0.751 | 1.161 | 0.178   | 0.684 0.144 | RI, GC/MS       |
| 53   | 1492 | 1485     | SH  | 0.751 | 0.074 | 0.013   | -     -     | -               |
| 54   | 1494 | 1443     | SH  | 0.751 | 0.095 | 0.016   | -     -     | RI, GC/MS       |
| 55   | -    | 1496     | SH  | -     | -     | -       | 0.071 0.014 | -               |
| 56   | 1500 | 1496     | SH  | 0.751 | 0.374 | 0.052   | -     -     | RI, GC/MS       |
| 57   | 1502 | 1505     | SH  | 0.751 | 0.407 | 0.068   | -     -     | RI, GC/MS       |
| 58   | 1505 | 1500     | SH  | 0.751 | 0.410 | 0.069   | 0.359 0.065 | RI, GC/MS       |
| 59   | 1512 | 1505     | SH  | 0.751 | 1.051 | 0.177   | 1.137 0.200 | RI, GC/MS       |
| 60   | 1515 | 1512     | SH  | 0.751 | 0.031 | 0.005   | -     -     | RI, GC/MS       |
| 61   | -    | 1518     | MO  | -     | -     | -       | 0.174 0.048 | -               |
| 62   | 1520 | 1513     | SH  | 0.751 | 0.688 | 0.106   | -     -     | RI, GC/MS       |
| 63   | 1530 | 1522     | SH  | 0.751 | 2.095 | 0.322   | -     -     | RI, GC/MS       |
| 64   | 1537 | 1454     | SH  | 0.751 | 0.043 | 0.007   | -     -     | RI, GC/MS       |
| 65   | 1539 | 1535     | SH  | 0.751 | 0.040 | 0.007   | -     -     | RI, GC/MS       |
| 66   | 1544 | 1537     | SH  | 0.751 | 0.099 | 0.019   | 0.131 0.023 | RI, GC/MS       |
| 67   | 1547 | 1536     | SH  | 0.751 | 0.088 | 0.017   | -     -     | RI, GC/MS       |
| 68   | 1550 | 1544     | SH  | 0.715 | 0.090 | 0.013   | 0.135 0.022 | RI, GC/MS       |
| 69   | 1567 | 1564     | SO  | 0.819 | 0.482 | 0.081   | 0.330 0.060 | RI, GC/MS       |
| 70   | -    | 1576     | SO  | -     | -     | -       | 0.150 0.026 | -               |
| 71   | 1579 | 1570     | SO  | 0.819 | 0.887 | 0.149   | 0.827 0.151 | RI, GC/MS       |
| 72   | 1587 | 1576     | SO  | 0.830 | 0.663 | 0.085   | 0.468 0.085 | RI, GC/MS       |
| 73   | 1593 | 1582     | SO  | 0.830 | 2.080 | 0.425   | 2.196 0.431 | RI, GC/MS       |
| 74   | 1600 | 1600     | SO  | 0.819 | 0.127 | 0.023   | 0.053 0.012 | RI, GC/MS       |
| 75   | 1609 | 1590     | SO  | 0.819 | 0.561 | 0.094   | 0.570 0.105 | RI, GC/MS       |
| Obs. | Lit. | Compound | C | RF | Column | Identification |
|------|------|-----------|----|----|---------|----------------|
|      |      | RI 1      | C 2 | RF 3 | HP-5MS | DB-HeavyWAX | HP-5MS | DB-HeavyWAX |
| 106  | -    | 2.5-Anhydro-1-O-octylhexitol | O | - | - | 0.133 | 0.049 | - | GC/MS |
| 76   | 1610 | Humulol   | SO | - | - | 0.092 | 0.021 | - | GC/MS |
| 77   | 1614 | Tetradecanal | A | 0.806 | 1.168 | 0.193 | 0.991 | 0.180 | RI, GC/MS | GC/MS |
| 78   | 1610 | Humulene oxide 2 | SO | 0.830 | 0.331 | 0.056 | 0.105 | 0.024 | RI, GC/MS | GC/MS |
| 79   | 1645 | Cubenol   | SO | 0.819 | 0.021 | 0.003 | 0.144 | 0.032 | RI, GC/MS | GC/MS |
| 80   | 1616 | Widdrol   | SO | 0.819 | 0.314 | 0.053 | 0.311 | 0.055 | RI, GC/MS | GC/MS |
| 81   | 1630 | α-Acorenol | SO | - | - | 0.221 | 0.039 | - | GC/MS |
| 82   | 1619 | 1,10-Diepicubenol | SO | 0.819 | 0.114 | 0.022 | 0.247 | 0.047 | RI, GC/MS | GC/MS |
| 83   | 1640 | α-epi-Muurolol | SO | - | - | 1.109 | 0.205 | - | GC/MS |
| 84   | 1628 | Caryophylladienol I | SO | 0.830 | 0.538 | 0.091 | 0.472 | 0.114 | RI, GC/MS | GC/MS |
| 85   | 1641 | α-Cadinol | SO | 0.819 | 3.417 | 0.573 | 2.300 | 0.418 | RI, GC/MS | GC/MS |
| 86   | 1645 | δ-Cadinol | SO | 0.819 | 0.284 | 0.048 | 0.231 | 0.042 | RI, GC/MS | GC/MS |
| 87   | 1662 | Longifolenaldehyde | SO | - | - | 0.115 | 0.021 | - | GC/MS |
| 88   | 1612 | Isoaromadendrene epoxide | SO | 0.830 | 0.460 | 0.078 | - | - | RI, GC/MS | - |
| 89   | 1685 | α-Bisabolol | SO | 0.819 | 0.141 | 0.026 | - | - | RI, GC/MS, Std | - |
| 90   | 1694 | (1R,7S)-Germacr-4(15).5.10(14)-trien-1β-ol | SO | 0.830 | 0.101 | 0.019 | 0.318 | 0.060 | RI, GC/MS | GC/MS |
| 91   | 1695 | Farnesol | SO | - | - | 0.075 | 0.017 | - | GC/MS |
| 92   | 1699 | 2-Pentadecanone | K | 0.799 | 0.053 | 0.010 | 0.047 | 0.011 | RI, GC/MS | GC/MS |
| 93   | 2201 | Geranylgeraniol | SO | 0.795 | 0.057 | 0.011 | - | - | RI, GC/MS | - |
| 94   | 1740 | Isolongifolol | SO | - | - | 0.111 | 0.026 | - | GC/MS |
| 95   | 1747 | 1-Bisabolone | SO | 0.830 | 0.051 | 0.010 | - | - | RI, GC/MS | - |
| 96   | 1717 | Cyperenone | SO | 0.841 | 0.097 | 0.019 | - | - | RI, GC/MS | - |
| 97   | 1798 | Hexadec-7-enal | A | 0.802 | 0.083 | 0.015 | - | - | RI, GC/MS | - |
| 98   | 1845 | Hexahydrofarnesyl acetone | SO | 0.782 | 0.240 | 0.038 | - | - | RI, GC/MS | - |
| 99   | 1877 | Hexadec-2-enal | A | 0.802 | 0.087 | 0.016 | - | - | RI, GC/MS | - |
| 100  | 1903 | Homosalate | E | 0.935 | 0.034 | 0.008 | - | - | RI, GC/MS | - |
| 101  | 1922 | Farnesyl acetone | SO | 0.806 | 0.337 | 0.056 | 0.316 | 0.055 | RI, GC/MS | GC/MS |
| 102  | 1960 | Hexadecanoic acid | FA | - | - | 0.724 | 0.132 | - | GC/MS |
| 103  | 1967 | Dibutyl phthalate | E | 1.015 | 0.027 | 0.006 | - | - | RI, GC/MS | - |
| 104  | 2114 | Phytol | O | 0.774 | 0.229 | 0.036 | 0.234 | 0.040 | RI, GC/MS | GC/MS |
| 105  | - | 2.2,4,7,9-Tetramethyldecahydro-1H-cyclobuta[e]inden-5-ol | SO | - | - | 0.124 | 0.028 | - | GC/MS |
Table 5. Cont.

| RI 1 | Compound | C 2 | RF 3 | Column 4 | Identification 5 |
|------|----------|-----|------|----------|------------------|
|      |          |     |      | HP-5MS DB-HeavyWAX HP-5MS DB-HeavyWAX |
| Obs. | Lit.     |     |      | (%) (%)  (%) (%)       |
| 106  | -        | -   | 2.5-Anhydro-1-O-octylhexitol O | - | - | 0.133 | 0.049 |

**Chemical classes**

| Aldehydes | Ketones | Fatty acids | Monoterpenes hydrocarbons | Oxygenated monoterpenes | Sesquiterpenes hydrocarbons | Oxygenated sesquiterpenes | Others |
|-----------|---------|------------|---------------------------|-------------------------|-----------------------------|---------------------------|--------|
| 1.338     | 0.053   | -          | 14.683                    | 20.730                  | 45.838                      | 12.639                    | 0.229  |

Total identified (%) 95.571 96.676

1 Retention indices: Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values [32,33]; 2 C = chemical class: A—aldehydes, E—esters, FA—fatty acid, K—ketones, MH—monoterpenes hydrocarbons, MO—oxygenated monoterpenes, O—others, SH—sesquiterpenes hydrocarbons, SO—oxygenated sesquiterpenes; 3 RF = response factor; 4 column = composition of essential oil detected on HP-5MS and DB-HeavyWAX columns; (%) = relative percentage content; c = content is expressed as concentration in milligram per 1 kg of dry plant material; 5 identification method: GC/MS = mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.0), RI = the retention index was matching literature database; Std = constituent identity confirmed by coinjection of authentic standards; 6 retention indices were not calculated for compounds identified only by DB-HeavyWAX column; 7 literature data not available; 8 not detected.

Table 6. Chemical composition of *Xanthostemon verdugonianus* leaf essential oil.

| RI 1 | Compound | C 2 | RF 3 | Column 4 | Identification 5 |
|------|----------|-----|------|----------|------------------|
|      |          |     |      | HP-5MS DB-HeavyWAX HP-5MS DB-HeavyWAX |
| Obs. | Lit.     |     |      | (%) (%)  (%) (%)       |
| 1    | 1341     | 1335 | γ-Elemen | SH 0.751 | 0.062 | 0.007 | - |
| 2    | 1377     | 1374 | Isoledene | SH 0.751 | 0.063 | 0.007 | - |
| 3    | 1388     | 1389 | β-Elemen | SH 0.751 | 3.015 | 0.350 | 19.519 | 3.648 |
| 4    | 1419     | 1409 | α-Gurjunene | SH 0.751 | 32.285 | 3.741 | 19.519 | 3.648 |
| 5    | 1425     | 1418 | Caryophyllene | SH 0.751 | 6.386 | 0.739 | 2.987 | 0.559 |
| 6    | 1444     | 1443 | Aromandendrene | SH 0.751 | 0.263 | 0.035 | 0.195 | 0.037 |

1 Retention indices: Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40); 2 C = chemical class: E—esters, FA—fatty acid, K—ketones, MH—monoterpenes hydrocarbons, MO—oxygenated monoterpenes, O—others, SH—sesquiterpenes hydrocarbons, SO—oxygenated sesquiterpenes; 3 RF = response factor; 4 column = composition of essential oil detected on HP-5MS and DB-HeavyWAX columns; (%) = relative percentage content; c = content is expressed as concentration in milligram per 1 kg of dry plant material; 5 identification method: GC/MS = mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.0), RI = the retention index was matching literature database; Std = constituent identity confirmed by coinjection of authentic standards; not detected.
Table 6. Cont.

| RI | Compound | C | RF | HP-5MS (%) | DB-HeavyWAX (%) | Identification |
|----|----------|---|----|-------------|-----------------|----------------|
|    |          |   |    | HP-5MS      | DB-HeavyWAX     |                |
| 7  | 1455     | 1479 | γ-Himalchalene | SH | 0.751 | 0.063 | 0.007 | 0.060 | 0.011 | RI, GC/MS | GC/MS |
| 8  | 1460     | 1452 | Humulene | SH | 0.751 | 0.724 | 0.082 | 0.417 | 0.079 | RI, GC/MS, Std | GC/MS |
| 9  | 1478     | 1477 | γ-Gurjunene | SH | 0.751 | 2.097 | 0.242 | 1.028 | 0.194 | RI, GC/MS | GC/MS |
| 10 | 1480     | 1479 | γ-Selinene | SH | 0.751 | 0.289 | 0.034 | 0.128 | 0.024 | RI, GC/MS | GC/MS |
| 11 | 1486     | 1484 | Isogermacone D | SH | 0.751 | 0.071 | 8000 | - | - | RI, GC/MS | GC/MS |
| 12 | 1492     | 1489 | β-Eudesmesene | SH | 0.751 | 0.235 | 0.027 | 0.148 | 0.028 | RI, GC/MS | GC/MS |
| 13 | 1503     | 1494 | β-Cyclogermacone | SH | 0.751 | 5.250 | 0.592 | 1.898 | 0.358 | RI, GC/MS | GC/MS |
| 14 | -        | 1496 | Viridiflorene | SH | - | - | - | 0.846 | 0.166 | - | GC/MS |
| 15 | -        | 1498 | α-Selinene | SH | - | - | - | 0.134 | 0.025 | - | GC/MS |
| 16 | 1519     | 1513 | γ-Cadinene | SH | 0.751 | 0.398 | 0.046 | 2.611 | 0.488 | RI, GC/MS | GC/MS |
| 17 | 1530     | 1522 | δ-Cadinene | SH | 0.751 | 2.463 | 0.280 | - | - | RI, GC/MS | - |
| 18 | -        | 1522 | Calamene | SH | - | - | - | 0.058 | 0.010 | - | GC/MS |
| 19 | 1538     | 1535 | Cubenene | SH | 0.751 | 0.047 | 0.005 | - | - | RI, GC/MS | - |
| 20 | 1544     | 1537 | α-Cadinene | SH | 0.751 | 0.107 | 0.012 | 0.254 | 0.048 | RI, GC/MS | GC/MS |
| 21 | -        | 1541 | α-Copaen-11-ol | SO | - | - | - | 0.914 | 0.191 | - | GC/MS |
| 22 | 1577     | 1567 | Palastrol | SO | 0.819 | 1.930 | 0.238 | 1.123 | 0.231 | RI, GC/MS | GC/MS |
| 23 | -        | 1567 | Maitol | SO | - | - | - | 0.091 | 0.019 | - | GC/MS |
| 24 | 1586     | 1576 | Spathuleneol | SO | 0.830 | 0.582 | 0.075 | 0.428 | 0.089 | RI, GC/MS | GC/MS |
| 25 | -        | 1583 | Caryophyllene oxide | SH | - | - | - | 0.089 | 0.019 | - | GC/MS |
| 26 | 1593     | 1590 | Globulon | SO | 0.819 | 1.297 | 0.160 | 0.624 | 0.123 | RI, GC/MS | GC/MS |
| 27 | -        | 1595 | Cubeban-11-ol | SO | - | - | - | 0.267 | 0.055 | - | GC/MS |
| 28 | 1601     | 1600 | Viridilol | SO | 0.819 | 1.017 | 0.125 | 0.493 | 0.092 | RI, GC/MS | GC/MS |
| 29 | -        | 1600 | Rosilol | SO | - | - | - | 0.100 | 0.020 | - | GC/MS |
| 30 | 1614     | 1602 | Ledol | SO | 0.819 | 3.629 | 0.445 | 1.517 | 0.312 | RI, GC/MS | GC/MS |
| 31 | -        | 1619 | 1,10-di-epi-Cubinol | SO | - | - | - | 0.074 | 0.015 | - | GC/MS |
| 32 | 1622     | 1630 | α-Acenasol | SO | 0.819 | 0.108 | 0.012 | - | - | RI, GC/MS | GC/MS |
| 33 | 1634     | 1755 | α-Vetivol | SO | 0.830 | 0.781 | 0.079 | 0.734 | 0.153 | RI, GC/MS | GC/MS |
| 34 | 1651     | 1640 | α-epi-Murolol | SO | 0.819 | 2.644 | 0.334 | 0.612 | 0.126 | RI, GC/MS | GC/MS |
| 35 | 1655     | 1645 | δ-Cadinol | SO | 0.819 | 0.397 | 0.047 | - | - | RI, GC/MS | - |
| 36 | -        | 1650 | β-Eudesmol | SO | - | - | - | 0.138 | 0.029 | - | GC/MS |
| 37 | 1661     | 17 | 1-(3-Methyl-2-cyclopenten-1-yl)-1-cyclohexene | O | 0.765 | 1.023 | 0.118 | - | - | GC/MS | - |
| 38 | 1665     | 1652 | α-Cadinol | SO | 0.819 | 3.730 | 0.473 | 2.628 | 0.534 | RI, GC/MS | GC/MS |
| 39 | -        | 1662 | Longifolenaldehyde | SO | - | - | - | 0.444 | 0.093 | - | GC/MS |
| 40 | 1684     | 1678 | Alloaromadendrene oxide-(2) | SO | 0.830 | 0.062 | 0.008 | - | - | RI, GC/MS | - |
| 41 | 1704     | - | γ-Gurjuneneoxide-(2) | SO | 0.830 | 0.152 | 0.019 | - | - | GC/MS | - |
| 42 | 1710     | 1711 | Valerenol | SO | 0.830 | 0.214 | 0.027 | 0.067 | 0.014 | RI, GC/MS | GC/MS |
Table 6. Cont.

| Obs. | Lit. | Compound | C | RF | HP-5MS (%) | DB-HeavyWAX (%) | c (%) | c (%) | Identification |
|------|------|----------|---|----|------------|----------------|------|------|---------------|
| 43   | -    | 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenol | SO | 0.830 | 0.318 | 0.042 | - | - | GC/MS |
| 44   | 1746 | 1723     | Isolongifolen-9-one | SO | 0.841 | 0.077 | 0.010 | - | - | RI, GC/MS |
| 45   | 1754 | 1730     | 2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one | SO | 0.841 | 0.057 | 0.008 | 0.078 | 0.017 | RI, GC/MS |
| 46   | 1765 | 1766     | Costol | SO | 0.830 | 0.118 | 0.016 | 0.148 | 0.031 | RI, GC/MS |
| 47   | 1785 | 1717     | Cyperenone | SO | 0.841 | 22.653 | 2.745 | 52.694 | 10.958 | RI, GC/MS |
| 48   | 1835 | -        | Spiro[tricyclo[4.4.0.0(5,9)]decane-10,12′-oxirane], 1-methyl-4-isopropyl-7,8-dihydroxy-, (8S) | O | 0.999 | 0.073 | 0.011 | 0.081 | 0.012 | GC/MS |
| 49   | 1910 | -        | Neointermedeol | MO | - | - | - | 0.423 | 0.087 | - |
| 50   | -    | -        | Tricyclo[5.3.1.1(2,6)]dodecan-11-ol, 11-methyl+12-methylene- | MO | - | - | - | 0.065 | 0.014 | - |

Chemical classes

- Oxygenated monoterpenes
- Sesquiterpenes hydrocarbons
- Oxygenated sesquiterpenes
- Others

Total identified (%) 94.757 96.114

1 Retention indices: Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values [32,33]; 2 C = chemical class: MO—oxygenated monoterpenes, O—others, SH—sesquiterpene hydrocarbons, SO—oxygenated sesquiterpenes; 3 RF = response factor; 4 column = composition of essential oil detected on HP-5MS and DB-HeavyWAX columns; (%)= relative percentage content; c = content is expressed as concentration in milligram per 1 kg of dry plant material; 5 identification method: GC/MS = Mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database; Std = constituent identity confirmed by coinjection of authentic standards; 6 retention indices were not calculated for compounds identified only by DB-HeavyWAX column; 7 literature data not available; 8 not detected.
Table 7. Chemical composition of *Nigella sativa* seed supercritical CO\(_2\) extract.

| Obs. | Compound       | C \(^2\) | RF \(^3\) | Column \(^4\) | Identification \(^5\) |
|------|----------------|---------|----------|---------------|-----------------------|
|      |                |         |          | HP-5MS \(\%\) | DB-HeavyWAX \(\%\) | HP-5MS \(c\) | DB-HeavyWAX \(c\) |
| 1    | o-Cymene       | MH      | -        | -             | -                     | 0.113       | 0.010  | -     | GC/MS |
| 2    | D-Limonene     | MH      | 0.765    | 0.376         | 0.015                 | 0.089       | 0.009  | RI, GC/MS | GC/MS |
| 3    | 4-methoxy thujane | MO   | 0.852    | 0.130         | 0.003                 | 0.240       | 0.028  | RI, GC/MS | GC/MS |
| 4    | Carvone        | MO      | 0.907    | 0.473         | 0.022                 | -           | -      | RI, GC/MS | -     |
| 5    | Thymoquinone   | K       | 1.071    | 0.700         | 0.037                 | -           | RI     | GC/MS | -     |
| 6    | Anethole       | MO      | 0.824    | 0.106         | 0.004                 | -           | RI     | GC/MS | -     |
| 7    | 2,4-Decadienal | A       | 0.887    | 0.080         | 0.003                 | 0.096       | 0.012  | RI, GC/MS | GC/MS |
| 8    | α-Terpinyl acetate | E   | 0.957    | 0.204         | 0.009                 | -           | RI     | GC/MS | -     |
| 9    | Caryophyllene  | SH      | 0.751    | 0.160         | 0.006                 | 0.066       | 0.006  | RI, GC/MS, Std | GC/MS |
| 10   | Tetradecanoic acid | FA   | -        | -             | -                     | 0.186       | 0.021  | -     | GC/MS |
| 11   | Sandaracopimaradiene | DH | 0.744    | 0.119         | 0.003                 | -           | RI, GC/MS | -     |
| 12   | Hexadecanoic acid | FA   | -        | -             | -                     | 9.897       | 1.097  | RI, GC/MS | GC/MS |
| 13   | Ethyl hexadecanoate | E  | 0.845    | 0.205         | 0.009                 | 0.102       | 0.011  | RI, GC/MS | GC/MS |
| 14   | Oleic acid     | FA      | -        | -             | -                     | 19.576      | 2.208  | -     | GC/MS |
| 15   | Ethyl linolate | E       | 0.846    | 5.023         | 0.138                 | 1.582       | 0.174  | RI, GC/MS | GC/MS |
| 16   | Ethyl oleate   | E       | 0.838    | 2.782         | 0.072                 | 0.265       | 0.030  | RI, GC/MS | GC/MS |
| 17   | Linoleic acid  | FA      | 0.863    | 71.657        | 3.019                 | 59.245      | 6.713  | RI, GC/MS | GC/MS |
| 18   | Disoocetyl phthalate | E  | 0.900    | 0.334         | 0.014                 | -           | RI     | GC/MS | -     |
| 19   | 1,2,15,16-Diepoxyhexadecane | O | -        | -             | -                     | 0.090       | 0.010  | -     | GC/MS |
| 20   | β-Monoolein    | E       | -        | -             | -                     | 0.761       | 0.091  | -     | GC/MS |

Chemical classes

|                | C \(^2\) | RF \(^3\) | HP-5MS \(\%\) | DB-HeavyWAX \(\%\) | HP-5MS \(c\) | DB-HeavyWAX \(c\) |
|----------------|---------|----------|---------------|-------------------|-------------|-------------------|
| Aldehydes      | 0.080   | -        | 0.086         |                   |             |                   |
| Ketones        | 0.700   | -        | -             |                   |             |                   |
| Fatty acids    | 71.657  | -        | 88.904        |                   |             |                   |
| Esters         | 8.548   | -        | 2.710         |                   |             |                   |
| Monoterpane hydrocarbons | 0.376 | 0.202 | -             |                   |             |                   |
| Diterpene hydrocarbons | 0.119 | - | -             |                   |             |                   |
| Oxygenated monoterpenes | 0.709 | 0.240 | -             |                   |             |                   |
| Sesquiterpene hydrocarbons | 0.160 | 0.066 | -             |                   |             |                   |
| Others         | 0.090   | -        | -             |                   |             |                   |

Total identified (%) 82.549 92.308

1 Retention indices: Obs = retention indices determined relative to a homologous series of \(n\)-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values [32,33].
2 C = chemical class: A—aldehydes, DH—diterpene hydrocarbons, E—esters, FA—fatty acid, K—ketones, MH—monoterpene hydrocarbons, MO—oxygenated monoterpenes, O—others, SH—sesquiterpene hydrocarbons; RF = response factor; \(\%\) = composition of essential oil detected on HP-5MS and DB-HeavyWAX columns; \((\%) = relative percentage content; c = content is expressed as concentration in milligram per 1 kg of dry plant material; Identification method: GC/MS = mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.0), RI = the retention index was matching literature database; Std = constituent identity confirmed by coinjection of authentic standards; retention indices were not calculated for compounds identified only by DB-HeavyWAX column; literature data not available; not detected.
3. Discussion

Based on the presented results, this study clearly demonstrates that the vapors of VPDPs can significantly affect results of microplate-based bioassays, as shown by the differences between the values observed for the plates sealed with vapor barrier and nonsealed plates covered with their lid only. Similar phenomenon has previously been observed in experiments assaying antistaphylococcal, toxic, and antifungal potentials of volatile agents such as thymoquinone, phenol, and plant EOs [28,34,35]. According to these findings, it is apparent that the results of assays evaluating biological activities of volatile agents in nonsealed microtiter plates might be unreliable. Unsealed wells are exposed to losses of bioactive compounds by evaporation, which can cause false-negative results [36]. On the other hand, vapors transition to adjoined wells can produce false-positive results of the tests [27], which is evident especially when the multiplate design of experiments is used. For this reason, the microplate layout is the important aspect affecting the accuracy of the results in nonsealed experiments. Considering the plate layouts, the results of the antimicrobial assay showed that the samples situated in rows closer to the most active volatile agents (8-hydroxyquinoline and thymoquinone) possessed lower or no growth-inhibitory effect in plates sealed with vapor barrier in comparison to nonsealed plates. Similarly, in MTT assay, the samples tested in the wells next to 8-hydroxyquinoline or thymoquinone were evaluated to be so highly toxic that their IC\textsubscript{50} values were not detected in nonsealed microplates as these values were below the lowest concentration tested. The same case occurred in our previous study [29] when high toxicity of carvacrol, eugenol, and thymol was observed in nonsealed experiments, whereas nontoxic potential of these compounds was determined in EVA Capmat sealed plates. Moreover, single-plate layouts with samples tested in one replicate in one microtiter plate are also affected by vapor losses of active agents and their transition to adjoining wells. Beside the design of microplate layout, a duration of the assay is a crucial parameter affecting the results of assessment of biological potential of volatiles. In the study on antistaphylococcal effect of thymoquinone, the increasing concentration of this compound during 5 h was detected by GC/MS in the microplate wells that were initially thymoquinone free [27]. Therefore, in the case of short-term tests such as DPPH assay, the influence of the vapors did not occur to such an extent because its incubation lasts only 30 min, in contrast to respective incubation times 24 and 72 h of standard antimicrobial and cytotoxicity assays.

As far as biological activity of VPDPs tested in this study is considered, N. sativa seed supercritical CO\textsubscript{2} extract is only one previously assessed for its antimicrobial effect. It exhibited growth-inhibitory activity with MIC values range of 16–128 µg/mL against standard strains of C. albicans, E. faecalis, Escherichia coli, Pseudomonas aeruginosa, and S. aureus [37]. In the case of A. elegans, the result can be supported by our previous study on A. elegans leaf EO where MIC value 512 µg/mL was determined against S. aureus [38]. Only weak antimicrobial potential of C. iners leaf methanol extract against C. albicans, E. coli, P. aeruginosa, and S. aureus was described by Mustafa et al. [39] with MIC values ranging from 780 to 25,000 µg/mL. Data on antimicrobial activity of X. verdugonianus are completely missing, nevertheless recent study concerting related plant species X. youngii from Thailand determined its antistaphylococcal activity with MIC value 1250 µg/mL [40]. Growth-inhibitory effects of capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone were evaluated against various S. aureus strains by several authors with respective MIC values >50, 60, 4, and 16 µg/mL [41–44], which are corresponding to our results in nonsealed plates.

There is a lack of data on antioxidant potential of EOs obtained from the abovementioned plant species with the exception of C. iners leaf oil that was observed to possess antioxidant effect with IC\textsubscript{50} value of 218.88 µg/mL [45]. Other study has previously reported DPPH radical scavenging activity of ethanol extract from A. elegans leaves with IC\textsubscript{50} value of 97.58 µg/mL [46]. However, EOs of these both species did not produce any activity in our study. In case of N. sativa supercritical CO\textsubscript{2} extract, our result might be considered as similar to Solati et al. [47] who observed a low level of antioxidant activity of this extract with IC\textsubscript{50} value of 2590 µg/mL. In general, the results of DPPH assay in our study evaluating antioxidant activity of volatile compounds are in correspondence with those obtained by other authors except Karakaya et al. [48] who detected antioxidant effect of caryophyllene
Although, N. sativa × (99%, CAS 148-24-3, 1.66 \text{ mm Hg at 25 } ^\circ\text{C}) seeds are known for their high content of thymoquinone, as described, e.g., in study [38]. According to the results of Mustafa et al. [52] who evaluated acute toxicity of C. iners leaf methanol extract using a brine shrimp assay, this plant species is considered safe, although high toxic potential was found in our study for C. iners seed EO. In case of N. sativa supercritical CO$_2$ extract, a medium cytotoxic effect was observed against human breast cancer cells with IC$_{50}$ value of 53.34 \mu g/mL [53]. Numerous assays for testing the cytotoxicity of plant-derived compounds have previously been performed on various human cancer cell lines from tissues such as a bone marrow, a colon epithelium, and a peripheral blood. Similar to our results, all compounds tested here, i.e., capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone, have been observed to possess a certain degree of the cytotoxicity with respective IC$_{50}$ values of 18.3, 57.7, 1.3, and 3.0–8.0 \mu g/mL [54–57].

The biological properties of EOs and supercritical CO$_2$ extract tested in this study have been attributed to their chemical composition primarily rich in monoterpenes, sesquiterpenes, and fatty acids. The chemical profile of C. iners leaf oil and N. sativa seed supercritical CO$_2$ extract has previously been described, whereas literature about chemical analysis of EOs from A. elegans seeds and X. verdugonianus leaves is not available. When comparing analytical data in this study with previously published works on C. iners leaf oil, its chemical composition corresponds to results of Son et al. [58], who detected \beta-caryophyllene, caryophyllene oxide, and humulene as the main components. Although, N. sativa seeds are known for their high content of thymoquinone, as described, e.g., in study of Venkatachallam et al. [59], the supercritical CO$_2$ extract analyzed in our study was observed to contain a high level of linoleic acid and other fatty acids. This finding was confirmed by [47,60] who also detected dominant prevalence of linoleic acid (60.74\%) and low amount of thymoquinone (0.28–1.42\%). In our previous study [38], we identified caryophyllene oxide (24.70/30.50\%), \alpha-pinene (9.70/10.50\%), isolongifolol methyl ether (4.20/3.80\%), and linalool (4.10\%) as major compounds of A. elegans leaf oil, which resembles chemical profile of EO obtained from its seeds. In addition to determination of raw percentages of peak areas, concentration of components in 1 kg of dry plant material was computed using predicted relative response factors with aim to increase the accuracy and reliability of the volatile compounds’ quantification. This approach is important in technological processes with several applications in the field of chemical analysis of VPDPs as it enables the quantification of volatile compounds by GC/MS with flame-ionization detection without having authentic compounds available, and also, it can avoid time-consuming calibration procedures [61].

4. Materials and Methods

4.1. Chemicals and Reagents

With aim to evaluate agents of different volatility characterized by distinct values of a vapor pressure, following plant-derived compounds: capsaicin (95\%, CAS 404-86-4, 1.32 \times 10^{-8} \text{ mm Hg at 25 } ^\circ\text{C}), caryophyllene oxide (99\%, CAS 1139-30-6, 7.00 \times 10^{-3} \text{ mm Hg at 25 } ^\circ\text{C}), 8-hydroxyquinoline (99\%, CAS 148-24-3, 1.66 \times 10^{-3} \text{ mm Hg at 25 } ^\circ\text{C}), and thymoquinone (99\%, CAS 490-91-5, 6.00 \times 10^{-2} \text{ mm Hg at 25 } ^\circ\text{C}) were assayed. Ciprofloxacin (98\%, CAS 85721-33-1), fluconazole (98\%, CAS 86386-73-4), oxacillin (86.3\%, CAS 7240-38-2), and tetracycline (98–102\%, CAS 60-54-8) were used as positive antibiotic controls. Other chemicals used were as follows: DPPH (CAS 1898-66-4), n-hexane
(CAS 110-54-3), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, CAS 53188-07-1), dimethyl sulfoxide (DMSO, CAS 67-68-5), methanol (CAS 67-56-1), MTT (CAS 298-93-1), and Tween 20% (CAS 9005-64-5). α-Bisabolol (CAS 23089-26-1), camphene (CAS 79-92-5), carvone (CAS 6485-40-1), caryophyllene (CAS 87-44-5), geraniol (CAS 106-24-1), humulene (CAS 6753-98-6), linalool (CAS 126-91-0), methyl octanoate (CAS 111-11-5), myrcene (CAS 123-35-3), α-pinene (CAS 7785-70-8), β-pinene (CAS 18172-67-3), α-terpinene (CAS 99-86-5), γ-terpinene (CAS 99-85-4), and terpinolene (CAS 586-62-9) were used as analytical standards. With exception, methanol and DMSO purchased from Penta (Prague, Czech) and n-hexane from Merck KGaA (Darmstadt, Germany), all other chemicals were obtained from Sigma-Aldrich (Prague, Czech).

4.2. Plant Material

The seeds of *A. elegans* and leaves of *C. iners* and *X. verdugonianus* were collected in the foothills of Mount Pangasugan located on the island Leyte (Philippines) in April 2018. The seeds of *N. sativa* were purchased in local spice store U Salvatora (Prague, CZ). The plants were authenticated by ethnobotanist Ladislav Kokoska from the Department of Tropical Crop Sciences and Agroforestry, the Faculty of Tropical Agrisciences, Czech University of Life Sciences (CUZ), Prague (CZ), and by taxonomist Edwino S. Fernando from the Institute of Biology Jose Vera Santos Memorial Herbarium, College of Science, University of the Philippines, Diliman (PHL). The voucher specimens of *A. elegans, C. iners,* and *X. verdugonianus* and voucher sample of *N. sativa* seeds were deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources, CUZ Prague (CZ). Dried plant material was ground and homogenized by Grindomix apparatus (GM 100 Retsch, Haan, Germany). The residual moisture content was evaluated gravimetrically at 130 °C by Scaltec SMO 01 analyzer (Scaltec Instruments, Gottingen, Germany) in triplicates. A detailed botanical description and physicochemical characteristic of plant samples including scientific name, family, voucher specimen/sample number, area of collection, part used, isolation technique for obtaining of EOs and supercritical CO₂ extract, their yield, and color are summarized in Table 8.

4.3. Hydrodistillation

Essential oils were obtained from *A. elegans, C. iners,* and *X. verdugonianus* by hydrodistillation of ground dried plant material in 1 L of distilled water for 3 h using a Clevenger-type apparatus (Merci, Brno, Czech) according to the procedures described in the European Pharmacopoeia [62]. The essential oils were stored in sealed glass vials at 4 °C. The data on yields (v/w, based on the dry plant weight) of obtained essential oils are shown in Table 8.

4.4. Supercritical Fluid Extraction

Supercritical CO₂ extraction of *N. sativa* seeds was carried out using Speed SFE Helix system (Applied Separations, Allentown, PA, USA). Initially, 10 g of ground material were filled into the 100 mL stainless steel extraction vessel between two layers of glass wool and subsequently installed into the extraction module. The extraction process was than performed using following parameters: isocratic pressure 200 Bar, temperature 40 °C and CO₂ flow rate 5 LPM. The extracts were stored in sealed glass vials at 4 °C. The properties and yield (w/w, based on the dry plant weight) of obtained extracts are shown in Table 8.

4.5. Bacterial Strains and Culture Media

The following four bacterial and one yeast standard strains of the American Type Culture Collection (ATCC) were used: *C. albicans* ATCC 90028, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213. All strains were purchased from Oxoid (Basingstoke, UK). Cation-adjusted Mueller-Hinton broth (MHB) (Oxoid) equilibrated to pH 7.6 with a Trizma base (Sigma-Aldrich) was used as the cultivation and assay medium for all bacteria tested, whereas further supplementation by 1% of glucose (Sigma Aldrich) was done in case of *E. faecalis.*
**Table 8.** Botanical description and physicochemical characteristic of plant species and samples tested.

| Scientific Name                          | Family          | Voucher Specimen/Sample Number | Area of Collection       | Part Used | Isolation Technique | Yield % | Color                |
|-----------------------------------------|-----------------|-------------------------------|--------------------------|-----------|---------------------|---------|----------------------|
| *Alpinia elegans* (C.Presl) K.Schum.    | Zingiberaceae   | 02509KBFR7                    | Mt Pangasugan, PHL       | Seed      | HD                  | 0.52    | Yellow               |
| *Cinnamomum iners* Reinw. ex Blume      | Lauraceae       | 02577KBFRC                    | Mt Pangasugan, PHL       | Leaf      | HD                  | 0.52    | Pale yellow          |
| *Nigella sativa* L.                     | Ranunculaceae   | 02604KBFR3                    | U Salvatora, Prague, CZ  | Seed      | SFE                 | 5.80    | Pale greenish yellow |
| *Xanthostemon verdugonianus* Náves ex   | Myrtaceae       | 02581KBFR7                    | Mt Pangasugan, PHL       | Leaf      | HD                  | 2.86    | Pale yellow          |
| Fern.-Vill.                             |                 |                               |                          |           |                     |         |                      |

1 HD: hydrodistillation, 2 SFE: supercritical fluid extraction.
Stock cultures of bacterial strains were cultivated in broth medium at 37 °C for 24 h prior to testing. For the preparation of inoculum, the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using a Densi-La-Meter II (Lachema, Brno, Czech) to obtain a final concentration of 10^8 CFU/mL.

4.6. Cell Cultures

Human colon cancer cells Caco-2 obtained from ATCC (Rockville, MD, USA) were propagated in Eagle’s Minimum Essential Medium (EMEM) obtained from Biowest (Nuaille, FR) supplemented with 10% fetal bovine serum, 1% sodium bicarbonate, 1% sodium pyruvate, 5 mM glutamine, 1% Minimum Essential Medium nonessential amino acids, and 1% penicillin-streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin). The components for cells’ cultivation were purchased from Sigma-Aldrich. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in the air.

4.7. Antimicrobial Assay

The in vitro antibacterial potential of EOs, supercritical CO₂ extract, and volatile compounds was determined using a broth microdilution method according to the guidelines of the CLSI [63]. Each sample of volatile agents was dissolved in DMSO and diluted in MHB in a range of 2–1024 µg/mL using an automated pipetting platform Freedom EVO 100 equipped with a four-channel liquid handling arm (Tecan, Mannedorf, Switzerland). Plates were inoculated with bacterial suspension and incubated at 37 °C for 24 h sealed/nonsealed with vapor barrier EVA Capmat (Micronic, Aston, PA, USA). Bacterial growth was measured spectrophotometrically using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) at 405 nm. The MICs were determined as the lowest concentrations that inhibited bacterial growth by ≥80% compared with that of the agent-free growth control and expressed in microgram per milliliter. DMSO assayed as the negative control did not inhibit any of the strains tested. The susceptibilities of *C. albicans*, *P. aeruginosa*, and *S. aureus*, to fluconazole, ciprofloxacin, and oxacillin, respectively, and susceptibilities of *E. faecalis* and *E. coli* to tetracycline were checked as positive antibiotic controls [64]. All experiments were carried out in triplicate in three independent experiments and the results were expressed as median/modal MIC values. The multiplate design of broth microdilution assay when eight different samples were tested in one microtiter plate is described in Figure 3.

![Figure 3. Scheme of multiplate design of broth microdilution assay. Ns: *Nigella sativa*, Ci: *Cinnamomum iners*, Ae: *Alpinia elegans*, Xv: *Xanthostemon verdugonianus*, Tq: thymoquinone, Hq: hydroxyquinoline, C: capsaicin, Co: caryophyllene oxide—nine serial twofold dilutions of volatile agents tested, ATB: eight serial twofold dilutions of positive antibiotic control, G: growth control (inoculated broth, 100% growth of bacteria), S: sterility control (noninfected medium control, 0% growth of bacteria), X: empty wells (not used in data calculation).](image-url)
4.8. Antioxidant Assay

The DPPH radical scavenging assay was performed using a slightly modified method previously described by Sharma and Bhat [65]. Initially, EOs, supercritical CO₂ extract, and plant-derived compounds were dissolved in DMSO and diluted in methanol to obtain concentration of 1024 µg/mL. Subsequently, serial dilutions of each sample were prepared in absolute methanol (100 µL) in 96-well microtiter plates using the automated pipetting platform Freedom EVO 100. Trolox was used as a standard reference material and pure methanol as blank control. The radical-antioxidant reaction was started after adding 75 µL of absolute methanol and 25 µL of freshly prepared 1 mM DPPH in methanol to each well, creating a range of concentrations from 0.25 to 512 µg/mL (final volume of 200 µL). The plates were kept in the dark at room temperature for 30 min nonsealed/sealed with vapor barrier EVA Capmat. Absorbance was measured at 517 nm using Cytation 3 microplate reader. All tests were performed in triplicates at three independent experiments. Results were expressed as IC₅₀ with standard deviation (±SD) in microgram per milliliter. The single-plate design of DPPH assay when two samples in triplicates are tested in one microplate is presented in Figure 4.

![Figure 4](image-url)

**Figure 4.** Scheme of single-plate designs with triplicates of two samples in one microtiter plate for DPPH assay. Ns: *Nigella sativa*, Tq: thymoquinone, Ci: *Cinnamomum iners*, Hq: 8-hydroxyquinoline, Ae: *Alpinia elegans*, C: capsaicin, Xv: *Xanthostemon verdugonianus*, Co: caryophyllene oxide—nine serial twofold dilutions of volatile agents tested; BL: blank control (pure methanol, 0% of radical inhibition); TRX: six twofold dilutions of positive Trolox control.

4.9. Cytotoxicity Assay

Cell viability was measured using a modified MTT cytotoxicity assay originally developed by Mosmann [66]. Caco-2 cell lines were seeded in 96-well plates at a density of 2.5 × 10³ cells per well. After 24 h, the cells were treated with twofold serially diluted samples (0.25–512 µg/mL) of EOs, supercritical CO₂ extract, and compounds dissolved in DMSO and cultivated for 72 h with/without vapor barrier EVA Capmat (Figure 5). Thereafter, MTT reagent (1 mg/mL) in EMEM solution was added to each well and the plates were incubated for an additional 2 h at 37 °C in a humidified atmosphere of 5% CO₂ in the air. The media with MTT were removed and the intracellular formazan
product was dissolved in 100 µL of DMSO. The solvent used did not affect the viability of the intestinal cells. The absorbance was measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Mannedorf, Switzerland), and the viability was calculated in comparison to an untreated control. Three independent experiments (two replicates each) were performed for every test. The single-plate design when four different samples in duplicates are tested in one microtiter plate is shown in Figure 6. The results of the cytotoxicity effect were calculated by GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as average IC\textsubscript{50} value with standard deviation in microgram per milliliter. The levels of cytotoxic effects were classified according to the Special Program for Research and Training in Tropical Diseases (WHO—Tropical Diseases) [67] as cytotoxic (IC\textsubscript{50} < 2 µg/mL), moderately cytotoxic (IC\textsubscript{50} 2–89 µg/mL), and nontoxic (IC\textsubscript{50} > 90 µg/mL).

![Thiazolyl blue tetrazolium bromide cytotoxicity assay](image1)

**Figure 5.** Thiazolyl blue tetrazolium bromide cytotoxicity assay performed in (a) the microtiter plate sealed with vapor barrier EVA Capmat and (b) nonsealed microtiter plate covered with the lid only.

![Single-plate design](image2)

**Figure 6.** Scheme of single-plate designs with duplicates of four samples in one microtiter plate for thiazolyl blue tetrazolium bromide cytotoxicity assay. Ae: *Alpinia elegans*, Hq: 8-hydroxyquinoline, Xv: *Xanthostemon verdugonianus*, Co: caryophyllene oxide, Ns: *Nigella sativa*, Tq: thymoquinone, Ci: *Cinnamomum iners*, C: capsaicin—12 serial twofold dilutions of volatile agents tested.

### 4.10. GC/MS Analysis

For determination of the main components of EOs and supercritical CO\textsubscript{2} extract, GC/MS analysis was performed using the dual-column/dual-detector gas chromatograph Agilent GC-7890B system equipped with autosampler Agilent 7693, two columns, a fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 19091s-433) and a DB-HeavyWAX (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 122–7132), and a flame ionization detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B (Agilent Technologies, Santa Clara, CA, USA).
Operational parameters were as follows: helium as a carrier gas at 1 mL/min and injector temperature 250 °C for the both columns. The oven temperature was raised for the both columns after 3 min from 50 to 280 °C. Initially, after an isothermic period of 3 min, the heating rate was 3 °C/min until the temperature reached 120 °C. Subsequently, the heating velocity increased to 5 °C/min until it reached 250 °C, and after 5 min of holding time on 250 °C, the heating rate increased to 15 °C/min until it reached 280 °C. Heating was followed by the isothermic period of 20 min. The essential oils were diluted in n-hexane for GC/MS at a concentration of 20 µg/mL, and for a quantitative analysis, 1 µL of methyl octanoate was added as an internal standard. Precisely, 1 µL of each EO solution was injected in a split mode (split ratio 1:50). The mass detector was set to the following conditions: ionization energy 70 eV, ion source temperature 230 °C, scan time 1 s, and mass range 40–600 m/z.

Identification of the constituents was based on the comparison of their retention indices, retention times and spectra with the National Institute of Standards and Technology Library ver. 2.0.f (NIST, USA) [32], as well as with authentic standards (Sigma-Aldrich) and literature [33]. The RI were calculated for compounds separated by H5-5MS column using the retention times of n-alkanes series ranging from C8 to C40 (Sigma-Aldrich). For each EO and supercritical CO2 extract analyzed, the final number of compounds was calculated as the sum of components simultaneously identified using the both columns and the remaining constituents identified by individual column only. Quantitative data were computed as described in Cachet et al. [68] using the following formula:

\[ m_i = \text{RRF}_{\text{pred}} m_{\text{MO}} \frac{A_i}{A_{\text{MO}}}, \]

where \( m_i \) is the mass of the compound \( i \) to be quantified, expressed in milligram per 1 kg of the plant dry weight (DWP); \( \text{RRF}_{\text{pred}} \) predicted relative response factor of compound \( i \), \( m_{\text{MO}} \) mass of methyl octanoate (internal standard, IS), \( A_i \) and \( A_{\text{MO}} \) are the peak areas of the analyte and the IS, respectively, determined by the FID. Moreover, relative percentage contents of identified components have been determined using the FID data and indicated for the both columns.

5. Conclusions

The results of experiments presented in this study clearly demonstrate that the vapors of VPDPs can significantly affect the results of standard microplate-based bioassays. In series of experiments using EVA Capmat sealed and nonsealed microplates, antimicrobial, antioxidant, and cytotoxic activities of three EOs from Philippine less explored plant species (\textit{A. elegans}, \textit{C. iners}, and \textit{X. verdugonianus}), one supercritical CO2 extract from \textit{N. sativa} and four plant compounds (capsaicin, caryophyllene oxide, 8-hydroxyquinoline, and thymoquinone) were evaluated. It was confirmed that vapor transition causes false-positive results of the bioassays performed in nonsealed microtiter plates. The microplate layout and a duration of the assay were demonstrated as the crucial aspects defining level of the results affinity by the vapors of volatile agents. In several cases, no antimicrobial activity was detected in sealed plates, however, certain grown-inhibitory effect was found in nonsealed plates. As well as in the cytotoxicity assay, significant differences in results were recorded between sealed and nonsealed plates. Due to the strong effect of the vapors of the most cytotoxic agents, toxicity of the samples in adjoining wells was not detected in nonsealed plates. Only capsaicin, 8-hydroxyquinoline, and thymoquinone showed some level of antioxidant activity, while IC\textsubscript{50} values of thymoquinone were the most affected by vapors. However, in DPPH assay, the influence of the vapors was not occurred to such an extent because this is a short-term test. Additionally, we reported biological activities and chemical composition of EOs from \textit{A. elegans} seeds and \textit{X. verdugonianus} leaves, which were, according to our best knowledge, analyzed for the first time. Due to our findings, certain modifications of the conventional bioassays performed in microtiter plates are necessary for evaluation of biological properties of the volatile agents (e.g., using of vapor barrier) in order to protect against vapor transition and to obtain reliable results.
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**Sample Availability:** Samples of the compounds are available from the authors.

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