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# Antifungal profiling of Brazilian essential oils: Chemotype classification and correlation with bioactivity

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**ABSTRACT.** Seven Brazilian commercial samples of tea tree oil (TTO) from *Melaleuca alternifolia* were analyzed from the chemical perspective to identify their chemotypes, and their efficacy against *Candida albicans*, *Cryptococcus neoformans,* and *Aspergillus fumigatus* by the broth microdilution method was also explored. Principal component analysis (PCA), hierarchical cluster analysis (HCA), and partial least squares (PLS) were performed to identify similarities and differences among the samples and their correlation with the antifungal activity. According to the chemical composition, 86 % of the samples analyzed are classified as chemotype I, and one of them can suggest an essential oil adulterated with *Eucalyptus* species. PCA and HCA demonstrated that the TTO samples should be divided into three groups and the major compounds in each group were identified. According to PLS analysis, two samples showed the most antifungal activities against *C. albicans* and *C. neoformans* most likely related to the content of 1,8-cineole and *p*-cymene as an enhancer and an inhibitor, respectively.

**Keywords:** CG-MS; chemotypes; *Melaleuca alternifolia;* multivariate data analysis; antifungal activity.

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## Introduction

*Melaleuca alternifolia* is an endemic Australian plant of the family Myrtaceae, commonly known as the 'tea tree'. Indigenous Australian people have explored the essential oil properties of this medicinal plant for more than a century (Martoni & Blacket, 2021). Especially in the last two decades, tea tree essential oil (TTO) has been used in various treatments and has been gaining popularity and acceptance due to its uses in medical therapies. The great worldwide demand for TTO is around US$ 45 million p.a., leading to a shortage of raw material and intentional adulteration of up to 50 % of all commercialized samples through the oil dilution, addition of other products, and the insertion of other, less expensive essential oils, such as *Eucalyptus* spp. (ATTIA, 2017) (Johnson et al., 2022).

The chemical constitution of TTO presents therapeutic properties such as antifungal (Mertas et al., 2015), antibacterial (Berechet et al., 2020), anti-inflammatory, antiviral (Lee et al., 2017), treatment of acne vulgaris, seborrheic dermatitis (Ergun et al., 2020), chronic gingivitis, blepharitis (Kokoska et al., 2019), including carcinogenic, acaricide, and insecticide action (Tarach et al., 2020).

More specifically, TTO is used predominantly in pharmaceutical and cosmetic topical solutions to treat cutaneous infections (Borotová et al., 2022).

The Australian Tea Tree Industry Association (2017) recognizes at least 113 compounds in the TTO composition (ATTIA, 2017). There are six "chemotypes" within the *M. alternifolia* species, based on their essential oil composition (Binshabaib et al., 2022). Chemotype 1 is identified by the prevalent presence of terpinen-4-ol, while the other ones 2, 5, and 6, contain this compound in low levels. Chemotype 2 is influenced by the presence of terpinolene, and chemotype 6 shows 1,8-cineole predominantly (Johnson et al., 2022). The acceptable levels for 15 of the 113 compounds present in tea tree essential oil (TTO) include a content of 35% to 48% of terpinen-4-ol and a level of less than 10% of 1,8-cineole (Zibetti et al., 2018). These chemotypes are distinguished by divergent concentrations of their main monoterpene compounds, such as

1,8-cineole, terpinen-4-ol, $\alpha$-terpinene, $\gamma$-terpinene, and terpinolene (Binshabaib et al., 2022). These substances contribute to fungicidal properties (Souza et al., 2017).

Terpinen-4-ol, an oxygenated monoterpene, is the main active compound of the TTO, mostly being the focal point in biological activity studies. Therefore, the minimum content of this compound was set at 30 % to guarantee the fungicidal and antibacterial quality in commercialized TTO (Kairey et al., 2023). Later, Carson and collaborators (2006) proposed that no difference in bioactivity was made prominent both *in vitro* and *in vivo* when testing microbicidal efficacy in commercial samples of TTO, suggesting that any variation in bioactivity comes from different chemotypes.

On the other hand, the use of multivariate statistical analysis techniques like principal component analysis (PCA), hierarchical component analysis (HCA), and partial least squares (PLS) has allowed the identification of chemical markers, adulteration, and grouping in natural products and their impact on biological activities. Therefore, this study aimed to identify if the seven commercial TTO samples have their composition according to ISO4730:2017, testing the efficacy against *Candida albicans*, *Cryptococcus neoformans,* and *Aspergillus fumigatus*. PCA and HCA were used to identify the chemical differences among TTO samples, and PLS to find the main substances responsible for the antifungal activity of these essential oils.

## Experimental

### Tea tree oil samples

Seven samples of processed tea tree oil (TTO), purchased from a commercial website in Brazil were labeled as follows: Amantikir (M1), Plantlife (M2), WNF (M3), Vittoria (M4), BioEssência (M5), ViaAroma (M6), and Agência Homeopática Personalizada (M7). The samples authenticity was confirmed by ensuring that their all compound concentrations were within the acceptable range specified by the ISO4730: 2017 regulations (Zibetti et al., 2018).

### GC-MS analysis

The gas chromatography (GC) analyses were performed in an Agilent GC 6850 equipment coupled to an Agilent 5975C mass spectrometer (MS) containing a 30 m x 0.25 mm long HP-5MS column with a 0.25 µm film. The samples were prepared at a concentration of 1 mg $mL^{-1}$, and the injection volume was 1µL in split mode (1:10), with Helium as carrier gas at a rate of 1 mg $mL^{-1}$. The method used has an initial oven temperature of 40°C, maintained for 8 min, and a heating rate of 5°C $min^{-1}$ until reaching a temperature of 320°C, which was maintained at the end of heating for another 8 min. The temperatures of the injector, the quadrupole, and the ion source were 300, 180, and 280°C, respectively. Mass spectra detections were performed by electron impact ionization (EI) at 70 eV, in full-scan acquisition mode in the *m/z* range 50-800 at 2.66 scan $s^{-1}$.

The metabolite identifications were performed by comparison with the NIST spectral library (v2.0, 2008) using Match and R-Match comparison values above 900 and by standard retention time comparison.

Kovat's linear retention indices (LRIs) were calculated from the retention times of *n*-alkane standards ($C_8$-$C_{20}$) run under the same conditions (Capetti et al., 2020). The identification of each peak was assigned only when the similarity was above 90%. The samples were analyzed in gas chromatography with a flame ionization detector (GC-FID) using an Agilent CG 6850 system. GC was equipped with the same chromatographic conditions used in the CG-MS analysis.

The relative percentages of the identified compounds in the samples were obtained by peak area normalization.

Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) for identifying samples' similarities.

PCA and HCA were performed from the chemical profile (% w/w) of the seven commercial samples of essential oil of *Melaleuca alternifolia*. These analyses aimed to identify similarities and differences among the samples, as well as the chemical reasons for the samples' grouping. Scaling and mean-centering of the data were applied before PCA. Ward's method was used for HCA. The multivariate data analyses were executed in SIMCA 17 software (Trial version, Sartorius Stedim Data Analytics AB, Umeå, Sweden).

### Antifungal activity assay

The *in vitro* inhibitory activity of the extracts was evaluated by the broth microdilution method.

*Candida albicans* SC 5314*, Cryptococcus neoformans* H99, and *Aspergillus fumigatus* ATCC were used as

quality control strains of the assay using fluconazole (standard antifungal). After the incubation period, the lowest concentrations of extracts that inhibit 50% of fungal growth were visually determined ($IC_{50}$).

*Partial Least Squares (PLS) to identify the chemical reasons for antifungal activities*

PLS modeling was also carried out using SIMCA 17 software. The chemical profiles of extracts were taken as the matrix of the independent variables (X), whereas the antifungal activities were defined as the dependent variable matrix (Y). The samples were randomly distributed into two groups; 80% of the total was used for model calibration and 20% for validation. Moreover, to improve the fit of the model, a cross-validation algorithm was implemented using seven groups.

## Results and discussion

Tea tree oil is widely marketed around the world due to its biological potential, mainly against human pathogenic fungi. It is known that different chemotypes depend on the content of certain monoterpenes in their constitution. The intense consumption of this phytotherapeutic agent results in a large portion of these commercialized oils being adulterated, thereby altering their chemical constitution and microbiological potential. In this sense, seven brands of TTO available on Brazilian websites were analyzed to find out the chemotypes and whether there is a correlation between the chemical composition and the antifungal potential against human pathogenic fungi. The seven commercial samples (M1-M7) of essential oil of *Melaleuca alternifolia* (TTO) were analyzed by GC-FID and GC-MS under the same chromatographic conditions. This chemical analysis identified 44 chemical constituents (Table 1). All TTO samples have a higher content of oxygenated monoterpenes than monoterpenes, except for samples M4 and M7. Terpinen-4-ol (41.0- 46.85%) was the main component in all TTO evaluated, followed by γ-terpinene (10.85-17-67%), except in M4 (7.84 and 0.53, respectively)

A comparison of the terpinen-4-ol, 1,8-cineole, and terpinolene content in the seven samples analyzed, with values in the range of 22-40%, 0-17%, and 2-6%, respectively, indicates that samples code M1, M2, M3, M5, M6, and M7 are of the chemotype 1, according to the specifications of the parameters included in the ISO4730:2017 (13). According to the contents within ISO4730:2017 (13), sample M4 is outside the required quality standards, as α-terpinene (6-12%), aromadendrene (0.2-3.0%), and viridiflorene (0.2- 3.0%) are absent in this sample. The α-pinene content of 28.21% should be in the 1-4% range, and α-terpineol (21.65) should be between 2 to 5%. The terpinen-4-ol content (7.84%) should be in the range of 35-48%. On the other hand, the contents of these compounds for sample M4 do not allow for indicating the chemotype of this TTO. According to Borotová and collaborators, various conditions can affect the quality of essential oils, such as season, storage conditions, geographical locations, including the extraction method.

**Table 1**. Compounds identified in the seven TTO analyzed by GC-MS and their inhibitory $IC_{50}$ and $IC_{90}$ values (% v/v) against human pathogenic fungi.

| Compound's identifier code | Chemical compound name | RI | RI* | Content of chemical compounds in the samples (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | M1 | M2 | M3 | M4 | M5 | M6 | M7 |
| 1 | α-thujene | 931 | 934 | 0.60 | 0.92 | 1.00 | 0.26 | 0.70 | 0.84 | 1.00 |
| 2 | α-pinene | 938 | 939 | 2.81 | 2.35 | 2.57 | 28.21 | 2.95 | 2.57 | 2.79 |
| 3 | camphene | 953 | 952 | 0.00 | 0.00 | 0.00 | 0.69 | 0.00 | 0.00 | 0.00 |
| 4 | Sabinene | 976 | 974 | 0.23 | 0.00 | 0.77 | 0.25 | 0.27 | 0.45 | 0.20 |
| 5 | β-pinene | 980 | 986 | 0.60 | 0.64 | 0.68 | 2.57 | 0.62 | 0.69 | 0.70 |
| 6 | Myrcene | 991 | 990 | 0.26 | 0.64 | 0.60 | 0.49 | 0.40 | 0.43 | 0.63 |
| 7 | α-phellandrene | 1005 | 1007 | 0.19 | 0.00 | 0.53 | 0.00 | 0.35 | 0.19 | 0.43 |
| 8 | Δ3-carene | 1011 | 1009 | 0.00 | 0.00 | 0.00 | 8.23 | 0.00 | 0.00 | 0.00 |
| 9 | α-terpinene | 1018 | 1010 | 5.29 | 8.68 | 9.42 | 0.00 | 9.18 | 4.43 | 8.60 |
| 10 | *p*-cymene | 1026 | 1028 | 8.51 | 8.18 | 4.80 | 5.05 | 5.41 | 11.89 | 7.00 |
| 11 | limonene | 1031 | 1029 | 1.65 | 1.63 | 1.86 | 4.83 | 2.04 | 1.69 | 1.93 |
| 12 | 1,8-cineole | 1033 | 1038 | 1.97 | 1.17 | 2.87 | 6.03 | 1.91 | 3.92 | 3.24 |
| 13 | (Z)-β-ocimene | 1040 | 1046 | 0.10 | 0.00 | 0.00 | 0.00 | 0.15 | 0.00 | 0.00 |
| 14 | γ-terpinene | 1062 | 1060 | 11.58 | 17.67 | 17.15 | 0.53 | 16.91 | 10.85 | 17.44 |
| 15 | α-terpinolene | 1088 | 1079 | 2.51 | 3.31 | 3.47 | 0.78 | 3.36 | 2.09 | 3.50 |
| 16 | linalool | 1098 | 1101 | 0.41 | 0.00 | 0.35 | 0.57 | 0.36 | 0.40 | 0.18 |
| 17 | citronellal | 1153 | 1149 | 0.24 | 0.00 | 0.23 | 1.40 | 0.25 | 0.32 | 0.00 |
| 18 | terpinen-4-ol | 1177 | 1179 | 42.53 | 46.85 | 41.75 | 7.84 | 43.43 | 43.60 | 41.07 |
| 19 | α-terpineol | 1189 | 1180 | 5.55 | 3.50 | 3.78 | 21.65 | 5.04 | 4.31 | 3.89 |
| 20 | Nerol | 1228 | 1221 | 0.36 | 0.00 | 0.00 | 2.55 | 0.32 | 0.00 | 0.00 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 21 | citronellol | 1229 | 1228 | 0.15 | 0.00 | 0.00 | 0.00 | 0.00 | 0.22 | 0.00 |
| 22 | neral | 1240 | 1236 | 0.36 | 0.00 | 0.00 | 0.50 | 0.00 | 0.00 | 0.00 |
| 23 | geraniol | 1255 | 1250 | 0.32 | 0.00 | 0.00 | 0.36 | 0.00 | 0.53 | 0.00 |
| 24 | linalyl acetate | 1257 | 1249 | 0.23 | 0.00 | 0.00 | 0.44 | 0.00 | 0.43 | 0.00 |
| 25 | Geranial | 1270 | 1266 | 0.21 | 0.00 | 0.00 | 0.00 | 0.00 | 0.47 | 0.17 |
| 26 | α-cubebene | 1351 | 1351 | 0.00 | 0.00 | 0.00 | 1.50 | 0.00 | 0.00 | 0.00 |
| 27 | (Z)-β-caryophyllene | 1404 | 1407 | 0.36 | 0.00 | 0.40 | 0.00 | 0.34 | 0.38 | 0.36 |
| 28 | (E)-β-caryophyllene | 1418 | 1415 | 0.28 | 0.00 | 0.55 | 0.00 | 0.25 | 0.47 | 0.43 |
| 29 | (Z)-β-farnesene | 1438 | 1434 | 1.80 | 1.02 | 1.39 | 0.00 | 1.56 | 1.53 | 1.40 |
| 30 | (E)-β-farnesene | 1457 | 1458 | 0.58 | 0.00 | 0.67 | 0.00 | 0.49 | 0.70 | 0.65 |
| 31 | aromadendrene | 1461 | 1464 | 0.23 | 0.00 | 0.37 | 0.00 | 0.23 | 0.33 | 0.29 |
| 32 | γ-gurjunene | 1473 | 1471 | 0.17 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 33 | germacrene D | 1480 | 1477 | 0.21 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 34 | viridiflorene | 1493 | 1490 | 2.28 | 1.48 | 2.14 | 0.00 | 2.01 | 1.67 | 1.55 |
| 35 | δ-cadinene | 1524 | 1529 | 1.45 | 1.96 | 2.02 | 0.00 | 0.93 | 2.26 | 1.65 |
| 36 | ledol | 1564 | 1565 | 0.18 | 0.00 | 0.26 | 0.00 | 0.00 | 0.25 | 0.21 |
| 37 | spathulenol | 1575 | 1570 | 0.23 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 38 | globulol | 1583 | 1576 | 0.69 | 0.00 | 0.37 | 0.00 | 0.54 | 0.68 | 0.33 |
| 39 | viridiflorol | 1589 | 1590 | 0.26 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.00 |
| 40 | guaiol | 1595 | 1592 | 0.00 | 0.00 | 0.00 | 1.23 | 0.00 | 0.00 | 0.00 |
| 41 | α-cadinol | 1640 | 1635 | 0.27 | 0.00 | 0.00 | 0.00 | 0.00 | 0.37 | 0.00 |
| 42 | α-muurolol | 1645 | 1641 | 0.00 | 0.00 | 0.00 | 2.49 | 0.00 | 0.00 | 0.00 |
| 43 | β-eudesmol | 1649 | 1644 | 3.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 44 | α-eudesmol | 1652 | 1657 | 0.56 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Full compound analysis | | | | | | | | | | |
| Monoterpenes | | | | 36.3 | 45.2 | 45.7 | 57.9 | 44.2 | 40.0 | 47.5 |
| Oxygenated monoterpenes | | | | 50.4 | 50.4 | 46.1 | 35.3 | 49.4 | 50.3 | 45.3 |
| Sesquiterpenes | | | | 12.6 | 4.5 | 8.2 | 5.2 | 6.4 | 8.7 | 6.9 |
| Identified | | | | 99.3 | 100.0 | 100.0 | 95.5 | 100.0 | 99.1 | 99.6 |
| Antifungal activity | $IC_{50}$ *Candida albicans* (%) | | | 0.56 | 0.56 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 |
| | $IC_{50}$ *Cryptococcus neoformans* (%) | | | >1.12 | >1.12 | 0.56 | 0.56 | >1.12 | >1.12 | >1.12 |
| | $IC_{50}$ *Aspergillus fumigatus* (%) | | | >1.12 | >1.12 | >1.12 | >1.12 | >1.12 | >1.12 | >1.12 |

RI values from experimental data; RI* values from the literature cited in the last column.

According to the antifungal tests performed against *C. albicans*, *C. neoformans,* and *A. fumigatus*, TTO samples inhibited the fungal growth of the *C. albicans* at 0.28-0.56%. However, all commercial TTO samples analyzed in this work did not show activity against *C. neoformans* and *A. fumigatus*, except M3 and M4 samples that were active at 0.56% against *C. neoformans*. According to Oliveira et al. (2022) the TTO chemotype 1 showed antifungal action against *C. albicans* with $IC_{50}$ of 0.125 (% v/v), while against *C. neoformans* the $IC_{50}$ was 0.03 (%v/v). The observed activity of all samples against *C. albicans,* except for sample M4, must be associated with the major constituents, such as terpinen-4-ol, γ-terpinene, and α-terpinene, which are recognized to increase yeast cell permeability and membrane fluidity and disrupt its structure. Additionally inhibits germ tube formation or mycelial conversion and inhibits microorganism respiration (Borotová et al.,2022; Kairey et al., 2023).

According to PCA and HCA, three different groups of TTO samples are identified. The most dissimilar group (Group 1) comprises sample M4, this TTO is rich in α-pinene (Compound 9), Δ3-carene (Compound 8), limonene (Compound 11), 1,8-cineol and α-terpineol (Compound 19) compounds (Figure1) and (Table 2). The chemical data can be consistent with possible adulteration with oil from some *Eucalyptus* species since the content of α-pinene (28.21 %), 1,8-cineole (6.3 %), α-terpineol (21.65 %), and limonene (4.84 %) was observed (Francisconi et al., 2020). Groups 2 (M1 and M6) and 3 (Remaining samples) are very similar, these TTO samples hold average contents of almost identified chemical compounds, though Group 2 is abundant in *p*-cymene (Compound 10), γ-terpinene (Compound 14) and terpinen-4-ol (Compound 18) compounds, whereas Group 3 is characterized by a high level of myrcene (Compound 6).

The samples M4 and M3 showed the best antifungal activities against *C. albicans* and *C. neoformans* (Table 1), this finding was also confirmed by PLS analysis (Figure 2A). These could be supported by the high contents of characteristic compounds included in Group 1 (Table 2) in combination with linalool (Compound 16), neral (Compound 22), and linalyl acetate (Compound 24). The samples M1 and M2 had the lowest antifungal activities, and samples M5, M6, and M7 held an average performance against these biological agents. Besides, to identify the main chemical compounds responsible for antifungal activity applied variable

importance for the projection (VIP) in the first latent variable (first component) (Figure 2B). VIP values larger than 1 indicate important X-variable and bars represent their confidence intervals at a 95% level. Thus, the compounds 1,8 cineole (Compound 12) and *p*-cymene (Compound 10) are the main ones responsible for the antifungal activity, the first one as an enhancer and the last one as an inhibitor. The 1,8-cineol showed antifungal activity against *C. albicans* biofilm, including in combination with chlorhexidine (Simsek & Duman, 2017). The *Thymus camphoratus* oil with a higher content of 1,8-cineol and α-pinene was active against this microorganism biofilm with low toxicity (Alves et al., 2019).

According to Haines et al. (2022) and collaborators, the *p*-cymene and 1,8-cineol showed fungicidal activity against *Candida* species, and the *p*-cymene and 1,8-cineol combination resulted in a significant synergistic antifungal effect. 1,8-cineole and the combination of 1,8-cineole and limonene (1:1) showed MIC values of 23 mg $mL^{-1}$ and 2-3 mg $mL^{-1}$ against *C. neoformans* (Roana et al., 2021).

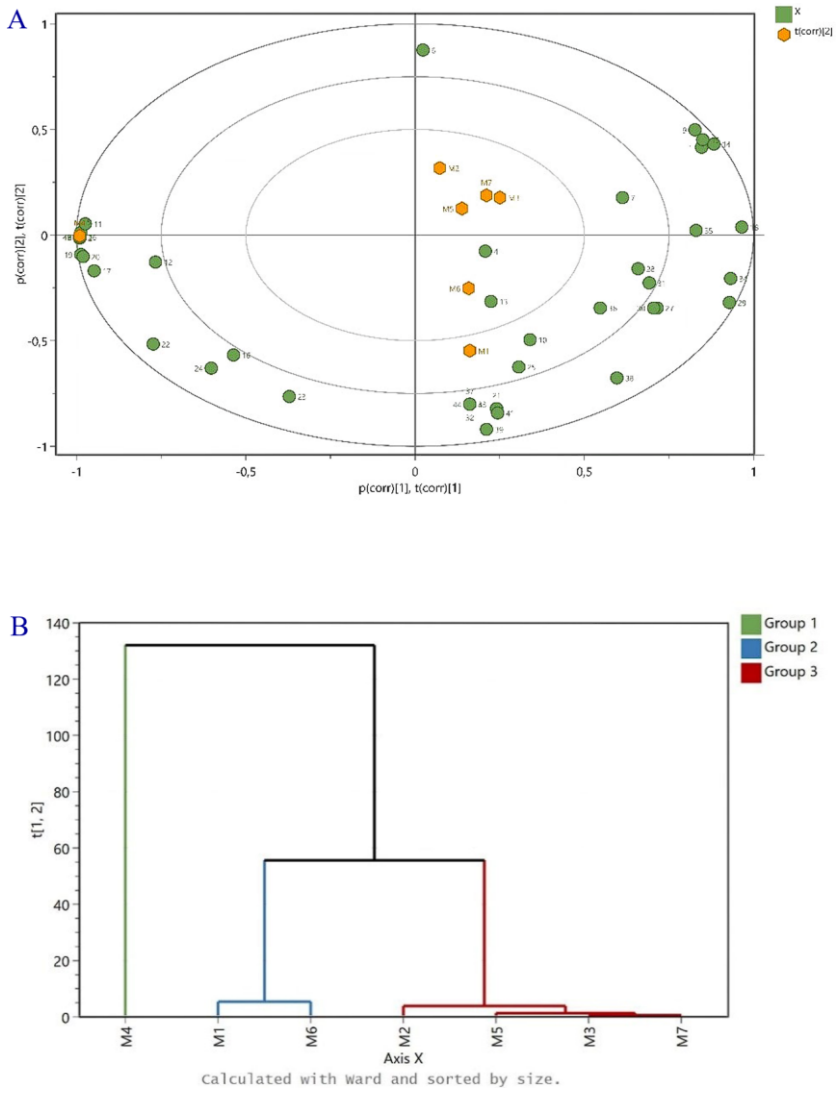


**Figure 1**. PCA and HCA for identifying samples' similarities. A: Biplot graph, co-chart of scores, and loading for simultaneous display and interpretation. B: Dendrogram of cluster analysis using HCA.

**Table 2**. The major compounds in each of the groups identified by HCA.

| HCA Group (TTO samples) | Chemical compound name |
|---|---|
| 1 (M4) | α-pinene<br>camphene<br>β-pinene<br>Δ3-carene<br>limonene<br>1,8-cineole<br>citronellal |

| | |
|---|---|
| | α-terpineol |
| | nerol |
| | α-cubebene |
| | guaiol |
| | α-muurolol |
| 2 (M1, M6) | *p*-cymene |
| | citronellol |
| | geranial |
| | γ-gurjunene |
| | germacrene D |
| | spathulenol |
| | globulol |
| | viridiflorol |
| | α-cadinol |
| | β-eudesmol |
| | α-eudesmol |
| 3 (M2, M3, M5, M7) | myrcene |

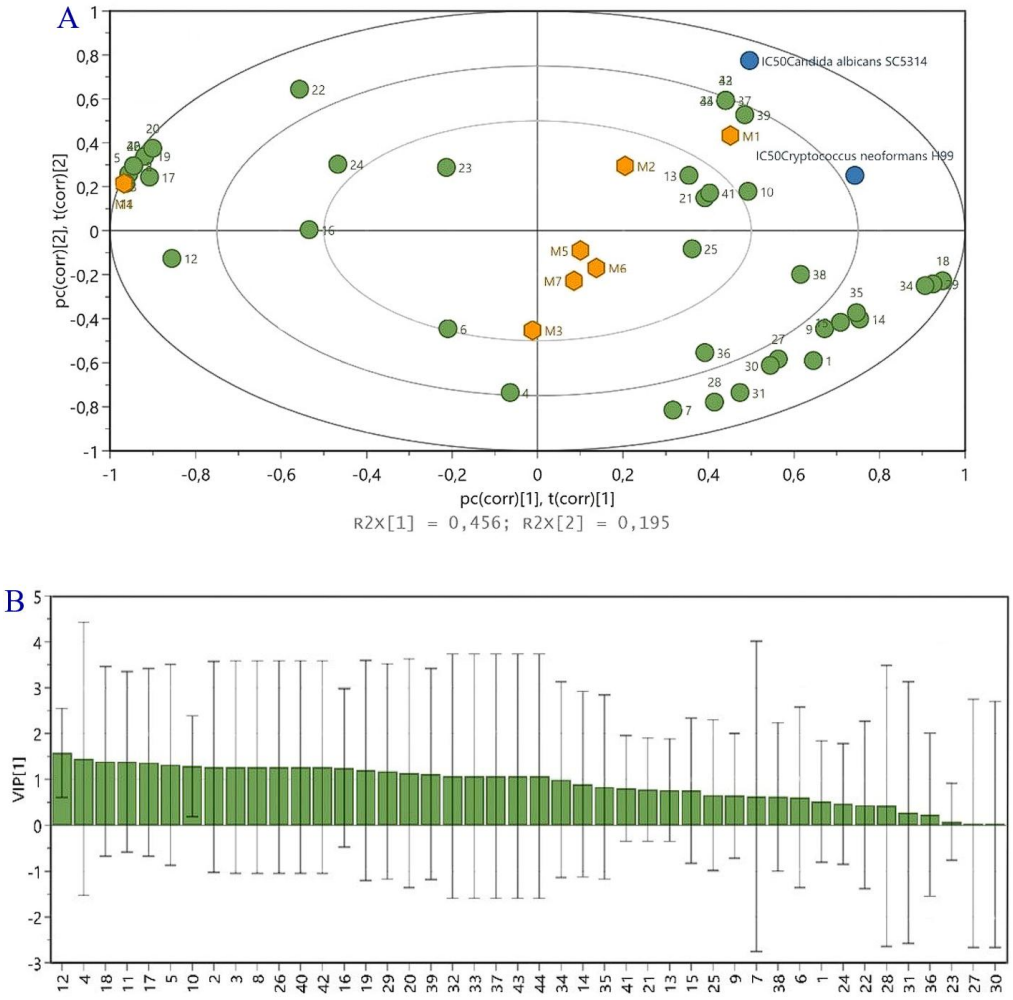


**Figure 2**. PLS analysis. A: Biplot graph, co-chart of scores for X and Y variables, as well as loading for simultaneous display and interpretation. B: The variable importance for the projection plot for the first component.

## Conclusion

Based on the chemical profile and the content of terpinen-4-ol, 1,8-cineole, and terpinolene by GC-MS and GC-FID of seven commercial samples of TTO showed that all of them are chemotype 1, except the M4 sample. According to the chemical analysis, this sample cannot be characterized as the chemotypes described in the literature and can be an adulterated product with essential oil of some *Eucalyptus* species. The PCA and HCA analysis identified three different groups of TTO samples, and the most dissimilar group 1 comprises

only sample M4. All TTO samples were active against *C. albicans*. However, the M3 and M4 samples were more effective than the others due to antifungal activities against *C. albicans* and *C. neoformans* and likely compounds responsible for this biological attribute were defined by PLS analysis.

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