Plant Volatile Secondary Metabolites: Citral Containing Essential Oils as Potential Tyrosinase Inhibitors

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Introduction

Tyrosinase is a copper containing enzyme characteristic of several bacteria fungi, animals, and plants. In human, tyrosinase is the key enzyme in the biosynthetic pathway of melanin, the biological pigment found in hair, skin and in the eye iris where it plays a crucial role in the absorption of free radicals and in the protection of the cell DNA from ionizing radiations. However excessive melanin production is responsible for minor aesthetic problems (i.e. freckles and solar lentigo) as well as for serious dermatological conditions including cancer and post inflammatory melanoderma. The downregulation of tyrosinase is a widespread approach to treat such disorders [1] and plant extracts have often revealed to be valuable sources of tyrosinase inhibitors as three out five of the most medically/cosmetically employed tyrosinase inhibitors are plant specialized metabolites (i.e. hydroquinone, α-arbutin and aloesin) [1]. Up to date, phenolic compounds have mostly been investigated as potential tyrosinase inhibitor while fewer studies have evaluated the tyrosinase inhibition activity of plant volatile terpenoids. Citral (a mixture of two isomers, *cis*- and *trans*-3,7-dimethyl-2,6-octadienal, also known as neral and geranial, in the typical 1/3 and 2/3 ratio) has proven to be a potential tyrosinase inhibitor as it blocks the enzymatic activity of mushroom tyrosinase, a fungal source of tyrosinase employed for preliminary and high throughput screenings as it is relatively cheap and readily available [2]. Citral is an important fragrance ingredient present in considerable amount in the essential oils (EOs) obtained from different botanical species including *Cymbopogon schoenanthus* (L.) Spreng., *Litsea cubeba* (Lour.) Pers., *Melissa officinalis* L. and *Verbena officinalis* L. To the best of author knowledge, only *L. cubeba* EO has been investigated for its tyrosinase inhibitory activity [3].

Aims

This study aims at 1) evaluating the *in-vitro* tyrosinase inhibitory activity of *Cymbopogon schoenanthus*, *Litsea cubeba*, *Melissa officinalis* and *Verbena officinalis* EOs to assess whether the different chemical composition may influence the EO overall inhibitory activity due to possible synergistic and/or competitive interactions among their components 2) at identifying the other possible bioactive components contributing to the investigated activity through a bio-guided fractionation approach.

Experimental

Materials and reagents

Essential oils (EOs) used in this study were supplied by Witt Italia SpA (Poirino, Italy). Pure standard sample of citral was from Sigma Aldrich (Milan, Italy). Solvents were all HPLC-grade from Sigma Aldrich (Milan, Italy). Phosphate saline buffer, tyrosinase from mushroom, L-tyrosine and kojic acid were also from Sigma Aldrich.

***In vitro* tyrosinase inhibition test**

Mushroom tyrosinase solution 200 UmL^{-1} (27.9 μgmL^{-1}) was prepared in sodium phosphate buffer (pH 6.8) and aliquots were stored at -18 °C and thawed just before the experiments. Tyrosine solution 0.1 $mgmL^{-1}$ was prepared in sodium phosphate buffer (pH 6.8) and renewed daily. Three solutions of Kojic at concentrations of 1.06, 0.53, 0.27 $mgmL^{-1}$ and, five solutions of increasing concentration for each investigated EO (i.e. 5.0, 10.0, 30.0, 50.0 $mgmL^{-1}$) were prepared in DMSO and stored at 4 °C. The *in vitro* spectrophotometric test was performed in a closed 4 mL vial to avoid the loss in the surrounding environment of any essential oil components given their volatile nature. The reaction components were placed in the vial following the described order: 1 mL of mushroom tyrosinase solution, 1 mL of sodium phosphate buffer solution, 10 µL of essential oil/kojic acid solution and finally 1 mL of tyrosine solution. After mixing by vortex the reaction mixture was incubated 25 °C for 6 minutes after which the final absorbance at 475 nm was recorded. The absorbance corresponding to 100% of the tyrosinase activity ($A_{control}$) was measure performing the experiments as described in the previous paragraph only replacing the essential oil/kojic acid solution with 10 µL of pure DMSO. The percentage of enzymatic inhibition was measured as follow:

$$\%\ \text{of enzymatic Inhibition} = \frac{(A_{\text{control } t_{6\,min}} - A_{\text{control blank}}) - (A_{\text{Sample } t_{6\,min}} - A_{\text{Sample blank}})}{(A_{\text{control } t_{6\,min}} - A_{\text{control blank}})} \times 100$$

Flash Column Chromatography

Fractionation of essential oils was carried out on a flash column chromatography system PuriFlash 450 (Sepachrom, Italy), equipped with UV and ELSD detectors. Stationary phase: sphera silica Daily 50µm (Sepachrom, Italy); mobile phase: petrolether (A) and ethylacetate (B), flow 25 ml/min. Linear gradient elution was adopted from 100% of A to 80% of A and 20% of B in 20 minutes.

Analysis conditions

GC-MS analyses were carried out with a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) installed on a Shimadzu 2010 GC unit coupled to a Shimadzu QP2010 Mass spectrometer. *GC conditions*: injector temperature: 280°C, injection mode: split, ratio: 1/20; carrier gas: helium, flow rate: 1 mL min^{-1}; column: Mega SE 52 (95 % polydimethylsiloxane, 5 % phenyl) 25 m×0.25 mm dc×0.25 µm df, from MEGA (Milan, Italy). Temperature program: from 50°C (1 min) to 250°C (5 min) at 3°C min^{-1}. MSD conditions: MS operated in EI mode (70 eV), scan range: 35 to 350 amu; dwell time 40 ms, ion source temperature: 230°C; quadrupole temperature: 150°C; transfer line temperature: 280°C. Essential oil markers were identified by comparing both their linear retention indices (*I*'s), calculated *versus* a C_9-C_{25} hydrocarbon mixture, and their mass spectra to those of authentic samples, or from commercially available mass spectral libraries (Adams, 2007).

Results and Conclusions

1. Chemical composition of the EOs

All EOs were analysed using gas chromatography, with FID and MS detectors, to characterize their **phytochemical profile**.

The **quantitative determination** of **neral** and **geranial** in each EO was performed **by external standard method**.

Figure 1 reports the compounds relative percentage abundances in each investigated EO; in all them, neral and geranial - contained in the same ratio independently of the EO - are the most abundant compounds, although present at different concentrations: *L. cubeba* and *C. schoenanthus* EOs contain similar citral concentration, greater than those observed in *V. officinalis* and *M. officinalis* EOs. *L. cubeba* and *V. officinalis* EOs present higher amounts of limonene compared to *M. officinalis* and *C. schoenanthus* Eos. However, unlike the others, *M. officinalis* EO hydrocarbon fraction is considerably marked, containing mainly the sesquiterpene trans-β-caryophyllene.

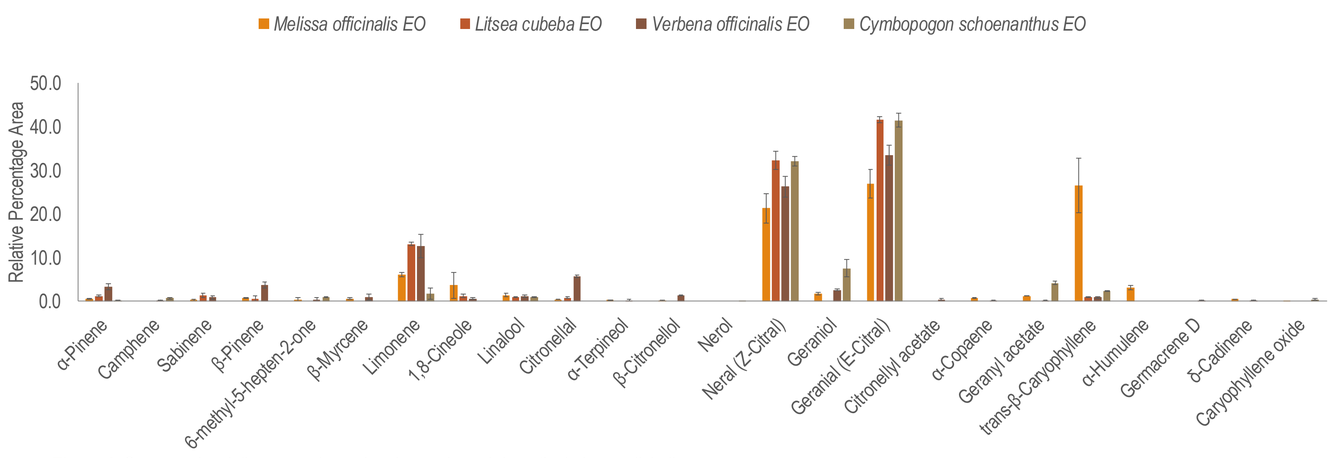

Figure 1: Compounds relative percentage abundances in each investigated essential oil

2. Mushroom tyrosinase inhibitory potential of *Cymbopogon schoenanthus*, *Litsea cubeba*, *Melissa officinalis* and *Verbena officinalis* EOs

Table 1: Dose – response curves and IC_{50} values of each investigated EO

Inhibitor Dose-response curve	IC_{50} (μgmL^{-1})
Litsea cubeba EO y = 0.2879x + 14.002 R^2 = 0.9997	125.0
Verbena officinalis EO y = 0.2148x + 13.951 R^2 = 0.9987	167.8
Melissa officinalis EO y = 0.1278x + 3.6708 R^2 = 0.9965	362.5
Cymbopogon schoenanthus EO y = 0.2124x + 3.9733 R^2 = 0.9977	216.7
Citral y = 0,3679x + 1,8094 R^2 = 0,9951	131.0
Kojic acid (Positive control) 8.2392x + 4.4503 R^2 = 0.9969	5.5

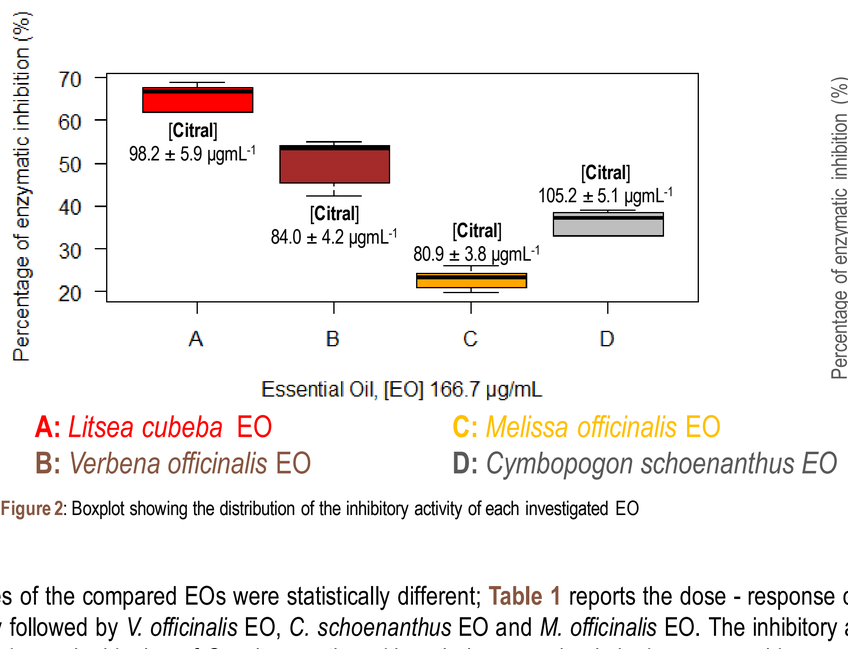

Figure 2: Boxplot showing the distribution of the inhibitory activity of each investigated EO

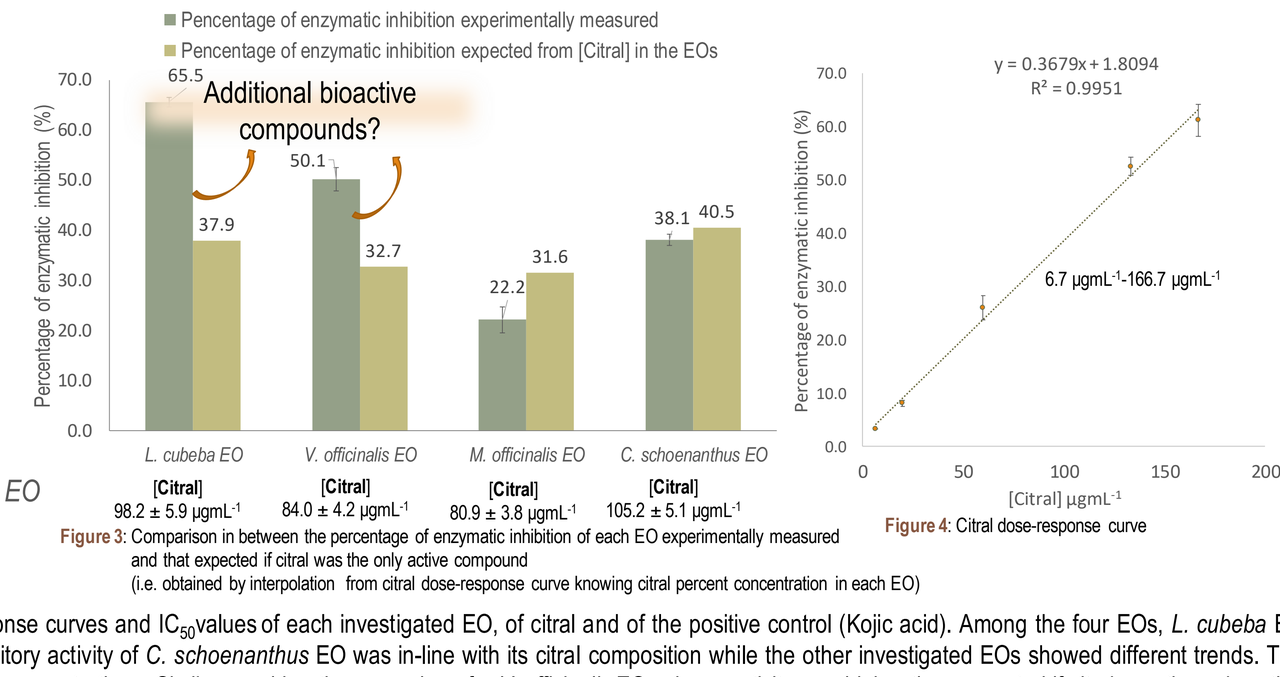

Figure 3: Comparison in between the percentage of enzymatic inhibition of each EO experimentally measured and that expected if citral was the only active compound (i.e. obtained by interpolation from citral dose-response curve knowing citral percent concentration in each EO)

Figure 4: Citral dose-response curve

Mushroom tyrosinase inhibitory activities of the compared EOs were statistically different; **Table 1** reports the dose - response curves and IC_{50} values of each investigated EO, of citral and of the positive control (Kojic acid). Among the four EOs, *L. cubeba* EO displayed the greatest inhibitory activity followed by *V. officinalis* EO, *C. schoenanthus* EO and *M. officinalis* EO. The inhibitory activity of *C. schoenanthus* EO was in-line with its citral composition while the other investigated EOs showed different trends. The inhibitory activity of *L. cubeba* EO was almost double that of *C. schoenanthus* although they contained citral at comparable concentrations. Similar consideration were done for *V. officinalis* EO, whose activity was higher than expected if citral was the only active compound. Contrary, *M. officinalis* inhibitory activity was lower than expected suggesting that it might contain specialized metabolites hampering the inhibitory activity of citral.

3. Bio-guided fractionation of the essential oils containig additional biactive compounds to citral. A case study: *Litsea cubeba*

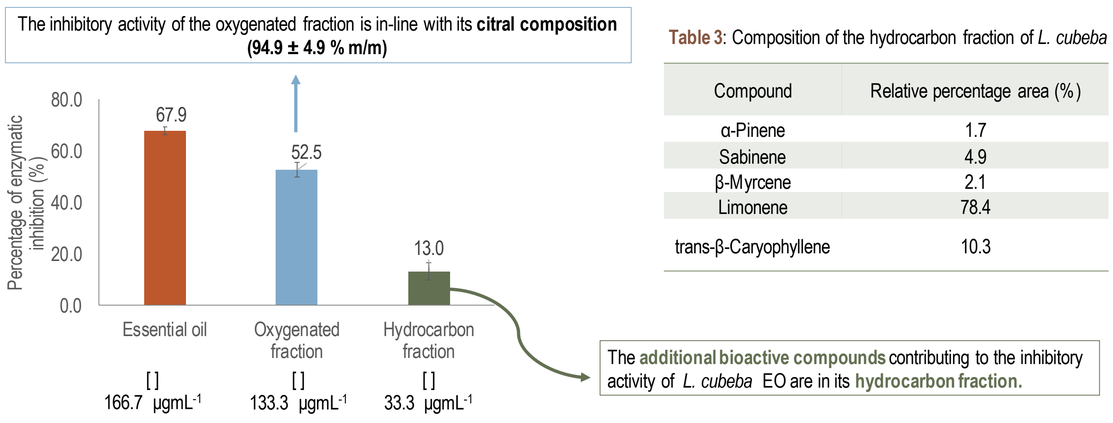

Figure 3: Percentage of enzymatic inhibition of *L. cubeba* EO and its oxygenated and hydrocarbon fractions

Table 3: Composition of the hydrocarbon fraction of *L. cubeba* EO

Compound	Relative percentage area (%)
α-Pinene	1.7
Sabinene	4.9
β-Myrcene	2.1
Limonene	78.4
trans-β-Caryophyllene	10.3

Conclusions

This study highlighted significant differences among the mushroom tyrosinase inhibitory activities of the four investigated EOs.

C. schoenanthus EO inhibitory potential was in-line with its citral composition while that of *M. officinalis* was lower than expected from its citral content. *L. cubeba* and *V. officinalis EO* contain further bioactive components, in addition to citral, contributing to overall investigated biological activity. *L. cubeba* EO bio-guided fractionation revealed that its further bioactive compounds are contained in its hydrocarbon fraction which displays an additional tyrosinase inhibitory activity to that of citral. *L. cubeba* EO hydrocarbon fraction largely consists of limonene which could be the additional bioactive compound, also according to literature data [3]. This could explain *V. officinalis* higher activity compared to what expected (if citral was the only active specialized metabolite) as it presents limonene in comparable amount to that in *L. cubeba* EO. Further studies are being carried out to further characterize the inhibitory activity of the oxygenated and hydrocarbon fractions of *V. officinialis* EO and to better comprehend why the inhibitory potential of *M. officinalis* EO is lower than what expected from its citral content.

Acknowledgements

The authors are indebted with **Dr. Gloria Giussani (Witt Italia SpA, Italy)** for supplying the essential oils.

References

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