PhD THESIS

In vitro studies on the bio-functionality of bioactive compounds in volatile oils

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(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

The global citrus processing industry produces large amounts of by-products, particularly citrus peels, which are a rich source of essential oils (CEOs). These oils are naturally abundant in bioactive compounds such as limonene, linalool, β-cis-ocimene, and terpenes, known for their potent antioxidant, antimicrobial, anti-inflammatory, and cytotoxic properties. Despite their promising biofunctional profile, CEOs remain underutilized in food and pharmaceutical applications due to their volatility, poor water solubility, and instability under environmental conditions such as heat, oxygen, and pH. These limitations necessitate innovative valorization strategies that preserve and enhance CEO functionality.

This Ph.D. thesis focuses on the formulation, characterization, biological activity, and safety evaluation of nano-emulsified citrus essential oils (CEO-NEs) as multifunctional ingredients for food preservation, health promotion, and therapeutic purposes. By incorporating advanced nano-formulation techniques and comprehensive biological and toxicological assessments, the thesis aims to support the safe and sustainable integration of citrus by-products into food and nutraceutical systems, aligning with circular economy and clean-label principles.

Main Objectives of the Thesis

The overarching goal of this thesis is to valorize citrus essential oils by transforming them into nano-emulsified systems with enhanced biological efficacy and safety for potential use in food, nutraceutical, and pharmaceutical applications. The specific objectives are:

O1. Formulation and physicochemical characterization of CEO-NEs: Develop stable nano-emulsions from various citrus oils (bergamot, lemon, tangerine, orange, grapefruit, and Tacle®) using food-grade surfactants, and evaluate their size, morphology, surface charge, and stability under different storage conditions.

O2. Chemical profiling of pure and nano-emulsified essential oils: Identify the volatile and bioactive compound composition using GC-MS, and determine changes in chemical profiles induced by nano-encapsulation.

O3. Biological activity assessment: Evaluate the antioxidant, antimicrobial, anti-inflammatory, and cytotoxic potential of CEO-NEs through in vitro assays using microbial strains and mammalian cell lines.

O4. Toxicological hazard identification and characterization: Use in silico and in vitro models to predict and experimentally confirm the metabolic fate, mutagenic, and genotoxic risks of CEO-NEs, focusing on bergamot nano-emulsions (BeNEs) as the model system.

O5. Elucidation of mechanisms of action through molecular docking: Apply computational docking methods to explore how CEO components interact with bacterial and inflammatory molecular targets.

Methodology

The research employed a multidisciplinary approach, combining nano-formulation, analytical chemistry, biological assays, computational modeling, and toxicological testing:

- **Nano-emulsion preparation**: Citrus essential oils were formulated into oil-in-water nano-emulsions via spontaneous emulsification and ultrasonication, stabilized by Tween 80 and ethanol. Emulsions were characterized using DLS, SEM, TEM, and UV-Vis.
- **Chemical analysis**: Essential oils and CEO-NEs were analyzed by GC-MS to determine volatile profiles and dominant compounds (e.g., D-limonene, β-linalool).
- **Biological assays**:
    - Antioxidant activity: Assessed using DPPH and ABTS radical scavenging assays across different temperatures and time points.
    - Antimicrobial activity: MICs were determined against *E. coli*, *S. aureus*, *L. monocytogenes*, and *Salmonella enteritidis*.
    - Cytotoxicity: Evaluated in B16-F10 melanoma and Hs27 fibroblasts using MTT assays.
    - Anti-inflammatory effects: Tested on RAW 264.7 macrophages by measuring NO inhibition.
- ***In silico* predictions and molecular docking**: Tools such as Biotransformer 3.0, ADMET Lab, and PASS Online were used for metabolic and toxicity predictions. Docking simulations explored interactions of CEO components with DNA gyrase, COX-2, NF-κB, and urease.

- ***In vitro* toxicological testing**: The Ames test (five *Salmonella* strains) and micronucleus assay (L5178Y cells) were performed to evaluate genotoxicity and mutagenicity of BeNEs with and without metabolic activation.

Results

- **Formulation and Physicochemical Properties**: All CEO-NEs exhibited nanometric droplet sizes (43–100 nm) with moderately negative zeta potentials (−10 to −20 mV), indicating colloidal stability. Tangerine NEs showed the highest instability during storage, while BeNEs remained stable.
- **Chemical Composition**: CEO-NEs retained key volatiles with D-limonene as the dominant compound (up to 87%), followed by β-linalool and β-cis-ocimene, especially in Tacle® EO. Nano-emulsification altered compound proportions, enhancing biological activity.
- **Biological Activities**:
  - Antioxidant capacity was strongest in tangerine NEs, maintained or improved during 30-day storage.
  - Antimicrobial activity was most pronounced in bergamot NEs, with MIC = 1.95–4.1 μL/mL against *E. coli*.
  - Anti-inflammatory effect of Tacle® NEs showed up to 78.34% NO inhibition without cytotoxicity.
  - Cytotoxicity was selective for melanoma cells, with minimal effect on fibroblasts.
- **Toxicological Evaluation**:
  - *In silico* analysis predicted potential respiratory and skin sensitization for some compounds (e.g., γ-terpinene), while BeNEs showed the lowest overall predicted toxicity.
  - *In vitro* biotransformation confirmed metabolic pathways (oxidation, glucuronidation), validating predictions.
  - Ames test revealed a mild mutagenic response in TA98 strain with S9 activation.
  - Micronucleus assay confirmed no genotoxicity under tested conditions.
- **Molecular Docking**: β-linalool and limonene demonstrated high binding affinity to DNA gyrase and inflammatory proteins, supporting their dual action against infection and inflammation.

General Conclusions

1. Citrus EO nano-emulsions are multifunctional agents with enhanced antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities, enabled by improved solubility and bioavailability.
2. Nano-encapsulation protects and enhances the stability and activity of volatile compounds, particularly D-limonene and β-linalool.
3. Bergamot and Tacle® NEs are the most promising, the former for antimicrobial activity, the latter for anti-inflammatory effects and safety.
4. In silico and in vitro toxicity assessments confirmed the safety of BeNEs, although some metabolic activation may induce low-level mutagenicity, requiring further in vivo validation.
5. The research supports the integration of CEO-NEs into clean-label food preservation systems, offering natural alternatives to synthetic additives and aligning with circular economy goals.

Originality and Personal Contributions

This thesis introduces novel insights into the formulation, safety, and functionality of citrus essential oil nano-emulsions, particularly from Tacle® and bergamot, which are underexplored in the literature. Key contributions include:

- The first in-depth toxicological characterization of CEO-NEs using a tiered EFSA-aligned approach, combining in silico, in vitro, and molecular modeling techniques.
- Demonstration of selective bioactivity—antimicrobial effects against *E. coli*, anti-inflammatory effects in macrophages, and cytotoxicity toward melanoma cells—validating CEO-NEs as multifunctional food-grade ingredients.
- Introduction of Tacle® EO nano-emulsions as a safe and potent candidate for future nutraceutical and therapeutic applications.
- Development of a screening pipeline for natural food additives, applicable to other plant-derived by-products.

This work contributes to the valorization of citrus processing waste, offering a sustainable path to develop high-value, health-promoting ingredients suitable for functional foods and biomedical innovations.

Furthermore, citrus essential oil nano-emulsions, particularly those derived from bergamot, may be regarded as potential food additives.