UNIVERSITÀ DEGLI STUDI DI SALERNO

PhD Program

UNIVERSITÀ DEGLI STUDI DI SALERNO

Dipartimento di Farmacia

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PhD Thesis in

Onconutraceutics and personalized medicine: isolation, characterization, pharmacokinetics and biological evaluation of antiproliferative compounds from complex natural matrices

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Abstract

The PhD research project aims to the study of novel *onconutraceuticals* obtained from natural matrices for their potential use in the prevention and as valid support to the pharmacological therapies for the treatment of cancer pathologies and chronic disease. This PhD thesis is mainly focused on the development and application of analytical techniques suitable for the characterization in detail of the chemical diversity of target compounds, to evaluate their biological properties and to elucidate the fate of key molecules after assumption.

A combined approach consisting of two high resolution analytical techniques, namely Online comprehensive two dimensional liquid chromatography-tandem mass spectrometry (LC × LC) and direct infusion Fourier transform ion cyclotron mass spectrometry (DI-FT-ICR MS) has been developed and applied for the accurate profiling of *Humulus lupulus* L. phytocomplex. A reversed phase × reversed phase approach with a shifted gradient in the second dimension provided increased peak capacity and was able to resolve with satisfactory selectivity multiple compound classes, as well isomeric compounds. Hyphenation with an Ion Trap-Time of Flight analyzer led to the identification of 101 compounds in 70 minutes. On the other hand, the ultra-high mass accuracy, resolution and the isotopic fine structure provided by FT-ICR-MS was very useful and complementary to LC × LC-MS/MS for the assignment of molecular formula, leading to more confident identification results (**Chapter II**). Subsequently, the possible use of hop secondary metabolites as natural immunomodulators and adjuvants in chemotherapy protocols has been evaluated. After fractionation by semi-preparative Liquid Chromatography, three different fractions were obtained. The phytocomplex and the fractions were tested to verify the ability to modulate the Lymphocytes CD3+ and Natural Killer compartment. Cytofluorimetric analysis revealed that a fraction containing bitter acids was able to up-regulate of NKG2D and NKp44 activating receptors. A further simplification yield a fraction mainly composed by Humulinones and Cohulupone derivatives, that at the concentration of 0.1 µg/mL induced selective activation of Nkp44 receptor and enhanced the cytolytic activity of NK cells against leukemia cell line K562 (**Chapter III**). In addition to immunomodulant potential of hop bioactive compounds, the mechanism for ingested breastmilk and probiotic to act in a complementary manner for the prevention of necrotizing enterocolitis (NEC) in very premature infants has been investigated. In detail, molecular cutoff fractionation and ultra-high-performance liquid chromatography-tandem mass spectrometry were used to identify indole-3-lactic acid (ILA), a metabolite of breastmilk tryptophan, as the anti-inflammatory molecule of *Bifidobacterium longum* subsp *infantis* (*B. infantis*) secretions. ILA was tested on human fetal small intestinal cell line, necrotizing colitis enterocytes and also fetal human organoids, providing to be able to reduce the

inflammatory cytokine IL-8 response after IL-1β stimulus through the interaction with the factor aryl hydrocarbon receptor (AHR) and TLR-4 of premature enterocytes surface, preventing the transcription of IL-8 (**Chapter IV**).

Moreover, an ultra high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) method has been developed and validated to assess the metabolic stability of hop α- and β-acids and the detection of their metabolites *in vitro* and *in vivo*. Mice liver microsomes were used to assess metabolic stability, *in vitro* $t_{1/2}$ and clearance values calculated, showing a slow and moderate metabolism for α-acids (avg $t_{1/2}$: 120.01 min, avg CL_{int} 11.96 μL/min/mg), while β-acids were metabolized faster (avg $t_{1/2}$: 103.01min, avg CL_{int} 13.83 μL/min/mg). Furthermore, phase I metabolites and phase II glucuronide were characterized both *in vitro*, and *in vivo*, in mouse plasma and urine after oral administration, by a combined full scan/data dependent/targeted neutral loss (FS/DDA/tNL) strategy. As a result, 12 phase I metabolites, including 2 novel potential di-oxidated metabolites (M6, M7) of humulones were detected. In addition, the tNL was able to detect for the first time 10 glucuronide conjugates of α-acids, comprising 7 glucuronide derivatives of oxidized phase I metabolites (M16-M22) (**Chapter V**).

Since UHPLC-HRMS methods are unable to report the spatial distribution of biomolecules of interest, the final part of the project has been focused on the application of matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI). A comparison of tissue stabilization protocols to highlight the *post mortem* degradation differences has been performed to image key neurotransmitters, metabolites and neuropeptides in rat brain. Although the heat stabilization did not showed differences in the levels of precursors dopamine, norepinephrine, and serotonin, their related metabolites (DOPAL, DOPAC, HVA, MOPAL, DOPEG and 5-HIAA) were all significantly lower, revealing a reduced neurotransmitter turnover ratios in heat stabilized brains compared to fresh frozen. *In situ* markers associated with the stabilization of enkephalin, dynorphin, and tachykinin derived neuropeptides were also imaged. Moreover, heat stabilization enabled the detection of very low-abundant neuropeptides such as the C-terminal flanking peptide, neuropeptide gamma, and nociception, providing evidence for the potential use of the heat stabilization prior to MALDI-MSI analyses to improve the examination of the *in vivo* state of neuronal chemical messengers in brain tissues (**Chapter VI Section I**). Finally, MALDI-MSI technique has been applied to the simultaneously mapping of hop α- and β-acids and their metabolites in rat liver sections after oral administration. Parents compounds of α-acids and phase I mono-oxidized metabolites were distributed across liver sections, revealing higher relative intensity of Humulinone derivatives respect the related precursor compound (**Chapter VI Section II**).