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Chemical Fingerprinting of Medicinal and Aromatic Plant Extracts: HP-TLC Bioautographic Assays as Preliminary Research Tool to Match Chemical and Biological Properties

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Chemical plant fingerprinting generally identifies the distribution of compounds within a plant matrix defining its chemical imaging in relation to the different extraction approach adopted. The chemical picture obtained with fingerprinting target needs to represent the highest number of chemical compounds as possible, giving the most realistic portrait of the chemical identity of the plant matrix. The fingerprinting of plant matrices is a research approach aimed to point out chemical evidences related to biological researches and applications of natural compounds in many productive fields, from human, animal health and phytoiatry to environmental biomonitoring and bioremediation.

The chemical portrait given by a fingerprinting is representative of the ecosphere where the plant grows, evidencing secondary metabolite patterns which are driven in quality and amount of compounds by the sensitivity of the plant to ecological variables. A chemical fingerprinting could allow to better understand the grown capacity of a plant species in a particular environment, to point out and select that particular genotype able to express a particular chemical phenotype useful for a particular applicative field for e.g. the production of bioactive compounds against particular human or plant pathogens to determine pollution levels and risk assessment for ecosystems by the presence/accumulation of pollutants or particular qualitative and quantitative expression of secondary metabolites [1]. The plant fingerprinting focused on the secondary metabolites is particularly relevant because of the important applicative relapses that this particular chemical portrait concerns. Plant secondary metabolites, infact, represent from a biological point of view the capacity of plant species to relate to environment complexity and to control many metabolic functions, for e.g. the known role of supporting plant electron transports [2]. These aspects are particularly important for studying the physiology of officinal plants to improve their cultivation and quality. Moreover, from applicative points of view, for e.g. those relating pharmaceutical-healthy applications and agricultural relapses of plant compounds, the study of secondary metabolism through mapping the quality and amount of the occurring molecules to have a complete chemical fingerprinting, may lead to discover more effective and safer bioactive chemicals, and officinal plants with a chemical profile more fitting with specific applicative needs [3].

For all these last applicative aspects, with particular relevance to health and plant defense/biostimulation (phytoiatric applications), also chemical fingerprinting related to some primary metabolites (for e.g. poly-unsaturated fatty acids, proteins, fixed oils, waxes and butters) has been considered [4].

However, besides the general considerations about the meaning of chemical fingerprinting, it should be stressed that its scientific importance and applicative relevance are linked to the extraction approaches adopted. In general, a complete chemical fingerprinting of a plant matrix is given by the chemical qualitative and quantitative profile of large polarity spectrum of the extraction strategies. A complete chemical fingerprinting of the secondary metabolites profile of a plant matrix is given by the combination of the qualitative and quantitative evidences of low, medium and high polarity extracts obtained using specific solvents and strategies with appropriate physical-chemical properties. However, for the most known plant species, as for example those with medicinal and aromatic properties, the chemical fingerprinting is limited to that secondary metabolites fractions known for traditional healthy properties and uses, generally characterizing aqueous or hydro-alcoholic preparations [5].

Chemical fingerprinting of the plant secondary metabolism could be performed through different methods, all able to report a graphical representation of the qualitative and quantitative content of secondary occurring molecules. Chromatographic approaches are obviously the most useful tool to reach the fingerprinting target, from thin layer to column chromatography performed by high performance liquid or gas chromatography strategy. All these techniques must be coupled to specific detectors and mass spectrometry instruments giving technical opportunities to quantify signals and identifying their chemical quality. In particular, the most used techniques are the high performance thin layer chromatography coupled to UV-visible plates visualizer and mass spectrometer (HP-TLC-MS), high performance thin layer chromatography coupled to diode array detector and mass spectrometer (HPLC-DAD-MS) and gas chromatography coupled to flame ionization detector and mas spectrometry (GC-FID-MS). In recent times, nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) has been purposed as additional chemical fingerprinting strategy to check identity, authenticity and quality of officinal plant extracts [6].

All these methods applied to chemical fingerprinting as characterization of medicinal and aromatic plants, besides their specific different methodology, need to have high accuracy and precision as common denominator, and this is only possible for specialized laboratories [1]. Other important and common property of all the fingerprinting strategies in the need to be quick and, if possible, inexpensive. Moreover, the importance of chemical fingerprinting is useful not only for determining and improving the quality of medicinal plants, but also for monitoring their transformation processing chain and the final products [4,7].

Medicinal and aromatic plants are essential sources of extracts,

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fractions and bioactive molecules with important applicative relapses, from those therapeutic as well as nutraceutical to that phytoiatric ones, where the use of natural compounds for plant defense or bio-stimulants for improving cultivated plants yield and quality is strongly demanded for preventing environmental problems and improving integrated agriculture. All these aspects are linked by the common target to answer the increasing demand of natural products, often due to the fact that the use of drugs and synthetic agro-pharmaceuticals in human medicine and agriculture respectively cause selective pressure leading to the spread of resistant etiologic mutants and weakness of innate defense capacities [8].

As previously already touched on, the nature and amount of active compounds isolated from the plant raw material depends on the type of extraction strategy, polarity of the solvent mixture, extraction time, temperature and pressure [9]. The importance of chemical fingerprinting, characterized by rapid, highly representative and inexpensive methods, is crucial for checking the quality of the extraction process and of the plant extracts. In relation to these aspects, the increasing interest to search for naturally occurring bioactive molecules in the complicated composition of natural materials challenges to rapidly screen the composition of phytocomplexes and the presence of biologically active chemical classes [10].

In this scenario of modern quality and functional demands regarding medicinal and aromatic plants and their products, bio autographic methods represent one of the solutions to match chemical fingerprinting of phytocomplexes to biological evidences of their chemical compounds In fact, bio autography is mainly known as a research strategy hyphenated with planar chromatography techniques aimed to detect bioactive components characterizing plant extracts, pointing out their efficacy directly on the chromatographic support used for their separation. The method that most matches manageability, rapidity, relative precision, cheapness with the target of chemical fingerprinting pointing out the most effective compounds is thin layer chromatography coupled to bio-autographic approaches (TLC-bioautoraphy). However, TLC bio-autography has been known since 1946but the modern and complex scenario regarding new applications of medicinal and aromatic plants leads the research towards the renewal of the application of this technique, improving the quality and applications of the results provided. TLC bioautography permits the separation of complex mixtures and, at the same time it points out the active compounds and fractions on the TLC plate [11].

The most important application of TLC bioautography that meets the modern targets involving medicinal and aromatic plant research is to be found in the fast screening of a large number of plant samples for their bioactivity-guided fractionation and isolation of the most active compounds.

The most used tests applied to TLC plates regard antimicrobial (antibacterial and antifungal) assays and antioxidant (DPPH, ABTS, for e.g.) ones [8,10]. Test microorganism cultures are capable of growing directly on the TLC plate, so each step of the assay is performed on the sorbent. As the common antimicrobial screening, TLC-bioautography must be carried out under controlled conditions: for e.g. solvent, sample application, compound resolution, type of microorganism, incubation time, etc. may strongly influence the result [8]. The same considerations could be made for antioxidant tests [10]. However, since TLC involves an amount of product on the plate and cannot deal with the concentrations of each occurring constituents, it is only a semi-quantitative method. Therefore, the full quantification and determination of biological efficacy should require supplementary tests in solution to reach IC_{50} or MIC (minimum inhibitory concentration) values for pure compounds [11].

However, bioactivity results about plant extracts on other non bioautographic supports (for e.g. agar plates) do not distinguish between active and inactive components, pointing out only the sum or synergic biological actions of non identified components. TLC bioautography instead identifies the really active components leading to their isolation and specific quantification, determination of specific activities through IC_{50} or MIC values, and searching for possible synergic interactions through the assaying of artificial mixtures with more efficacy and safety capacities.

In light of these premises, our research group set up pharmaceutical biology studies based on thin layer chromatography plates (HP-TLC) coupled to bio-autography of medicinal plant extracts to obtain the best separation of the compounds together with the best evidence of their possible biological activity, driving further in-depth investigations toward a more focused chemical identification and quantification, and a wider biological activity profile. We optimized HP-TLC bioautography for antimicrobial (antibacterial and antifungal) activities employing different bacterial, filamentous fungi and yeast strains pathogens for humans and plants. In parallel, HP-TLC bioautography has been set up to point out antioxidant properties with DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radicals. Our research strategy is planned to have HP-TLC bioautography as preliminary chemical and biological matched data of medicinal plant extracts giving us the opportunity to point out the chemical compounds with antibacterial, antifungal, antioxidant capacity; to focus on the chemical isolation and identification of fractions characterized by specific bioactive molecules; to check biological capacities of pure single compounds, or combinations, assaying possible synergic interactions; to explore in-depth other biological capacities with supplementary assays i.e. cytotoxic, mutagen and mutagen-preventive activity, etc. Related to those preliminary evaluated through HP-TLC bioautography. Here following are reported brief details about studies about essential oils and other kind of solvent extracts where HP-TLC bioautography has been performed because of its key role in driving medicinal plant researches. In case of essential oils, the HP-TLC bioautography has been performed to explore possible applications for human health products; in case of other solvent extracts, instead, the chromatographic bioautography has been done to verify possible phytoiatric applications.

First case study

Essential oils obtained from plant crude drugs of different geographical origin (for e.g. *Cryptocaryamassoia*, Lauraceae, from Indonesian regions; *Piper aduncuum*, Piperaceae; *Croton lechleri*, Euphorbiaceae from Amazonian Ecuador). *Cryptocaryamassoia* (Lauraceae) essential oil evidenced on HP-TLC plates relevant antimicrobial activity related to massoia lactones, as single pure compounds, versus Gram negative strains (*Klebsiellaoxytoca*), Gram positive bacteria (*Enterococcus faecalis)* and yeasts (*Candida albicans*). Benzylbenzoate and benzyl salicylate contributed towards specific efficacy against *E.faecalis*. The evidences will also drive the research towards in-depth investigations (cytotoxicity, mutagenicity) of the active isolated compounds and their different qualitative and quantitative combinations. In this case, genotoxic activities through Ames and SOS chromotest have been performed with negative responses. Cytotoxic activity of each compound identified and isolated and of the whole essential oil has been instead checked against CaCo-2

colon cancer cell lines. Particularly effective have been resulted C10 and C12 massoia lactones, with particular reference to the corresponding unsaturated compounds. Benzylbenzoate and benzyl salicatefractions showed a 100 folds lower bioactivity than the whole essential oil and massoia lactones, driving further research to investigate the synergic interactions among the biomolecules.

The case of *Piper aduncum* (Piperaceae) essential oil, characterized by high abundance of dillapiole (45.92%), highlighted this compound as the main responsible of highly selective antibacterial activity against *Staphylococcus aureus*.

Amazonian *Croton lechleri* essential oil evidenced antibacterial activity against *Escherichia coli* on HP-TLC mainly due to sesquicineole (17.29%). DPPH-(HP)TLC) bioautographic assays evidenced a lower radical scavenging capacity (IC_{50}) with respect to commercial thyme essential oil and BHA (butylated hydroxyl anisole), pointing out, however, that the *Croton lechleri* essential oil fraction characterized by α-calacorene, β-calacorene and δ-cadalene was the most involved in the bioactivity. However, caryophyllene oxide (1.24%) and 1,10-di-epi-cubenol (4.75%) showed the best antioxidant capacity with HP-TLC-DPPH assay.

Second case study

126 extracts obtained from different plant biomasses from industrial (agro-food) and agricultural by-products investigated through HP-TLC bioautography for achieving preliminary suggestions about their possible role as source of bioactive molecules (Ager-Innovapero, Grant n° 2010-2107). In this case ethanol, chloroform and acetone extracts have been assayed for matching chemical fingerprinting and biological activity, giving preliminary but clear suggestions about the most promising extracts in terms of bioactivity potential and composition. Among all plant sources, those most interesting were those of *Malusdomestica* (leaves) *Juglansregia* (fruit esocarp) and *Allium sativum* (peels and roots). The compounds identified as bioactive with HP-TLC bioautographic approach targeted to antimicrobial capacities weremainly phloretin in *Malusdomestica*, alliin in *Allium sativum*, and juglone and chlorogenic acid in *Juglansregia* extracts.

HP-TLC profiles of *Juglansregia*ethanol, chloroform and acetone extracts showed always the presence of juglone and chlorogenic acid. Juglone, identified in all the three kind of solvent extracts, has been checked as particularly abundant in chloroform extracts, while chlorogenic acid has been detected in the ethanol and acetone ones. The chromatographic bioautography evidenced that both the biomolecules exhibited interesting results against fungal phytopahtogens *B. Cinerea* and *N. galligena*. On the basis of these results, it has been attested by agar dishes assays that the bioactivity is certainly enhanced by synergic interactions since the whole extracts exhibited an efficacy 10% and 53% higher than juglone and chlorogenic acid as pure compounds. Non remarkable efficacy has been evidenced on HP-TLC plates against phytopathogen bacteria.

HP-TLC bioautographic assays on the three *Malusdomestica* extracts highlightedthe presence of phloretin and phloridzin, with particular evidence in ethanol and acetone extracts. Phloretininhibited mycelial growth about 97% and 98% of *N. Galligen a*and *B. cinerea*strains respectively, evidencing its potential role as antifungal biomolecule in agricultural treatments. Phloridzin, instead, did not evidence any remarkable bioactivity. Non remarkable biological efficacy emerged against bacterial strains, with the sole exception of *A. tumefaciens* which evidenced a weak sensitivity in presence of *M. domestica* chloroform extracts, due to minor compounds evidenced by HP-TLC bioautography to be identified.

HP-TLC analysis of ethanol, chloroform and acetone *Allium sativum* extracts evidenced mainly the presence of four identified bands other than the know nalliin. The bioautography coupled to HP-TLC pointed out a weak bioactivity of alliin both against phytopathogen fungi and bacteria, with pale growth inhibition areas corresponding to the separated bands to be identified.However, agar dishes tests evidenced higher efficacy of the whole extracts than pure separated compounds, suggesting the further investigation of synergic interactions among alliin and minor secondary occurring biomolecules.

In conclusion, as regards to all these considerations it could be stated that, even if the TLC bio-autographic approach dates at 1946, it represents a modern and rediscovered research tool which capacity to match chemical fingerprinting – even if with semi-quantitative indications and biological activities that however need to be analyzed by supplementary tests drives further in-depth studies towards more targeted results. High performance TLC plates and more specific elution methods allow separating in a more well-defined way the bands, evidencing consequently bioactivities through bioautography clearer. The fields of application of HP-TLC bio-autographic approach concerning medicinal and aromatic plants are many, from the identity and quality control of plant crude drugs and extracts, of crude drugs transformation processes and final products,to research for interesting biomolecules and their possible synergic interactions for new products, answering for new and modern needs of efficacy and safety.

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