 doi:10.20944/preprints202207.0355.v1

Review

Thin-Layer Chromatography (TLC) in Screening of Botanicals – its Versatile Potential and Selected Applications

Teresa Kowalska and Mieczysław Sajewicz

* Kowalska; e-mail address: teresa.kowalska@us.edu.pl
* Sajewicz; e-mail address: mieczyslaw.sajewicz@us.edu.pl

Abstract: The aim of this paper is to present a comprehensive overview of the main aims and scopes in screening of botanicals, a task which thin-layer chromatography (TLC) is on an everyday basis confronted with and engaged in. Stunning omnipresence of this modest analytical technique (both in its standard format (TLC) and the high-performance one (HPTLC), either hyphenated or not) for many analysts might at a first glance appear chaotic and random, with an auxiliary rather than leading role in research, and not capable of issuing meaningful final statements. Based on these reflections, our purpose is not to present a general review paper on TLC in screening of botanicals, but a blueprint rather (illustrated with a selection of practical examples), which highlights a sovereign and important role of TLC in accomplishing the following analytical tasks: (i) solving puzzles related to chemotaxonomy of plants, (ii) screening a wide spectrum of biological properties of plants, (iii) providing quality control of herbal medicines and alimentary and cosmetic products of biological origin, and (iv) tracing psychoactive plants under forensic surveillance.

Keywords: TLC screening of plants; TLC screening of psychoactive plants; TLC-direct bioautography; effect-directed detection; chemotaxonomy of plants; quality control of medicinal plants; quality control of alimentary products; quality control of cosmetic products

1. Introduction

Thin-layer chromatography both in its standard (TLC) and high-performance (HPTLC) format is known as a versatile and high-throughput liquid chromatography technique, with a wide range of important practical applications. These applications can roughly be divided into those in service of botany, phytochemistry and medicine (handling rather fundamental issues like contributing to chemotaxonomy of plants, or searching for enzyme inhibitor templates) on one hand, and the more practical goals on the other. Practical applications are closely related to authentication and quality control of herbal medicines, alimentary herbs and spices, and forensic control of these ethnobotanicals which as illicit highs are subject to criminal law.

Among the most often employed screening TLC procedures, we find those which focus on drug control of synthetic pharmaceuticals, with special emphasis laid on substandard and fake drugs which are illegally traded in developing countries [1-5], yet this purely pharmaceutical issue is out of scope of our present overview. Construction of a chemistry-based taxonomy of plants is a meaningful help in plant systematics. Contribution of TLC is significant in this sense that the thin-layer chromatographic screening of plant extracts facilitates identification of chemotaxonomic markers and the entire chemotaxonomic profiles and hence, it facilitates determination of botanical taxa [6-9]. The TLC-based screening methods often target medicinal plants in the search for various different physiological properties of botanical material (e.g., the free radical scavenging, antimicrobial, and enzymes inhibiting activity [10-12]). Hyphenation of TLC with other analytical techniques which allow an *in-situ* (i.e., directly on the chromatographic plate surface) recognition of biological properties of an individual compound or compound fraction has

 doi:10.20944/preprints202207.0355.v1

evolved into the so-called TLC-EDA strategy (with EDA held for the effect-directed analysis) [13, 14], which in particularly favourable cases might suggest novel structural motifs for synthetic medicines. Two more areas of application of the TLC-based screening approach are quality control of plant medicines, botanical alimentary and cosmetic products (e.g., [15-17]) and psychoactive ethnobotanicals, which in many cases are illicit and subject to criminal law. In the latter case though, the TLC methods sometimes tend to remain unpublished, or provide incomplete analytical details (e.g., [18]).

It is not an aim of this paper to provide a traditional and exhaustive review on applications of TLC to screening botanicals in their various different roles and functions but instead, to provide a comprehensive blueprint equipped with a selection of practical examples, which clearly defines distinct competences of this versatile, efficient and high-throughput analytical technique. It will also be an added value of this paper and an undoubted joy for its authors, if it manages to light a spark of inspiration with some of those who are interested in plant analysis and search for simple, reliable and cost-effective approaches in this field.

2. Thin-layer chromatography in chemotaxonomy of plants

Although slightly more than one third of a million species of plants are known to man today, taxonomy was recognized as a formal subject in the early 19th century only and then it was understood as the science of identifying, naming, and classifying plants. The earliest systems of plant classification, certainly used even in prehistorical times, were based on one or a few easily observable characters of plants, such as their habit (trees, shrubs, herbs, etc.), or floral characters (particularly the number of stamens and carpels). These classification systems were based on arbitrarily selected and easily observable features, and they are therefore viewed as artificial. Natural classification systems are based upon overall resemblances, mostly in gross morphology, thus, utilizing as many taxonomic characters as possible, to group taxa. The most advanced classification systems also use as many taxonomic characters as possible, yet in addition to phylogenetic (i.e., evolutionary) interpretations. Chemotaxonomy is an advanced approach to plant classification, which is based on plant biochemistry and chemistry, as it supplements morphological evidence at another (mostly molecular) level of structural organization. Within the framework of this approach, relations are investigated between the classes of plants and the occurrence of specific substances or substance groups in plant tissues [19, 20]. Thin-layer chromatography has proved an excellent tool for fast screening of plant material for chemotaxonomic purposes by providing an easy and reliable access to the plant fingerprints, which is a very important step 1 in chemotaxonomic procedure exerted with aid of TLC. Relatively simple, inexpensive and fast TLC methods of fractionating complex plant extracts and obtaining respective TLC fingerprints permit easy perceiving similarities among different plant species and make a solid base for the consecutive chemotaxonomic steps [21]. In the following paragraphs, selected examples are given of TLC contributions to chemotaxonomy of plants.

In paper [8], an example is presented of the TLC application to solving a taxonomic problem with certain plants from the sandstone region of southern KwaZulu-Natal and Pondoland areas, identified as a distinct centre of endemism in South Africa and therefore called the Pondoland Centre (PC). This region abounds in species from the *Maytenus* genus (family of Celastraceae), out of which at least four species seem to be endemic to the region. Thus an idea arose about a possible split of representatives of the endemic *Maytenus* genus into more natural and closer related complexes of species.

Knowing that a vast number of the secondary plant metabolites (like sesquiterpenoids, triterpenoids, alkaloids and flavonoids) have earlier been isolated from the *Maytenus* genus specimens from around the globe, the authors decided to explore chemotaxonomic potential of the secondary metabolites fractions extracted from the leaves of the *Maytenus* genus representatives, characteristic of the PC region and to fingerprint them by means of TLC. Thus the leaves of fourteen *Maytenus* genus species originating from

 doi:10.20944/preprints202207.0355.v1

the PC region underwent an extraction with methanol and then the fingerprint analysis of the obtained extracts was carried out on the silica gel pre-coated chromatographic plates using four solvent systems of an increasing polarity (light petroleum–ethyl acetate (8:3, v/v), light petroleum–ethyl acetate chloroform–formic acid (8:7:5:1, v/v/v/v), chloroform–ethyl acetate–formic acid (5:4:1, v/v/v), and chloroform–methanol–water (12:3:1, v/v/v)), to obtain fingerprints targeting different groups of secondary metabolites. The chromatograms were acquired photographically. Based on these fingerprints, the authors succeeded in splitting certain *Maytenus* genus species into smaller and closer related complexes (e.g., *M. oleosa* and *M. undata* make one such cluster), or to the contrary, they attributed separate status of non-clustering species to *M. peduncularis*, *M. acuminata*, and *M. cordata*.

In paper [9], another example was given of the TLC-based chemotaxonomic approach to eight specimens of plants from the family of Lauraceae belonging to two genera, *Cinnamomum* and *Litsea*. The investigation set included four representatives of the *Cinnamomum* genus (*C. bejolghota*, *C. camphora*, *C. tamala* and *C. verum*) and four representatives of the *Litsea* genus (*L. assamica*, *L. glutinosa*, *L. laeta* and *L. monopetala*). Habitat of these plants is in the sub-Himalayan Terai and Duars regions of North Bengal, and the bark extracts of these plants have many medicinal uses which include antioxidant, anti-microbial and anti-inflammatory ones. The reason for the TLC-based research was that the indicated members of the Lauraceae family show variations and similarities in different morphological aspects which pose certain problems with identification of these plants, so that they have been earlier many times re-classified, but with no satisfactory effect.

In the reported experiment, bark samples were collected from all plants and extracted with methanol to obtain secondary metabolites, and they were also hydrodistilled for essential oils. The TLC analyses of the methanol extracts were carried out on the silica gel pre-coated chromatographic plates, using separate solvent systems for different groups of secondary metabolites. For quantification of anthraquinones, ethyl acetate–methanol–water (100:13.5:10, v/v/v) was used and visualization of chromatograms was carried out at 365 nm in UV light. For quantification of flavonoids and phenolics, ethyl acetate–formic acid–acetic acid–water (100:11:11:27, v/v/v/v) was employed and visualization was carried out at 365 nm in UV light (for flavonoids) and in visible light (for phenolics). Moreover, chromatograms of flavonoids and phenolics underwent the DPPH• test for antioxidant potential of individual fractions. Last not least, TLC was also used to fingerprint essential oils and then visualization of chromatograms was carried out by spraying the plates with anisaldehyde followed by heating, while detection was carried out in visible light. Based on multiple test repetitions and high amounts of the collected fingerprint results, cluster analysis was applied to analyse the obtained data, which confirmed chemotaxonomic correlation of the two genera (*Cinnamomum* and *Litsea*) of the Lauraceae family, and the *Cinnamonum* genus proved distinctly separate from the *Litsea* genus. It was also shown that *C. camphora* makes an independent entity, while close relationship is observed among *C. tamala*, *C. verum* and *C. bejolghota*. In the case of *Litsea*, four genera were divided into two dendrogram branches, one branch representing *L. laeta* and *L. glutinosa*, and the other standing for *L. monopetala* and *L. assamica*.

In paper [22], a comparison was made of TLC fingerprints for the popular medicinal and culinary herbs from the Mediterranean region, which all belong to the Lamiaceae family, yet to the three different Lamiaceae genera, *Salvia*, *Dracocephalum* and *Thymus* (and from each individual genus, two different species were selected). Thus the following six kinds of plants were examined, *S. triloba*, *S. staminea*, *D. moldavica* (variety with white flowers), *D. moldavica* (variety with blue flowers), *T. vulgaris*, and *T. serpyllum*. All six plant kinds underwent selective multistep liquid extraction for phenolic acids and flavonoids, following procedure given in book [23], and the detailed protocol was elaborated based on an additional information derived from the literature [24-27]. Thus the plant extracts were divided into six fractions of phenolic compounds, i.e., the (i) free phenolic acids, (ii)

 doi:10.20944/preprints202207.0355.v1

bonded phenolic acids liberated through acidic hydrolysis, (iii) bonded phenolic acids liberated through basic hydrolysis, (iv) flavonoid aglycones, (v) low-polar flavonoid glycosides, and (vi) polar flavonoid glycosides.

An aim of this study was to compare six fingerprints (i)-(vi) for each of the six aforementioned kinds of plants, in order to find out if these fingerprints could allow distinguishing among the genera of these closely related species. It was found out that chromatographic fingerprints of fraction (iv), i.e., flavonoid aglycons, were the only ones which permitted correctly ascribing the investigated species to the *Salvia*, *Dracocephalum* and *Thymus* genera, in spite of the lowest intensity of the respective signals on the chromatograms. Thus, the chromatograms of flavonoid aglycones proved their chemotaxonomic importance as marker fingerprints for individual plant genera.

In paper [28], the TLC method was introduced for chemotaxonomic differentiation between two medicinal plants listed in Chinese Pharmacopoeia, i.e., field thistle (*Cirsium setosum*) and Japanese field thistle (*Cirsium japonicum*). Both plants belong to the family of Compositae and they are important components of traditional Chinese medicines, internally and externally used to treat diverse kinds of bleeding and inflammation. They are herbaceous perennials and the aerial parts of both plants, which are used medicinally, are difficult to distinguish morphologically, while differentiation of the dried and cut crude plants is even more challenging. Simple TLC method proposed in [28] permits an unambiguous differentiation though of *C. japonicum* and *C. setosum* by their flavonoid fingerprints. To this effect, plant material was extracted with methanol and then the chromatograms of the extracts were developed with use of the silica gel pre-coated aluminium sheets and ethyl acetate-formic acid-acetic acid-water (12:1.5:1.5:4, v/v/v/) as mobile phase. Two characteristic flavonoids, i.e., pectolinarin and linarin, were used as chemotaxonomic markers and external standards for analysis. Visualization was carried out by spraying the plates with the natural products spray reagent (a 1% solution of 2-aminoethyldiphenylborinate in methanol) and after gentle heating, the plates were inspected in UV light at 366 nm for fluorescent spots of linarin (yellow) and pectolinarin (brown). This procedure allowed distinguishing between *C. setosum* (containing in its extract linarin only) and *C. japonicum* (containing both, pectolinarin and linarin).

3. Thin-layer chromatography coupled with bioassays

Thin-layer chromatography hyphenated with a number of bioassays is an excellent strategy for rapid screening of various different botanicals (and in the first instance, medicinal plants and culinary herbs and spices), mainly for their free radical scavenging activity and antimicrobial and enzymes inhibiting properties [29-31]. This trend of coupling TLC with a variety of other analytical tools is effective and very promising for the future, hence it definitely is on a rising tide now and far from having said its last word yet. According to a newly coined terminology, the discussed strategy is often referred to as TLC-EDA, where EDA holds for the effect-directed analysis. Certain bottlenecks in development of new couplings (or hyphenations) are due to technical demands caused by increasingly more sophisticated analytical tools, but on the other hand, inventiveness of researchers is hard to overestimate and numbers of new technical solutions are steadily growing. In this section, we will present a selection of illustrative and practical enough TLC-EDA examples in the three main application fields, focused on (i) free radical scavenging properties of selected fractions of the secondary plant metabolites, (ii) antimicrobial properties of the secondary metabolites fractions, and (iii) well pronounced enzymes inhibiting properties with certain botanicals.

3.1. Thin-layer chromatography in screening of botanicals for their free radical scavenging activity

 doi:10.20944/preprints202207.0355.v1

The α,α'-diphenyl-β-picrylhydrazyl (DPPH•) free radical scavenging method offers flexible approach to assessment of antioxidant potential of a compound, an extract, or another biological source. This is the simplest method, wherein the prospective compound or plant extract is brought into contact with the DPPH• solution and the result is recorded after certain period of time. This method has initially been devised for the instrumental (basically spectrophotometric) techniques, but with time it has undergone various modifications to suit different requirements, although the basic concept remains essentially the same. Paper [32] presents the genesis and development of the DPPH• method, including basic mechanism which stands behind it. This mechanism can be presented in the following manner: If we represent the DPPH• radical by Z• and the free radical scavenging molecule (e.g., phenolic acid) by AH, then the primary reaction is given below:

$$Z\bullet + AH \Leftrightarrow ZH + A\bullet \quad (1)$$

the other one for resolution of the medium and highly polar ones (ethyl acetate-water-formic acid-acetic acid (100:26:11:11, v/v/v/v)). As reference compounds, the authors used the gallic acid, hiperoside, rutin, caffeic acid, chlorogenic acid and rosmarinic acid standards. Developed plates were sprayed with the vanillin–sulphuric acid reagent (to produce chemical fingerprints) and with DPPH• solution (to generate the free radical scavenging fingerprints). With four *Salvia* species, it was revealed that their strong free radical scavenging properties were not only due to polar flavonoids and phenolic acids present in the extracts, but also due to the other free radical scavengers in the less polar fractions. It was also established that due to similarities in chromatographic and free radical scavenging fingerprints of *S. triloba* and *S. officinalis*, the former one can be regarded as a pharmacopoeial species candidate. The developed method was validated for its specificity, precision (repeatability and intermediate precision), stability and robustness, according to the recognised AOAC guidelines for the qualitative TLC procedures [37].

In paper [38], a report was given on thirty six herbal species from the Lamiaceae family belonging to the two plant genera (*Salvia* and *Thymus*) which underwent a multistep extraction described elsewhere [23-25]. Six fractions derived from each plant underwent the TLC-DPPH• test to reveal these plants and plant fractions with the most strongly pronounced antioxidant properties and caffeic acid was selected as an external standard to monitor these properties. It was shown that caffeic acid most abundantly appeared in the fractions derived from all the investigated herbs in the course of basic hydrolysis, but it never appeared in the fractions derived from the acidic one. Moreover, it was once again established that all the analysed fractions of *S. officinalis* and *S. triloba* demonstrate similar free radical scavenging activity, which might serve as a starting point for further studies and eventual proclamation of *S. triloba* as a medicinal plant in its full right and deserving its monograph in herbal pharmacopoeia.

Paper [39] presents the HPTLC-EDA approach to assessment of biological properties (the antioxidant and antidiabetic potential) of the ethanol and ethyl acetate extracts derived from ten macroalgae species (three Chlorophyta, four Phaeophyta and three Rhodophyta) originating from the Blue Lagoon beach in Malaysia. Chromatograms were developed with use of the HPTLC quality silica gel as stationary phase and *n*-hexane-ethyl acetate-acetic acid (20:9:1, v/v/v) as mobile phase. The experiments were carefully performed and the method was validated for the contents of the standards employed. A comparison was made of antioxidant potential depending on the extractant used and it was established that on average, higher antioxidant activity was observed with the ethyl acetate extracts, although the phenolic content was higher in the ethanol extracts. Although the authors did not comment on this observation, it seems justified to suppose that in the case of the discussed macroalgae, not only the phenolic compounds, but also those which are less polar characterize with well pronounced antioxidant properties (a similar conclusion was inferred in paper [36]).

 doi:10.20944/preprints202207.0355.v1

In paper [40], an up-to-the-date review was provided of a selection of the most important studies on natural antioxidants in foods (including beverages), food ingredients, and dietary supplements, performed with aid of the TLC-DPPH• test (and of TLC hyphenated with some other bioautographic techniques). In one way or another, all examples collected in this review refer to botanicals and to their antioxidant potential, and the review provides a considerable amount of 73 references to original research apers.

3.2. Thin-layer chromatography in screening of botanicals for their antimicrobial properties

In review paper [41], a thorough and inspiring spectrum of different TLC approaches is given to characterize plants for their biological properties. It focuses on antimicrobial and antifungal assays, enzyme inhibition, antioxidant testing, and free radical scavenging activity, and it comes with 66 references to the original research papers. A similar review taking on a vast range of the TLC approaches to screening botanicals for their different biological properties is also given in paper [42], which dispenses a solid number of 99 references to the original research papers. Apart from general reviews which attempt to cover an entire spectrum of the TLC applications to screening different biological properties of botanicals, we also have reviews which cover selected kinds of TLC applications and a good example is paper [43], which focuses on screening of antimicrobial properties and is implemented with 75 references. An interesting paper [44] (published in Journal of Visual Experiments) provides a step-by-step explanation (implemented with a nice selection of instructive figures) on how to practically perform the TLC separation followed by a bioassay of plant extract (known as the dot-blot test), to identify antimicrobial compounds. Combination of TLC and the dot-blot test is known as the TLC-direct bioautography (the TLC-DB) approach. As a working example given in [44], the TLC separation of phenolics extracted from the red clover (*Trifolium pratense* cv. Kenland) plant is presented, followed by screening of the separated fractions for their activity against *Clostridium sticklandii*, a hyper ammonia-producing bacterium (HAB) that is native to bovine rumen.

A kind of precursor of the TLC-DB approach is the dot blot test alone, performed with aid of the thin-layer chromatographic adsorbent, yet without preliminary chromatographic fractionation of the sample considered. In that way, an information is derived on an overall antibacterial potential of the plant extract, but without pointing out to any specific fraction or individual compound derived from the scrutinized sample. A good practical example of such approach is given in paper [45]. The authors applied this test to compare antibacterial activity of 18 thyme (*Thymus*) specimens and species (originating from the same gardening plot and harvested in the same period of time). To this effect, polar fractions of the secondary metabolites were derived from each thyme plant, which were then drop-wise deposited on the silica gel pre-coated chromatographic plates, yet without developing the chromatograms. Then the well described dot-blot procedure was performed for antibacterial activity against the Gram-positive *Bacillus subtilis* strain. It was established that all investigated extracts exhibited antibacterial activity, yet distinct differences in the size of the bacterial growth inhibition zones were observed among the compared thyme species. Based on the results obtained, *T. citriodorus* "golden dwarf" and *T. marschallianus* were selected as prominent targets for further investigations and possible inclusion in herbal pharmacopeia, which was an essential scientific novelty of this study.

Practical illustration of the TLC-DB assay is provided in paper [46] and it focuses on two medicinal plants belonging to the European ethnopharmacy, i.e., on *Matricaria recutita* L. (chamomile) and *Achillea millefolium* L. (yarrow). To this effect, tinctures from aerial flowering parts of these plants were prepared by seven days of maceration in 70% ethanol (according to Polish Pharmacopoeia VI), then chromatographically developed and the chromatograms with separated fractions were tested against eight bacterial strains, i.e., *Staphylococcus epidermidis*, *S. aureus*, the methicillin-resistant *S. aureus*, *Escherichia coli*, *Pseudomonas syringae* pv. *maculicola*, *Xanthomonas campestis* pv.*vesicatoria*, *Aliivibrio fischeri*, and *Bacillus subtilis*. As a result, considerable antibacterial properties were for the first time

 doi:10.20944/preprints202207.0355.v1

confirmed with two compounds found in the examined tinctures, i.e., with apigenin and α-linolenic acid, and their identity was additionally confirmed by means of LC/MS.

Rapid screening of botanicals for their antimicrobial properties is advantageous, especially with plants originating from sub-tropical and tropical regions which are more abundant in flora and relatively less researched than flora originating from the temperate climatic zones. In that way, a shortcut verification can be assured of healing potential with traditional local medicines, which in terms of accessibility and use are much ahead of Western medicines. Working example is provided in paper [47], which focuses on antibacterial properties of the leaf extract derived from the Philippine *Piper betle* L. plant belonging to the family of Piperaceae, which is recognized in India, Sri Lanka, Malaysia, Philippines and the other subtropical countries for its antibacterial, cytotoxic, hepatoprotective and many other advantageous pro-health properties. From the research data obtained from instrumental techniques (more advanced than the TLC-DB assay), it has already been known that the methanol, ethanol and supercritical CO_2 leaf extracts from *P. betle* are exceptionally active against a number of the multi-drug resistant (MDR) bacteria. In the discussed study, the ethanol leaf extract of *P. betle* was separated with use of TLC into eight fractions, which then underwent the dot-blot test. To this effect, the TLC system used consisted of the silica gel stationary phase and ethyl acetate-*n*-hexane (7:3, v/v) mobile phase. Two spots with R_F values of 0.86 and 0.13 showed inhibitory activities against two Gram-positive MDR bacteria, i.e., the methicillin-resistant *Staphylococcus aureus* and the vancomycin-resistant *Enterococcus*. The spot with R_F=0.86 also showed inhibitory activity against two Gram-negative MDR bacteria, i.e., the carbapenem-resistant Enterobacteriaceae, *Klebsiella pneumoniae* and the metallo-β-lactamase-producing *Acinetobacter baumannii*. With aid of the GC/MS technique, six compounds contained in the spots showing antibacterial activity were identified, with four of them never before having been mentioned in medical literature.

Paper [48] presents the most up-to-the-date overview of the TLC-DB applications to phytochemistry, and its two considerable advantages are that it provides chronological order of development of this technique (nicely combined with dynamic development of its application range) and that it is largely focused on applications of TLC to scrutinize Traditional Chinese Medicines (TCM), to which task it is actually the best placed. What sets this most recently published review apart from the others is that it provides preliminary information on attempts to identify natural products with an anti-COVID potential inherent of medicinal plants. In fact, it points out to paper [49], which connects plant material with its possible efficiency in alleviating and/or combating the COVID-19 risks (which in the first instance are respiratory syndromes). Namely, in paper [49], a report is given on the *in silico* study of eleven Indian herbal plants with putative inhibitory properties against COVID-19. From this study, it comes out that components of two plants, *Nyctanthes arbortristis* (harsingar) and *Aloe barbadensis* Miller (*Aloe vera*), are the most promising ones which might display an anti-COVID-19 potential. For this reason, these two plants should be selected for future steps of the experimental studies.

3.3. Thin-layer chromatography in screening of botanicals for their enzyme inhibiting potential

Enzymes are important pharmacological targets and hence, drug discovery programmes often include enzyme targets in the primary screening assays for drug candidates. These assays most often utilize human or mammal enzymes and involve measurements of product formation by a wide variety of advanced instrumental techniques, whereas the use of the TLC-DB approach to discover the plant-derived enzyme inhibitors can be a cost-effective and a rapidly performing alternative. Similar to discovering botanicals with strongly pronounced antioxidant and antimicrobial properties, TLC is also used to discover enzyme inhibitors of botanical origin.

Nowadays, one of the most acute health conditions among ageing populations worldwide is the Alzheimer's disease and for this reason, the inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) currently form the basis of the newest

 doi:10.20944/preprints202207.0355.v1

drugs available for management of this disease. In view of a considerable potential of plants, the TLC bioautographic assays were performed for screening plant extracts in the search for natural cholinesterase inhibitors. Review on these inhibitors which are available from natural botanical sources [50], implemented with 76 references to the original research papers, introduces a long list of plants recognized for their cholinesterase inhibiting activity. These plants have long been used in ethnomedicines, particularly for the memory-related disorders, and their efficiency is attributed to specific alkaloids, terpenes, sterols, flavanoids and glycosides. The most potent cholinesterase inhibitors are observed with the components of plants from the families of Buxaceae, Amaryllidaceae and Lycopodiaceae, although certain activity has also been established with the extracts from plants belonging to the families of Lamiaceae, Chenopodiaceae, Papaveraceae, Apocynaceae, and Labiatae. A number of papers have been published on this subject matter (e.g., [51-55]), and basic principle of all these methods is that the enzyme (cholinesterase) converts 1-naphthyl acetate into naphthol which reacts with Fast Blue B salt to make a purple-coloured background on TLC plates. Thus upon the thin-layer chromatographic fractionation of the plant extract, the plate is dried for complete removal of liquid mobile phase and then it is sprayed with the enzyme stock solution. Then it is again dried yet kept in humid atmosphere for incubation at 37 ^{o}C for 20 min and eventually, it is sprayed again with solution of α-naphthyl acetate and Fast Blue B salt, to give purple colouration after 1-2 min. The AChE and BuChE enzyme inhibiting zones (i.e., fractions derived from the plant extracts) produce white spots on the purple background.

Among first reports on a possibility of screening plant extracts for the detection of the cholinesterase inhibition zones was that issued by Verpoorte et al. [51], who used acetylcholinesterase inhibitors from the Amaryllidaceae extracts in the TLC-DB test instead of synthetic galanthamine. Working conditions of the TLC-DB assay (which in fact follows a very detailed protocol) were tested upon two alkaloids (galanthamine and physostigmine) and the results were reported in paper [52]. Although galanthamine and physostigmine are nowadays produced synthetically and are used for the treatment of cognitive decline in mild to moderate Alzheimer's disease, both alkaloids have originally been isolated from plants and more specifically, from the bulbs and plants of *Galanthus nivalis* (known as common snowdrop) and *Physostigma venenosum*, a leguminous plant endemic to tropical Africa, respectively. Over time, working conditions of the original TLC-BD method were modified and in paper [53], an improved methodology was proposed focusing on concentration of enzyme, the reagents, and the reaction time. As a consequence, consumption of enzyme was reduced by 85% and the detection limits were remarkably decreased. In paper [54], a comparison was reported between performance of the TLC-DB assay for inhibitors of cholinesterase and the 96-well plate assay based on the Ellman's method. For the majority (83%) of the 138 test compounds of natural and synthetic origin, the results obtained with the two assays have converged and both screening assays were considered as suitable for the generation of new hits. The TLC-DB methodology for screening of plant extract in the search for new botanical inhibitors of cholinesterase is still in use and in paper [55], a report is given on examination of the never before tested single components from the ginger (*Zingiber officinale*) extract. The experiment led to recognition of three active inhibitors among volatile constituents of this plant, i.e., *ar*-curcumene, α-sesquiphellandrene, and α-zingiberene. Identification was possible owing to the TLC-HPLC-MS interface analysis of active zones and the GC-MS analysis of tested samples. In paper [56], another report was given on the TLC-DB test of the methanol extract of *Schisandra chinensis* for the cholinesterase inhibition effect and the dibenzocyclooctadiene lignans contained in this plant were proved as responsible for the inhibition.

Along with the Alzheimer's disease, obesity (i.e., an abnormal or excessive fat accumulation) is also considered as one of great threats to human health on a global scale. It tends to aggravate the chances of acquiring diseases such as type 2 diabetes, hypertension, fatty liver, and cancer, which in turn reduces both life expectancy and the quality of life. Pancreatic lipase is a key enzyme for digestion of triacylglycerols, and inhibition of lipase

 doi:10.20944/preprints202207.0355.v1

has until now been the most explored strategy for treatment of obesity. In recent years, increasingly more medicinal herbs have been reported to show inhibitory activities against pancreatic lipase and a discovery of lipase inhibitors among herbal medicines may provide potential alternative for the treatment of obesity. In paper [57], a report was made on the TLC-DB screening of methanolic extracts from the *Camellia sinensis* (L.) Kuntz, *Rosmarinus officinalis* L. and *Morus alba* leaves, for their ability to inhibit pancreatic lipase. Upon the thin-layer chromatographic fractionation of the plant extracts in the TLC system composed of silica gel as stationary phase and ethyl acetate-methanol-water (60:30:10 v/v/v) mobile phase, chromatographic plates were sprayed with α-naphtyl acetate and the enzyme solutions before incubation at 37 ^{o}C for 20 min. Finally, solution of Fast Blue B salt was sprayed onto the TLC plates giving a purple background colouration. Orlistat (a synthetic lipase inhibitor drug) was used as a reference analyte. The pancreatic lipase inhibiting zones of the plant extracts and the reference drug zone appeared as white spots on the purple background. In that way, it was demonstrated that the extracts from *C. sinensis* and *R. officinalis* exert an inhibitory effect upon pancreatic lipase, whereas *Morus alba* lacks an analogical activity.

In paper [58], an alternative TLC-DB procedure was proposed to screening pancreatic lipase and to this effect, the *p*-nitrophenyl butyrate (PNPB) and bromothymol blue system were used to detect the lipase inhibition fractions derived from three unexplored species of *Streptomyces* (*S. tendae*, *S. aurantiacus* and *S. albaduncus*), with orlistat used as a positive control of procedure performance. Upon developing orlistat and supernatants derived from the *Streptomyces* samples on the silica gel pre-coated chromatographic plates (with chloroform-methanol (90:10, v/v) for orlistat and benzene-methanol (60:40, v/v) for *Streptomyces*), the plates were air dried till the mobile phase was evaporated completely. Then a complex yet well described procedure involving the porcine pancreatic lipase enzyme was implemented and finally, the inhibitory zones were visualized as blue spots against the greenish-yellow background.

In paper [59], a thorough report was given on usage of the TLC-DB test to screening the Chinese medicine Ophiopogonis Radix (which is the dried root of *Ophiopogon japonicus* (L.f) Ker-Gawl.) for its ability to inhibit pancreatic lipase. To this effect, the HPTLC method was used which employed the two-step gradient elution with two different mobile phases for simultaneous analysis of seven constituents of Ophiopogonis Radix. Direct bioautography was performed for plant samples originating from two different regions of China (i.e., the Sichuan and the Zhejiang province) and/or from different growth years. Visualization of the enzyme inhibiting effect was performed in a standard manner by spraying the developed and incubated plates with the solution of α-naphthyl acetate and Fast Blue B salt. The lipase inhibition essays showed that five compounds contained in the plant extracts (i.e., ophiopogonin D, ophiopojaponin C, ophiopogonin D', ophiopogonin C' and methylophiopogonanone B) demonstrated an inhibitory effect, yet its strength largely depended on the plant vegetation region and the growth year. In that way, not only confirmation was obtained of the Ophiopogonis Radix potential as an active lipase inhibitor, but a quality control method was also proposed for this herbaceous material.

Review paper on application of the TLC-DB approach as a tool for rapid detection of enzyme inhibitors is given in paper [60]. A statement is made that the TLC-DB methods have been developed for several classes of enzymes including oxidoreductases, hydrolases and isomerases, and there is a potential for developing functional methods for the other classes of enzymes as well. Paper [60] provides a summary of the known TLC-DB methods and their applications for determination of enzyme inhibitors in plant extracts and a comparison of effectiveness of different methodological approaches. It also indicates the current state and perspective of development of the TLC-DB methodology used for screening of botanicals for their enzyme inhibiting potential and its possible future applications.

 doi:10.20944/preprints202207.0355.v1

4. Thin-layer chromatography in screening of medicinal and culinary herbs, and in quality control of alimentary and cosmetic products of botanical origin

4.1. Thin-layer chromatography in screening of medicinal and culinary herbs

There are three main factors which limit rational use of medicinal herbs, and these are uncertainty on their effectivity, uncertainty on their safety and variation in their quality. Uncertainty regarding effectivity of herbal medicines is not an issue which can be tackled with use of the TLC-based methodology, as it remains entirely within the competence of medical sciences. Thin-layer chromatography is an excellent practical tool though which allows issuing binding opinions regarding quality of herbs, as economical motivation of fraud on herbs (either medicinal, or culinary) is widely known and it can seriously endanger public health. Authentication and quality control of herbal medicines is of a particular importance in all these regions where herbal ethno-medicines play a prevalent role in health care of the population. In fact, similar precautions refer to culinary herbs and spices, which – contrary to ethno-medicines of a local importance only – can also be quite expensive and hence, become a temptation for the fraudulent actions. Moreover, culinary herbs and spices are abundantly traded worldwide. A comprehensive review on TLC in quality control and safety of botanicals is given in book chapter [61].

In paper [62], an example is given of an impressive versatility of TLC in quality control of three medicinal herbs recognized by Korean Pharmacopoeia IX, which are radix of *Angelica gigas* Nakai (Umbelliferae) from Korea, fruit of *Evodia rutaecarpa* Bentham (Rutaceae) from China and fruit of *Schisandra chinensis* Baillon (Schisandraceae) from China. Firstly, herbs were extracted with use of the methods listed in Korean Pharmacopoeia IX, and then extracts were developed on the silica gel pre-coated plates with use of carefully selected mobile phases. Extract of the *Angelica gigas* radix was developed with hexane-ethyl acetate-methanol (3:2:1, v/v/v) and visualisation of chromatograms was made in UV light at 365 nm. Marker compounds for *Angelica gigas* were decursin and decursinol. Extract of the *Evodia rutaecarpa* fruit was developed with dichloromethane-methanol-formic acid (40:1.5:2, v/v/v) and visualization of chromatograms was made with the ethanolic H_2SO_4 in UV light at 365 nm. Marker compounds for *Evodia rutaecarpa* were evodiamine and rutaecarpine. Extract of the *Schisandra chinensis* fruit was developed with toluene-ethyl acetate-formic acid (7:3:0.5, v/v/v) and visualization of chromatograms was made with the ethanolic H_2SO_4 in UV light at 365 nm (for gomisin A and gomisin N), and at 254 nm (for schisandrin). Mass spectra of the spots were registered directly on the chromatograms by placing the developed plates between the DART ion source and the TOF analyser, in the so-called TLC-TOF-DART/MS hyphenation mode (the approach which was first proposed in paper [63]). The *m*/*z* signals detected in the mass spectra of marker compounds and then confirmed as present in the mass spectra of the three medicinal herb extracts were an ultimate authentication step for these plants.

Paper [64] presents a similar example of the quality control of selected herbs belonging to the Chinese medicinal system, yet with use of another hyphenated TLC-MS approach. The authors targeted eight medicinal plants including *Sophorae Flavescentis* Radix, *Angelicae Sinensis* Radix, *Acori Tatarinowii* Rhizoma, *Phellodendri Chinensis* Cortex, *Picrasmae* Ramulus et Folium, *Gynura Japonica*, *Rhei* Radix and Rhizome, and *Dendrobii* Caulis. Botanical material was first extracted, then the extracts were developed (along with respective marker compounds) by means of TLC, and an ultimate identification step was performed with use of laser-ablation of the developed chromatographic plates and direct analysis in the real-time mass spectrometry system (the TLC-LA-DART/MS hyphenation mode). Also in this case, taxonomic markers of individual herbs were identified on chromatographic plates in form of characteristic *m*/*z* signals, which allowed an ultimate authentication of the herbs under scrutiny. Details of the performed extraction and the TLC analysis are given in supplementary material for paper [64].

Still different hyphenation of TLC with one more advanced instrumental detector, i.e., SERS (where SERS holds for the surface-enhanced Raman spectroscopy), used to be

 doi:10.20944/preprints202207.0355.v1

applied as the TLC-SERS hyphenation mode for rapid screening of botanical material. In paper [65], an example is given of the TLC separation combined with the SERS identification of four main *β*-carboline alkaloids (harmalol, harmaline, harmane and harmine), characteristic of the seed extract from Syrian rue (*Peganum harmala*). Plant itself has been recognized in the Mediterranean basin from the times immemorial for its well pronounced medicinal properties in curation of dull eyesight and recently, *β*-carbolines have drawn attention of the medical world for their significant antitumor activity. Moreover, from seeds of this plant the reddish dye can be obtained which has been traditionally used in Western Asia for dyeing carpets and wool fabrics. For the sake of experiment, extraction of alkaloids from the *Peganum harmala* seeds was carried out with methanol. Then the seed extracts and four commercial alkaloid standards were spotted on to the silica gel pre-coated plates and chromatograms were developed with $CHCl_3$-CH_3OH-10%NH_3 (80:20:1.5, v/v/v). Small amounts (0.8 μL and 0.1 μL, respectively) of the Ag colloid and the 0.5 M KNO_3 solution were dropped upon each spot visualized in UV light and a good quality Raman spectra were recorded at four different excitation wave-lengths 1064, 785, 633 and 488 nm, while the spots were still wet. Finally, Raman spectra of the four *β*-carboline alkaloid standards were considered as fingerprints which allowed easy identification of the same compounds in the seed extract.

Expensive TLC systems hyphenated with mass spectrometric, or Raman spectroscopic detectors are often not available in laboratories oriented on fast screening and authentication of botanicals, and such hyphenated systems are especially missing in laboratories of developing countries. However, expensive systems are not always a necessary precondition to perform a quality screening of herbal material and often simple TLC equipment is just enough in this regard. In paper [66], an example is given of the quality control of popular medicinal and culinary herbs, *Heterotheca inuloides, Citrus aurantium, Peumus boldus, Equisetum arvense, Eucalyptus globulus, Ginkgo biloba, Mentha piperita, Aloe vera, Salvia officinalis* and *Cassia senna*, with use of TLC alone. Each plant was extracted with the ethanol–water solution (90:10, v/v), following a carefully elaborated extraction procedure. For each herbal extract, separate TLC system was developed and appropriate standards were procured, which allowed development of the calibration curves, establishing of the LOD and LOQ values for each standard and in fact, enabled development of a validated authentication and quality control method for each herb considered.

Screening and authentication of medicinal and culinary herbs and spices is a very specific challenge, due to the fact that they are most often traded on an international scale in a fragmented or pulverized form, which facilitates adulteration, usually by partial replacement of a more expensive botanical with a cheaper substitute. In paper [16], a TLC method was presented for the quality control of ground black pepper (*Piper nigrum* L.), which used to undergo deliberate falsifications with cheaper botanical substituents, e.g., with the ground chilli pepper. First step was elaboration of the calibration curve for the piperine standard as a taxonomic marker for black pepper (which is absent from the chilli pepper and allows differentiation between the two). Thus a series of six different piperine aliquots from 0.10 to 0.60 μg piperine $spot^{-1}$ in the 0.10 μg piperine $spot^{-1}$ intervals, was deposited on the silica gel pre-coated TLC plates and acetone-*n*-hexane (3:2, v/v) was used as mobile phase. Then the investigated black pepper sample underwent an exhaustive extraction with dichloromethane at 70°C, in order to completely remove piperine and prepare a blank black pepper matrix. Eventually, the elaborated method was used in the experiment which consisted in spiking the blank black pepper matrix at four different concentration levels with piperine, followed by a single extraction run and determination of piperine obtained in the extracts from the spiked blank matrix with use of the developed TLC method. Efficiency of the method was positively confirmed upon a number of control experiments.

4.2. Quality control of alimentary and cosmetic products of botanical origin

 doi:10.20944/preprints202207.0355.v1

Quality control of alimentary and cosmetic products of botanical origin is a very broad analytical field which covers different issues, like adulteration with cheaper botanicals and prohibited synthetic dyes, different kinds of contamination, e.g., with heavy metals, pesticides and other agrochemicals, etc. Thin-layer chromatographic methods are abundantly developed for practically each possible application area, and in this section a few examples are given out of a really vast number of different practical cases.

In paper [67], the first from-start-to-end TLC method was presented of fingerprinting the *Cistus incanus* L. raw herbal material, with a purpose to further using it for rapid screening, authentication, and quality control of the traded *C. incanus* L. herbal teas. The efficiency of this method was tested upon twelve *C. incanus* L. samples of different origin (Turkey, Albania and Greece) and of an unknown vegetation region and harvesting period, randomly purchased from a local market. Samples were first extracted by means of the accelerated solvent extraction (ASE) with chemometrically optimized solvent extraction mixture and temperature (methanol–water, 27:73, v/v; 130°C), to derive polar fraction from the plant samples. Then, the extracts were developed in the two different thin-layer chromatographic systems, both using the silica gel pre-coated plates, yet two different mobile phases: (i) ethyl acetate–formic acid–acetic acid–water (100:11:11:13, v/v/v/v), and (ii) ethyl acetate–dichloromethane–formic acid–acetic acid–water (100:10:10:10:11, v/v/v//v). The developed and dried chromatograms were densitometrically scanned in the fluorescence mode at the wavelength λ = 366 nm. Visual inspection of both sets of the obtained chromatographic fingerprints (i.e., the densitograms of herbal extracts) confirmed authenticity of all investigated samples as the *C. incanus* L. species, yet revealed a considerable difference in terms of contents of polar fraction, regarded as a specific marker of pro-health properties of herbal teas. These differences could be due to a vast number of reasons (e.g., genetic differences among the plants, different environmental and climatic conditions of the herbs growing, different harvesting periods, different drying and storage conditions, etc.).

Paper [68] describes development of a novel TLC method of authentication of the anthocyanins- and anthocyanidins-containing alimentary products, in despite of great vulnerability of these botanical pigments due to their confirmed chemical instability, and especially in contact with silanols of the silica gel type stationary phase. In order to overcome this difficulty and to ensure stability of anthocyanins in the course of analysis, the reversed-phase chromatographic system was developed based on the RP-18 stationary phase (which ensures protective retention mechanism with the non-localized adsorption on octadecyl ligands), acetic acid as the mobile phase component and triple development of chromatograms. Two anthocyanins (cyanin and keracyanin) and two anthocyanidins (pelargonidin and delphinidin) were used as phytochemical standards. First development was carried out with mobile phase I (acetonitrile-methanol-glacial acetic acid (16:4:0.15, v/v/v)) to the distance of 90 mm from the lower plate edge. Second development was carried out with mobile phase II (methanol-glacial acetic acid (20:0.15, v/v)) to the distance of 70 mm from the lower plate edge. Third development was carried out with mobile phase III (methanol-glacial acetic acid (20:0.45, v/v)) to the distance of 60 mm from the lower plate edge. For cyanin and keracyanin, densitometry was performed in the absorbance mode at the wavelength 545 nm, for pelargonidin at the wavelength 450 nm, and for delphinidin at the wavelength 555 nm. The developed and validated method was successfully used to identify and quantify cyanin, keracyanin, pelargonidin and delphinidin in selected alimentary products (syrups, juices and herbal infusions).

In paper [69], a novel and validated TLC method was presented for the analysis of two isomeric biphenyl neolignans, magnolol and honokiol, which are derived from the *Magnolia officinalis* bark and are regarded as traditional Oriental medicines. Nowadays, the *Magnolia* bark extracts and powders are abundantly added to a variety of pro-health dietary supplements traded in the form of tablets, capsules and liquids. To this effect, the magnolol and honokiol standards were prepared in methanol, and silica gel and *n*-hexane-ethyl acetate-ethanol (16:3:1. v/v/v) were used, respectively, as stationary and mobile

 doi:10.20944/preprints202207.0355.v1

phase. Densitometric scans of chromatograms were performed in the absorbance mode, at the wavelength 290 nm. Based on the developed calibration curves for each individual neolignan, the contents of magnolol and honokiol were assessed in six dietary supplements from a local market (four supplements were with label declaration of the neolignans quantity per one table/capsule/vial, and two supplements missed any declaration). In two preparations neither magnolol, nor honokiol were detected, either due to their absence in these two samples, or because of their contents falling below the LOD values established for the method. Thus the developed TLC method was shown as an efficient and reliable tool for quantitative determination of the two magnolia neolignans, magnolol and honokiol, in dietary supplements. The obtained quantitative results once again clearly suggest a necessity of stricter quality control of dietary supplements to exclude marketed products with doubtful constitution and hence, doubtful physiological effects also.

Two examples of the TLC application to quality control of cosmetic raw materials are given in papers [70, 71]. Recently, growing interest has been observed in preventive and "anti-ageing" medicine, and for this reason great attention has been attracted by *trans*-resveratrol known for its excellent antioxidant properties and hence, as an excellent additive to cosmetic preparations. For economic reasons, the main sources of *trans*-resveratrol for cosmetic industry are plants and plant extracts, and the most popular plant is common grape vine (*Vitis vinifera* L.). In paper [70], a simple and validated TLC method was proposed to determine *trans*-resveratrol in such botanical raw materials with wide application in modern cosmetics, as red wine, dry red wine, extract from red wine, extract from skin of red grapes, extract from the American blueberry juice, extract from fruit wine used in "wine spas", etc. The TLC method was developed in the reversed phase mode with the RP-18 pre-coated plates and methanol-water (6:4, v/v) mobile phase. Densitometric detection was carried out in the fluorescence mode (the irradiation wavelength λ = 340 nm), which enabled quantification of *trans*-resveratrol in the cosmetic raw materials of natural origin. The presence of *trans*-resveratrol in the analysed samples was additionally confirmed by visualization of chromatograms with anisaldehyde as a selective visualizing agent.

In paper [71], two thin-layer chromatographic methods were presented which were developed to facilitate detection of two anthocyanins (cyanin and keracyanin) and two anthocyanidins (pelargonidin and delphinidin) in a selection of homemade and commercial fruit juices, and in infusions prepared of the dried plants, with an applicability to cosmetics. As stationary phase, microcrystalline cellulose was used in form of the commercially pre-coated chromatographic plates. For the detection of pelargonidin in the investigated juices and infusions, conc. hydrochloric acid-80% formic acid-water (9:46:90, v/v/v) was proposed. For the detection of the remaining three pigments (cyanin, keracyanin and delphinidin) in the analogical samples, 80% formic acid-water-*n*-butanol (16:19:65, v/v/v) was used. Upon drying, the chromatograms were densitometrically scanned, and they were also assessed in the daylight, the UV light (at 254 nm), and upon visualization in the ammonia vapours (as in basic environment, the investigated plant pigments change their colour from pink or red to blue or navy blue). Fifteen products with alimentary and cosmetic applications were tested for the contents of the discussed plant pigments, and among them the home-made raspberry, blueberry, chokeberry and elderberry juices, analogical products from local discounts and pharmacies, home-made infusions of dried blueberries, hibiscus flower, etc.

5. Planar chromatography in screening of psychoactive plants

Psychoactive plants and fungi have been with mankind since prehistoric times. Some anthropologists even suggest that these organisms might have played an evolutionary role in mental development of humans [72]. There is firm evidence that they have been included in spiritual practices and ancient rituals of many different cultures worldwide. Some of these practices have survived until our time (e.g., among the tribes of the South

 doi:10.20944/preprints202207.0355.v1

American and Mesoamerican Indians), thus enabling diverse anthropologic and medical surveys of this phenomenon [73, 74]. Two fungi that are particularly popular for their hallucinogenic properties in the aforementioned regions are the psilocybin mushroom (also known as the psychedelic or "magic" mushroom, *Psilocybe semilanceata*) [75] and sage of the diviners (*Salvia divinorum*) [76]. In many different parts of the Old World, consumption of psychoactive plants also took place, as documented over the centuries since the Neolithic era [77]. Nowadays, in modern societies worldwide a need for easily available recreational substances has developed in an effort to temporarily alleviate growing challenges and tensions of everyday life. An intensified intercontinental tourism enhanced this trend by bringing together tourists from the better off regions with whole populations in developing countries who make recreational use of local herbs known for containing psychoactive components (e.g., the marijuana-containing cannabis plant in the Indian subcontinent and most of the South Asian region, the cocaine-containing coca leaves in Latin America, the amphetamine-like cathinone contained in the khat leaves from the Arabian Peninsula and the Horn of Africa, etc.) As a result, a burning need has emerged for rapid and efficient screening of psychoactive plants and an interesting review on this subject matter is provided in paper [78]. The authors of this paper have focused on the screening methods for psychoactive plants with use of a variety of analytical techniques, including TLC. In this section, we present selected examples of the paper and thin-layer chromatographic applications to screening psychoactive plants and fungi for their most characteristic ingredients.

Argyreia nervosa (from the family of Convolvulaceae, known under a number of different common names like Hawaiian baby wood rose) is a perennial climbing vine native to the Indian subcontinent and with time introduced to many places worldwide including Hawaii, Africa, and the Caribbean. Powerful psychoactive properties of the Hawaiian baby wood rose are due to the ergoline alkaloids contained in its seeds. Two reports have been released on applications of the preparative layer chromatography and paper chromatography to analyse ergoline alkaloids contained in this plant. First report originates from as early as 1965 [79]. Following the alkaloids-targeting liquid extraction of plant seeds, fraction of the ergoline alkaloids was isolated by preparative layer chromatography. Eventually, these compounds were separated by means of paper chromatography, using butanol–acetic acid–water (4:1:1, v/v/v) as mobile phase. It was established that the content of ergoline alkaloids in the Hawaiian baby wood rose seeds equals to ca. 3 mg per gram of seeds, with one eighth of this amount being lysergamide. Second report on analysis of ergoline alkaloids contained in the same plant by means of the preparative layer chromatography followed by paper chromatography comes from 1973 [80]. In this case, nineteen indole alkaloids were identified by the thin-layer and paper chromatographic procedures. Lysergene, festuclavine, setoclavine, isosetoclavine, agroclavine, elymoclavine, ergine, and isoergine were first isolated by means of the column chromatography and then characterized by TLC and IR. Penniclavine, chanoclavine-I, chanoclavine-II, ergometrine, ergometrinine, lysergic acid α-hydroxyethylamide, isolysergol, racemic chanoclavine-II, molliclavine, lysergol, and isolysergic acid α-hydroxyethylamide were identified by TLC alone. It needs to be added that the pericarp of the Hawaiian baby wood rose was also analysed for contents of the ergoline alkaloids and the revealed pattern was the same one as in the seeds, yet concentrations of individual compounds in the pericarp were at least by two magnitude orders lower than in the seeds.

Catha edulis (from the family of Celastraceae, known under the common name of khat, or qat) is a flowering plant native to Ethiopia and the Horn of Africa. Khat contains the alkaloid cathinone, a stimulant which is known to cause an excitement and euphoria upon chewing its evergreen leaves. Because of a significant psychotropic potential of khat (widely used as "natural amphetamine"), the demand for specific, sensitive and rapid method for determination of its psychoactive principle, the monoamine alkaloid S-(-)-cathinone, in the plant material resulted in elaboration of an efficient TLC screening method [81]. For this purpose, the plant sample was extracted with methanol-0.1 N HC1

 doi:10.20944/preprints202207.0355.v1

(90:10, v/v) and the TLC analysis was performed with use of the silica gel pre-coated HPTLC plates and ethyl acetate–methanol–ammonia (25%) (85:10:5, v/v/v) as mobile phase. Densitometric scans of the chromatograms were performed in the absorption/reflectance mode, at the wavelength 205 nm.

A review paper on TLC in the analysis of the hemp plants (commonly known as cannabis, belonging to the family of Cannabaceae, and having three recognized genera, *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*), on their chemical composition and synthetic cannabinoids contains 84 references [82]. This paper focuses on most important examples of the analysis of the cannabis variants and their components, and also on synthetic cannabinoids related to their medical and recreational uses. It is known that cannabis plants have been recognized for their medicinal properties for thousands of years now. Over 700 varieties of cannabis that contain hundreds of compounds are currently known, which include fatty cannabinoids that are the main biologically active constituents and volatile terpenes that have distinct odours. The most important component of all cannabis genera is tetrahydrocannabinol (THC), which provides euphoric effects and makes them popular for use as recreational drugs, alternative medicines, and clinical research drugs. Although the TLC methods cited in this review paper complement more expensive and more difficult to perform HPLC and GC methods (basically HPLC/MS and GC/MS), the TLC methods alone are also used and they are especially valuable and often sufficient for the separation, detection and identification of cannabinoids in the resources-limited countries. Valuable information in this respect is contained on the website of the HPTLC Association [83] and it contains examples of the NP and RP separations of cannabinoids which allow their fingerprinting and identification. For the needs of the NP HPTLC analysis, the cannabis plant is extracted by sonication with the methanol-hexane (9:1, v/v) mixture, and for the needs of the RP HPTLC analysis, it is extracted with pure methanol. The recommended NP HPTLC analysis ought to be performed on the silica gel stationary phase with *n*-heptane-diethyl ether-formic acid (75:25:0.3, v/v/v) as mobile phase. Visualization is recommended by the spray or dip derivatization with solution of Fast Blue salt. The recommended RP HPTLC analysis ought to be performed on the RP-18 stationary phase with methanol-water-acetic acid (70:15:15, v/v/v) as mobile phase.

Pausinystalia johimbe (from the family of Rubiaceae) is native to the tropical West Africa, and widely grown in Cameroon, with yohimbine being the major psychoactive alkaloid present in the bark of this plant. Upon extraction of the stem bark of *P. johimbe* with methanol, HPTLC was successfully employed for quantification of yohimbine [84]. For this purpose, chromatographic system was composed of the silica gel pre-coated HPTLC plates and toluene–methyl acetate–diethyl amine (7:2:1, v/v/v) as mobile phase. Quantification was performed by densitometric scanning of the chromatograms at 285 nm in the reflectance-absorbance mode. The response to yohimbine was linear over the concentration range of 400 to 1200 ng per band and the method was validated for selectivity, linearity, accuracy, recovery, precision, and robustness.

Piper methysticum (from the family of Piperaceae, known under the common name of kava or kava kava) is a crop of the West Pacific Islands (Hawaii, Samoa, Fiji, Pohnpei etc.). Root of this plant is used to produce a drink with sedative, anaesthetic and euphoriant properties, largely consumed on festive occasions and regarded as an identity symbol of populations of the South Pacific Islands. Active ingredients of kava called kavalactones are known for various psychotropic effects, including the anxiolytic, sedative, and hypnotic action. Kavalactones and flavokavins are the most characteristic components of kava and they are used as targets for rapid TLC screening of the kava raw material before export, due to a steadily growing international trade on this particular plant. The HPTLC detection and quantification method for routine assessment of flavokavins contained in the cultivars of kava is given in paper [85]. Upon extraction of the dried and pulverized plant material with acetone, the HPTLC system used for separation of the extract components includes the silica gel F_{254} pre-coated HPTLC plates and hexane-dioxane (8:2, v/v) as mobile phase Visual inspection and documentation of the chromatograms is carried

 doi:10.20944/preprints202207.0355.v1

out at 254 and 366 nm. Densitometric quantification is carried out in the reflectance mode at 366 nm. In paper [86], the HPTLC identification and quantification method applied to nine compounds from the groups of kavalactones and flavokavins was proposed, based on the same HPTLC system as that introduced in paper [85].

Salvia divinorum ("sage of the diviners") from the family of Lamiaceae; known under a number of common names like seer's sage, magic mint, lady salvia, or simply salvia) is a plant species with transient psychoactive properties when its leaves containing the opioid-like terpenoids are consumed by chewing, smoking, or as a tea. Native habitat of salvia is a remote and hilly Sierra Mazateca region of Mexico. In paper [87], the TLC-GC/MS method was introduced for rapid screening of herbal products containing *Salvia divinorum* for the contents of salvinorin A, the most potent diterpenoid present in this herb. The TLC analysis was carried out following the procedure incompletely described in paper [18]. The only known details are that the crushed fresh or dried leaves were extracted with acetonitrile and the TLC analysis was carried out against the salvinorin A standard on the Whatman silica gel precoated chromatographic plates.

Psilocybe mexicana is a psychedelic mushroom from the family of Hymenogastraceae, genus *Psilocybe*, and its first known usage was by the natives of the North and Central America over 2000 years ago, in the so-called mushroom cult. There are also numerous other fungi belonging to the same genus which naturally produce psychoactive compounds psilocybin, psilocin and baeocystin like, e.g., the tiny *Psilocybe baeocystis* fungus, which grows under such plants like rhododendrons and rose bushes, and is common in the Pacific Northwest. The most potent and the most largely distributed in the temperate regions of the Northern Hemishphere (and particularly in Europe) is *Psilocybe semilanceata*, commonly known as the liberty cap. In paper [88], the first report is given on the TLC analysis of psilocybin and psilocin contained in *Psilocybe baeocystis* (Singer and Smith). Frozen fungi were first ground and then macerated in methanol for 12 h, following a well elaborated working protocol. For the purpose of the TLC analysis, the authors experimented with three different stationary phases, i.e., silica gel, microcrystalline cellulose and alumina, and with nine different mobile phases. Eventually, the best separation results were achieved on silica gel as stationary phase and with butanol-acetic acid-water (12:3:5, v/v/v) as mobile phase. Visualization was first performed in the UV light, then the spots were encircled with a pencil and finally the plates were sprayed with the visualizing reagent (10% *p*-dimethylaminobenzaldehyde in conc. hydrochloric acid). Thin-layer chromatography was also used to discover a new mushroom from the *Psilocybe* genus, i.e., *Psilocybe germanica* sp. nov. [89]. The authors used their own extraction protocol to isolate compounds of interest from the mushroom, which in principle also based on methanol as an extractant. For the purpose of the TLC analysis, silica gel was employed as stationary phase and two mobile phases: (i) *n*-butanol-acetic acid-water (2:1:1, v/v/v) and (ii) methanol-aqueous ammonia (25%) (100:1.5, v/v) were used, both providing a positive separation result. Detection of the compounds of interest was carried out against the psilocybin, baeocystin and psilocin standards, and it was based on a comparison of the retardation factor (R_F) values for the standards with those for respective fractions derived from the mushroom extract.

Summing up, the contents of this review paper cover a vast area of TLC applications to a large number of important and demanding analytical tasks within the framework of screening the botanicals, in which TLC in most cases plays a sovereign and important role. Our review is illustrated by original research papers and also by selected overview papers focused on narrower thematic scopes. They were chosen in an arbitrary manner following personal experience and preferences of the authors, as it has been our intent to emphasize recent achievements in the field discussed, yet at the same time to present some of the most interesting and hopefully inspiring cases of method development. To make it easier for our readers to navigate through the collected material and to facilitate a targeted access to the area of primary interest, in Table 1 we point out to these references cited in our

 doi:10.20944/preprints202207.0355.v1

review which we regard as most important within each of the seven TLC application types mentioned therein.

Table 1. Scoping review of references on thin-layer chromatography (TLC) in screening of botanicals.

Applicability areas to screening botanicals	Selection of topical reads
Chemotaxonomy of plants	[6]-[9], [19]-[28], [90]-[92]
Screening of botanicals for free radical scavenging activity	[10], [12], [14], [32]-[40]
Screening of botanicals for antimicrobial properties	[12], [14], [41]-[49]
Screening of botanicals for enzyme inhibiting potential	[12], [14], [50]-[60]
Quality control of medicinal and culinary herbs	[16], [61]-[66]
Quality control of alimentary and cosmetic products of botanical origin	[15], [17], [67]-[71]
Screening of psychoactive plants	[18], [72]-[89]

6. Conclusions

It was an aim of this study to present a multifaceted picture of huge versatility of thin-layer chromatography in plant research and in solving important and diverse practical tasks, by emphasizing four main directions of its applicability. Two directions, (i) thin layer chromatography in chemotaxonomy of plants and (ii) screening of a wide spectrum of biological properties of plants, have a considerable cognitive potential. The former one is able to contribute to plant chemosystematics and hence, to the development of botany, ethnobotany and related disciplines. The latter one is able to contribute to a wide number of research areas ranging from environmental issues to medicine. Further two applicability directions, (iii) thin layer chromatography in screening of medicinal and culinary herbs, and in quality control of alimentary and cosmetic products of botanical origin and (iv) planar chromatography in screening of psychoactive plants, focus on purely analytical aspects related to quality control of botanicals.

Research output in each of the four aforementioned directions differs in terms of the number of published papers and undoubtedly for diverse reasons. Obvious advantage of TLC which is a relatively low cost technique which combines high speed and easy availability of conclusive results enjoys a highest output with papers which introduce new methods of screening medicinal and culinary herbs and spices, and quality control of alimentary and cosmetic products. Not very high output of papers is observed with planar chromatographic screening of psychoactive plants, which probably is due to sensitivity of the issue itself and also due to higher rapidity with the other instrumental techniques as an urgency demand for forensic purposes. Chemotaxonomy of plants by means of the TLC methods is represented by a good number of scientific reports, although current trends in this field start reorienting plant taxonomy toward genetic taxonomy, owing to available new analytical tools able to reveal genetic profiles of studied organisms [90-92]. Screening of biological properties of plants is a quite exciting and relatively new research field based on the TLC methods. It characterizes with a steadily growing number of published papers which augurs a very positive further direction of its development.

Based on the above reflections, this study has obviously not been planned as a review paper in a traditional sense of the word, but instead it presents a blueprint rather, which highlights most important roles and functions of TLC in screening of botanicals, indicates further directions of the development in the field, and provides short selection of adequately chosen and hopefully inspiring illustrative examples.

References

[1] Kenyon A.S., Flinn P.E., Layloff T.P., 1995. Rapid screening of pharmaceuticals by thin-layer chromatography: Analysis of essential drugs by visual methods. J. AOAC Int. 78:41-49.

[2] Kaale E., Risha P., Layloff T., 2011. TLC for pharmaceutical analysis in resource limited countries. J. Chromatogr. A, 1218:2732-2736.

[3] Shewiyo D.H., "Development and validation of HPTLC methods to assay pharmaceutical formulations", the Ph.D. Thesis, Department of Pharmaceutical Biotechnology and Molecular Biology, Center for Neurosciences, Free University of Brussels, Brussels, Belgium, 2012.

[4] Kaale E., Risha P., Layloff T., Sherma J., Chapter 14:"Screening of substandard and fake drugs in underdeveloped countries by TLC", in: (Eds, Komsta Ł., Waksmundzka-Hajnos M., Sherma J.) "Thin-Layer Chromatography in Drug Analysis", CRC Press, Taylor & Francis Group, Boca Raton, Fl, USA, 2014; pp. 247-266.

[5] O'Sullivan C, Sherma J., 2012. A model procedure for the transfer of TLC pharmaceutical product screening methods designed for use in developing countries to quantitative HPTLC-densitometry methods. Acta Chromatogr. 24:241-252.

[6] Kaltsikes P.J., Dedio W., 1970. A thin-layer chromatographic study of the phenolics of the genus *Aegilops*. I. Numerical chemotaxonomy of the diploid species. Can. J. Bot., https://doi.org/10.1139/b70-260

[7] Kaltsikes P.J., Dedio W., 1970. A thin-layer chromatographic study of the phenolics of the genus *Aegilops*. II. Numerical chemotaxonomy of the polyploid species. Can. J. Bot., https://doi.org/10.1139/b70-261

[8] Rogers C.B., Abbot A.T.D., van Wyk A.E., 1999. A convenient thin layer chromatographic technique for chemotaxonomic application in Maytenus (Celastraceae). S. Afr. J. Bot. 66:174-176.

[9] Mandal P., Choudhury D., Ghosal M., Das A.P., 2016. TLC based chemotaxonomic approach of some laurels present in sub-Himalayan Terai and Duars region of West Bengal, India. Int. J. Pharm. Sci. Rev. Res. 41:193-196.

[10] Cieśla Ł., Waksmundzka-Hajnos M., 2010. Application of thin-layer chromatography for the quality control and screening the free radical scavenging activity of selected pharmacuetical preparations containing *Salvia officinalis* L. extract. Acta Pol. Pharm.-Drug Res. 67:481-485.

[11] Kagan I.A., Flythe M.D., 2014. Thin-layer chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. J. Vis. Exp. 85 | e51411 | Page 1 of 8.

[12] Wang M., Zhang Y., Wang R., Wang Z., Yang B., Kuang H., 2021. An evolving technology that integrates classical methods with continuous technological developments: Thin-layer chromatography bioautography. Molecules *26*(15), 4647; https://doi.org/10.3390/molecules26154647

[13] Schönborn A., Grimmmer A., 2013. Coupling sample preparation with effect-directed analysis of estrogenic activity – proposal for a new rapid screening concept for water samples. J. Planar Chromatogr. – Modern TLC 26:402-408.

[14] Agatonovic-Kustrin S., Morton D.W., 2020. Hyphenated TLC as a tool in the effect-directed discovery of bioactive natural products. Appl. Sci. 2020, 10, 1123; doi:10.3390/app10031123.

[15] Sherma, J., 2000. Thin-layer chromatography in food and agricultural analysis. Review. J. Chromatogr. A 880:129-147.

[16] De Mey E., De Maere H., Dewulf L., Paelinck H., Sajewicz M., Fraeye I., KowalskaT., 2014. Application of accelerated solvent extraction (ASE) and thin layer chromatography (TLC) to determination of piperine in commercial samples of pepper (*Piper nigrum* L.). J. Liq. Chromatogr. Relat. Technol. 37:2980-2988.

[17] Łata E., Fulczyk A., Kowalska T., Sajewicz M., 2017. Thin-layer chromatographic method of screening the anthocyanes containing alimentary products and precautions taken at the method development step. J. Chromatogr. A 1530:211-218.

[18] Kowalczuk A.P., Raman V., Galal A.M., Khan I.A., Siebert D.J., Zjawiony J.K., 2014. Vegetative anatomy and micromorphology of *Salvia divinorum* (Lamiaceae) from Mexico, combined with chromatographic analysis of salvinorin A. J. Nat. Med. 68:63–73.

[19] Waksmundzka-Hajnos M., Sherma J., Kowalska T., 2008. Overview of the field of TLC in phytochemistry and the structure of the book, Chapter 1, in: Thin-Layer Chromatography in Phytochemistry, CRC Press, FL, USA, pp. 3-14.

[20] Singh R., 2016. Chemotaxonomy: A tool for plant classification. J. Med. Plants Stud. 4:90-93.

[21] Schibli A., Reich E., 2005. Modern TLC: A key technique for identification and quality control of botanicals and dietary supplements. J. Planar Chromatogr. – Modern TLC 18:34-38.

[22] Staszek D., Orłowska M., Waksmundzka-Hajnos M., Sajewicz M., Kowalska T., 2013. Marker fingerprints originating from TLC and HPLC for selected plants from the Lamiaceae family. J. Liq. Chromatogr. Relat. Technol. 36:2463-2475.

[23] Jerzmanowska Z., 1967, Plant Material. The Isolation Methods, PWN, Warszawa, (in Polish).

[24] Świątek L., 1977. The iridoid phenolic acids and glycosides in certain Polish medicinal herbs from the *Plantago* genus. Herba Polon. 23:201–210.

[25] Ibrahim R.K., Towers G.H., 1960. The identification by chromatography of plant phenolic acids. Arch. Biochem. Biophys. 87:125–127.

[26] Świątek L., Dombrowicz E., 1984. Phenolic acids in the bitter raw materials. Part I. Analysis of the *Artemisia absinthium* herb and the *Gentian* root. Farm. Pol. 40:729–732.

[27] Schmidtlein, H., Hermann K., 1975. Quantitative analysis for phenolic acids by thin layer chromatography. J. Chromatogr. 115:123-128.

[28] Ganzera M., Pöcher A., Stuppner H., 2005. Differentiation of *Cirsium japonicum* and *C. setosum* by TLC and HPLC-MS. Phytochem. Anal. 16:205-209

[29] Sherma J., 2021. Thin-layer chromatography in the determination of synthetic and natural colorants in foods, Chapter 4, in: (Eds Grinberg N., Carr P.W.), Advances in Chromatography Vol. 56 CRC Press, Taylor & Francis Group, Boca Raton, FL, SA; pp. 109-136.
[30] Agatonovic-Kustrin S., Morton D.W., 2020. Hyphenated TLC as a tool in the effect-directed discovery of bioactive natural products. Appl. Sci. 10, 1123; doi:10.3390/app10031123
[31] Choma I., 2013. Thin-layer chromatography hyphenated with bioassays. J. AOAC Int. 96:1165-1166.
[32] Kedare S.B., Singh R.P., 2011. Genesis and development of DPPH method of antioxidant assay. J. Food Sci. Technol. 48:412-422.
[33] Glavind J., Holmer G., 1967. Thin-layer chromatographic determination of antioxidants by the stable free radical α,α'-diphenyl-β-picrylhydrazyl. J. Am. Oil Chem. Soc. 44:539–542.
[34] Li P., Anu H., Jari S., Yrjönen T., Vuorela H., 2005. TLC method for evaluation of free radical scavenging activity of rapeseed meal by video scanning technology. http://www.regional.org.au (accessed in July, 2022)
[35] Cieśla Ł., Kryszeń, Stochmal A., Oleszek W., Waksmundzka-Hajnos M., 2012. Approach to develop a standardized TLC-DPPH· test for assessing free radical scavenging properties of selected phenolic compounds. J. Pharm. Biomed. Anal. 70:126-135.
[36] Cieśla Ł., Staszek D., Hajnos M., Kowalska T., Waksmundzka-Hajnos M., 2011. Development of chromatographic and free-radical scavenging activity fingerprints by thin-layer chromatography for selected *Salvia* species. Phytochem. Anal. 22:59-65.
[37] Reich E., Schibli A., DeBatt A., 2008. Validation of high-performance thin-layer chromatographic methods for the identification of botanicals in a cGMP environment. J. AOAC Int. 91:13–20.
[38] Cieśla Ł., Staszek D., Kowalska T., Waksmundzka-Hajnos M., 2013. The use of TLC-DPPH· test with image processing to study direct antioxidant activity of phenolic acid fractions of selected Lamiaceae species. J. AOAC Int. 96:1228-1232.
[39] Agatonovic-Kustrin S., Morton D.W., 2017. High-performance thin-layer chromatography-direct bioautography as a method of choice for alpha-amylase and antioxidant activity evaluation in marine algae. J. Chromatogr. A 1530:197-203.
[40] Sherma J., 2018. Review of the determination of the antioxidant activity of foods, food ingredients, and dietary supplements by thin layer chromatography-direct bioautography, spectrometry, and the dot-blot procedure. J. AOAC Int. 101:1285-1294.
[41] Marston A., 2011. Thin layer chromatography with biological detection in phytochemistry. J. Chromatogr. A 1218:2676-2683.
[42] Dewanjee S., Gangopadhayay M., Bhattacharya N., Khanra R., Dua T.K., 2015. Bioautography and its scope in the field of natural product chemistry. J. Pharm. Anal. 5:75-84.
[43] Choma I.M., Jesionek W., 2015. TLC-direct bioautography as a high throughput method for detection of antimicrobials in plants. Chromatography 2:225-238.
[44] Kagan I.A., Flythe M.D., 2014. Thin-layer chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. J. Vis. Exp. (85):51411; doi: 10.3791/51411.
[45] Orłowska M., Kowalska T., Sajewicz M., Jesionek W., Choma I.M., Majer-Dziedzic B., Szymczak G., Waksmundzka-Hajnos M., 2015. A comparison of antibacterial activity of selected thyme (*Thymus*) species by means of the dot blot test with direct bioautographic detection. J. AOAC Int. 98:871-875.
[46] Jesionek W., Móricz Á.M., Ott P.G., Kocsis B., Horváth B., Choma I.M., 2015. TLC-direct bioautography and LC/MS as complementary methods in identification of antibacterial agents in plant tinctures from the *Asteraceae* family. J. AOAC Int. 98:857-861.
[47] Valle D.M. Jr., Puzon J.J.M., Cabrera E.C., Rivera W.L., 2016. Thin layer chromatography-bioautography and gas chromatography-mass spectrometry of antimicrobial leaf extracts from Philippine *Piper betle* L. against multidrug-resistant bacteria. Evidence Based Complementary and Alternative Medicine, Vol. 2016, Article ID 4976791, 7 pages; http://dx.doi.org/10.1155/2016/497679
[48] Wang M., Zhang Y., Wang R., Wang Z., Yang B., Kuang H., 2021. An evolving technology that integrates classical methods with continuous technological developments: Thin-layer chromatography bioautography. Molecules 2021, 26, 4647.
[49] Abiri R., Abdul-Hamid H., Sytar O., Abiri R., de Almeida E.B.. Sharma S., Bulgakov V., Arroo R., Malik S., 2021. A brief overview of potential treatments for viral diseases using natural plant compounds: The case of SARS-CoV. Molecules 2021, 26, 3868.
[50] Ahmed F., Ghalib R.M., Sasikala P., Mueen Ahmed K.K., 2013. Cholinesterase inhibitors from botanicals. Pharmacogn. Rev. 7:121-130.
[51] Rhee I.K., van de Meent M., Ingkaninan K., Verpoorte R., 2001. Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. J. Chromatogr. A 915:217-223.
[52] Marston A., Kissling J., Hostettmann K., 2002. A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. Phytochem. Anal. 13:51-54.
[53] Yang Z., Zhang X., Duan D., Song Z., Yang M., Li S., 2009. Modified TLC bioautographic method for screening acetylcholinesterase inhibitors from plant extracts. J. Sep. Sci. 32:3257-3259.
[54] Di Giovanni S., Borloz A., Urbain A., Marston A., Hostettmann K., Carrupt P.-A., Reist M., 2008. In vitro screening assays to identify natural or synthetic acetylcholinesterase inhibitors: thin layer chromatography versus microplate methods. Eur. J. Pharm. Sci. 32:109-119.
[55] Czernicka L., Ludwiczuk A., Rój E., Marzec Z., Jarzab A., Kukula-Koch W., 2020. Acetylcholinesterase inhibitors among *Zingiber officinale* terpenes – extraction conditions and thin layer chromatography-based bioautography studies. Molecules, 2020, *25*(7), 1643.1680
[56] Sobstyl E., Szopa A., Ekiert H., Gnat S., Typek R., Choma I.M., 2020. Effect directed analysis and TLC screening of Schisandra chinensis fruits. J. Chromatogr. A 1618 (2020) 460942
[57] Hassan A.M.S., 2012. TLC bioautographic method for detecting lipase inhibitors. Phytochem. Anal. 23:405-407.

[58] Bayineni V.K., Suresh S., Singh G., Kadeppagari R-K., 2014. Development of a bioautographic method for the detection of lipase inhibitors. Biochem. Biophys. Res. Commun. 453:784-786.
[59] Hua X., Hong H.-J., Zhang D.Y., Liu Q., Leong F., Yang Q., Hu Y.-J., Chen X.-J., 2022. Rapid screening of lipase inhibitors from *Ophiopogonis* radix using high-performance thin layer chromatography by two step gradient elution combined with bioautographic method. Molecules 2022, *27*, 1155.
[60] Legerská B., Chmelová D., Ondrejovič M., Miertuš S., 2022. The TLC-bioautography as a tool for rapid enzyme inhibitors detection - A review. Crit. Rev. Anal. Chem. 52:275-293.
[61] Waksmundzka-Hajnos M., Móricz Á.M., Hawrył A., Sajewicz M., Kowalska T., 2022. Applications of thin-layer chromatography in the quality control of botanicals, Chapter 22 in: (Ed. Poole C.) Instrumental Thin Layer Chromatography, 2nd Edition, Elsevier (in press)
[62] Kim H.J., Jee E.H., Ahn K.S., Choi H.S., Jang Y.P., 2010. Identification of marker compounds in herbal drugs on TLC with DART-MS. Arch. Pharm. Res. 33:1355-1359.
[63] Morlock G., Schwack, W., 2006. Determination of isopropyl-thioxanthone (ITX) in milk, yoghurt and fat by HPTLC-FLD, HPTLC-ESI/MS and HPTLC-DART/MS. Anal. Bioanal. Chem. 385:586-595.
[64] Chen Y., Li L., Xu R., Li F., Gu L., Liu H., Wang Z, 2021. Characterization of natural herbal medicines by thin-layer chromatography combined with laser ablation-assisted direct analysis in real-time mass spectrometry. J. Chromatogr. A 1654, 462461; https://doi.org/10.1016/j.chroma.2021.462461
[65] Pozzi F., Shibayama N., Leona M., Lombardi J.R., 2013. TLC-SERS study of Syrian rue (*Peganum harmala*). J. Raman Spectrosc. 44:102:107.
[66] Durón R.-R., Almaguer L.C., Garza-Juárez A., Salazar Cavazos M.L., Waksman de Torres N., 2009. Development and validation of thin-layer chromatography methods for the quality control of herbal products. Acta Chromatogr. 21:203-215.
[67] Szeremeta D., Knaś M., Długosz E., Kowalska T., Sajewicz M., 2017. Thin-layer chromatographic fingerprinting of the nonvolatile fraction extracted from the medicinal herb *Cistus incanus* L. J. Liq. Chromatogr. Relat. Technol. 40:304-310.
[68] Łata E., Fulczyk A., Kowalska T., Sajewicz M., 2018. Vulnerability of anthocyanins to the components of a thin-layer chromatographic system and comprehensive screening of anthocyanes in alimentary products. J. Chromatogr. A 1572:137-144.
[69] Łata E., Fulczyk A., Ott P.G., Kowalska T., Sajewicz M., Móricz Á.M., 2020. Thin-layer chromatographic quantification of magnolol and honokiol in dietary supplements and selected biological properties of these preparations. J. Chromatogr. A 1625, 461230; https://doi.org/10.1016/j.chroma.461230
[70] M., Kozik V., Kowalska T., Sajewicz M, 2015. Thin-layer chromatographic quantification *of trans*-resveratrol in cosmetic raw materials of botanic origin. J. Planar Chromatogr. – Modern TLC 28:167-172.
[71] Skorek M., Pytlakowska K., Sajewicz M., Kowalska T., 2017. Thin-layer chromatographic investigation of plant pigments in selected juices and infusions of cosmetological importance and their antioxidant potential. J. Liq. Chromatogr. Relat. Technol. 40:311-317.
[72] Murray, J.D., 2003. Shamanism and rock art, Chapter 12.5, in: Mathematical Biology. II. Spatial Models and Biomedical Applications, 3rd Edition, Springer, New York, NY, USA, pp. 657–659.
[73] Dobkin de Rios, M., and Grob, C.S., 2015. Ritual uses of psychoactive drugs, in: Encyclopaedia of Psychopharmacology, (Eds Stolerman, I.P. and Price, L.H.), Springer, Berlin, pp. 1474–1479.
[74] Dobkin de Rios, M., 2009. Psychedelic Journey of Marlene Dobkin de Rios: 45 Years with Shamans, Ayahuasqueros, and Ethnobotanists, Park Street Press, Rochester, Vermont.
[75] Metzner, R., Ed., 2005. Sacred Mushroom of Visions: Teonanácatl: A Sourcebook on the Psilocybin Mushroom, 2nd Edition, Park Street Press, Rochester, Vermont.
[76] Prisinzano, T.E., 2005. Psychopharmacology of the hallucinogenic sage *Salvia divinorum*. Life Sci. 78:5
[77] Merlin, M.D., 2003. Archeological evidence for the tradition of psychoactive plant use in the Old World. Econ. Bot. 57: 295–323.
[78] Lukić V., Micić R., Arsić B., Nedović B., Radosavljević Ž., 2021. Overview of the major classes of new psychoactive substances, psychoactive effects, analytical determination and conformational analysis of selected illegal drugs. Open Chem. 19:60-106.
[79] Hylin J.W., Watson D.P., 1965. Ergoline alkaloids in tropical wood roses. Science 148:499–500.
[80] Chao J.-M., Der Marderosian A.H., 1973. Ergoline alkaloidal constituents of Hawaiian baby wood rose, *Argyreia nervosa* (Burm. F.) Bojer. J. Pharm. Sci. 62:588-591.
[81] Mathys K., Brenneisen R., 1993. HPLC and TLC profiles of phenylalkylamines of khat (*Catha edulis* Forsk.) confiscated in Switzerland. Pharm. Acta Helv. 68:121-128.
[82] Sherma J., Rabel F., 2019. Thin layer chromatography in the analysis of cannabis and its components and synthetic cannabinoids. J. Liq. Chromatogr. Relat. Technol. 42:613-628.
[83] HPTLC Association Website, https:www.hptlc-association.org (accessed on July 2nd, 2022).
[84] Singh A., Alvi N.I., 2011. High-performance thin-layer chromatographic quantification of yohimbine in the stem bark of *Pausinystalia yohimbe*. J. Planar Chromatogr. – Modern TLC. 24:253-256.
[85] Lebot V., Do T.K.T., Legendre J., 2014. Detection of flavokavins (A, B, C) in cultivars of kava (*Piper methysticum*) using high performance thin layer chromatography (HPTLC). Food Chem. 151:554-560.
[86] Lebot V., Michalet S., Legendre J., 2019. Kavalactones and flavokavins profiles contribute to quality assessment of kava (*Piper methysticum* G. Forst.), the traditional beverage of the Pacific. Beverages 2019, *5*(2), 34; https://doi.org/10.3390/beverages5020034

[87] Wolowich W.R., Perkins A.M., Cienki J.J., 2006. Analysis of the psychoactive terpenoid salvinorin A content in five Salvia divinorum herbal products. Pharmacotherapy 26:1268-1272.

[88] Beug M.W., Bigwood J., 1981. Quantitative analysis of psilocybin and psilocin and *Psilocybe baecystis* (Singer and Smith) by high-performance liquid chromatography and by thin-layer chromatography. J. Chromatogr. A 207:379-385.

[89] Gartz J., Wiedemann G., 2015. Discovery of a new caerulescent *Psilocybe* mushroom in Germany: *Psilocybe germanica* sp.nov. Drug Test. Anal. 7:853-857.

[90] Liu Z., Liu Y., Liu C., Song Z., Li Q., Zha Q., Lu C., Wang C., Ning Z., Zhang Y., Tian C., Lu A., 2013. The chemotaxonomic classification of *Rhodiola* plants and its correlation with morphological characteristics and genetic taxonomy. Chem. Cent. J. 7**,** 118 (2013); https://doi.org/10.1186/1752-153X-7-118

[91] Hayano-Kanashiro C., Gámez-Meza N., Medina-Juárez L.Á., 2016. Wild pepper *Capsicum unnuum* L. var. *glabriusculum*: Taxonomy, plant morphology, distribution, genetic diversity, genome sequencing, and phytochemical compounds. Crop Sci. 56:1-11; https://doi.org/10.2135/cropsci2014.11.0789

[92] van Bemmelen van der Plaat A., van Treuren R., van Hintum T.J.L., 2021. Reliable genomic strategies for species classification of plant genetic resources. BMC Bioinformatics, 22, 173; https://doi.org/10.1186/s12859-021-04018-6