

Application based Studies of HPTLC-bioautography in Evaluation of Botanicals: a Review

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Abstract—HPTLC is a widely used tool in standardization of herbs because of its ability to estimate the presence of active components in samples. HPTLC-bioautography is a technique for isolating active phytoconstituents on chromatograms based on their target. A large number of bioactive compounds with therapeutic and nutritional relevance can be found in medicinal plants. It allows the quick, simple detection and isolation of bioactive compounds. It is a novel approach for evaluating these bioactive compounds having pharmacological effects. The technique is hyphenated with NMR/MS or Raman spectroscopy to determine the structural properties of compounds. This review summarizes and highlights the importance of this technique in today's lifestyle, existing bioautographic technologies, and their application for detecting various pharmacological and other biological activities in plants. It also explains the current state and prospects of HPTLC-bioautography as well as its potential applications.

Keywords: HPTLC-bioautography, α - or β -amylase enzyme, α - or β -glucosidase DPPH solution, LC-MS, NMR

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With changing pattern of lifestyle and modernization, the objective standards for evaluating the safety, quality, and efficacy of these medicinal plants are essential. Plants have medicinal properties owing to their phytochemicals, and the concentration of phytochemicals changes with time and region. Variation in collection and processing can affect their efficacy. As there is no availability of different methods or protocols for the collection and evaluation of botanicals, these methods need revivification. Many improvements can be made to the basic method of thin-layer chromatography (TLC) to automate various steps, achieve better resolution, and allow for more accurate quantitative measurements. High-performance thin-layer chromatography (HPTLC) is one of the useful methods for the separation of phytochemicals from complex matrices allowing simultaneous isolation of various compounds. This technique is cost-effective and requires less amount of sample. HPTLC not only greatly reduces time and consumption of organic solvents, but also provides adequate resolution. Chromatographic analysis hyphenated with the biological detection method is known as bioautography. Bioautography means target directed isolation of active molecules on the chromatogram. HPTLC-bioautography is the technique used for the separation as well as evaluation of the phytochemical constituents possessing medicinal properties in the plant extracts. HPTLC-bioautography is superior in the following aspects.

Firstly, HPTLC can only be used for the separation of complex molecules, but with bioautography we can separate as well as identify or verify target compound in a complex sample. This technique is also useful in the identification or verification of pharmacological activities in medicinal plants. Secondly, both HPTLC and bioautography are cost-effective techniques, but bioautography is effect directed analysis (EDA). It is also known as a target directed analysis method.

Bioautography is a form of chromatography employing a biological detection and is used to detect pharmacological activities in a plant extract.

In the process, the first step is called screening required to identify the presence or absence of target compounds in the sample. This technique is hyphenated with nuclear magnetic resonance (NMR) spectroscopy, tandem mass spectrometry (MS/MS), or Raman spectroscopy for the structural confirmation of target compounds. After separation of a complex mixture by HPTLC-bioautography, target compound is isolated, and the sample is analyzed by one of the spectroscopic methods for the structural confirmation. In today's lifestyle, the use of antibiotic residues and their metabolites has increased in food items or nutritional supplements. Due to their overuse, the resistance to the drugs or medicines is increased, hence it is necessary to monitor the antibiotic residues

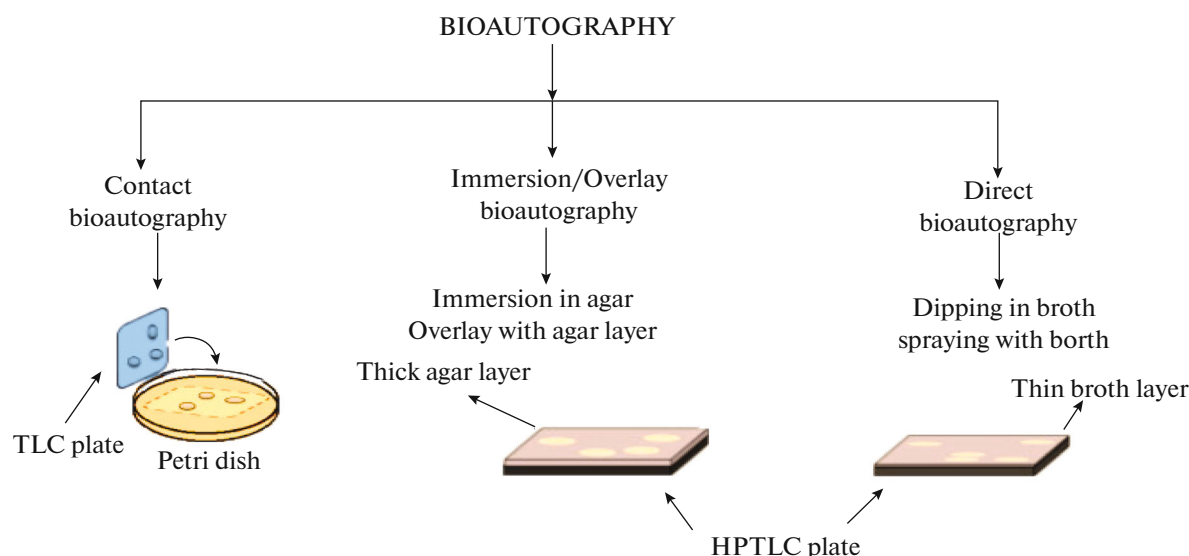


Fig. 1. Classification of high-performance thin-layer chromatography-bioautography.

and their metabolites in food supplements, and this technique acts as a monitoring tool for it.

Bioautography was introduced by Levi and Goodall [1] for the purification of penicillin by paper chromatography in 1946. Since that time, there were many and continuous developments in this technique. In 1961, thin-layer chromatography-based bioautography was introduced by Nicolaus et al. [2]. In 1973, the first review on this technique was written by Betina in which both paper chromatography and thin layer chromatography techniques were discussed [3]. After Betina, Rios [4] did study on bioautography and introduced the versions of bioautography like contact, direct, and immersion based on the parameters affecting bioautography. After him, Botz, Nagy, and Kocsis [5] found out the history of this technique and mentioned the scope and various applications of this technique in their literature. Their focus was mainly on the direct version of this technique.

Bioautography is divided into three main types: contact bioautography, immersion bioautography, direct bioautography (DB).

Contact bioautography. Paper chromatograms or HPTLC plates are placed on the inoculated agar surface in direct contact with the surface (Fig. 1) for several minutes to hours to allow diffusion. After that, the plate is removed, and agar plate is placed for incubation.

The principle of this technique is the transfer of antimicrobial agents from developed plate to agar plate. In this type of bioautography, a developed plate or chromatogram is positioned face down in such a way that it contacts with the inoculated agar layer for a specific period to allow diffusion. The developed plate or chromatogram is then removed, and agar layer is

incubated. Antimicrobial substances are indicated by zones of inhibition formed or developed on agar plate.

Incubation time for growth lies between 16 and 24 h, but can be reduced up to 5–6 h by spraying with 2,6-dichlorophenol indophenol or 2,3,5-tetrazolium chloride.

For example, antibacterial activity of *Dodonaea viscosa* using contact bioautography technique was studied by Muhammad Khurram and others [6]. *Dodonaea viscosa* was analyzed for antibacterial potential against three gram-negative bacteria, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and four gram-positive bacteria, i.e., *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Bacillus cereus*. Four different extractions were made in *n*-hexane, dichloromethane (DCM), ethyl acetate, and *n*-butanol, and solvent systems used for each type of extraction were methanol–chloroform (3 : 200), methanol–chloroform (3 : 20), *n*-butanol–acetic acid–water (12 : 3 : 5), *n*-butanol–acetic acid–water (12 : 3 : 5), respectively. Geitie et al. [7]. reported no activity against gram-negative organisms, but they found promising antimicrobial activity against both gram-positive and gram-negative organisms. Only aerial parts of the plants were collected and evaluated. The bacterial strains were cultured on nutrient agar, and a single isolated colony of each test bacteria was picked and transferred to nutrient broth for an 18-h incubation period except *Salmonella typhi* and *Micrococcus luteus* which require 4 and 48 h of incubation, respectively. One hundred microliters of each inoculum was evenly spread using a sterile glass spreader. The seeded plates were allowed to dry in a laminar flow environment. The pieces of TLC plates were placed aseptically on the bacterial lawn and left for two hours to allow the materials to diffuse onto the seeded

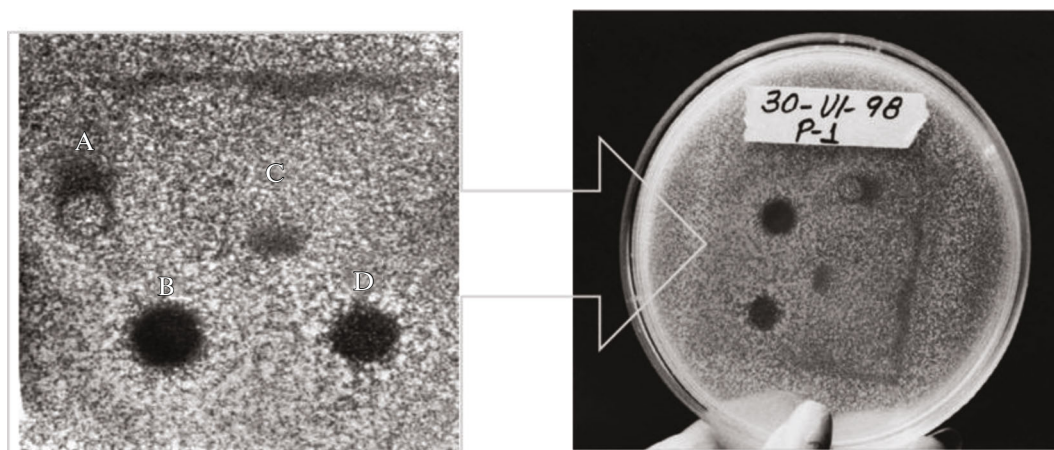


Fig. 2. (A, C) Milk samples – unidentified antibiotics, (B) erythromycin working standard solution, (D) control milk fortified with erythromycin [8]. This figure is reused with permission of Elsevier.

plates. TLC plates were removed from the surface using sterile forceps and incubated in an inverted position for 24 h in the case of all organisms except *M. luteus* which was incubated for 48 h. The areas of inhibition were marked, and the corresponding retention factor (**Rf**) values were recorded. The results show that it is effective against *B. subtilis*, *M. luteus*, and *P. aeruginosa*. In contact bioautography, *S. aureus* failed to produce any zone of inhibition, which is most likely due to the synergistic action of the compounds present in the crude extract [6].

Ramirez et al. [8] showed various antibiotic residues in cow's milk using HPTLC-contact bioautography technique. Milk extracts and antibiotic standard solutions were applied alternately on the HPTLC plates, and the solvent system used was dichloromethane–acetone–methanol–glycerin (64 : 20 : 15 : 1). Developed HPTLC plate was gently placed face down on a Petri dish, and the silica layer contacted with the inoculated media for 25 min. Inoculated media used was molten Mueller–Hinton agar which was inoculated with 0.3 mL of bacterial suspension. The bacterial suspension used was *Bacillus subtilis*. The Petri dish was then inverted and pressed slightly to separate the HPTLC plate from agar. Inverted Petri dishes were then incubated for 18–24 h at 37°C. Inhibition zone diameters from fortified milk extracts were observed (Fig. 2) and measured with Vernier caliper, and values were interpolated from the calibration curve. HPTLC plates and inoculated media confirmed that a contact period of 25 min was required to produce a good response compared to 15 min previously reported for TLC-bioautography [8]. Each tested antibiotic showed that inhibition zone diameter was only dependent on drug amount and independent of applied volume. From this study, Ramirez et al. also found that described method of HPTLC-bioautography is suitable for the detection of ampicillin, benzylpenicillin, dicloxacillin, chloramphenicol, and erythromycin res-

idues in milk samples at the tolerance levels, which would allow its use in routine monitoring of these antibiotics in milk.

Immersion bioautography. This technique is a combination of contact and direct bioautography. TLC-bioautography assay detects the presence of bioactive secondary metabolites on the developed chromatograms. With respect to antimicrobial secondary metabolite detection, both direct and contact bioautography have their own disadvantages such as contamination and improper diffusion, respectively. The agar overlay TLC-bioautography assay is the most trusted, cost-effective, simple, and sensitive assay available. Additionally, it has the advantage of detecting antimicrobial metabolites in microbial extracts that are viable against bacteria and fungi. In the agar overlay TLC-bioautography, the colored formazan is produced due to dehydrogenase activity of microorganisms that convert vital dyes into a chromogenic product by the reduction process. The colorless zone indicates the inhibition of test pathogens due to the presence of fractionated active metabolites on the chromatogram; here, the dye remains colorless. Already developed plate is immersed in agar medium or placed in direct contact with agar medium or covered with it. Molten, seeded agar medium is covered over the chromatogram. The inhibition or growth bands are visible after solidification, incubation, and staining. Plates were placed at low temperature for a few hours before incubation to allow for better diffusion. Visualization is done as in direct bioautography [9]. Gram-negative bacteria can be grown on agar solutions containing the red-colored bacterium *Serratia marcescens*. A red-colored gel is incubated at room temperature overnight, and inhibition zones appear as white or pale-yellow areas on a red background.

For example, the agar overlay assay has been used for yeasts (*Candida albicans*) and can also be used for bacteria (*B. subtilis*, *Pseudomonas aeruginosa*, and

Staphylococcus aureus). The detection threshold was established for the following compounds: itraconazole, miconazole, griseofulvin, ampicillin, and chloramphenicol. Samples were applied on TLC plates and developed in the suitable solvent system. The solvent system used for itraconazole was methanol-chloroform (7 : 3), while for miconazole, griseofulvin, ampicillin, and chloramphenicol methanol-chloroform (8 : 2) mixture was used. Chromatograms were dried with a hairdryer for complete removal of solvents. All TLC plates were run in duplicate, one of them was used as the reference chromatogram. UV active spots were detected at 254 and 366 nm. The reference chromatograms were stained with Godin reagent. Chromatograms were placed on a hot plate maintained at 35°C. Approx. 10 mL of the inoculum was rapidly distributed over the TLC plate with a sterile pipette. After solidification of the medium, TLC plates were incubated overnight at 30°C in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution of thiazolyl blue and incubated for 4 h at 30°C. Clear inhibition zones were observed against a purple background. Adopted principle of this technique is as follows. The inoculated medium is rapidly poured onto the developed chromatogram. The medium solidifies as a thin layer due to the TLC plate heating to 35°C. Overnight incubation in a humid environment allows yeast to grow. Zones of inhibition are visualized by the detection of dehydrogenase activity with a tetrazolium salt. Active compounds appear as clear spots against a colored background [10–12].

Debashis Halder et al. [13] studied the antibacterial activity of four varieties of lactobacilli isolates (*L. animalis*, *L. acidophilus*, *L. plantarum*, *L. rhamnosus*) isolated from 4 different curd samples, i.e., commercial curd 1 (open sample prepared from cow milk), home-made cow milk curd, commercial curd 2 (sachet sample prepared from cow milk), commercial curd 3 (cup sample prepared from cow milk). To isolate lactic acid bacteria from samples, Man, Rogosa, and Sharpe (MRS) broth was inoculated with freshly collected curd samples and incubated for 24–48 h at 35°C. Isolated lactobacilli were grown (for 24 h at 37°C) in MRS broth with sodium chloride supplementation of 2, 4, and 6.5%. Isolated strains were inoculated on separate MRS agar plates and incubated at 37°C for 48 h, after that soft Muller-Hinton agar was overlaid with 0.8% agar pre-mixed with 10⁸ colony-forming units (CFU) of the indicator stains (one on each MRS agar plate) and incubated after solidification of the overlaid agar medium at 37°C for 24 h. Zone diameter of inhibition (ZDI) was measured and interpreted in accordance with Shokryazdan et al. [11]. ZDI > 20 mm were considered as strong, 10–20 mm as intermediate, and 10 mm—weak inhibitions as shown in Fig. 3 [12].

Direct bioautography. As shown in Fig. 4, in this bioautographic technique, separation and microbial

detection of compounds are performed on the same chromatographic plate. In a proper broth, microorganisms are placed for growth. In that broth, the developed TLC plate is dipped and incubated in a humid atmosphere. Broth medium covered on silica surface becomes a source of nutrients and promotes the growth of microorganisms directly on it. Inhibition zones of microorganisms are formed in the places where antimicrobial agents are spotted. To visualize these zones, dehydrogenase deactivating agents were used (most commonly used agent is tetrazolium salt). Due to the conversion of dehydrogenase of living microorganisms by tetrazolium salt into intensely colored formazan, creamy white spots appear against white background.

Direct bioautography is the most popular method for the identification of herbal medicinal products and pharmaceutical formulations. It is mostly used for the evaluation of antibacterial properties of such plants as *Thymus vulgaris* and *Salvia officinalis* [14]. In this technique, samples containing these drugs were applied on a TLC plate, the plate was dipped in the bacterial suspension and left aside for incubation. Growth of the bacteria was observed on the plate surface where antibacterial substance was present. Then, bioautography was carried out against gram-positive bacteria *Bacillus subtilis*. After spraying tetrazolium salt, purple colored formazan was observed.

Antimicrobial detection. In HPTLC-bioautography performed using TLC plates, samples were applied on the surface of the silica plates and stored on their side to dry the sample, and then plates were put into the chamber with a suitable solvent system for development (Fig. 5). When solvent system runs through the plate, the samples get isolated. The samples were observed by spraying suitable agents or plates in a UV chamber. Generally used bacterial strains were gram-negative (*Escherichia coli*) and gram-positive (*Bacillus subtilis*).

Nowadays, current antibiotics show an increase in antimicrobial resistance in human and veterinary use so that the discovery of new antibiotics is an important goal. Bioautography is used to detect antibiotics in food samples, environmental plants, and dietary supplements (Table 1) [20–24].

In the last two decades, two main trends can be observed. The first one is estimation of food quality. In recent years, bioautography has been a popular tool for the estimation of food quality, for the determination of antibiotics, pesticides in food supplements such as milk, fruits or juices, and water [22]. The second trend is related to plant analysis. Due to the increase in the use of herbal medicines for the treatment of many diseases owing to fewer side effects, bioautography becomes the most popular tool for analysis. Plants are rich in bioactive compounds so that continuous development is observed for the identification of these plants [25].

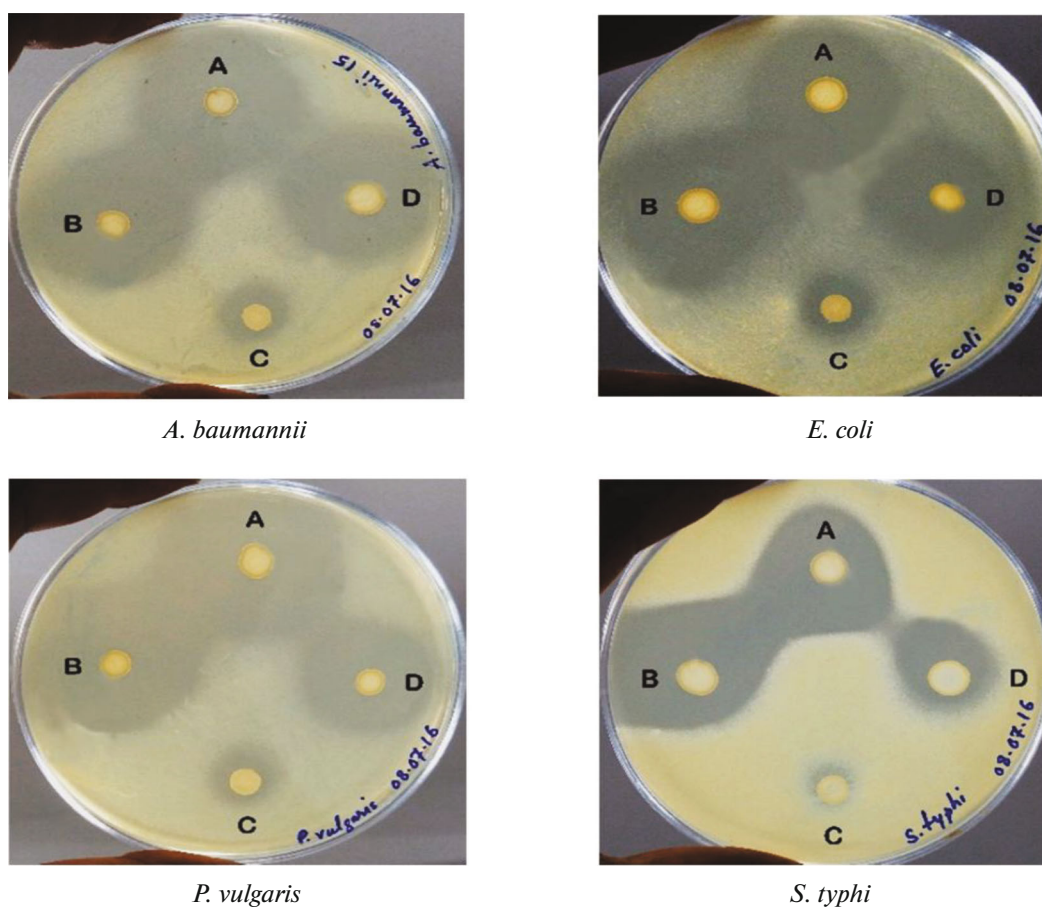


Fig. 3. Zones of inhibition around four test lactobacilli strains (grown on the plates as spot forms) against each of the four bacterial pathogens: (A) *L. animalis*, (B) *L. plantarum*, (C) *L. acidophilus*, (D) *L. rhamnosus* [13].

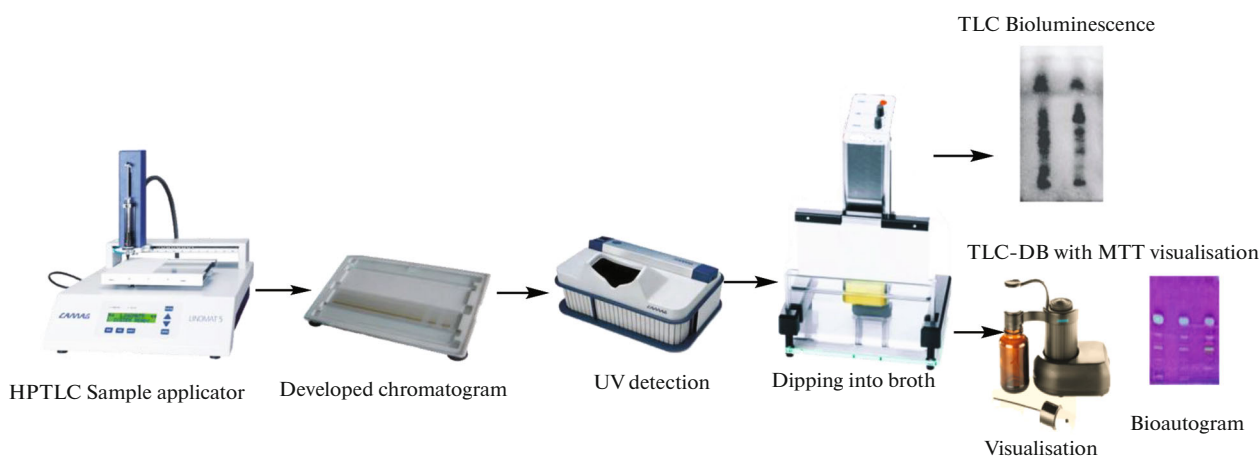


Fig. 4. Scheme of high-performance thin-layer chromatography-direct bioautography.

In the case of complex compounds or matrices, biological screening is performed on TLC plates which have already been developed, and it is the best way to obtain reliable information about the activity of single compounds. However, the main problem for this tech-

nique is to find the best solvent for the extraction of active compound from the matrix or to select a solvent system for well separation of the complex matrix. Bioautographic methods are often combined with analytical methods such as liquid chromatography-mass

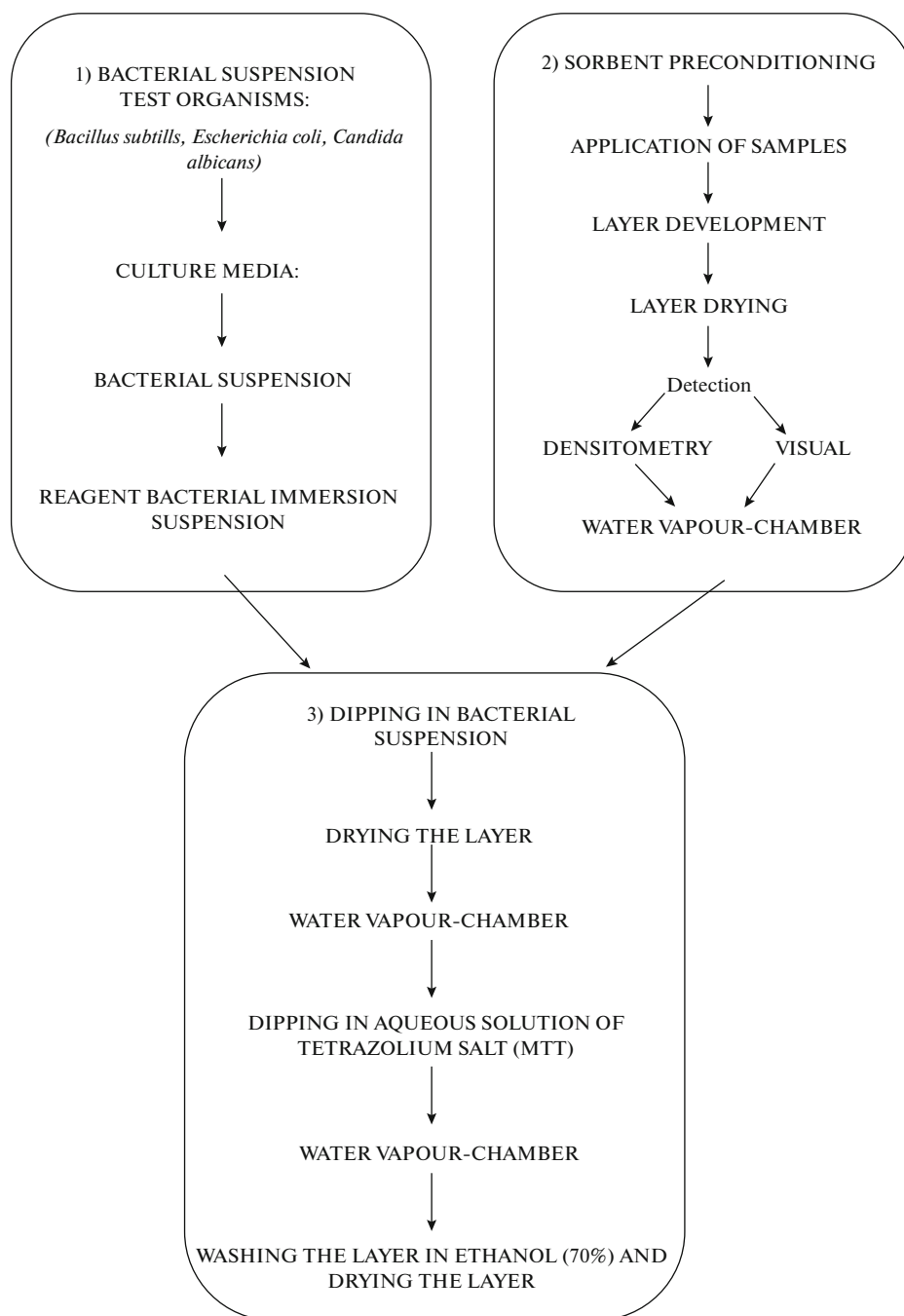


Fig. 5. Flow diagram for antimicrobial detection procedure.

spectrometry (LC-MS), NMR or IR spectroscopy to determine the structure of active constituents [26]. Hyphenation of HPTLC with bioluminescence detection is used in daily analysis of water toxicity in Germany. In this process, BioLuminizer is used to document the plate which has been subjected to bacterial growth, e.g., for *Aliivibrio fischeri*.

Aliivibrio fischeri is a bioluminescent bacterium which is exclusively used for toxicity studies. In Germany, it is used to test water toxicity. Water is passed

through the plate, and the plate is submerged in bioluminescent bacteria. Then, bacteria get coated on the surface of the plate [27]. By observing bacterial growth, we can state that the water is toxic or non-toxic. If bacteria grow the water is non-toxic, and if bacterial growth is inhibited or bacteria die the water is toxic [28–30].

Syzygium cumini plant was studied for its antimicrobial activity by Dr. Sopan Kharat and Mr. Vijay Mendhulkar [31]. Plant leaf extract was made in 5 dif-

Table 1. Some examples of antimicrobial TLC-bioautography (contact, overlay, direct) in the search for target compound

Type of sample	Investigated compounds	Type of microorganism	Bioassay	Reference
Cow's milk	Chloramphenicol, benzylpenicillin, ampicillin	<i>B. subtilis</i>	HPTLC contact bioautography	[12]
<i>Sclerocarya birrea</i>	Acetone and aqueous extracts	<i>Helicobacter pylori</i>	TLC overlay bioautography	[15]
<i>Myrtus communis</i>	Ethyl acetate, dichloromethane petroleum ether, methanol extracts	<i>Microsporum canis</i> , <i>Microsporum gypseum</i> , <i>Tricophyton</i>	TLC overlay bioautography	[16]
Marine sponge <i>Hymeniacidon perleve</i>	Norharman (beta-carboline alkaloid) from <i>Pseudoalteromonas piscicida</i>	<i>Staphylococcus aureus</i>	TLC overlay bioautography	[17]
Cow's milk	Cefacetril	<i>Bacillus subtilis</i>	TLC direct bioautography	[18]
Bilberry, blueberry	Malvidin	<i>Aliivibrio fischerii</i>	HPTLC direct bioautography	[19]
Marine sponges	Avarol, avarone aerophysinin, dienon	<i>V. fischerii</i>	HPTLC direct bioautography	[20]

ferent solvents, namely, methanol, ethanol, ethyl acetate, chloroform, water and studied against 5 microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, and *Salmonella typhi*). Plate was developed in an optimized solvent system consisting of ethyl acetate-formic acid-acetic acid-water (10 : 0.5 : 0.5 : 1.3) The plates were left for 1 h at room temperature and then incubated at 37°C for 24 h. Inhibition zones were observed and measured in millimeters.

Enzyme inhibition detection. Esterase inhibitors. Numerous bioautographic techniques were developed for the identification of such enzymes as acetylcholinesterase (**AchE**) and butyrylcholinesterase (**BchE**) and for glucosidases and xanthine oxidase (**XO**) inhibition [32, 33].

Drugs used in Alzheimer's disease therapy are focused on identifying the compounds with AchE inhibitory properties. Drugs having anticholinesterase properties prevent the destruction of neurotransmitter acetylcholine. When breakdown of acetylcholine is inhibited, the level of neurotransmitters can return to normal or near normal. According to the literature [33, 34], various plants were studied for their AchE and BchE activities which can be used in the treatment of Alzheimer's disease.

HPTLC-cholinesterase studies have also been used to detect insecticides, for example, organophosphate and carbamate. The most popular technique to visualize esterase inhibitors is the diazotization reaction. In this technique, already developed and dried HPTLC plate is sprayed with enzyme AchE or BchE, after that the plate is incubated and sprayed with 1-naphthyl acetate and fast blue salt which act as visualizing agents. Active components can be observed as a white spot against purple background. In modification, 1-naphthyl acetate is replaced by 2-naphthyl acetate.

Although 2-naphthyl acetate is more expensive than 1-naphthyl acetate, it gives deeper purple background.

For example, study of *Galbanum* plant for its AchE inhibitory activity was carried out by Adhami.H.R et al. [35]. Extract was prepared in dichloromethane, and the plate was developed in the solvent system consisting of chloroform-ethyl acetate-methanol (100 : 10 : 2). Chelidonine acts as a positive control in this system. Relative humidity (**RH**) during the development of the plates in the automatic developing chamber was maintained between 33.9 and 35.0%. Saturation time was 20 min. Galbanum shows AchE inhibition in a screening by bioautography. Compounds are separated on HPTLC plate, and respective zones are eluted for further HPTLC-MS/NMR studies.

Study of antidiabetic properties. Diabetes can be controlled by inhibiting two enzymes so that the concentration of glucose in blood reduces significantly. The mechanism is as follows.

Glucosidase inhibitors are the potential drugs used in the treatment of cancer, diabetes, viral infections (including human immunodeficiency virus (**HIV**)), obesity, and these drugs can be detected by bioautography based on glucosidase inhibitory activity [36]. Glucosidase inhibitors are useful in Type 2 diabetes mellitus, i.e., they prevent the digestion of carbohydrates. Carbohydrates are normally converted into monosaccharides (simple sugars) by glucosidase enzymes and enable monosaccharides to be absorbed through the intestine.

Developed and dried TLC plate is sprayed with α - or β -glucosidase and set for incubation. α -D-glucosidase requires incubation for about 1 h, while β -glucosidase requires much less time (about 20 min). Then, the mixture of substrate solution and visualizing agent (fast blue salt) is sprayed on the plate. In few minutes, fast blue salt gives purple coloration due to reaction

with 2-naphthol coming from the cleavage of 2-naphthyl- α/β -D-glucopyranoside [37].

Centela asiatica, *Aloe vera*, *Tinospora crispa*, *Phaleria macrocarpa*, *Curcuma aeruginosa*, *Xoncus arvensis*, *Caesalpinia sappan*, *Parcia speciosa*, *Gynura procumbens*, *Physalis peruviana*, and *Hibiscus sabdariffa* are some representative plants having antidiabetic activity which can be used in the treatment of diabetes [38].

α -Amylase inhibitors are useful in treating diabetes. Amylase inhibitors delay the breaking of starch (consisting of many glucose molecules) into maltose (glucose + glucose) in the small intestine. Therefore, the absorbance of glucose in blood reduces significantly. Starch is a complex molecule which cannot be directly absorbed into blood. Since it is excreted from the body, the blood sugar level is controlled. Thus, the diabetic condition is controlled by inhibiting α -amylase enzyme. *Syzygium cumini*, *Azadirachta indica*, *Aloe vera*, etc., are some traditionally used plants possessing antidiabetic properties. For the verification of this activity, Gram's iodine is used as the biomarker since it gives purple or dark blue color against a white background.

Procedure for study of amylase inhibitor activity is described above. Developed plate was dipped and saturated with enzyme solution and incubated for 30–60 min at 37°C in a humid chamber. After incubation, the plate was dipped again into 1% starch solution and incubated for 10–20 min for enzyme-substrate reaction. The plate was dipped in Gram's iodine solution. If blue spot against white background was observed, it confirmed the α -amylase inhibitory activity.

Detection of antioxidants. HPTLC is used in combination with 2,2-diphenyl-1-picrylhydrazyl (DPPH) for the identification of antioxidant properties and antifungal properties. This hyphenation has many advantages, such as quick results, a reduction in the time-consuming preparation of the sample, and most significantly, direct access to isolated target compounds on the HPTLC plate [39].

Two groups of TLC-DB experiments are applied to evaluate if free radical scavenging or antioxidant activity can be tested using 2,2-diphenyl-1-picrylhydrazyl radical or 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), where DPPH is more stable than ABTS. Generally, the developed HPTLC plate is sprayed with or dipped in a DPPH solution, and the color shift is observed, namely, white or light-yellow spots against a purple background. *n*-Hexane was suggested due to little effect on the radical antioxidant reaction. The use of silica gel plates and DPPH methanol solutions provide the most intensive results [40].

The DPPH assay shows the presence of radical scavengers as yellow spots/bands on a purple backdrop which are detected at 517 nm as at this wavelength DPPH has maximum absorption. For TLC-ABTS, active compounds appear as colorless or pink zones against a green background at 734 nm [41, 42],

because this wavelength provides maximum absorption for ABTS. It should be noted that DPPH has higher stability than ABTS on the TLC plate.

Chrysanthemum tinctoria which is used as a herbal tea from the ancient era has stronger antioxidant activity than two samples used to compare the antioxidant activities, i.e., *chrysanthemum moliforium* cv. "Gongju" and "Hangju". Samples were applied on TLC plate, the plate was developed in a solvent system consisting of toluene-ethyl acetate-formic acid-water (9 : 20 : 6 : 3) for 15 min and scanned at λ_{\max} of 365 nm. The developed plate was sprayed with 4% DPPH solution. *C. tinctoria* was observed to have stronger antioxidant activity than other samples due to the presence of 7 compounds, which is shown in Fig. 6 [43].

Another examined sample was *Morus alba* which is widely used as a medicinal plant in Asia. Root bark of that plant was most widely used in conditions like cough, edema, heat. It has different chemical composition and various activities like antioxidant, antiviral, anti-inflammatory, antitumor, antidiabetic, etc. After developing the plate in the solvent system of *n*-hexane, ethyl acetate and methanol (6 : 4 : 3.5, v/v/v), the plate was dipped in 1% methanol solution of DPPH, and light yellow bands against dark purple background were observed. The samples collected from Serbia showed a richer metabolic profile and bands of higher intensity compared with the plants collected from China. The samples collected from Serbia showed bands in hRf range between 50–80 [44].

These are the 7 samples extracted from plant *M. alba* from Serbian variety: sanggenon A (hRf = 77), sanggenon B (hRf = 70), sanggenon C (hRf = 65), moracin P (hRf = 63), kuwanon L (hRf = 58), sanggenon D (hRf = 57), sanggenon G (hRf = 55).

Out of these reference compounds moracin P, kuwanon L, sanggenons C, D, and G showed moderate radical scavenging activities, where sanggenon D and sanggenon G showed intensive yellow bands on the HPTLC plate of *M. alba* extracts of Serbian variety [44–46].

New possibilities. A complex isolation and detection method called Bio-Arena has been introduced by Tyihák et al. [47]. which combines the advantages of thin-layer chromatography or overpressure layer chromatography (OPLC) with bioautography. Bio-Arena is a quite simple, inexpensive, and reliable method, and modification in incubation time is the main advantage of this system. This system is generally used in investigating biochemical interactions between biologically active compounds [48]. For the detection of antibiotics separated by thin-layer chromatography, Merck developed the direct bioautography test called Merck Biodip® antibiotics. This kit is used to detect new antibiotics, monitor antibiotics in pharmaceutical formulations, manage food and feed, as well as detect antibiotics in waste water (Merck Biodip® antibiotics) [48–52]. Hyphenation gives answers to many ques-

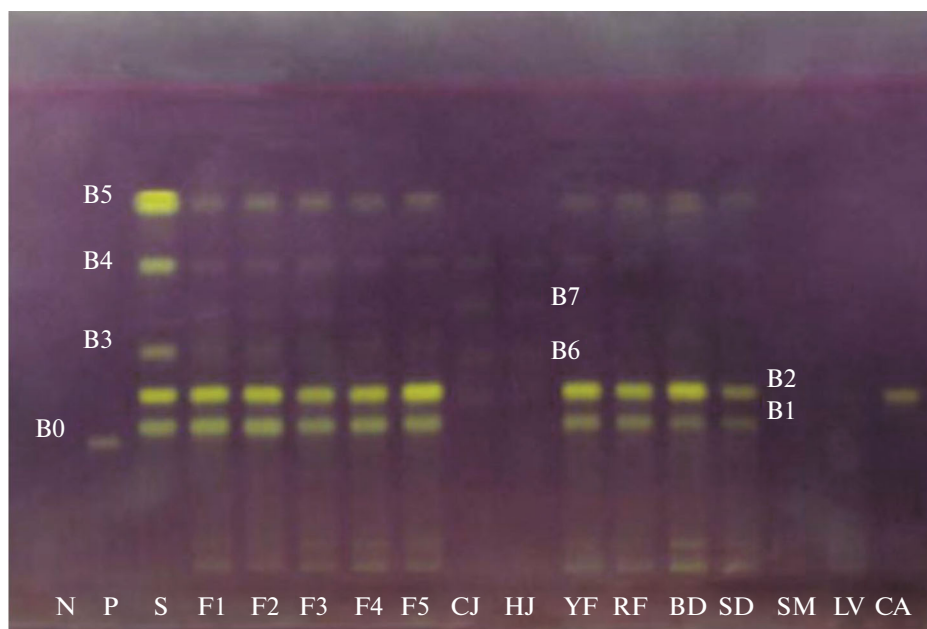


Fig. 6. High-performance thin-layer chromatograms of antioxidants in the collected samples of *C. tinctoria* and *C. morifolium*. Development of antioxidants colorized with 2,2-diphenyl-1-picrylhydrazyl methanol solutions, where N corresponds to the blank solvent used as negative control, P—L-ascorbic acid used as positive control, S—mixed standards; F1 to F5 and YF correspond to yellow flowers of *C. tinctoria*; GJ and HJ correspond to *C. morifolium* cv. “Gongju” and “Hangju”, respectively; BD, SD, SM, and LV stand for buds, seeds, stems, and leaves of *C. tinctoria*, respectively; CA—for chlorogenic acid; B0 to B7—codes of detected antioxidant bands, where B1—flavonmarine, B2—marine and chlorogenic acid, B3—5,7,3,5-tetrahydroxyflavanone-7-O-glucoside, B4—3,5-dicaffeoylquinic acid, B5—okanin, iso-okanin. B6 and B7 are unknown compounds [43]. This figure is reused with permission of John Wiley & Sons.

tions arising during the evaluation of target molecules. Hence, bioautography is hyphenated with MS, NMR, for further characterization of molecules or compounds. Structural elucidation may be done using NMR and IR, and for more selective detection this technique is coupled with Raman spectroscopy and mass spectrometry. In the future, test kits can be developed for the detection of the quality of dietary supplements, milk, fruits, and food.

Application of hyphenated techniques to samples of natural products. HPTLC can be hyphenated with MS/NMR for analysis of plant extracts for the structural conformation of active phytoconstituents [52]. Spectroscopic analysis of samples improves the confirmation by predicting their functional groups and elucidating their structure. Hyphenation with MS gives the confirmational peaks by using the molecular weight principle, and NMR finds out the molecular formula and structure of target compound. The procedure is divided into three steps described below.

In the first step (HPTLC, separation of compounds), parallel isolation of several complex compounds takes place, as this technique includes cost-effective identification of active compounds, increased reproducibility, and the highest resolution. TLC plates with an automated sample applicator are produced by CAMAG.

The second step is bioautographic detection. The use of a staining agent such as Gram’s iodine for the specific and fast reading of the TLC plate after the detection of inhibition zones on bioautograms suggested areas of concern on the duplicate TLC plate, and only these zones were extracted for further MS and NMR analysis.

The third step is LC-MS detection. HPTLC-bioautography in combination with LC-MS and NMR provides structural details of active compounds.

One such example is dereplication of ecumicin. *Nonomuraea* species strain a promising source for antituberculosis (**anti-TB**) active lead compounds, which led to the isolation of the new anti-TB macrocyclic tridecapeptide ecumicin. Ecumicin exerts potent, selective bactericidal activity against mycobacterium tuberculosis (**Mtb**) in vitro including non-replicating cells. The HPTLC-bioautography-MS/NMR approach was applied to the *Nonomuraea* extract to confirm the capability to identify the previously isolated ecumicin and gain sufficient structural information about the active principle(s) at the beginning of the isolation procedure. The ecumicin reference material and the enriched Sephadex LH-20 fraction were both spotted on a TLC plate. After detecting the growth inhibition zone on the bioautogram, the zone of interest was marked on the duplicate chromatogram plate and extracted with the TLC-MS interface. The

structural dereplication was performed by LC-MS and NMR analysis [53].

CONCLUSIONS

Bioautography is part of the effect directed analysis method by which the separation step is conducted using planar chromatography techniques. Using this technique, we can comparatively study the potential antioxidant, antifungal or any other physiological activities of various herbs or plants having medicinal activities. HPTLC-bioautography is better than only the HPTLC method since it allows to determine the pharmacological activities, while HPTLC can only separate complex matrices. This technique has many more possibilities and can be very useful in future for analysis of food, dietary supplements, and nutraceuticals. Popularity of this technique increased in the last two decades due to the increased pollution and adulteration of foods and dietary supplements, as this technique is useful in highlighting their purity. Hyphenation of HPTLC-bioautography with MS, NMR, and IR is useful for researchers in structural elucidation for the confirmation of target compounds.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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