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Review Article

Application of TLC Bioautography for Natural Bioactive Screening

Vishwa Patel[1]*, Niyati Acharya[2]

[1]*Assistant Professor, Department of Pharmaceutical analysis, Sardar Patel College of Pharmacy, Bakrol.*
[2]*Head of Department of Pharmacognosy, Institute of pharmacy, Nirma University.*

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ABSTRACT

Bioautography is a mean of targeted-directed isolation of active molecules on chromatogram. Bioautography is technique to detect substances affecting the growth rates to test organisms in complex mixtures and matrices. The antimicrobial, antioxidant and enzyme inhibitory activities can be performed on TLC bioautography. Bioautography methods are divided into three categories. 1)Agar diffusion or contact bioautography 2) Immersion or agar-overlay bioautography 3) Direct bioautography. High throughput method enabling analyses of many samples in parallel and the comparison of their activity. Both screening and semi-quantitative analysis is possible. The targeted compounds can be identified using spectroscopic methods, that can be performed directly on a TLC plate. This review summarizes known TLC-bioautography methods and their applications for determining the presence of enzyme inhibitors in extracts, preliminary screening, natural products possessing these biological activities and for the bioactivity-directed fractionation and isolation of active components from complex extracts. It also indicates the current state and perspective of the development of TLC-bioautography and its possible future applications.

INTRODUCTION

Planar chromatographic analysis hyphenated with the biological detection method is known as Bioautography [1].It is effective and inexpensive technique for the phytochemical analysis of plant extracts to identify bioactive lead/ scaffolds. It can thus be performed both in highly developed laboratories as well as in small laboraties which have maximum access to sophisticated equipment [2]. In 1964, Goodall and Levi introduced paper chromatography (PC)- based bioautography for the first time to estimate the purity of penicillin [3]. In 1961, Fisher and Lautner and Nicolaus et.al introduced thin layer chromatography (TLC) based bioautography [4].The first review on bioautography was written by Betina in 1973 [5]. TLC can separate many samples in parallel and has

***Corresponding Author:** Vishwa Patel
Address: Assistant Professor, Department of Pharmaceutical analysis, Sardar Patel College of Pharmacy, Bakrol.
Email ✉: vishwa091098@gmail.com
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an open layer that allows for solvent evaporation, it appears to be the perfect technique for hyphenation with bioautographic detection. Besides classical TLC, High performance thin-layer chromatography (HP-TLC), Over pressured-layer Chromatography (OPLC) and planar electro-chromatography (PEC) can also be easily linked to bioautography [6-12]. Thin-layer chromatography-direct bioautography (TLC-DB) is one of three variants of a TLC bioautography method (the others are contact and agar overlay bioautography) in which separation given assay visualization are performed directly on a TLC plate [13-15]. The major application of bioautography are the fast screening of a large number of samples for bioactivity like antibacterial, antifungal, antioxidant, enzyme inhibition, antimicrobial etc. In this review, the techniques and application of bioautography are discussed in detail with suitable examples.

Bioautography Methods:

Three bioautographic methods are 1) Agar diffusion or contact bioautography 2) Immersion or agar overlay bioautography 3) Direct TLC bioautography.

(Table:1 Types of Bioautography)

Bioautography	Basic Procedure	Specification
Agar diffusion or Contact bioautography	Inoculation with microbial strains. Allowing diffusion into agar media. Incubation.	Applying for chromatography silicic acid Fiber sheets and Chroma AR still helped to avoid these shortcomings.
Immersion or Agar-overlay bioautography	Solidification. Incubation. Staining with tetrazolium salt.	A combination of direct and contact bioautography is agar overlay. It is highly recommended to use agar overlay when direct bioautography cannot be completed.
TLC- Direct bioautography	Dipping of chromatogram in microbial strains. Spraying with tetrazolium salt incubation under humid condition for 48hr. Indicate antimicrobial leads.	Direct bioautography is usually perform without agar gel. Plant extracts can be quickly screened chemically and biologically using TLC-direct bioautography. "Agar spray assay" is a recently discovered bioautographic technique that can be thought of as an agar variant of direct bioautography.

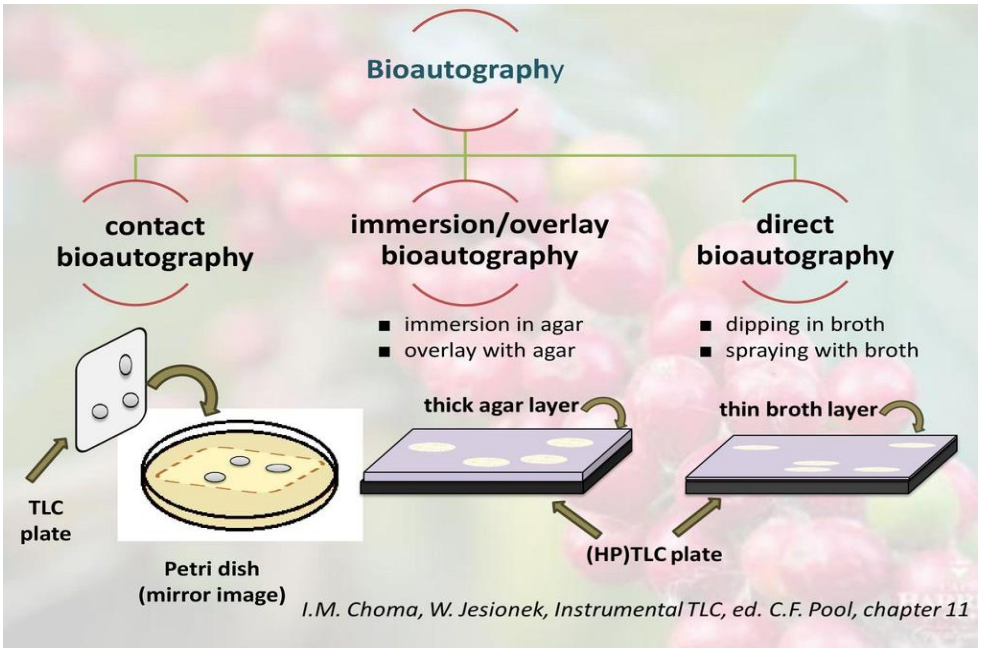

Figure 1: Scheme of bioautographic methods. (HP-TLC) (High Performance Thin layer chromatography)

2.1 Agar diffusion or contact bioautography [16-20]

The technique with the least usage is agar diffusion. It entails the antimicrobial agent being transferred by diffusion from the chromatogram (PC or TLC) to an agar plate that has already been inoculated with the pathogen under investigation. The agar plate is incubated and the chromatogram is taken out after a few minutes or hours to allow for diffusion. The areas where the antimicrobial chemicals come into touch with the agar layer are where the growth inhibition zones develop. The growth requires an incubation period of 16 to 24 hours, however, this can be shortened to 5 to 6 hours by spraying 2,6-dichlorophenol-indophenol or 2,3,5-tetrazolium chloride. Antimicrobials had to be diluted and lost during the process of moving them from chromatographic plates to agar, which followed the same general procedure. An other unique instance involves the bioautographic identification of 6-aminopenicilanic acid, a very weak antibiotic that needs to be transformed into benzyl penicillin by phenyl acetylation. This is achieved by chromatographic plates or paper with acetyl chloride under mildly alkaline conditions prior to bioautography. Microbiologists are accustomed to using this method to find antibiotics in bacteria, and several techniques have been applied to enhance its efficacy.

2.2 Immersion or agar overlay bioautography [21-25]

Combining contract and direct bioautography is known as agar overlay. Using this technique, a melted, seeded agar media is applied on the chromatogram. The growth or inhibition bands are visible after solidification, incubation, and staining (often with tetrazolium dye). An agar solution containing the red-colored bacterium Serratia marcescens can be used to identify gram-negative bacteria. After an overnight incubation period at room temperature, the red-colored gel takes on a white or pale yellow, indicating inhibitory zones. Through the use of a dehydrogenase activity, microbes transform the tetrazolium salt (MTT) into corresponding highly colored formazan, allowing other colorless zones of microbial growth inhibition to be seen. The agar overlay assay has

been applied to bacteria, including Bacillus subtilis, E. coli, Pseudomonas, and Staphylococcus aureus, as well as yeast, and Candida albicans.

2.3 TLC-DB (Thin layer chromatography-direct bioautography) [26-28]

A suspension of microorganisms growing in nutrient broth is applied to a designed TLC plate (TCL-DB of antimicrobials), which is subsequently incubated in a humid environment. The bacteria grows straight on a TLC plates surface, avoiding antimicrobial areas. 3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide, or MTT, is the most widely used tetrazolium dye. Addition of detergent (e.g., Triton X-100) to the dye can boost the reduction of tetrazolium salts many times. The purple formazan that results from the dehydrogenases of live microorganisms transforms tetrazolium salt. Inhibition zones, which are cream-colored patches that occur on a purple backdrop, indicate the presence of antimicrobial drugs. Gram-positive Bacillus subtilis Aerobic Bacteria and Gram-negative Escherichia coli are the most commonly utilized test bacteria. Additionally, TLC can be hyphenated with bioluminescence. Detection mostly employing Photobacterium phosphoreum, also known as luminous Vibrio phosphoreum, and Aliivibrio fischeri, formerly known as Vibrio fischeri. The metabolism of bacteria is disrupted by antibacterial agents and other bacterially toxic substances (such as pesticides, heavy metals, and mycotoxines), which ultimately results in the cessation of the bacteria's luminescence. The TLC plate was quickly immersed in a bacterial broth suspension that had been infected with luminous bacteria, and it was then viewed at 490 nm using a luminograph or CCD (cooled charged coupled device) camera. Toxic chemicals are indicated by variations in the emission of blue-green light (darker or brighter zones), which are linked to disruptions in essential cell activities. The technique can be regarded as a direct bioautography even though there are no alterations in bacterial growth.

Application Of Bioautography

The majority of applications for thin-layer chromatography bioautography (TLC-B) use microorganisms, such as fungi and bacteria, as testing organisms. The development of novel antibiotics is a particularly crucial goal in light of the growing microbial resistance to conventional antibiotics in both medicine and veterinary practice.

Ref. No	Name of plant material/extracts	Activity reported/bacterial strain	Chromatographic condition	Bioassay	Conclusion
[29]	Saraca indica linn (Acetone extract) **Family:** Caesalpiniaceae	**Activities:** Vibriocidal activity Antibacterial activity	**Mobile phase:** methanol: water (70:30 v/v) **Stationary phase:** Silica gel 60 F_{254} TLC plate	HPLC/TLC bioautography	The aqueous extracts of A. Sativum were the most efficacious, although the acetone extracts of S. indica and D. stramonium
		Bacteria:			

	Datura stramonium linn (Acetone extract) **Family:** Solanaceae Allium sativum Linn (Aqueous extract) **Family:** Liliaceae	Gram-negative bacteria *Vibrio cholerae* *Vibrio parahaemolyticus*	**Column:** Reverse phase Lichrosphere C18 column (250 mm × 4.6 mm,5 µm) **Wavelength:** UV 310 nm **Flow rate :** 0.7 ml/min		were more potent in most cases.
[30]	Erycibe obtusifolia (E.obtusoifolia) (Crude extracts) **Family:** Convolvulaceae	**Activities:** Antioxidant activity Xanthine oxidase inhibitor Antifungal activity **Fungus:** Penicillium italicum	**Mobile phase:** 0.1% formic acid **Stationary phase:** Silica gel 60 F_{254} TLC plate **Column:** XBridge C18 column (19 ×150 mm,5 µm) **Wave length:** UV 290 nm **Flow rate:** 1 mL/min.	DART-MS/TLC bioautography	Effective treatment for gout and disorders associated with oxidative stress is provided by E. obtusifolia.
[31]	Rumex crispus L. (R. crispus) Rumex sanguineus L. (R. sanguineus) **Family**: Polygonaceae	**Activity:** Anti-A. baumanni activity Antimicrobial activity **Bacteria:** Gram negative bacteria *Acinetobacter baumannii* *Klebsiella pneumoniae* *Emodin* *E-coli*	**Mobile phase:** Ethyl acetate:toluene:formic acid:water (80:10:5:5 v/v/v/v) **Stationary phases:** Silica gel 60 F254 aluminum plates **Column:** Zorbax Eclipse XDB-C18 column (50 mm×4.6 mm,1.8 µm) **Wave length:** UV 366 nm **Flow rate:** 0.8 ml/min	HPTLC-bioautography/LC-DAD-MS	The application of R. sanguineous and R. crispus extracts for wound healing.
[32]	Phlomis tuberosa (P. tuberosa) **Family**: Lamiaceae	**Activity:** α-glucosidase inhibitory activity	**Mobile phase:** ethyl acetate:methanol:water (15:2:1 v/v/v) **Stationary phase:** Silica gel 60 F254 TLC plates **Column:** Agilent Zorbax SB-C18 column (250 mm× 4.6 mm, 5 µm) **Wave length:** UV 366 nm	SEP BOX/TLC bioautography	P. tuberosa compounds showed much greater α-glucosidase inhibitory capabilities, suggesting that they could be used as substitute medications for diabetes mellitus.

			Flow rate: 1 mL/min		
[33]	Philippine Piper betle Linn (Ethanol extract) **Family:** Piperaceae	**Activities:** Antibacterial activity Antimicrobial activity Gram-positive MDR bacteria: *Methicillin resistant Staphylococcus aureus (MRSA*) and *vancomycin-resistant Enterococcus (VRE)* Gram-negative MDR bacteria: *Carbapenem-resistant Enterobacteriaceae (CRE) Klebsiella pneumoniae* and *metalloid-β-lactamase (MβL)*	**Mobile phase:** ethyl acetate: n-hexane (7 :3 v/v) **Stationary phase:** silica gel 60 F254 TLC plates **Column:**Elite-5 MS capillary column 30mm×0.25 mm, 25 μm **Wave length: for six bands:** UV 254 nm **For eight bands:** UV 366 nm **Flow rate:**1 mL/min	GC-MS/ TLC bioautography	The potential for P. betle to yield innovative therapeutic antibacterial compounds that can treat particular illnesses or ailments. phytochemical examination of P. betle's secondary metabolites, which include saponins, alkaloids, terpenoids, phenolic acids, and flavonoids.
[34]	Onopordon Macrocephalum Or Scotch thistle (Methanolic extract) **Family:** Asteraceae	**Activities:** Antioxidant activity Antimicrobial activity Antibacterial activity **Bacteria:** Gram- positive bacteria *Bacillus cereus Staphylococcus aureus*	**Mobile phase:** ethyl acetate:methanol:water:glacial acetic acid:formic acid (63:4:5:2:1v/v/v) **Stationary phase:** silica gel 60 F254 TLC plates **Wave length:** UV 245nm and 365nm	TLC bioautography	This plant is used in traditional medicine to treat a number of ailments linked to liver disorders and bacterial infections.
[35,36]	Green Tea (Camellia) (Methanol, ethanol & DMSO extracts) **Family**: Theaceae	**Activities:** Antibacterial activity Antioxidant activity **Bacteria:** Gram-positive *cocci S.aureus S. pyogene* Gram-negative *E. coli*	**Mobile phase:** Chloroform:ethyl acetate:acetic acid (50:50:1) **Stationary phase:** Silica gel 60F 264 TLC plate **Column:** Zorbax Eclipse XDB-C18 column (50 mm×4.6 mm,1.8 μm) **Wavelength:** UV 360 nm **Flow rate:**1 mL/min	TLC bioautography	The biologically active substances, including glycosides, alkaloids, phenols, and flavonoids. It has been demonstrated that polar solvents with bioactive components have antioxidant and antibacterial properties.

		S. marcescens			
[37]	Ficus carica Linn (Ethyl Acetate Extract) **Family:** Moraceae	**Activities:** Antibacterial activity **Bacteria:** *Staphylococcus aureus* *Escherichia coli*	**Mobile phase:** chloroform:methanol (7: 3) **Stationary phase:** silica gel F254 TLC plate **Wave length:** UV 254 and 366 nm	TLC-DB	Ethyl acetate has been used in numerous studies on the isolation of antibiotics. This is because ethyl acetate makes it simple to isolate antibiotics, which are typically semipolar.
[38,39]	Justicia wynaadensis (Nees) T.Anders (methanol extract) **Family:** Acanthaceae	**Activities:** Antibacterial activity Antimicrobial activity **Bacteria:** Gram negative bacteria *Klebsiella pneumoniae*	**Mobile phase:** petroleum ether :ethyl acetate (7:3) **Stationary phase:** Silica gel 60F_{254} TLC plate	TLC/GC-MS	The antibacterial properties of J. wynaadensis's methanolic extract against Klebsiella pneumoniae may be ascribed to fatty acids including stearic acid, myristic acid, palmitic acid, and linoleic acid as well as volatile components like phytol.
[40]	Salvia officinalis Linn. (S. officinalis L) (Sage Extract) **Family:** Lamiaceae	**Activities:** Antibacterial activity Antioxidant activity **Bacteria:** *Staphylococcus aureus* *Bacillus subtilis* *methicillin-resistant S. aureus (MRSA)*	**Mobile phase:** chloroform:diethyl ether:methanol (30:10:1 v/v) **Stationary phase:** aluminum-backed TLC Si_{60} F_{254} plates **Column:** Rt-β-DEXm (Restek) capillary column, 30 mm, 0.25mm **Wave length:** UV 365nm **Flow rate:**0.5 mL/min	LC-MS-MS/TLC-DB	Sage extract, a natural antioxidant utilized in the food sector, has excellent antioxidant qualities. Salvigenin and cirsimaritin are two examples of flavonoids that have anti-inflammatory, analgesic, and antioxidant qualities.
[41]	Acacia Senegal (Crude extract) **Family:** Fabaceae	**Activity:** Antioxidant activity Antibacterial activity **Bacteria:** *E-coil* *S.pyogenes* *V. Cholera*	**Mobile phase:** Chloroform **Stationary phase:** Silica gel 60F254 TLC plate	TLC-DB	Phytochemical examination to look for cardiac glycosides, phenols, flavonoids, and saponins.The plant was applied externally to sooth irritated areas and

					was used to treat diabetes.
[42]	Vetiver Zizanoides (ethanol extract) **Family:** Poaceae Phragmites karka. (diethyl ether extract) **Family:** Poaceae	**Activities:** Antimicrobial activity **Bacteria:** Gram positive bacteria *S. aureus* Gram negative bacteria *Salmonella paratyphi* *Klebsiella pneumonie* **Fungus:** *Spergillus niger* *Candidas albicans* **Activities:** Antimicrobial activity	**Mobile phase:** toluene: ethyl acetate (93:7 v/v) **Stationary phase:** pre-coated aluminium silica gel G 25 plates **Column:** Elite-5 MS 30×mm×0.25 mm, 25 μm capillary column **Wave length:** UV 235nm **Flow rate:**1 mL/min	TLC Bioautography	Vetiver zizanoides and Phragmites karka solvent extracts have the potential to be employed as antimicrobial drugs against infectious agents and to treat a variety of infectious disorders.
[43]	Manuka (Leptospermum scoparium) leaf and branch extracts	**Bacteria:** Gram positive bacteria *S. aureus and E. coli*	**Column:** GOLD C18 Column, 100 × 2.1 mm, particle size 1.9 µm Solvent A water/0.1% formic acid, Solvent B acetonitrile/0.1% formic acid Inject volume- 5 µL	TLC Bioautography & HR-ESI-MS, and -MS/MS	TLC-bioautography protocol for assessing the antibacterial ability of Leptospermum scoparium. The minimum effective dose (MED) was observed to be 0.29–0.59 µg/cm2 against S. aureus and 2.34–4.68 µg/cm2 against E. coli. The enhanced protocol demonstrated suitability for both S. aureus and E. coli.

CONCLUSION

In spite of wide employment of sophisticated chromatographic techniques coupled with on-line bioassays, bioautography is still proving its worth as a simple and inexpensive tool for simultaneous chemical-biological screening of natural sources. In other word, it offers the simplest mean of bioassay guided lead discovery from natural products. For the natural product the separation process is not easy, and if separated the amount is very less in maximum cases, so it is necessary to develop a process which can detect lead in a small amount and biological activity can also be measured successively. The standard experimental procedures are required for TLC antioxidant and antimicrobial assays. Some new enzymes should be attempted and adopted on TLC bioautography. The existing TLC methods for enzyme inhibition need more application studies to assess their screening capacity in the discovery of active compounds. The GC-MS or LC-MS approaches have gradually been coupled to TLC bioautography for fast structural characterization of active compounds. Considering these problems,

we can say that bioautographic detection technique would create a new era in separation science.

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