



## BIOAUTOGRAPHY- A MODERN ERA IN CHROMATOGRAPHIC TECHNIQUE

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### ABSTRACT

Bioautography is the technique which includes planar chromatography hyphenated with biological detection method. This is the used to detect to antimicrobial, antioxidant, enzyme inhibition etc. Bio autography is a growth promoting or inhibiting biological activity of the substance. Different technique involved in the Bioautography, they are 1) contact Bioautography 2) direct Bioautography 3) agar overlay method. Normally, chromatographic technique separated small amount of sample there we can't identify the biological activity. But, through the bio autographic technique we can detect the biological substance even in small amount. Therefore Bioautography is called modern era in separation technique.

**Key Words:** Hyphenated Technique, Thin Layer Chromatography, Direct Bioautography, Estrogenic Compounds Etc.

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gradually these lead on to more advanced instrumental techniques .we have seen development in many areas of analytical analysis and particularly the development of chromatographic and spectroscopic methods and the hyphenation techniques. (Martin fitzgerald et al.)Hyphenated techniques: Combination of techniques with modern development in metabolomic analysis and computational pattern recognition program open up a wider scope of application to medicinal plants analysis. Combination of MS with HPLC has proved a productive route to expanding analytical medicinal plant application not only in identification and fingerprinting but also further chemical characterization of individual compounds (Liu et al., 2011) characterized a spectrum of alkaloids components in the Chinese herb ku shen (sophara flavescens). Further combination of MS and NMR with HPTLC has been used to detect the acetyl cholinesterase inhibitors in galbanum in a search for natural product drug candidates (Hamid – reza et al., 2013). Bioautography is one of the hyphenated technique

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### INTRODUCTION

The analysis of medicinal plant had a long history and especially with regard to assessing a plant's quality. The first technique was organoleptic using the physical senses of taste, smell and appearance. Then

which is the combination of planar chromatography and biological detection system.

**BIOAUTOGRAPHY – DEFINITION:** Bioautography is a useful technique to bioactive compound in the plant extraction. Bioautography involves to determine the localization and target directed isolation of active constituents in a mixture. This methodology has been considered as the most efficacious assay for the detection of antimicrobial compounds. (Shahverdi et al., 2007). Currently, this technique extended to the research study of anti-tumour, anti-protzoa, enzyme inhibition, estrogenic compound etc.

#### History:

- 1946 – PC based Bioautography to estimate the purity of penicillin by good all and levy. (R.R goodall et al., 1946)
- 1961 – TLC based bioautography by fishner launter and nicolaus. (R. Fisher et al., 1961)
- 1973 – The first review on Bioautography published by betina. (V. Betina 1973)
- 2012 – 2018 the progression on the TLC Bioautography method for detecting antimicrobial and enzyme inhibitors activities. (Yichao zang et., 2020)

Although, the detection can be successfully improved by the application of advanced chromatographic technique like HPTLC, OPLC, planar electro chromatography. (Saikat dewanjee., 2015)

#### Major Application of Bioautography

Screening of a large number of samples for bioactivity such as

1. Antibacterial
2. Antifungal
3. Antioxidant
4. Enzyme inhibition
5. Estrogenic Activity

#### Factors Influencing the Bioautography:

- The solvent and concentration of sample
- Sample complexity
- Types of absorbent
- Sample application mode
- Resolution of separated compounds
- Test microorganism
- Living condition for test bacteria
- Precondition of sorbents, development techniques and condition of post chromatographic technique
- Time of incubation

#### Evaluation of Biological Activity by following Bioautographic Method:

- 1) Detection of antimicrobial agent  
Contact Bioautography Fig 1

Direct Bioautography  
Agar overlay method Fig 2

- 2) Detection of antioxidant agent  
Using DPPH  
Using ABTs  
Using Beta carotene

- 3) Enzyme inhibition  
Ache inhibitors  
A and  $\beta$  glucosidase inhibitors  
Xanthase inhibitors

- 4) Estrogenic Activity.

#### Detection of antimicrobial agents

##### Contact Bioautography

##### METHOD

- The chromatography is placed face down onto the inoculated agar layer for the specific period to enable diffusion.
- Then the chromatogram is removed and the agar layer is incubated. (E.Mayers et al., 1964)
- The zone of inhibition is the indicative of the antimicrobial substance.
- Incubation time for the growth 16-24 hrs. It can be reduced to 5-6 hrs by spraying 2, 6 dichlorophenol indophenols or 2, 3,5tetrazolinechloride. (A.A.shahat et al., 2008)

The compound separated from the contact bioautography

- ✓ Sphaerococcenol A, a bromoditerpene antibiotic. (S. Caccamese et al., 1989)
- ✓ Monensin, lasolacid, and salinomycin. (vanderkoop et al., 1990)
- ✓ Eight antifungal agents from bark of *bridelia retusa* (L. Jayasingh et al.,)
- ✓ Through HPTLC contact bioautography, (Ramirez et al.,) showed multiple antibiotic residues in cow's milk.

##### Direct Tlc Bioautography

##### METHOD

- The developed TLC plate is with or dipped into a fungal or bacterial suspension
- An inoculum of absorbance is 0.84 at 560nm was suggested for bacteria. (J.J.M. meyeret al.,)
- Suspension of 10 CFU/ml could be employed for both bacteria and fungi. (G. Schmourlo et al.,)
- The plate is incubated at 25 c for 48 hrs.
- Tetrazolium salts are used to visualize the microbial growth. These salts are converted by the dehydrogenases of living organism to intensity coloured formazan. (M.T.G Silva,) these salts are

sprayed onto the bioautogram and re incubated at 25°C for 24 hrs or at 37°C for 3-4 hrs. .

- Clear white zones against purple background on the TLC plate indicate antimicrobial activity. (D.K.B runyoro et al.)

#### Compounds separated by direct bioautography:

- *Pseudomonas cepacia* B37W produces an antifungal compound pyronitrin.
- Condaline – A has been isolated from the bark of *condaline buxifolia*
- Erythromycin

#### Immersion Or Agar Overlay Bioautography METHOD

- It is the combination of contact and direct Bioautography.
- The chromatogram is covered with a molten, seeded agar medium.
- After solidification incubation and staining with tetrazolium dye, the inhibition or growth bands are visualized. (J.B harbore et al.,)

For example gram negative bacteria

An agar solution containing red coloured bacterium *Serratia marcescens*. then zone appear as white, pale or yellow colour on a red background. (L/ Williams et al., 2001)

In colourless microorganism, zones are visualised with aid of a dehydrogenase activity – detecting reagent (tetrazolium salt). Metabolically active micro organism convert into intensely coloured formazan. (L. Williams et al., 2001)

The compound separated by this technique:

- Yeast such as *Candida albicans*.
- Antibacterial activity of isoflavonoids and sesquiterpenoids phytoalexin
- Several antimicrobial compounds were isolated from *tyrosen esculentum* husks, cotyledons and tubers.

#### Detection of antioxidant agents:

##### Bioautography using DPPH method

- The developed chromatogram is sprayed with solution of 0.21 DPPH in methanol/ethanol.
- The plate is examined in daylight after 30 minutes.
- Free radical scavengers appear as cream /yellow spots against a purple background the intensity of the yellow colour can be measured with chromatometer. (Rossi et al.,)

##### Bioautography Using ABTS Method

- The developed chromatogram is stained with ABTS
- The plate is examined for several hours

- Free radical scavengers appear as colourless or pink spots against green background. (Rossi et al.,)

#### Bioautography Using $\beta$ - Carotene (Fig 3)

- Chromatogram sprayed with 90.05 percent solution of  $\beta$ - carotene in chloroform.
- They left in room temperature for decolourization of the background.
- They can be placed under 366nm UV light.
- Orange/yellow spots on white background. ( D.E Pratt, E.E Miller)

These three methods are usually done by DIRECT TLC BIOAUTOGRAPHY METHOD.

#### Enzyme Inhibition:

Enzyme is important molecular target for lead discovery in primary screening assays. The uses of a TLC support for potential plant derived enzyme inhibitors are a regard method which is relatively free of disturbance due to solvent.

#### Acetyl Cholinesterase (AChE) Inhibitors:

##### Detection of Enzyme Inhibitors by Diazotization principle:

Alpha naphthyl acetate concentrate is converted into alpha naphthol by the enzyme. it reacts with fast blue B salt (chromogenic agent) to make purple coloured background on the TLC plates, while AChE inhibitors produce white spots. Enzyme inhibitors block alpha – naphthol formation, hence no purple colour formation.

- Samples are applied on silica gel and migrated
- After complete removal of solvent, acetyl cholinesterase solution sprayed.
- The enzyme is allowed to incubate for 20 minutes at 37°C.
- Then sprayed with a mixture of 1- ninthly acetate and fast blue B salt.
- After 1-2 minutes, AChE inhibitors show white spots on a purple background.

#### $\alpha$ And $\beta$ Glucosidase Inhibition:

It is potentially useful as anti diabetic, anti obesity, anti viral, anti adhesive, antibacterial or anti metastatic agents.

#### METHOD

Test which involves the cleavage of 2- naphthyl – beta – d- glucopyranoside or 2- naphthyl –  $\alpha$ - d- glucopyranoside by respective  $\alpha$ - or  $\beta$ - glucosidase inhibitors. The  $\beta$  naphthol which is formed reacts with fast blue B salt, to give a purple coloured diazo dye. For the detection of the active enzyme, the solutions of 2- naphthyl –  $\alpha$  – d- glucopyranoside (for  $\alpha$  – glucosidase ) or 2 – naphthyl –  $\beta$  – d- glucopyranoside in ethanol and fast blue B salt solution are mixed at a ratio of 1:1 for alpha

glucosidase or 1:4 for beta glucosidase. The mixture is sprayed onto the plate to give a purple colouration within 2-5 min. A TLC assay has previously been established for the detection of beta – glucosidase inhibitors. It is based on the hydrolysis of esculin into esculetin which reacts with FeCl<sub>3</sub> to provide a brown complex. (J. Kissling et al.,)

#### **Xanthine Oxidase Inhibition:**

The enzyme xanthine oxidase(XO)catalyses the oxidation of hypoxanthine and xanthine to uric acid and producing o<sub>2</sub> and h<sub>2</sub>O<sub>2</sub>.the inhibition of xanthine oxidase diminishes oxidative stress and exhibits prophylactic role in inflammation, arteriosclerosis, cancer, aging etc.. to identify XO inhibitors on TLC plates the enzymes is suspended in agar and distributed on the TLC plate. After solidification the plate is immersed in xanthine 38°C for 20 min in the dark. Enzymatic oxidation of xanthine produces o<sub>2</sub> which reduces the pale yellow tetrazolium salt to a formazan and hence a purple background is obtained.this method could determine the nanogram level of XO inhibitors.

#### **Detection Of Estrogenic Compounds By Bioautography**

Estrogenic receptors agonist include natural and synthetic hormones, phytoestrogens and chemicals such as metabolites of alkyl phenol extoxylate, bisphenol A, parabens, benzophenons and phthalates. Muller first observed that an estrogenic compound can be bioautographed using production capacity of beta glucosidase enzyme from yeast cell. This process is called HPTLC – YES procedure. (Fig 4) This is the receptor gene assay which had seen developed for the assessment of the estrogenic potency of individual substance. Here 4 – methyl umbelliferyl β – d- galactopyrnoside (MUG) is used as a flurogenic substrate on which the enzyme βgalactosidase works and converted it into 4- methyl umbelliferone. Chlorophenol red β-d- galactopyrnoside a chromogenic substrate also be used in place of MUG but the sensitivity of the test was the highest in the case of MUG. (E.J. Routledge et al.,)

**Figure 1: “Bioautography is a technique to isolate hit(s)/lead(s) by employing a suitable chromatographic process followed by a biological detection system.” (A marson et al., 1997)**

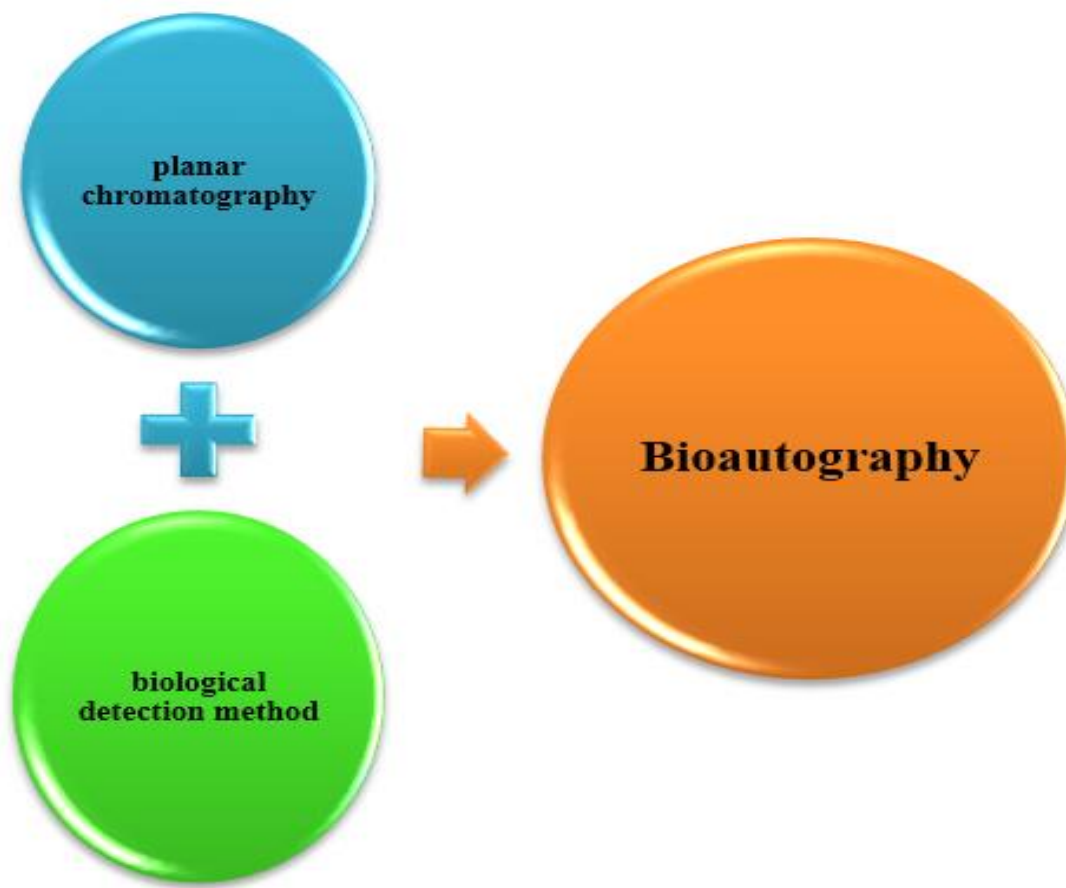


Figure 2: Planar chromatography analysis hyphenated with biological detection method is termed as bioautography. (M.B.muller et al., 2004)(Saikat dewanjee et al.,)

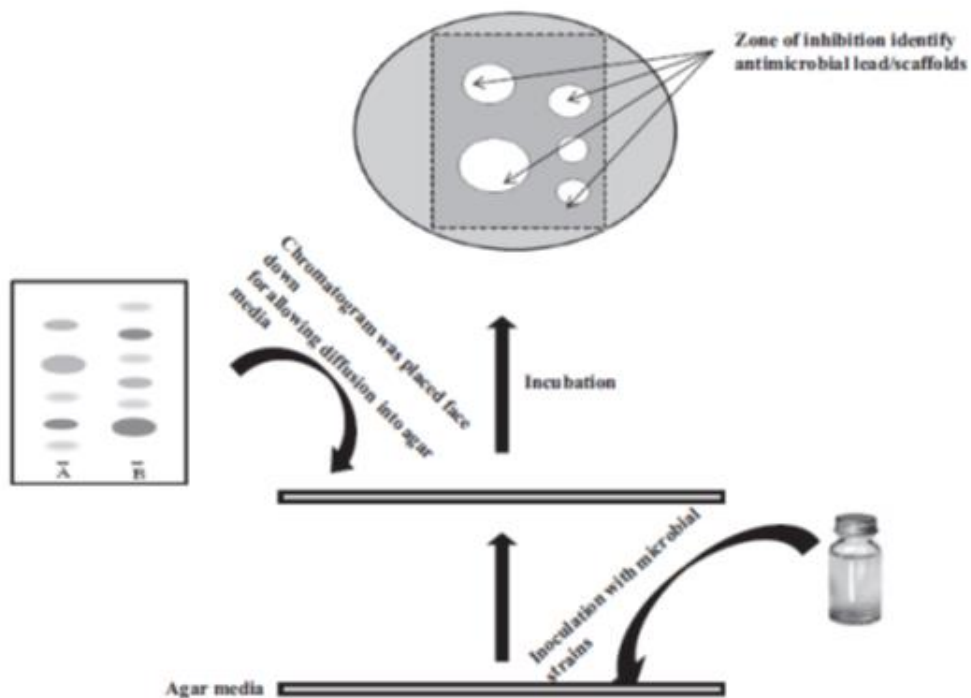


Fig. 1 Schematic overview of contact bioautography.

(Saikat dewanjee et al.,)

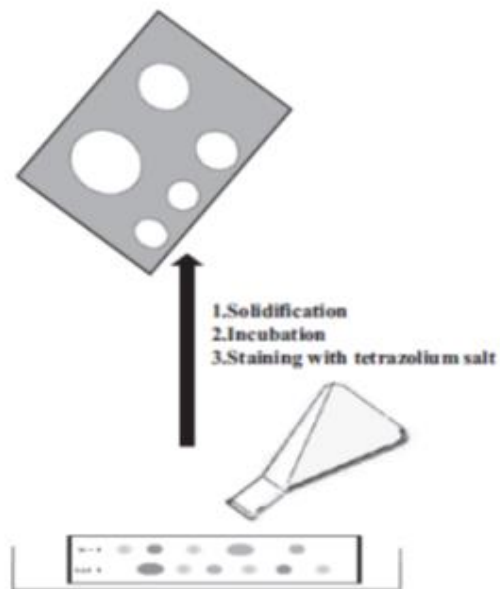
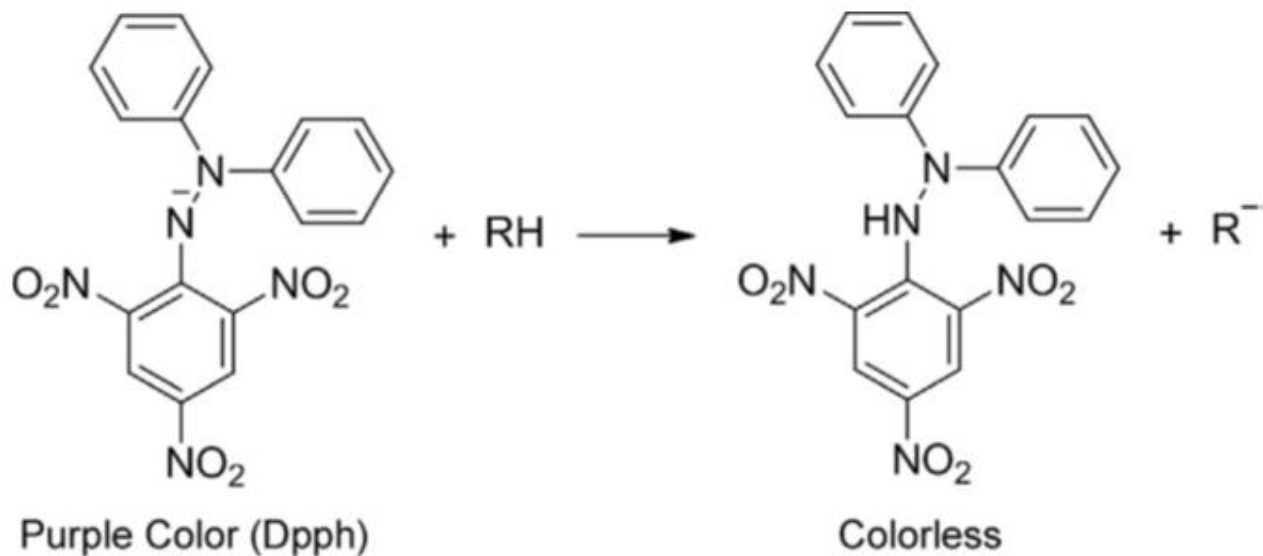


Figure 3: Agar overlay bioautography

(Saikat dewanjee et al.,)



(Saikat dewanjee et al.,)

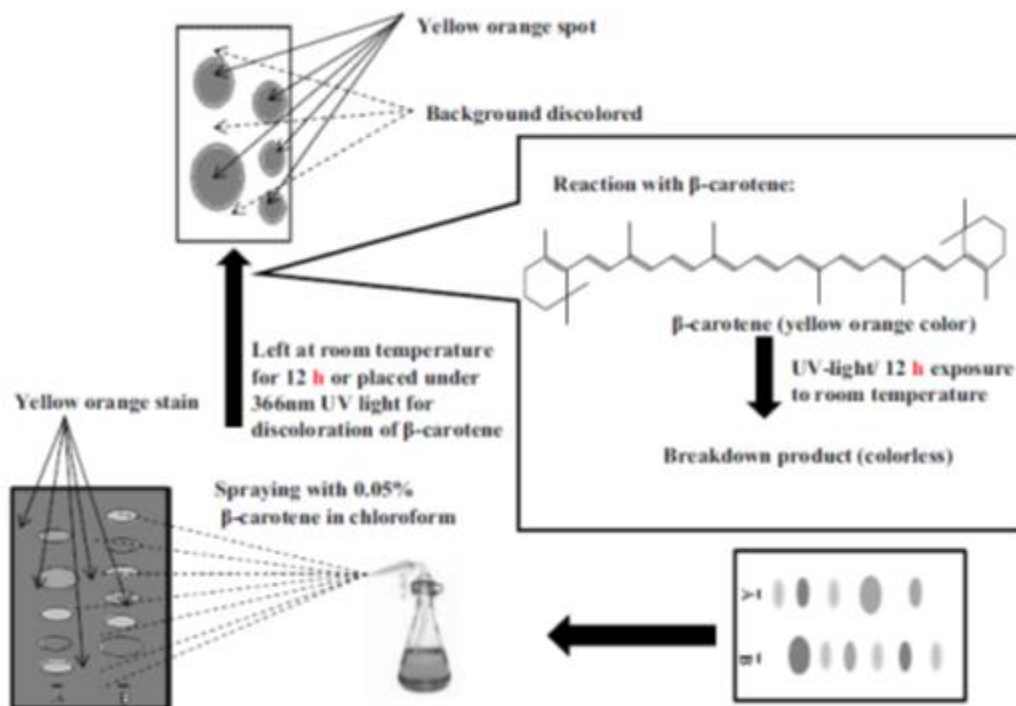
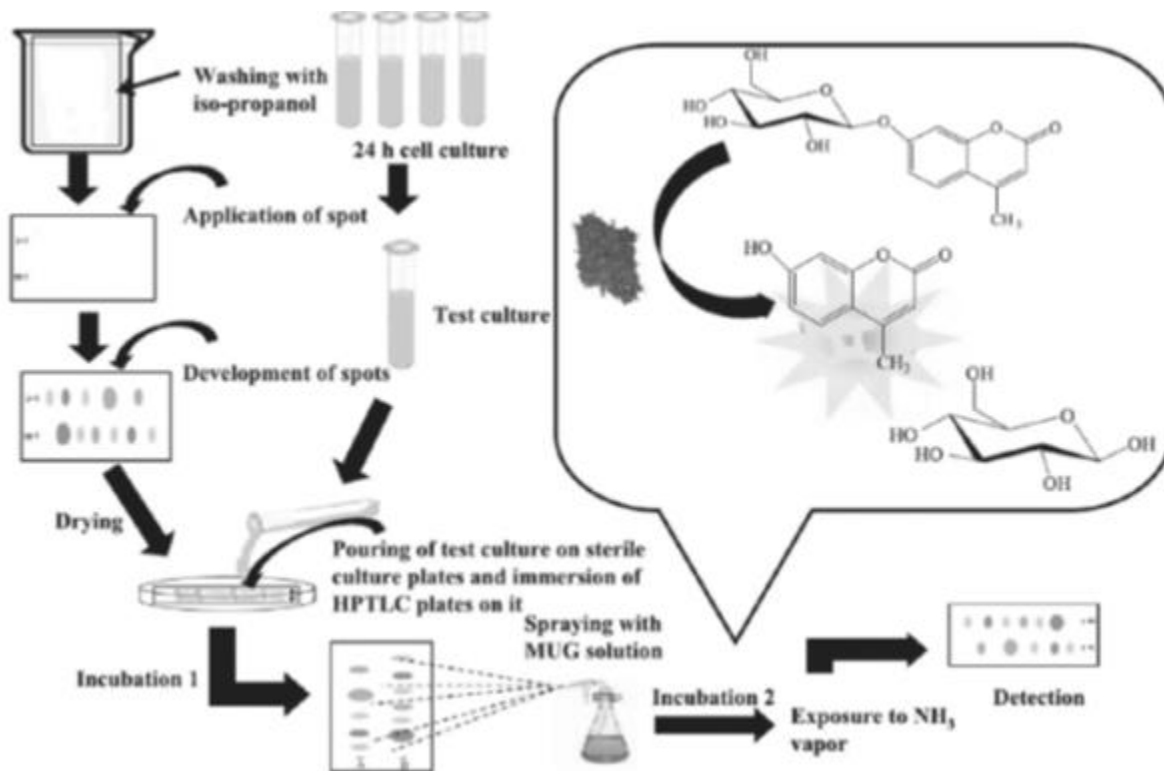


Figure 4: Inhibition of bleaching of beta carotene (Saikat dewanjee et al.,)



**Figure 5: HPTLC – YES Process**  
(Saikat dewanjee et al.,)

#### Advantages over other Chromatographic Technique:

Despite bioautography method is done after chromatographic technique, the plate removed from organic solvent before the process starts, because the organic solvent could damage the bioactive compounds. USESScreening for new antibiotic substance and antifungal, antitumor and anti protozoa compounds studying biological activities of substance originating from plants, microorganism. Investing of antibiotics and biologically active compounds in waste water, drinking water, body fluids, feed and food. Quality control of antibiotics Searching for antimicrobial compounds effective against plant pathogenic bacteria and fungi Detection and determination of toxic compounds.

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#### CONCLUSION:

Bioautography is simple and affordable tool for screening biological activity of natural sources. It is an easiest way of bioassay guided lead discovery from natural products. Chromatography separation process is not easy and the resulted separated product is very less in amount. So it is need to detect lead compound and biological activity in that lesser amount of product. But in bioautography technique we can detect the biological activity even in smaller amount of lead compound. So that, we can say that bioautographic detection technique would create a tremendous change in separation technique.

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