Antimicrobial techniques for natural products .

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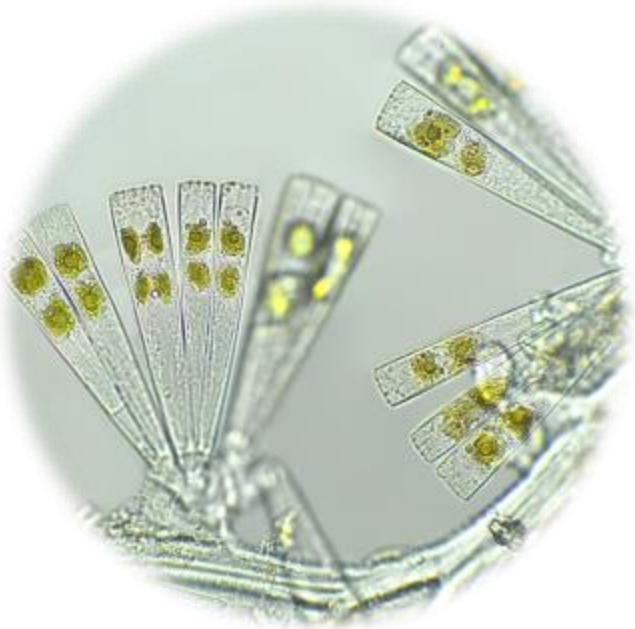
Antimicrobial agent

- Any substance of natural, synthetic or semisynthetic origin which at low concentrations kills or inhibits the growth of bacteria but causes little or no host damage

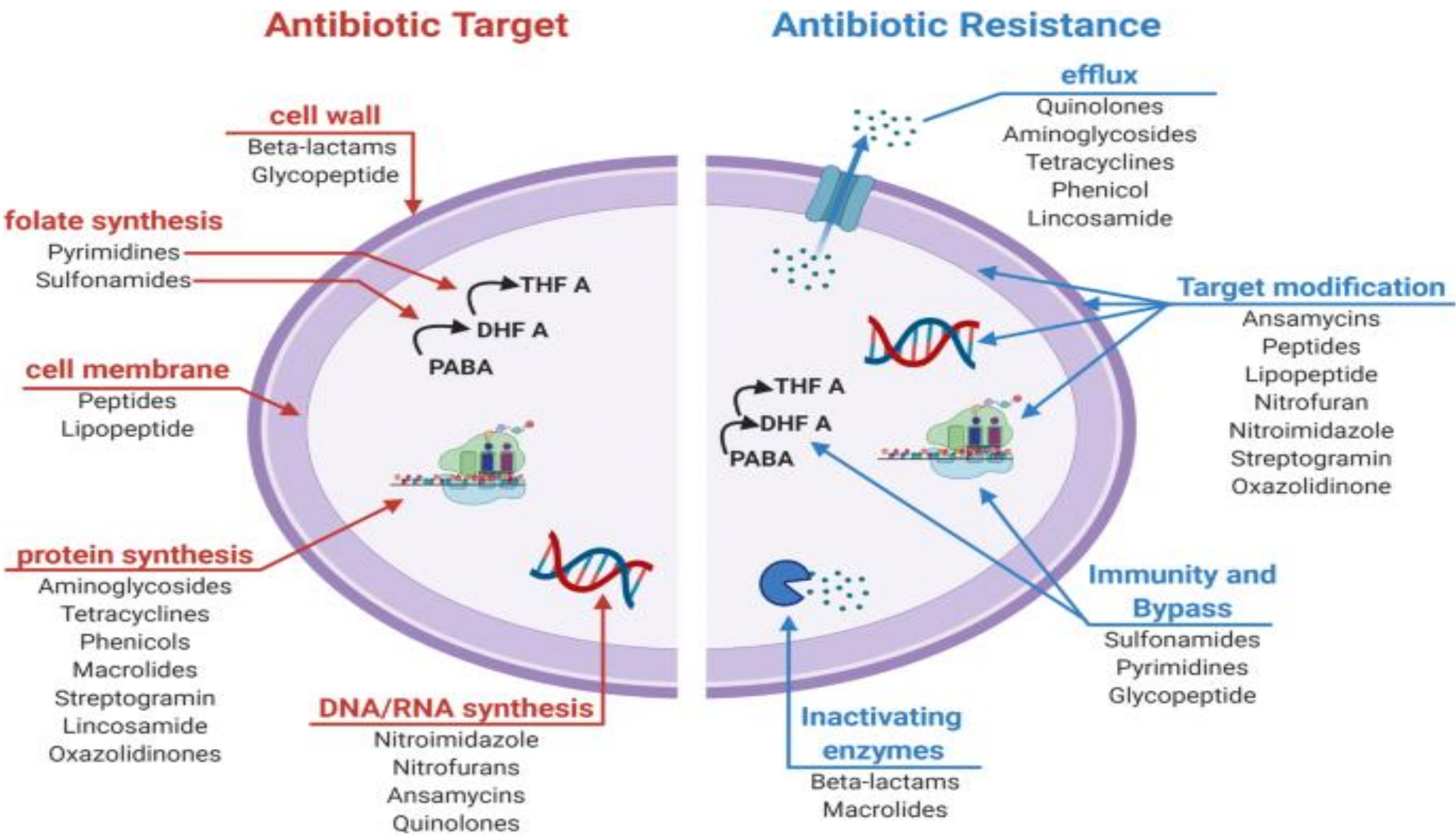


Natural sources of antimicrobial

- **Various types of bacteria such as Streptomyces, Actinomyces, Bacillus**
- **Fungi like penicillium , cephalosporium**
- **Algae**
- **Higher plant**
- **Lower and higher animals.**

Mechanisms of Action of Antimicrobials

- Inhibitors of Cell Wall Biosynthesis
- The components of the cell wall synthesis machinery are appealing antimicrobial targets because of the absence of counterparts in human biology, thereby providing intrinsic target selectivity.
- Inhibitors of Nucleic Acid Metabolism
- Inhibitors of Protein Synthesis


Antibiotic Target
cell wall
Beta-lactams
Glycopeptide
folate synthesis
Pyrimidines
Sulfonamides
THF A
DHF A
PABA
cell membrane
Peptides
Lipopeptide
protein synthesis
Aminoglycosides
Tetracyclines
Phenicols
Macrolides
Streptogramin
Lincosamide
Oxazolidinones
DNA/RNA synthesis
Nitroimidazole
Nitrofurans
Ansamycins
Quinolones
Antibiotic Resistance
efflux
Quinolones
Aminoglycosides
Tetracyclines
Phenicol
Lincosamide
Target modification
Ansamycins
Peptides
Lipopeptide
Nitrofuran
Nitroimidazole
Streptogramin
Oxazolidinone
THF A
DHF A
PABA
Immunity and Bypass
Sulfonamides
Pyrimidines
Glycopeptide
Inactivating enzymes
Beta-lactams
Macrolides

MAJOR GROUPS OF ANTIMICROBIAL COMPOUNDS FROM PLANTS

Useful antimicrobial phytochemicals can be divided into several categories,

- **Simple phenols and phenolic acids.**
- **Quinones.**
- **Flavones, flavonoids, and flavonols.**
- **Tannins**
- **Coumarins**
- **Terpenoids and Essential Oils**
- **Alkaloids**
- **Lectins and Polypeptides**

Class	Subclass	Example(s)	Mechanism	
Phenolics	Simple phenols	Catechol	Substrate deprivation	
		Epicatechin	Membrane disruption	
	Phenolic acids	Cinnamic acid		
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes	
	Flavonoids	Chrysin	Bind to adhesins	
	Flavones		Complex with cell wall	
		Abyssinone	Inactivate enzymes	
			Inhibit HIV reverse transcriptase	
	Flavonols	Totarol	?	
	Tannins	Ellagitannin	Bind to proteins,Enzyme inhibition , Complex with cell wall, Membrane disruption	

Coumarins	Warfarin		26, 9Interaction with eucaryotic DNA (antiviral activity)5, 113, 251	
Terpenoids, essential oils		Capsaicin	Membrane disruption	
Alkaloids		Berberine	Intercalate into cell wall and/or DNA	
		Piperine		
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption	
		Fabatin	Form disulfide bridges	
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	?	

Methods for *in vitro* evaluating antimicrobial activity

What are the main keys we need to know for determining the antimicrobial activity of any substance?

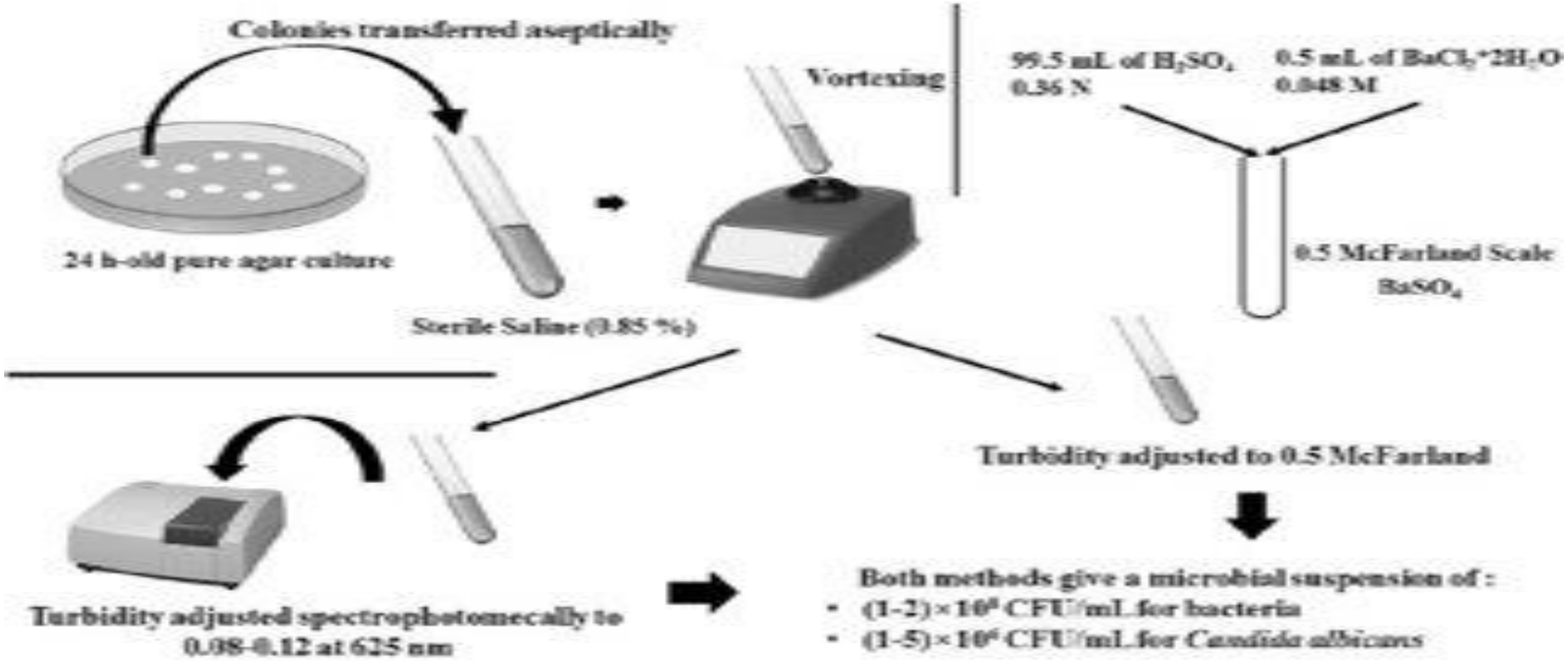
Media (MH, NB) (Mueller–Hinton agar)

- MHA is a microbiological growth medium that is commonly used for antibiotic susceptibility testing, specifically disk diffusion tests. It has a few properties that make it excellent for antibiotic use.
- First, it is a nonselective, nondifferential medium. This means that almost all organisms plated on it will grow.
- Second, it contains starch. Starch is known to absorb toxins released from bacteria, so that they cannot interfere with the antibiotics.
- Third, it is a loose agar. This allows for better diffusion of the antibiotics than most other plates. A better diffusion leads to a truer zone of inhibition.

- McFarland
- Bacterial and fungal strain
- The period of incubation
- Incubation condition
- MIC ,MLC,MBC

McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4)

0.5 McFarland microbial inoculum preparation by the direct colony suspension as recommended by CLSI guidelines



McFarland Standard No.	0.5	1	2	3	4
1.0% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1X10^8 CFU/mL)	1.5	3.0	6.0	9.0	12.0
% transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.08 to 0.1	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

Methods	Microorganism	Growth medium	Final inoculum size	Incubation temp. (°C)	Incubation time (h)
Disk-diffusion method	Bacteria	MHA	(0.5 McFarland) (1–2)×108 CFU/mL	35±2	16–18
	Yeast	MHA+ GMB	(0.5 McFarland) (1–5)×106 CFU/mL	35±2	20–24
	Molds	Non-supplemented MHA	(0.4–5)×106 CFU/mL	–	–
Broth microdilution	Bacteria	MHB	5×105 ×CFU/mL	35±2	20
	Yeast	RPMI 1640	(0.5–2.5)×103 CFU/mL	35	24–48
	Molds	RPMI 1640	(0.4–5)×104 CFU/mL	35	48 for most fungi
Broth macrodilution	Bacteria	MHB	5×105 CFU/mL	35±2	20
	Yeast	RPMI 1640	(0.5–2.5)×103 CFU/mL	35	46–50
	Molds	RPMI 1640	(0.4–5)×104 CFU/mL	35	48 for most fungi
Agar dilution	Bacteria	MHA	104 CFU/spot	35±2	16–20
Time-kill test	Bacteria	MHB	5×105 CFU/mL	35±2	0, 4, 18, and 24

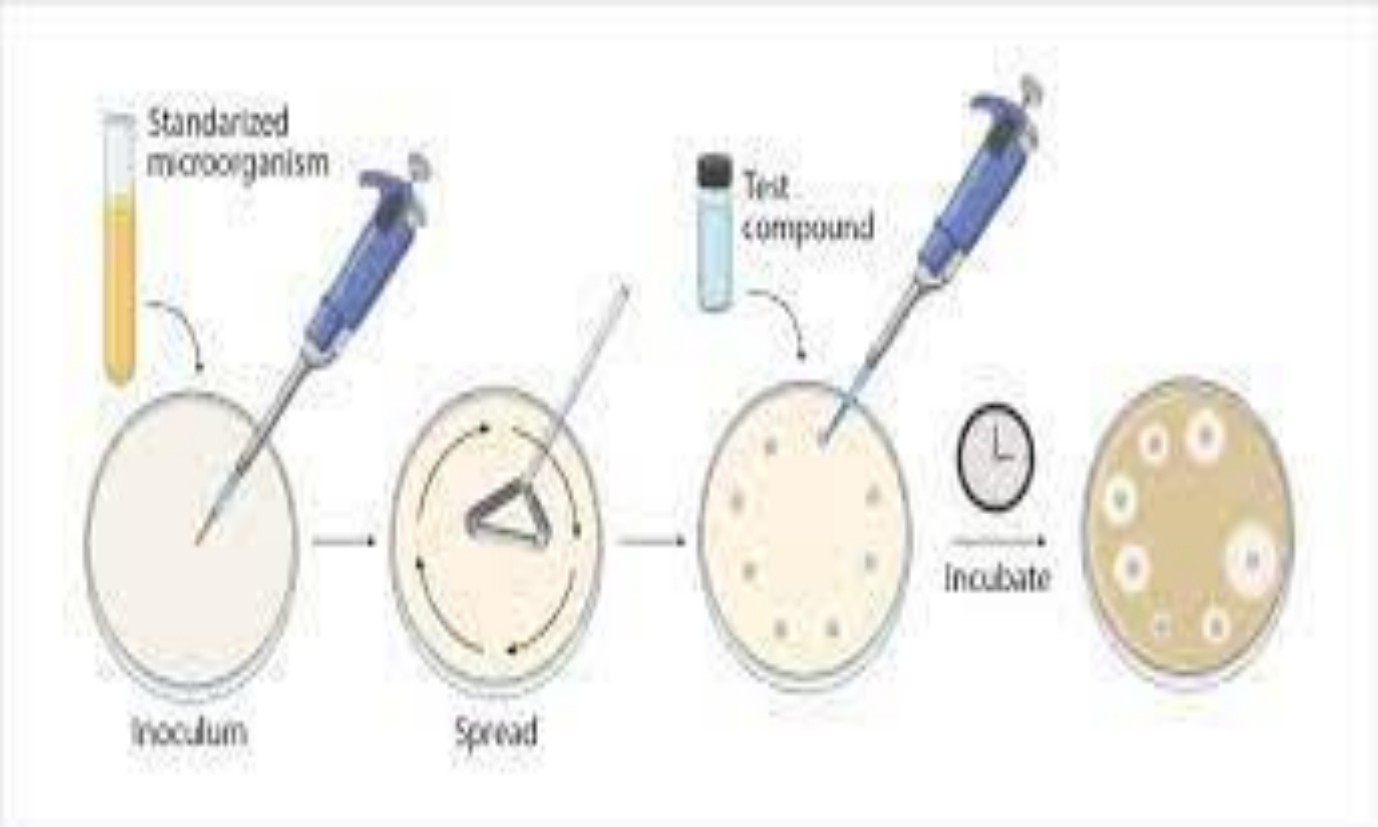
Important factors that affect on antimicrobial activity

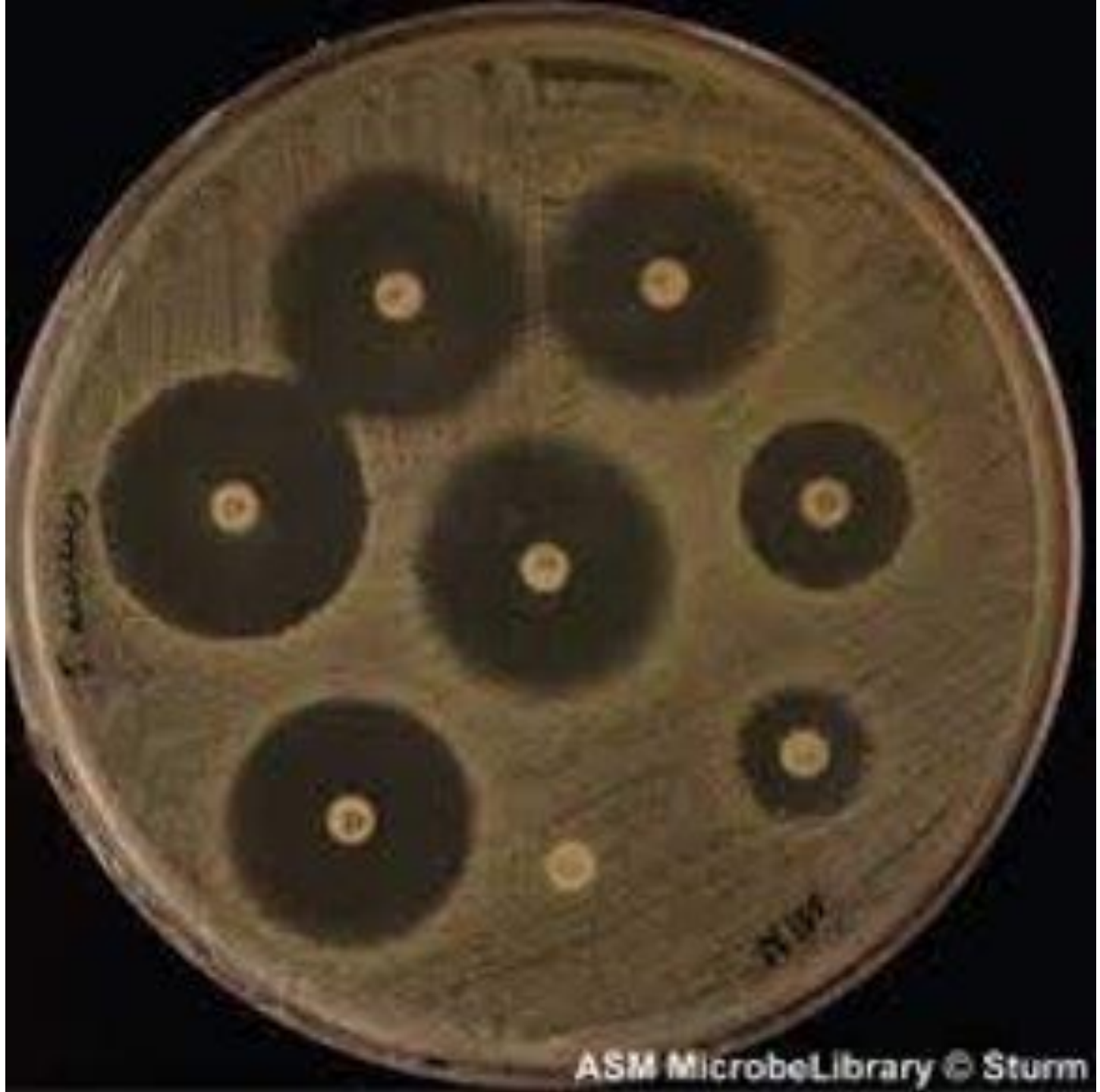
The organism and inoculum used for assays

- Organisms used
- Inoculum size
- **The solvent used for extraction and for dissolving dried extracts in the bioassay**
- **Positive and negative control treatments**
- The average MIC for different extractants that are miscible with water against fungi are DMSO 45%, acetone 51%, ethanol 30% and methanol 32%
- In several thousand assays we have never found any growth inhibition by the 25% acetone present in the first well

1-Diffusion methods

- 1-1- Agar disk-diffusion testing

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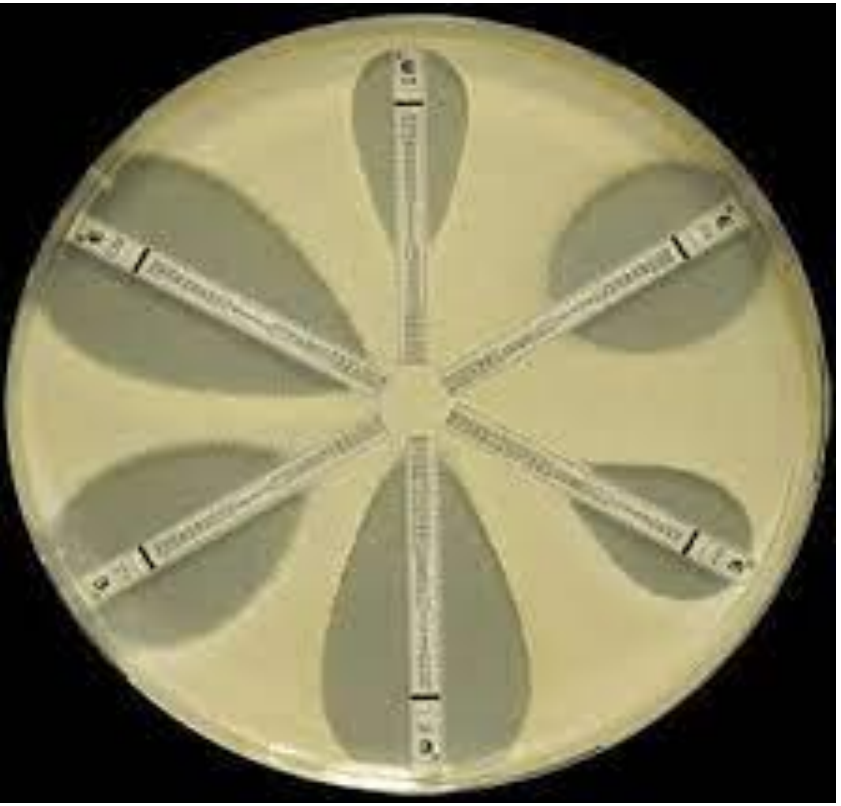
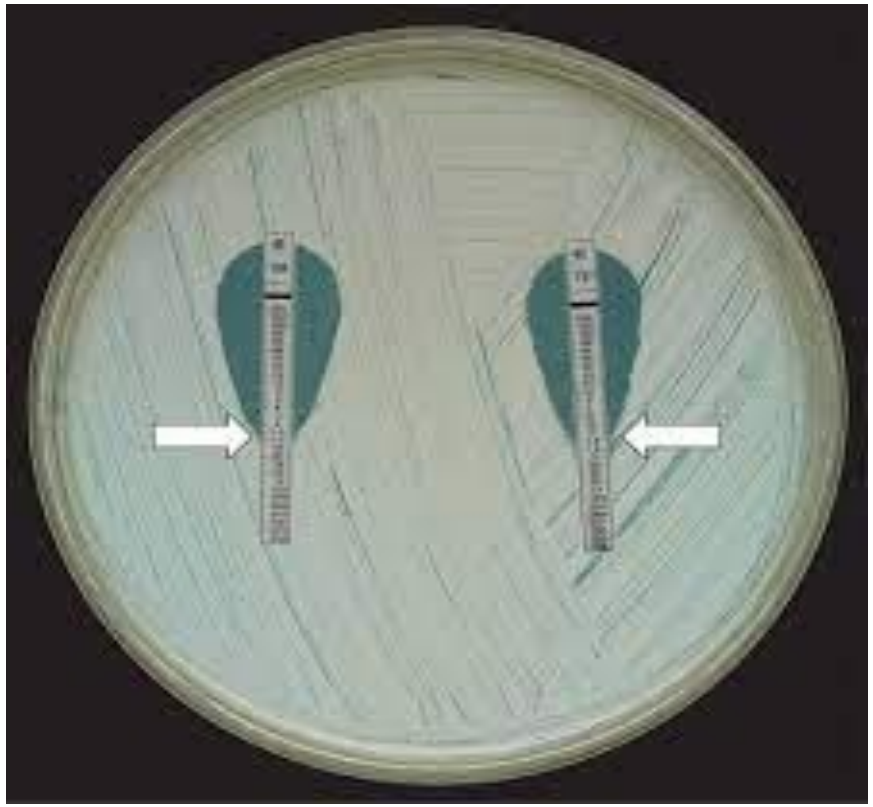
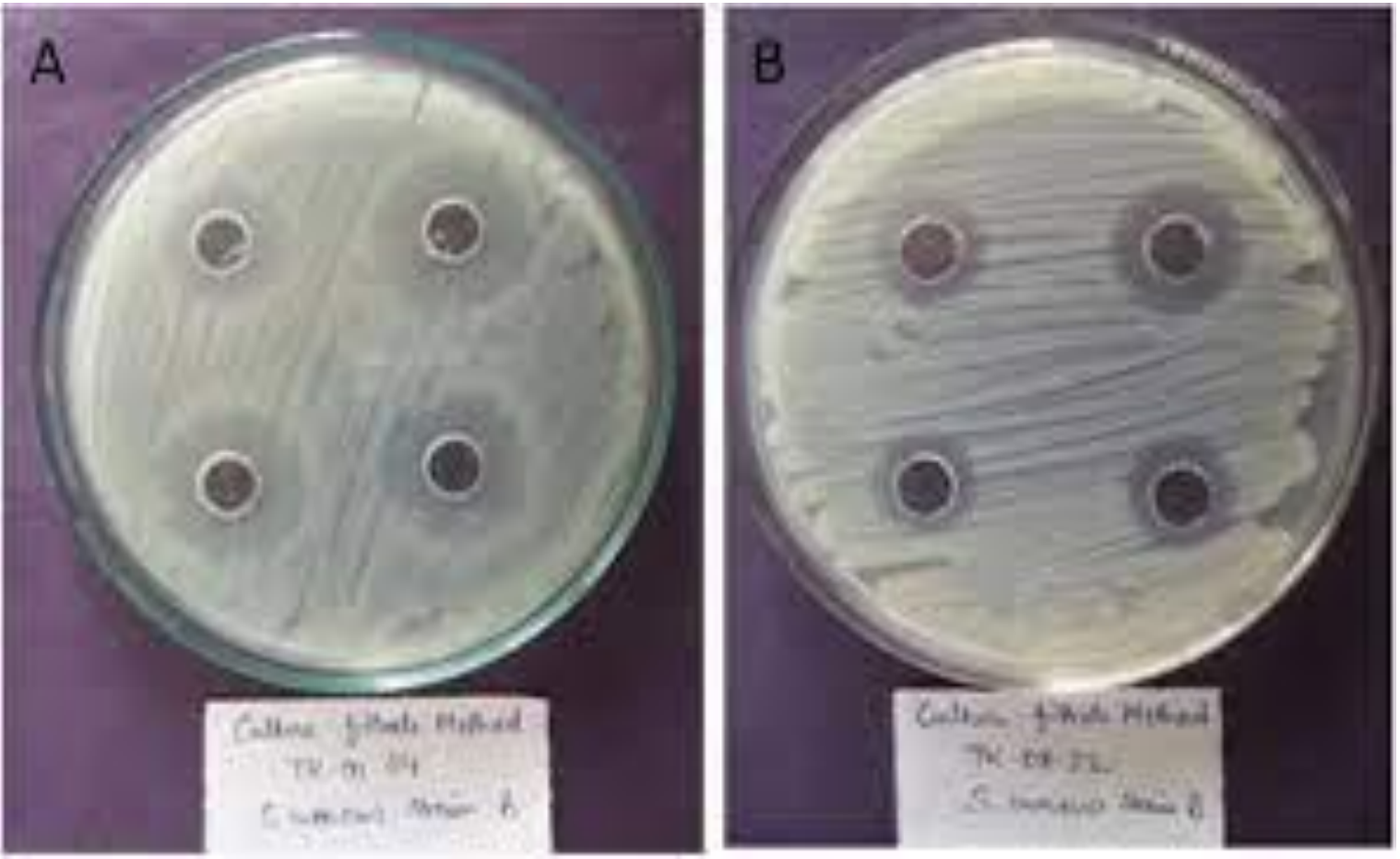
1-2 Antimicrobial gradient method(Etest)

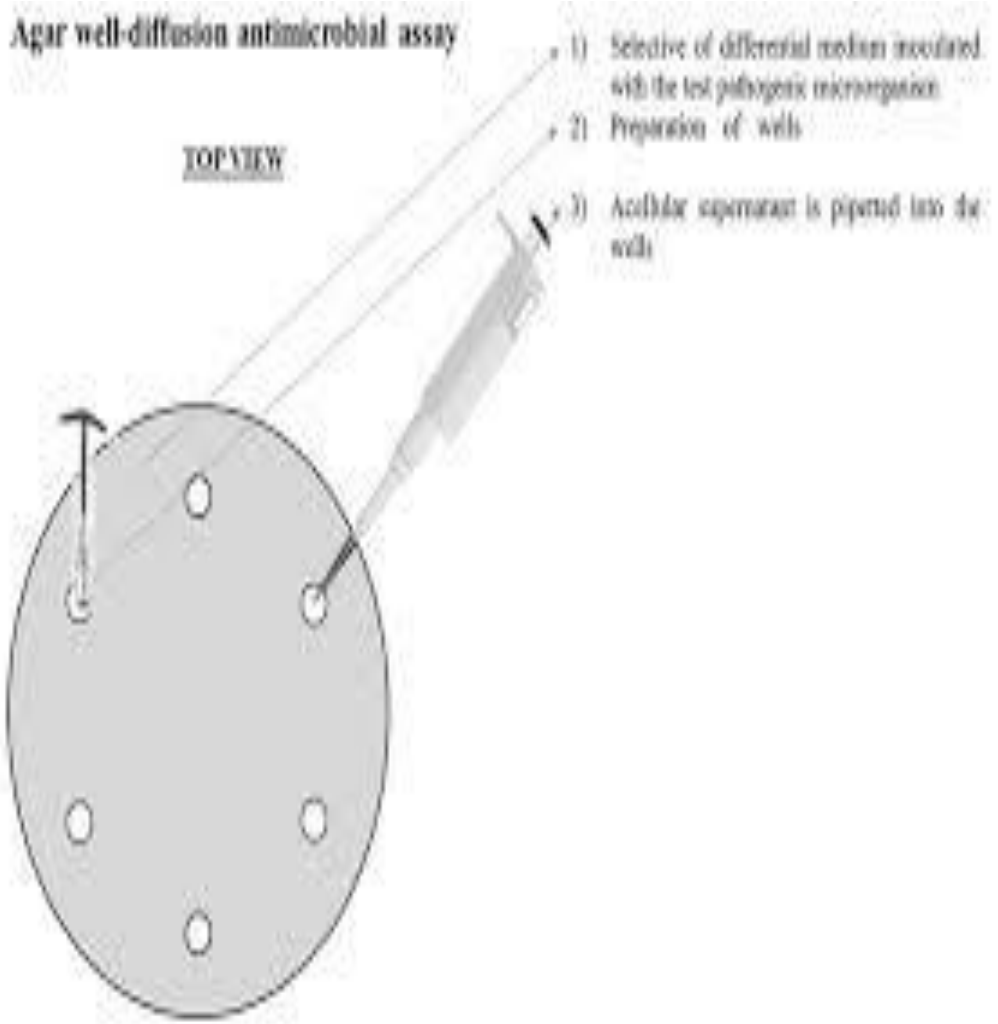




This method is used for the MIC determination of antibiotics, antifungals and antimycobacterials

1-3 Agar well diffusion method



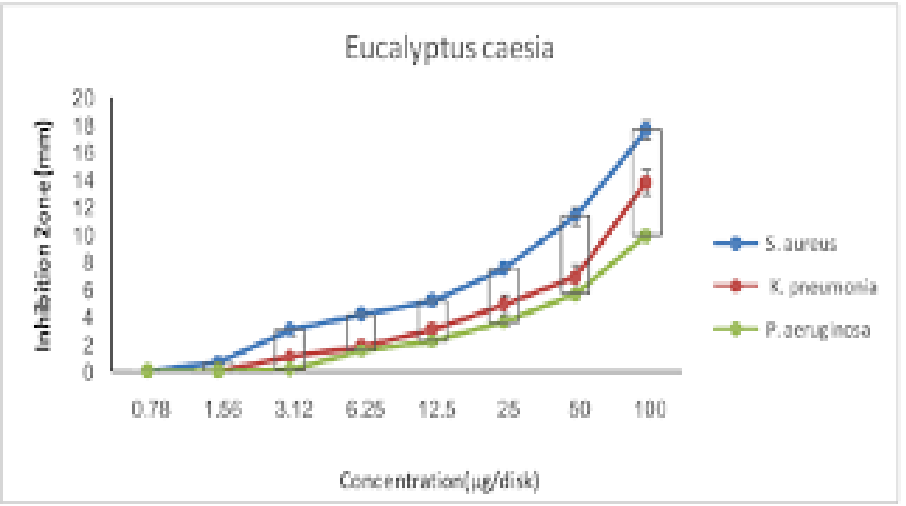


Factors influencing the zone of inhibition

- The concentration of the antibiotic in the test solution.
- The volume of the test solution in the hole or on the paper
- The density of the inoculum.
- The duration and temperature of the diffusion phase before incubation.
- The thickness of the agar medium.
- The composition of the medium
- Incubation temperature”

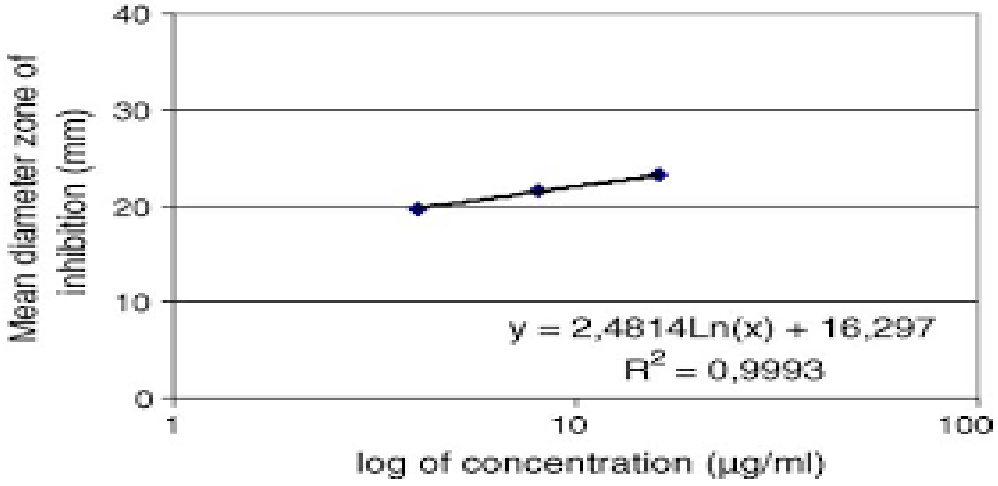
Dose-response relationship

Many scientists accept that there is a linear relationship between antimicrobial activity and the zone of inhibition in agar diffusion studies. In practise over a wide range of doses the square of the zone of inhibition has a linear relationship with the logarithm of the dose



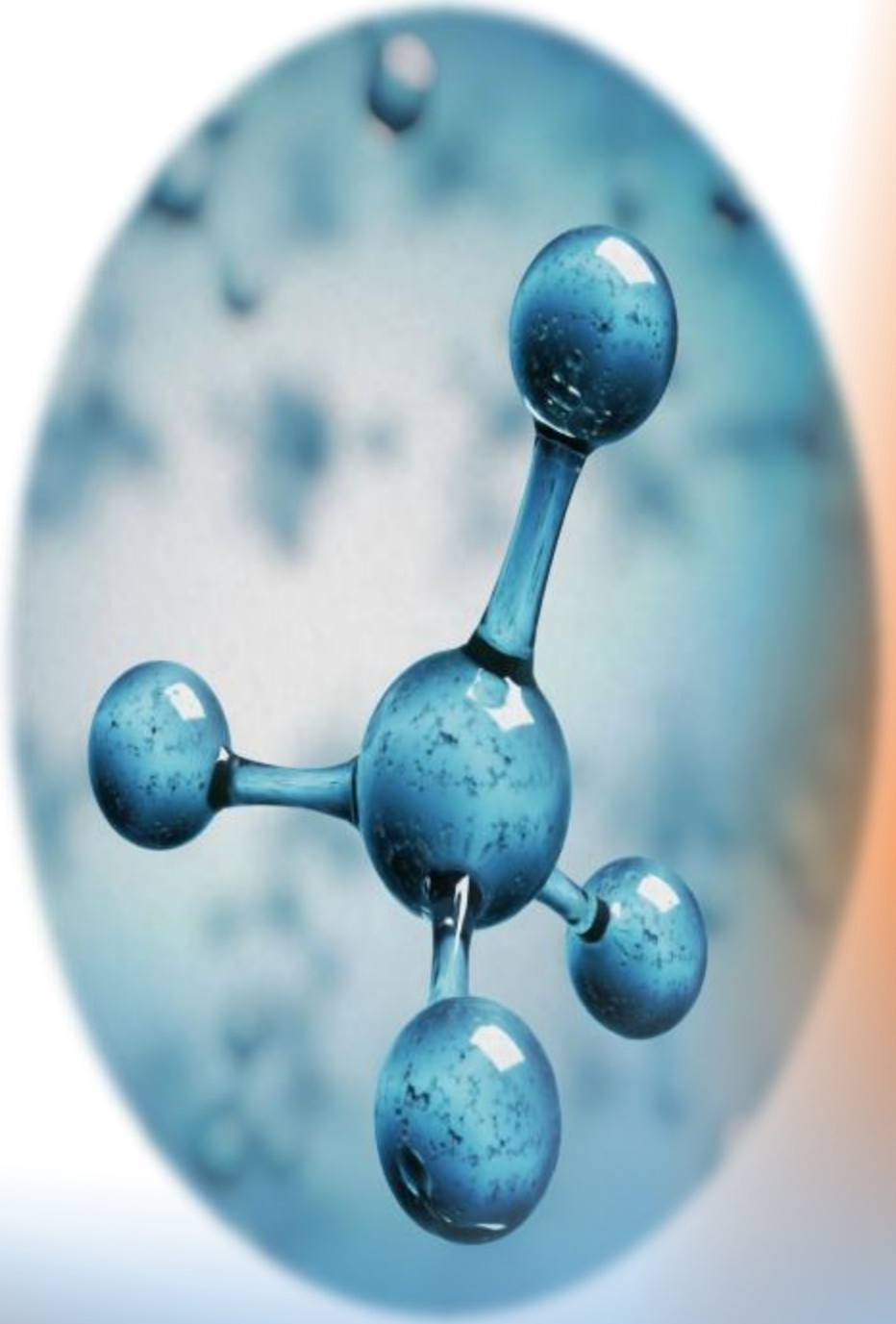
determining the zone of inhibition of a positive control antibiotic and then calculating the relationship

We must use the same concentration of the extracts with the positive control. and calculated the square of the zone of inhibition in assessing activity.



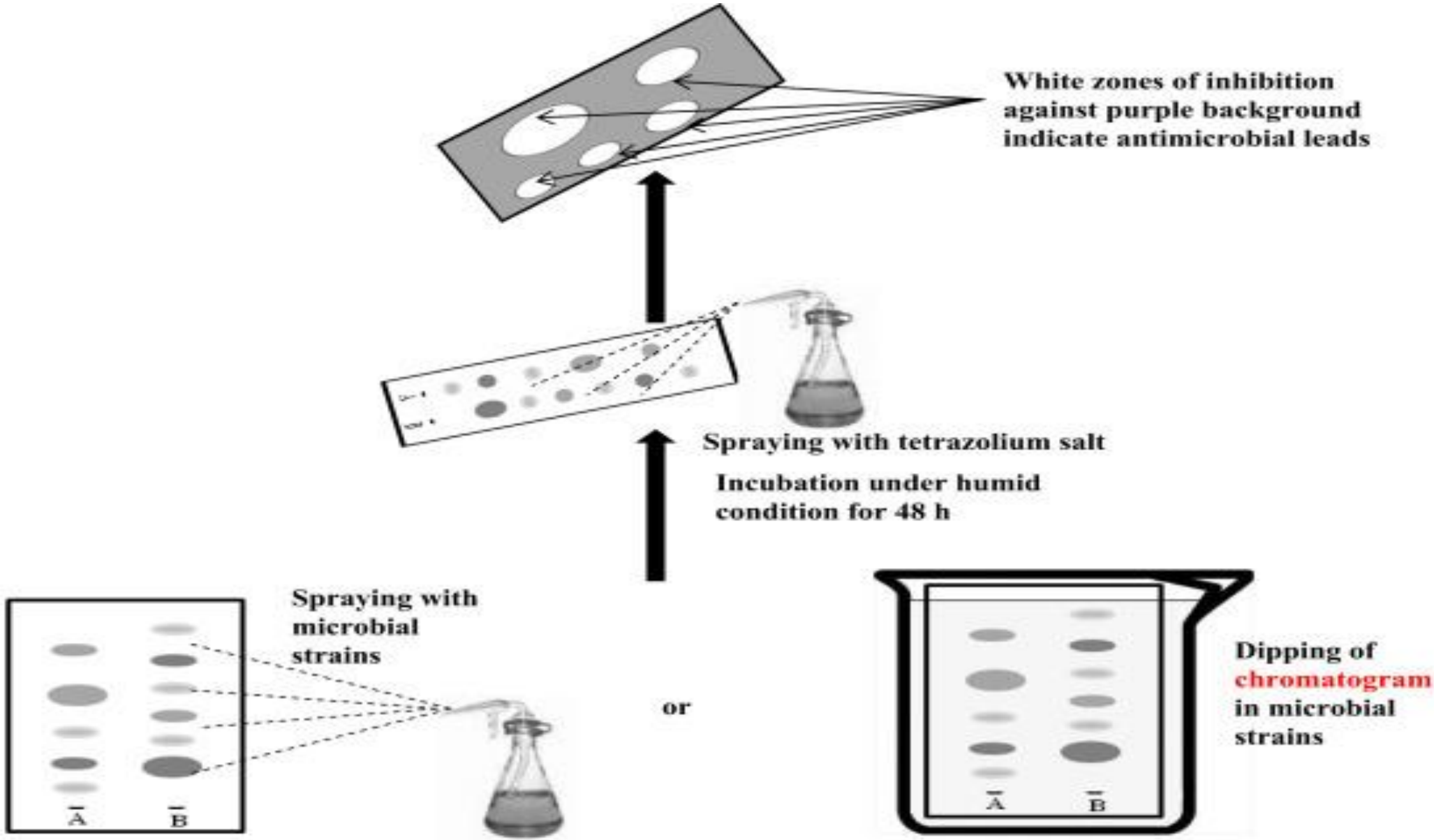
The physico-chemical problem associated with agar-dilution studies

- Because the agar is an aqueous preparation, non-polar compounds will not diffuse as well as polar compounds . In several cases it has been shown that the intermediate polarity compounds have the highest antimicrobial activity and polar extracts such as water does not extract antimicrobial compounds from many plants The agar-diffusion method could be useful with a single compound with a known polarity. Even in such a case if the polarity of the positive control differs much from that of the single compound, comparisons may not be valid.
- Because plant extracts frequently contain several antimicrobial compounds with different polarities the agar-diffusion method is therefore not useful to determine antimicrobial activity



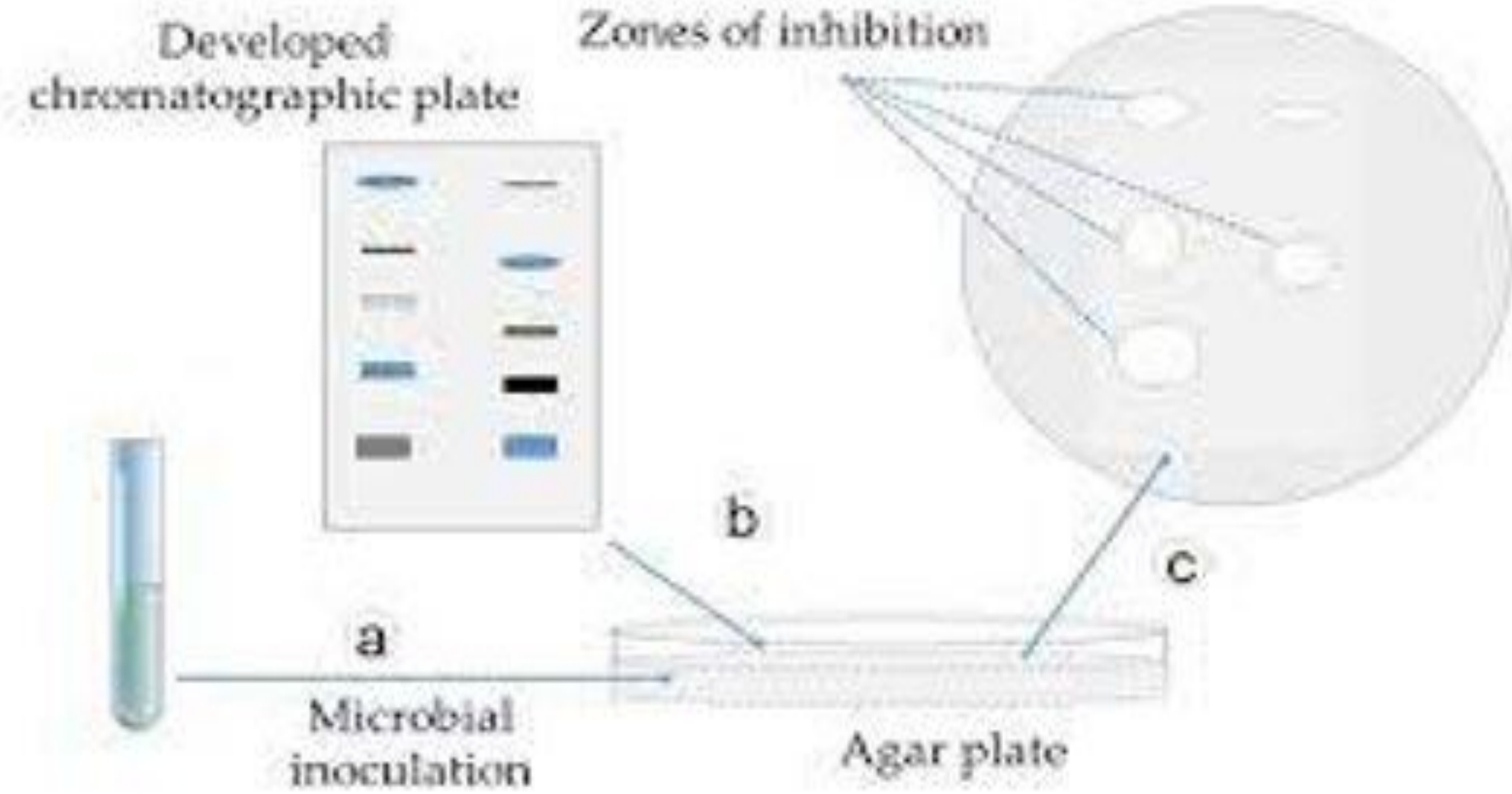
2-Thin-layer chromatography (TLC)–bioautography

- **2-1-Direct bioautography**
- is the most applied method among these three methods
- . The developed TLC plate is dipped into or sprayed with a microbial suspension.
- Then, bioautogram is incubated at 25 °C for 48 h under humid condition .
- For visualization of the microbial growth, tetrazolium salts are frequently used. These salts undergo a conversion to corresponding intensely colored formazan by the dehydrogenases of living cells . p-Iodonitrotetrazolium violet is the most suitable detection reagent.
- These salts are sprayed onto the bioautogram, which is reintubated at 25 °C for 24 h or at 37 °C for 3–4 h
- . The Mueller Hinton Broth supplemented with agar has been recommended to give a medium sufficient fluid to allow a best adherence to the TLC plate and maintain appropriate humidity for bacterial growth


White zones of inhibition against purple background indicate antimicrobial leads
Spraying with tetrazolium salt
Incubation under humid condition for 48 h
Spraying with microbial strains
A
B
or
A
B
Dipping of chromatogram in microbial strains

2-2-Agar diffusion (Also known as agar contact method)

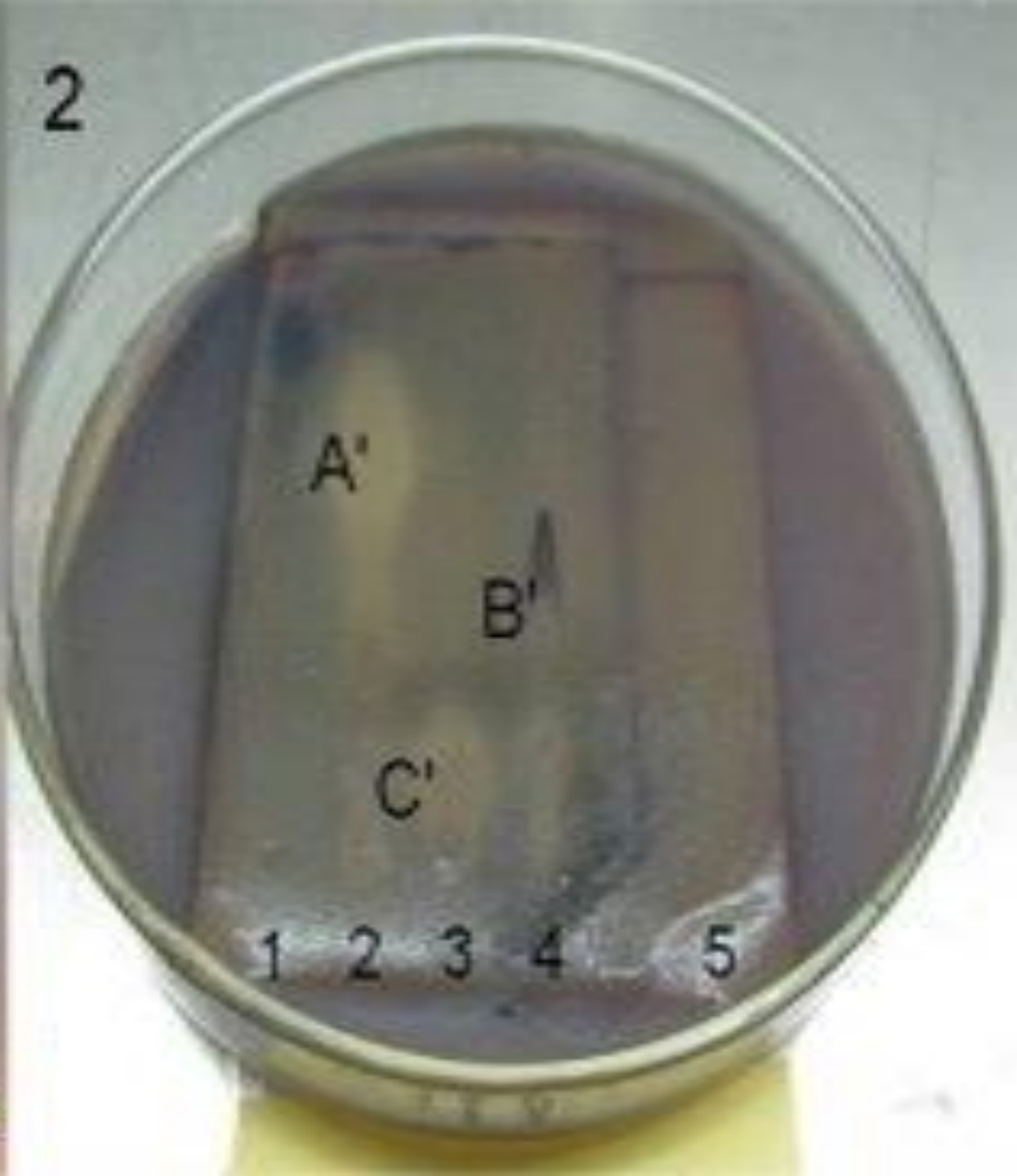
It is the least-employed one of the techniques. It involves the transfer by diffusion of the antimicrobial agent from the chromatogram (PC or TLC) to an agar plate previously inoculated with the microorganism tested. After some minutes or hours to allow diffusion, the chromatogram is removed and the agar plate is incubated. The growth inhibition zones appear in the places, where the antimicrobial compounds contact with the agar layer


Developed
chromatographic plate
Zones of inhibition
b
c
a
Microbial
inoculation
Agar plate

2-3-Agar overlay bioassay

- TLC plate is covered with a molten seeded agar medium
- The plates can be placed at low temperature for few hours before incubation(allow a good diffusion)
- After incubation under suitable conditions depending upon the test microorganism, staining can be made with tetrazolium dye
- Like direct bioautography, this method can be applied to all microorganisms such as *Candida albicans* and molds . It provides well-defined growth inhibition zones and is not sensitive to contamination


1
A
B
C
D
E
1 2 3 4 5


2
A'
B'
C'
1 2 3 4 5

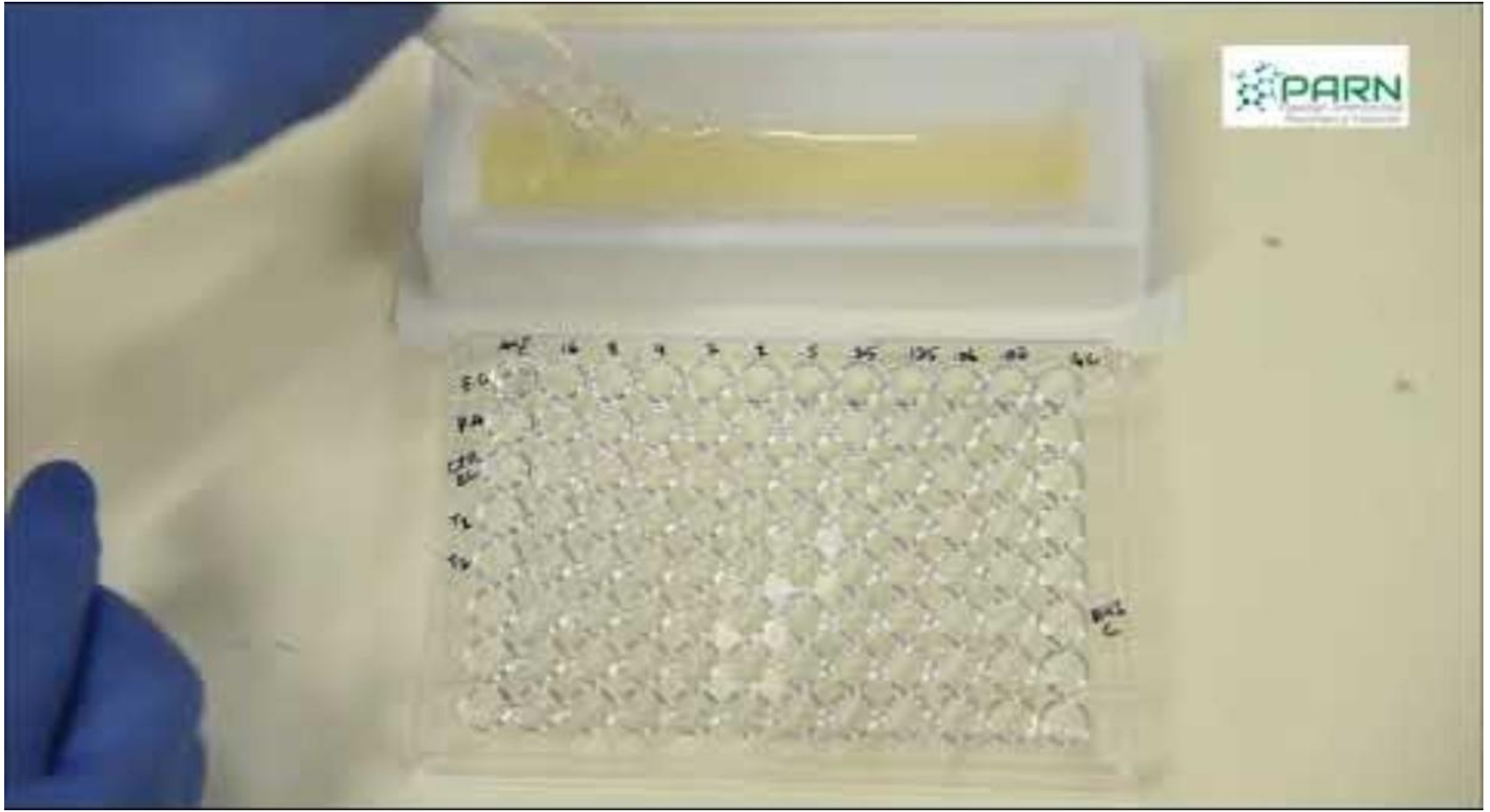
- TLC–bioautography is a simple, effective and inexpensive technique for the separation of a complex mixture, and at the same time, it localizes the active constituents on the TLC plate. Therefore, it can be performed both in sophisticated laboratories and small laboratories which only have access to a minimum of equipment.
- The TLC–bioautography offers a rapid technique for the screening of a large number of samples for bioactivity and in the bioactivity-guided fractionation.
- It can be used for detection of antimicrobials in environmental and food samples as well as for searching for new antimicrobial drugs.

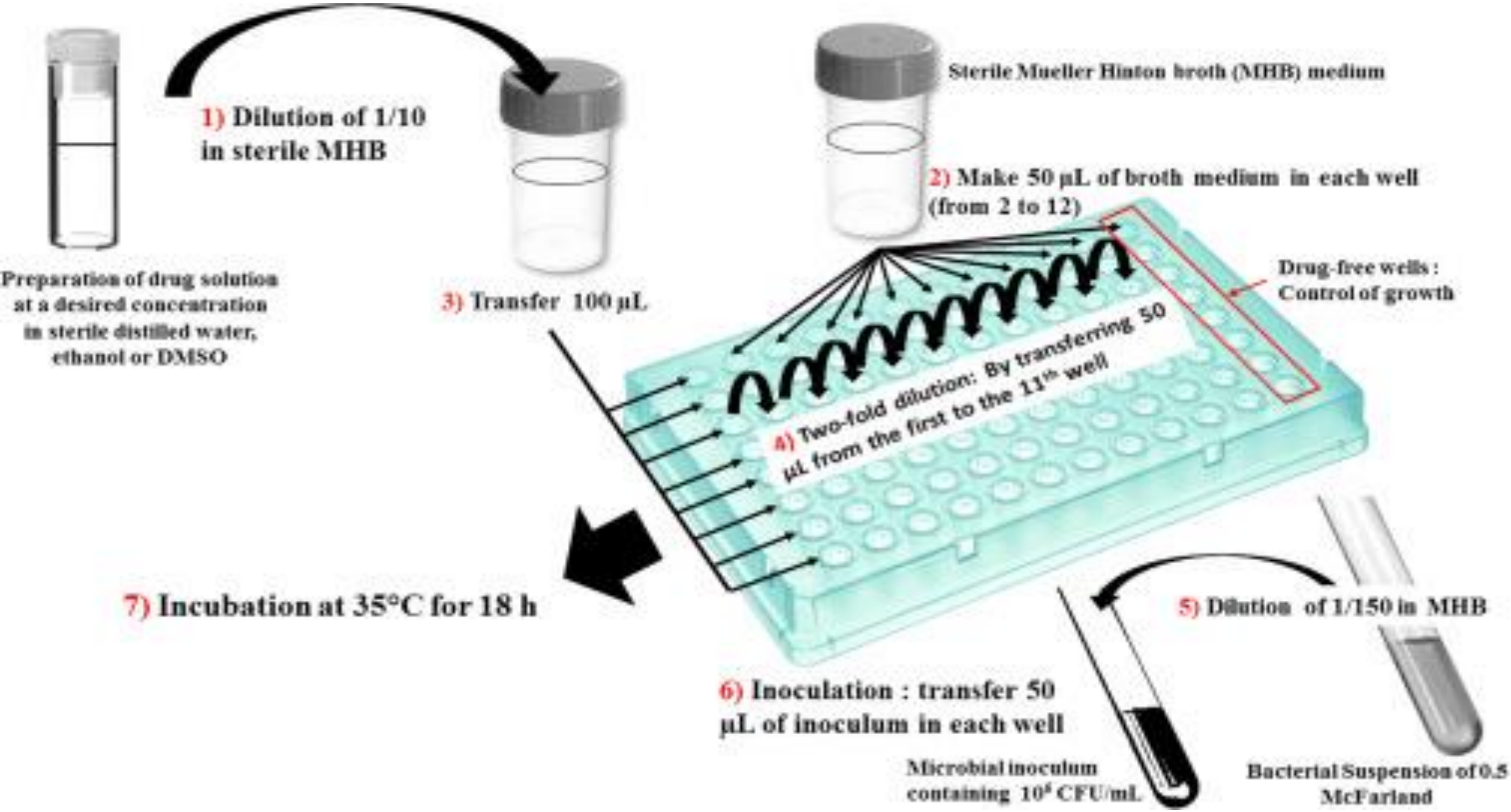
3-Dilution methods

- Dilution methods are the most appropriate ones for the determination of MIC values
- MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in µg/mL or mg/L. There are many approved guidelines for dilution antimicrobial susceptibility testing of fastidious or non-fastidious bacteria and yeast

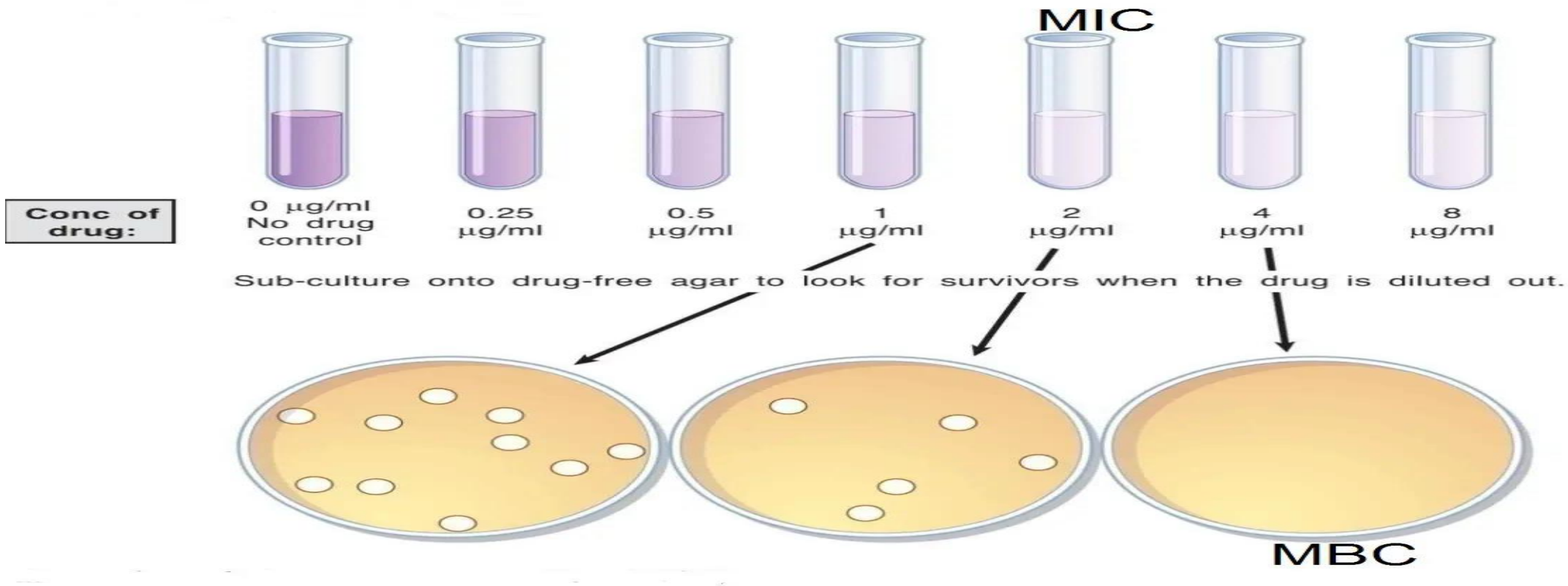
3.1. Broth dilution method

- Broth micro- or macro-dilution is one of the most basic antimicrobial susceptibility testing methods.
- The procedure involves preparing two-fold dilutions of the antimicrobial agent (e.g. 1, 2, 4, 8, 16 and 32 µg/mL) in a liquid growth medium dispensed in tubes containing a minimum volume of 2 mL (macrodilution) or with smaller volumes using 96-well microtitration plate (microdilution)
- Then, each tube or well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland.
- After well-mixing, the inoculated tubes or the 96-well microtitration plate are incubated (mostly without agitation) under suitable conditions depending upon the test microorganism




1) Dilution of 1/10 in sterile MHB
Preparation of drug solution at a desired concentration in sterile distilled water, ethanol or DMSO
Sterile Mueller Hinton broth (MHB) medium
2) Make 50 µL of broth medium in each well (from 2 to 12)
3) Transfer 100 µL
Drug-free wells : Control of growth
4) Two-fold dilution: By transferring 50 µL from the first to the 11th well
5) Dilution of 1/150 in MHB
6) Inoculation : transfer 50 µL of inoculum in each well
Microbial inoculum containing 10⁵ CFU/mL
Bacterial Suspension of 0.5 McFarland
7) Incubation at 35°C for 18 h

3.2. Agar dilution method



4-Time-kill test (time-kill curve)

- It is performed in broth culture medium using three tubes containing a bacterial suspension of 5×10^5 CFU/mL. The first and the second tubes contain the molecule or the extract tested usually at final concentrations of 0.25×MIC and 1×MIC, and the third one is considered as the growth control.
- The incubation is done under suitable conditions for varied time intervals (0, 4, 6, 8, 10, 12 and 24h. Then, the percentage of dead cells is calculated relatively to the growth control by determining the number of living cells (CFU/mL) of each tube using the agar plate count method. Generally, the bactericidal effect is obtained with a lethality percentage of 90% for 6 h, which is equivalent to 99.9% of lethality for 24 h.

Thank you for listen