Resazurin microtitre assay (REMA) for antibacterial and antifungal activity of herbs of three antidiarrhoeal formulations: Bilagyl® and Berbenterone® tablets and Berbenterone® suspension

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CONTENTS

INTRODUCTION

COMPOSITION OF FORMULATIONS

1. BILAGYL LEHYA

Each 10g of BILAGYL contains

1	Bilwaphal (*Aegle marmelos*)	2.222g
2	Sharkara (Sugar)	Q.S

2. BERBENTERONE TABLETS

Each tablet contains

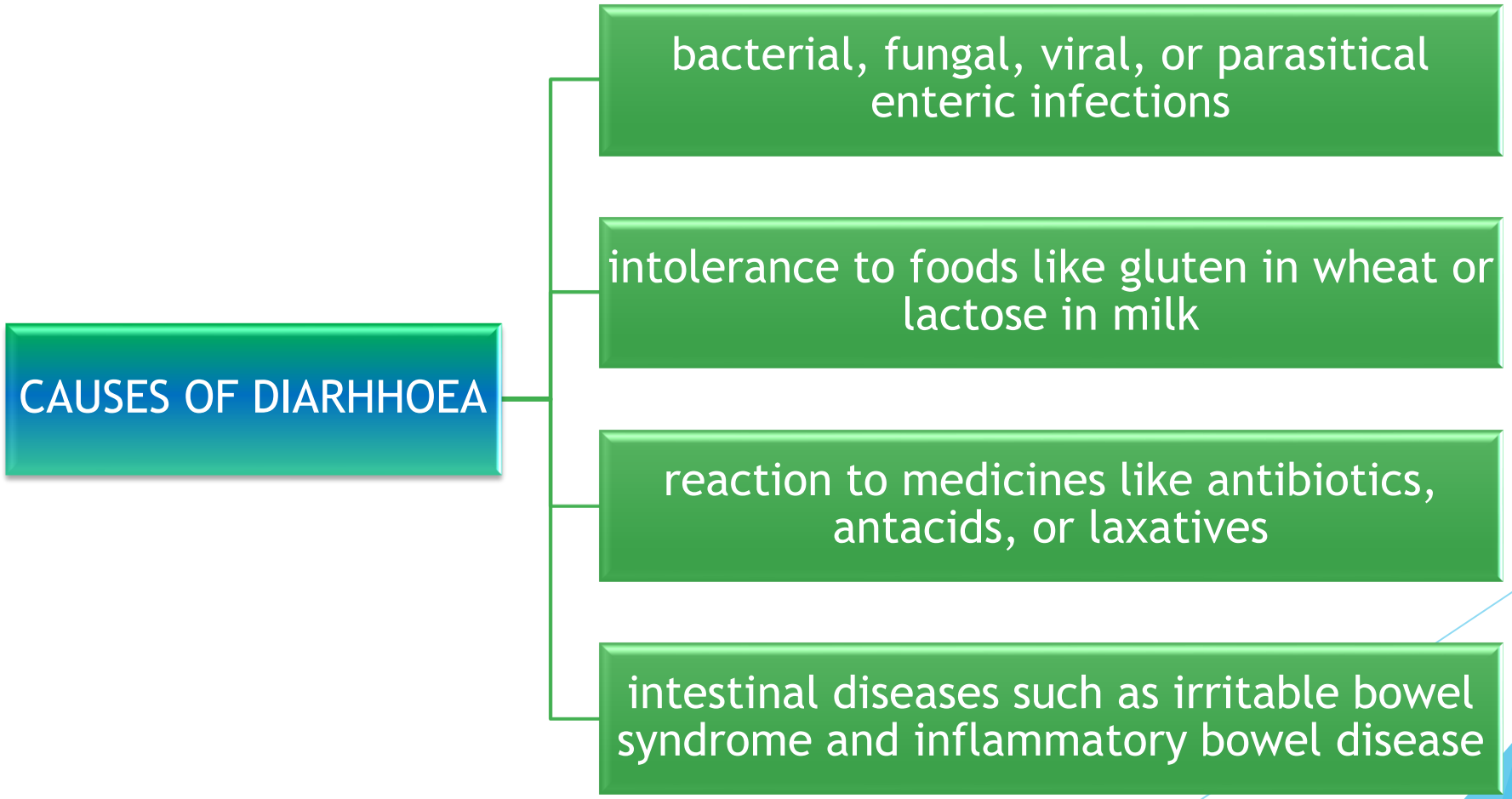
1	Dadimtvak (*Punica granatum*)	125mg
2	Maiphal (*Quercus infectoria*)	125mg
3	Jaiphal (*Myristica fragrans*)	100mg
4	Lavang (*Syzygium aromaticum*)	50mg
5	Kutajchal (*Holarrhena antidysenterica*)	1000mg
6	Daruharidra (*Berberis aristata*)	875mg

3. BERBENTERONE PAEDIATRIC SUSPENSION

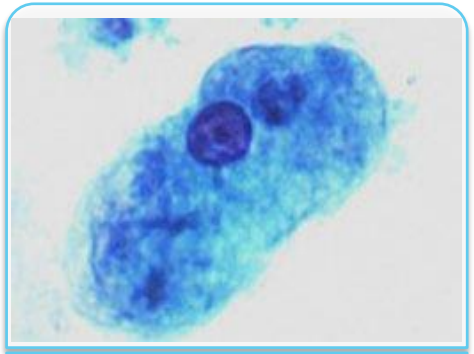
Each 5 mL of suspension contains

1	Daruharidra (*Berberis aristata*)	666mg
2	Kutajchal (*Holarrhena antidysenterica*)	666mg
3	Ativisha (*Aconitum heterophyllum*)	333mg
4	Nagarmotha (*Cyperus rotundus*)	333mg
5	Dadimtvak (*Punica granatum*)	333mg
6	Vidang (*Embelia ribes*)	1333mg
7	Sharkara (Sugar)	Q.S.

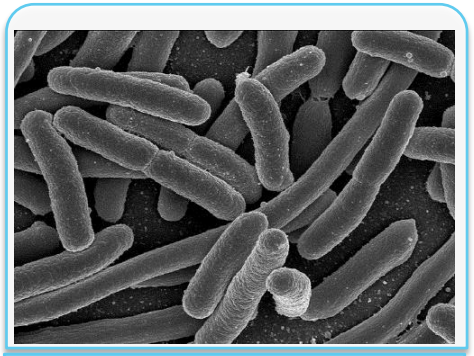
- Diarrhoea and dysentery - leading cause of mortality and morbidity in developing countries
- Diarrhoea - increase in the motility and imbalance in the absorption and secretion properties of the gastrointestinal tract.
- Dysentery is inflammation of intestine causing diarrhea with blood.



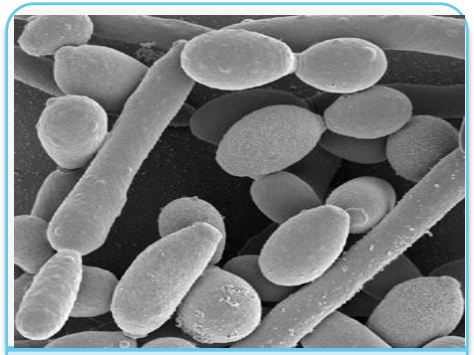
CAUSATIVE AGENTS OF INFECTIOUS DIARRHOEA



Entamoeba histolytica



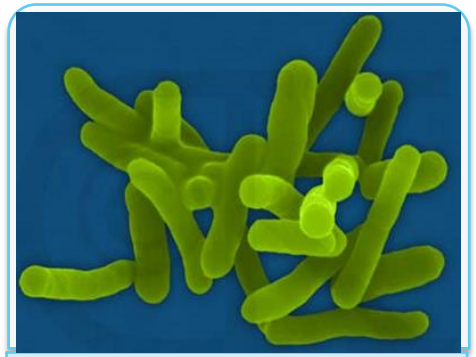
Escherichia coli



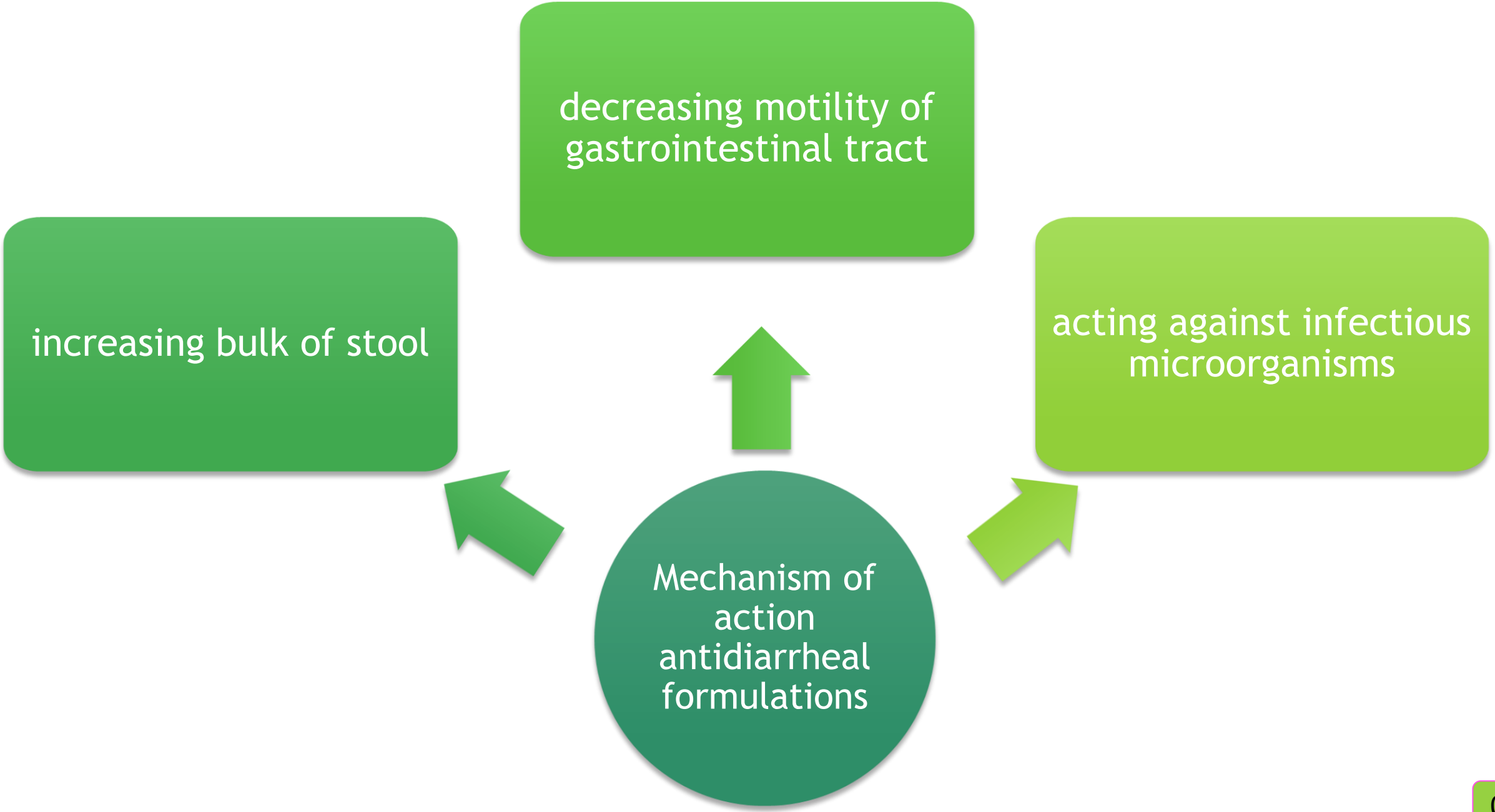
Candida albicans

Staphylococcus aureus

Salmonella typhi



Shigella flexneri


decreasing motility of gastrointestinal tract
increasing bulk of stool
acting against infectious microorganisms
Mechanism of action antidiarrheal formulations

IN VITRO ANTIMICROBIAL ANALYSIS

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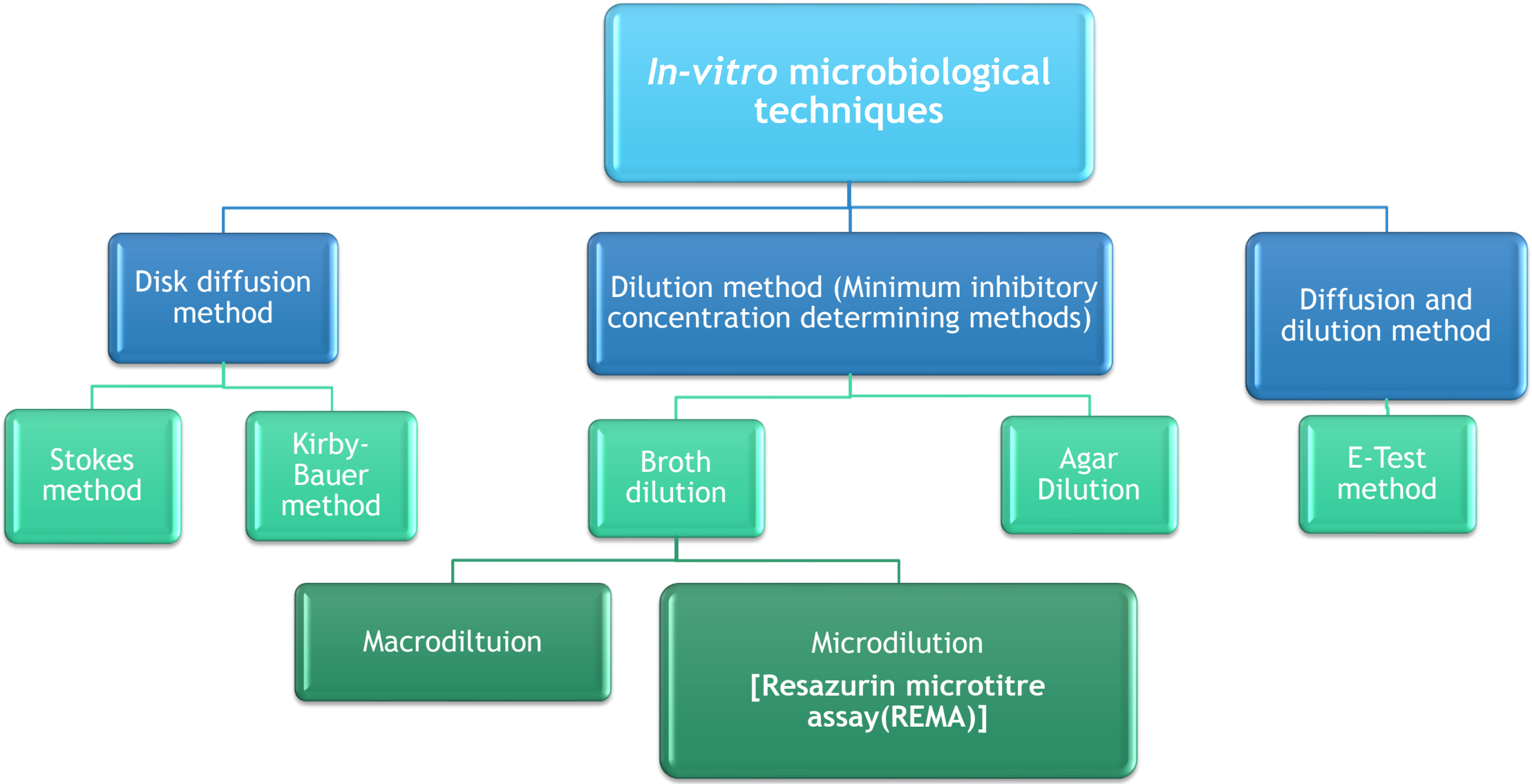
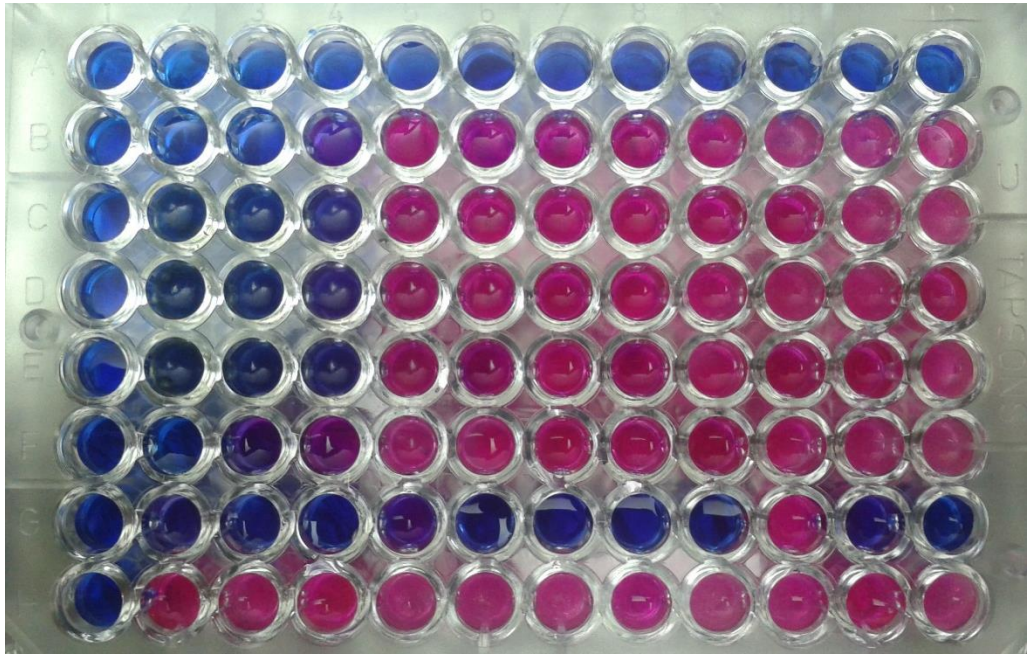
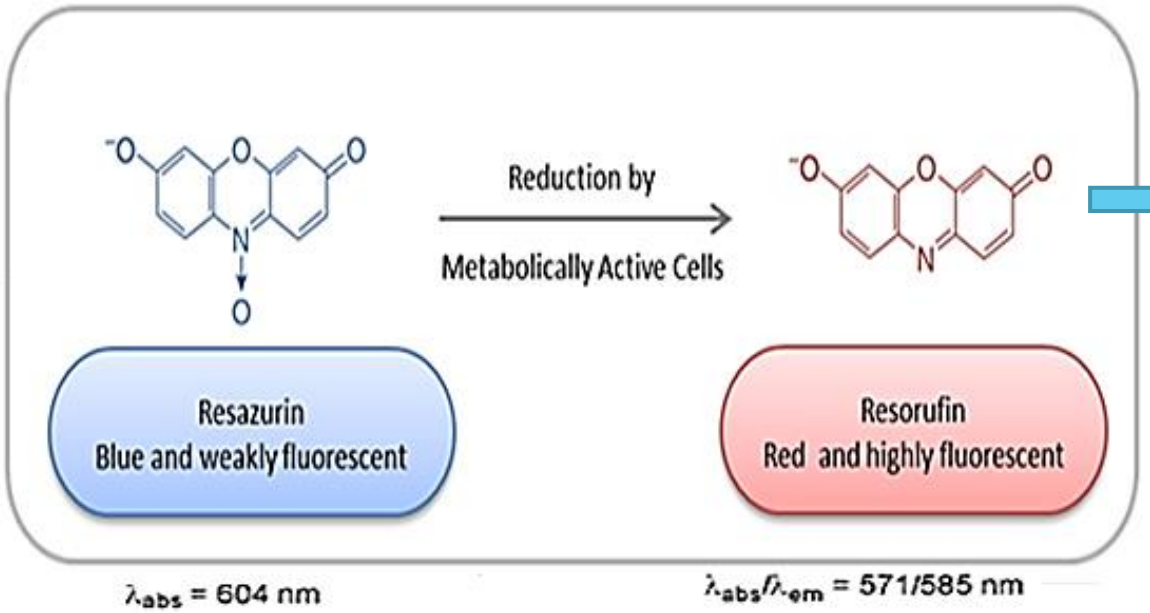


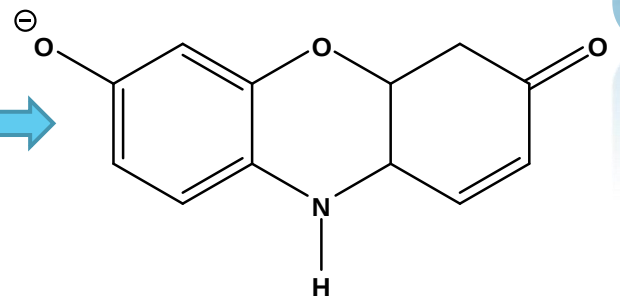


RESAZURIN MICROTITRE ASSAY (REMA)

RESAZURIN MICROTITRE ASSAY (REMA)

- PRINCIPLE:





Hydroresorufin (Colourless)

If over incubation is done

- **Resazurin** (7-Hydroxy-3-*H*-phenoxazin-3-one-10-oxide) is a blue dye
- It is weakly fluorescent irreversibly reduced to and highly red fluorescent **Resorufin**
- Used as an oxidation-reduction indicator in **cell viability assays**

ADVANTAGES OF REMA

- Resazurin is water soluble, non-toxic and very much stable in culture media, and also relatively inexpensive.
- Testing of non-polar samples or samples that do not easily diffuse into agar can be done.
- Visual detection is easier and determination of MICs can be done accurately.
- To obtain colour in other colorimetric assays, cells are broken down by using organic acids while in REMA colour change is direct.
- High-throughput screening can be done.

IMPORTANT ASPECTS

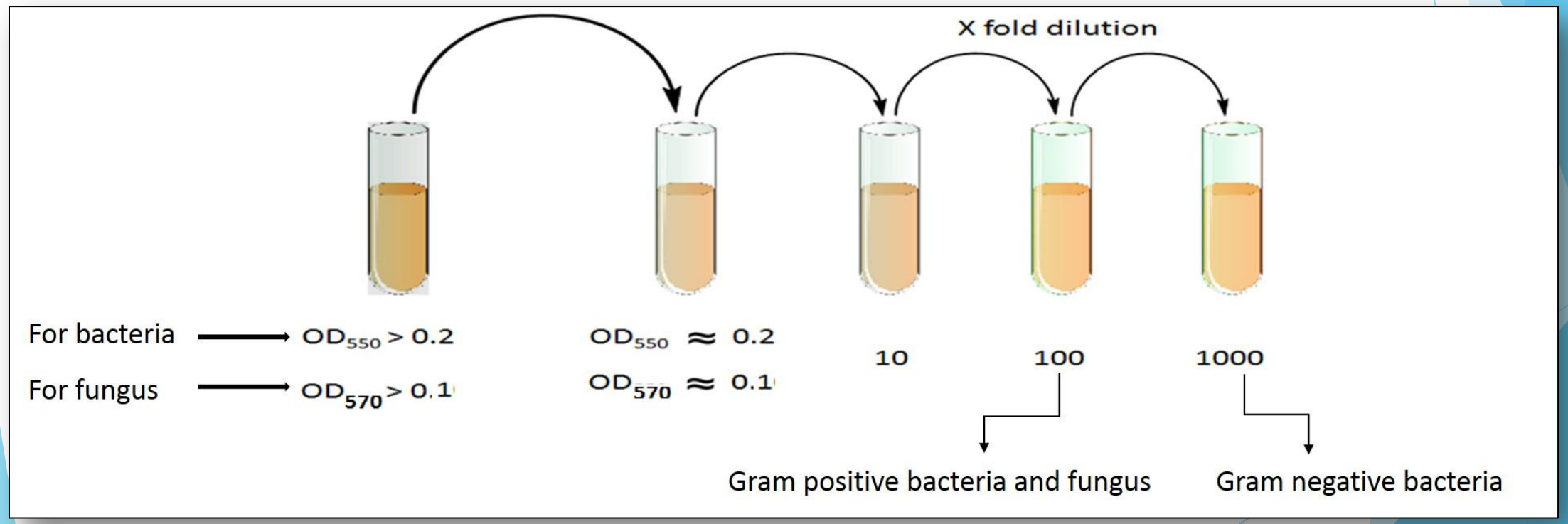
- Standard microbial culture
- Stabilization of microbial colony count:

Turbidity : McFarland standards
(Mixture of 1% $BaCl_2$ + 1% H_2SO_4)

Optical density : UV spectrophotometer

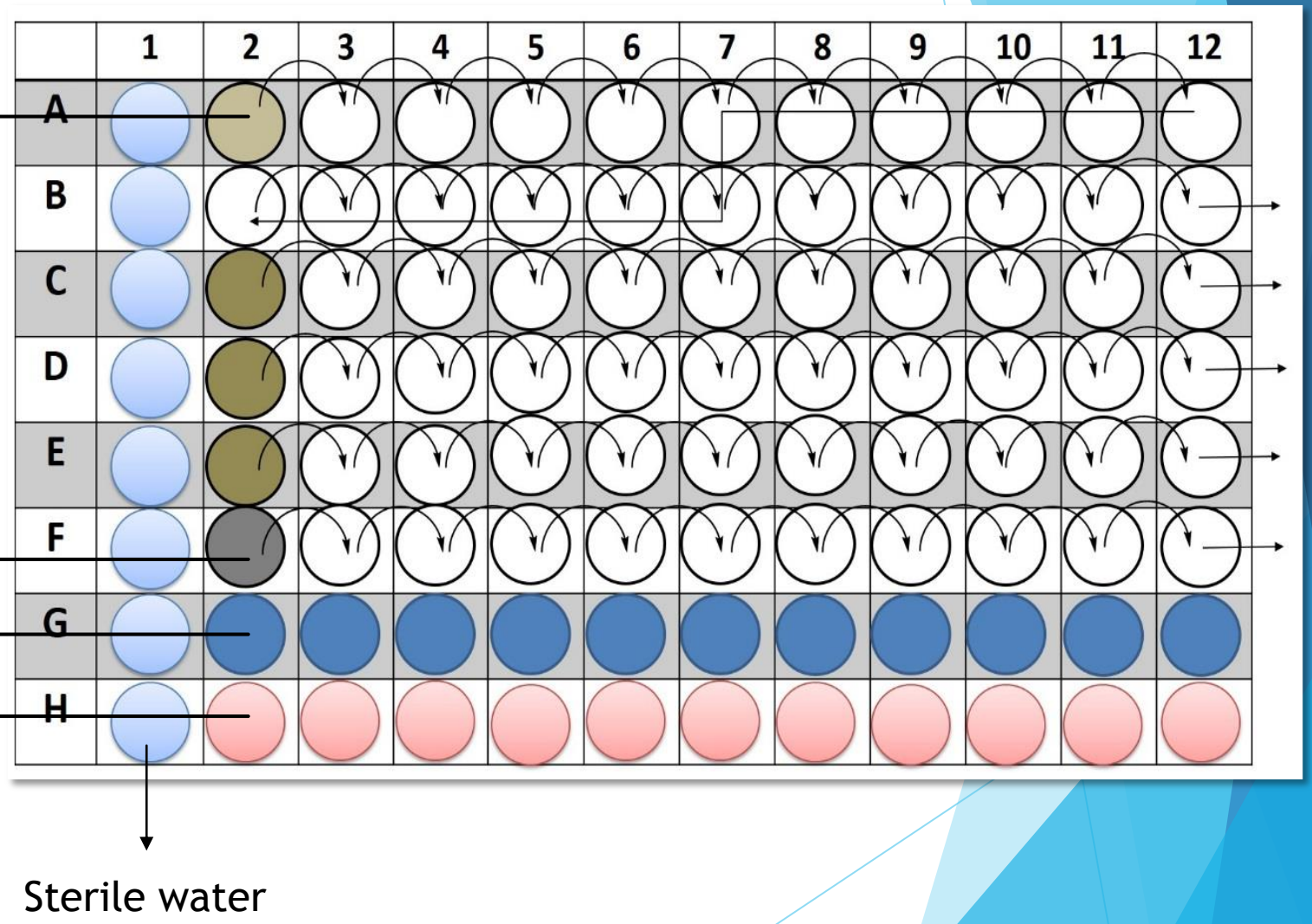
- Storage of Resazurin stock solution : Prevent exposure to light store at 4ºC
- Microtitre plate should be discarded after single use

- Optical density (OD) by using UV spectrophotometer
- OD is calculated mostly in between 400-600nm range
- For bacteria, OD_{550}= 0.2 then
  i. $1\text{-}2\times10^{9}$ CFU/ml for Gram negative bacteria and
  ii. $1\text{-}2\times10^{8}$ CFU/ml for Gram positive bacteria
- For *Candida albicans*, OD_{570} = 0.1 corresponds to $1\text{-}2\times10^{7}$CFU/mL



Cos, P.; Vlietinck, A. J.; Berghe, D. V.; Maes, L., **Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'**; Journal of Ethnopharmacology, **2006**, 106 (3), 290-302.

Plan for REMA



(n=6)

General procedure

✓100µl of medium in all wells.

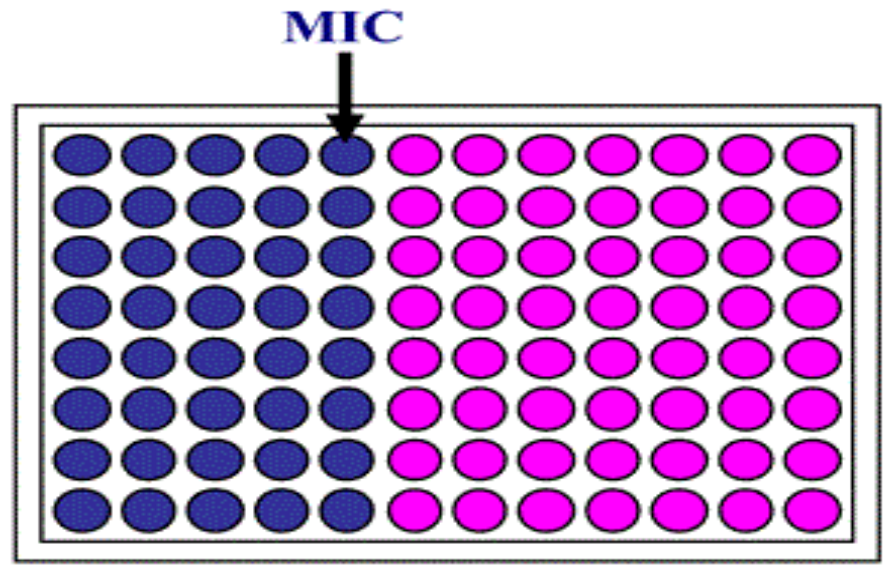
✓100µl of standard drug solution as well as test solution and serially diluted.

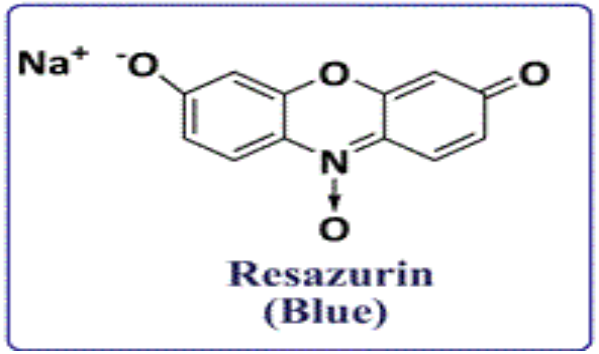
✓100µl of microbial susp. was added except negative control.

✓Plate was incubated at 37°C for 24-48hrs.

✓After incubation add 30µl of resazurin.

✓Incubate for 2-4 hrs at 37°C and observe color change





STANDARDIZATION OF REMA PROCEDURE

Standardization of REMA procedure for *Escherichia coli*

- Standard antibacterial agent - Ciprofloxacin - 5mg/ml and 1mg/ml
- Solvent used- Dimethyl sulphoxide (DMSO)
- <u>Standardization of bacterial count</u>-
- Growth medium: Soyabean-Caesin Digest Medium (Tryptone soya broth)
- Subcultures were grown at 37^0C for 24 hrs. and used for inoculation of the test culture.
- Test culture was incubated for 3-4 hrs at 37^0C and checked for OD_{550}
- Mid log phase bacterial culture (*E. coli* ATCC8739) -The test culture was inoculated into 10 mL of sterile growth medium for incubation up to 24hrs to obtain mid-log phase cultures

Trial 1

Time →	0 hr	4 hrs	16 hrs
OD_{550}	0.0021	0.0053	1.2708

Diluted with broth to get 0.2 OD (0.1830)

Trial 2

Time →	0 hr	6 hrs	20 hrs
OD_{550}	0.0080	0.0565	1.2860

Diluted with broth to get 0.2 OD (0.2169)

As *E coli* is Gram negative bacteria, 1000 fold dilution is used for REMA.

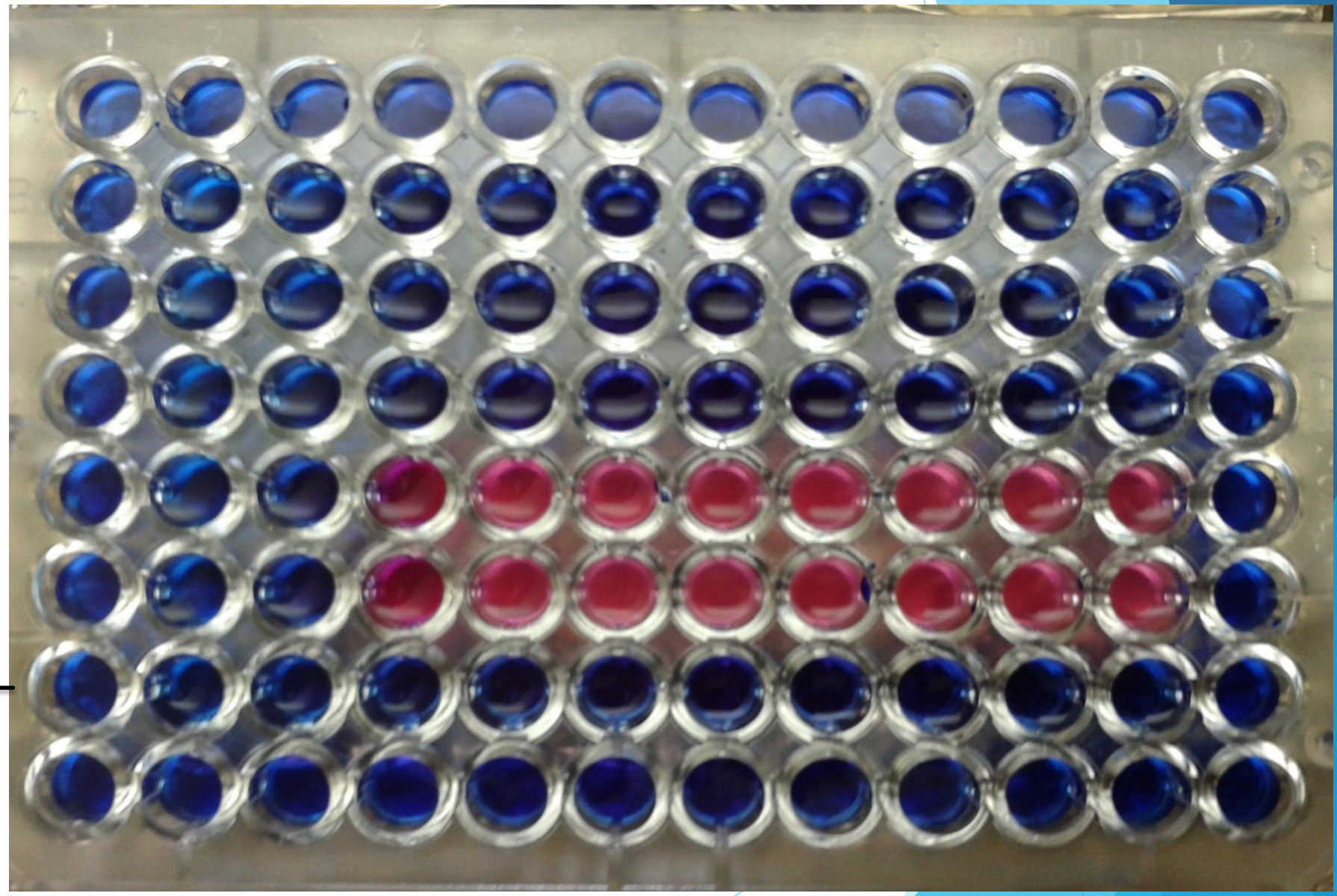
Result Standardization of *Escherichia coli*

Ciprofloxacin (5mg/mL) in duplicate

DMSO Control (with bacteria)

DMSO control (without bacteria)

Sterile water in all edge wells



- Growth medium: Mueller-Hinton Broth

Trial 1

Time →	0 hr	4 hrs
OD_{550}	0.0021	0.3866

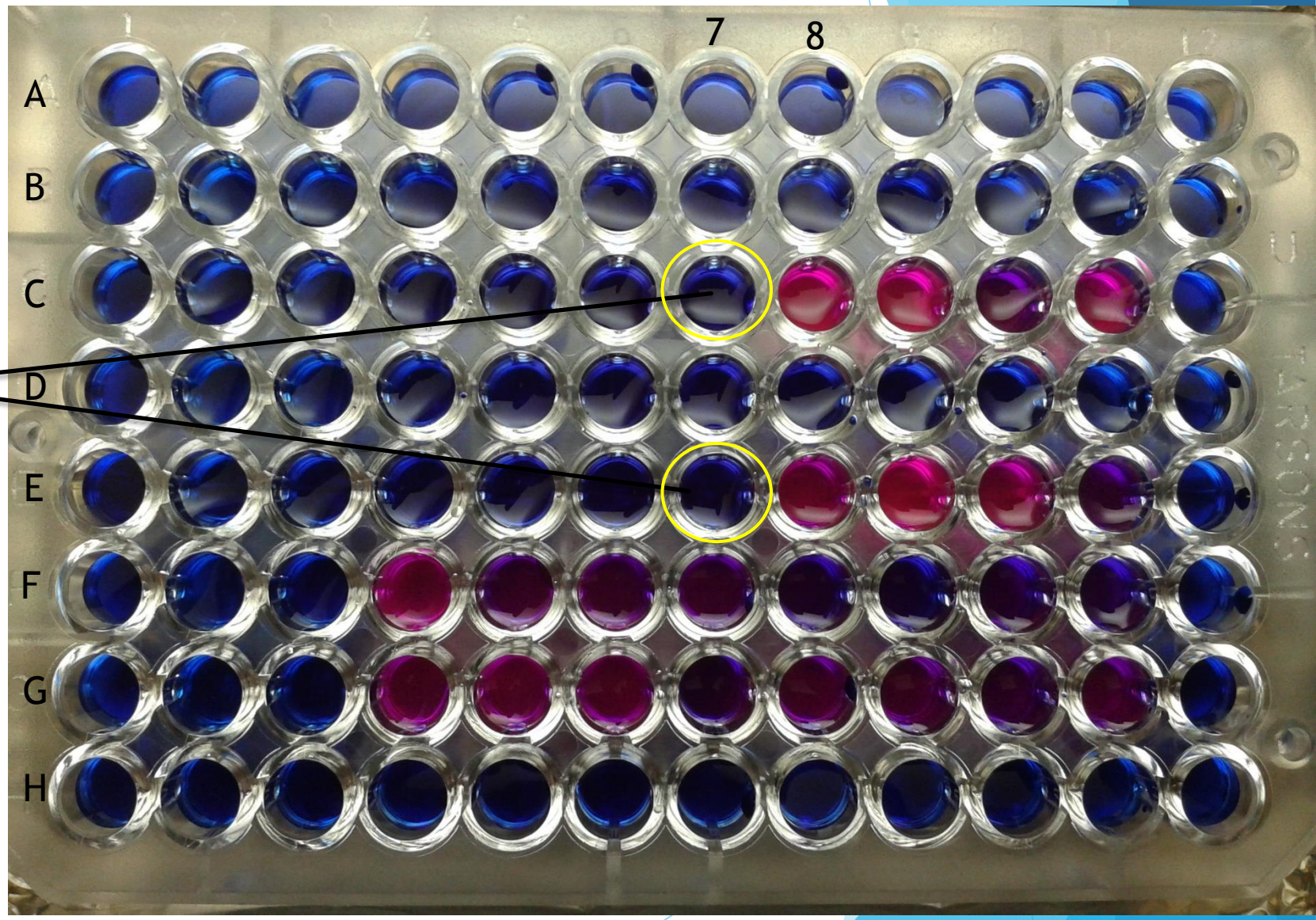
Diluted with broth to get 0.2 OD (0.1926 & 0.2102) ; 1000 fold dilution of 0.2102 is used for REMA

Trial 2

Time →	0 hr	8 hrs
OD_{550}	0.0021	0.8910

Conclusion:
By changing the media (from Soyabean-Caesin Digest media to Mueller-Hinton Broth), there was a significant reduction in the time required to achieve desired OD count.

Result Standardization of *Escherichia coli*



0.04 µg/ml

MIC = 0.02-0.04 µg/ml

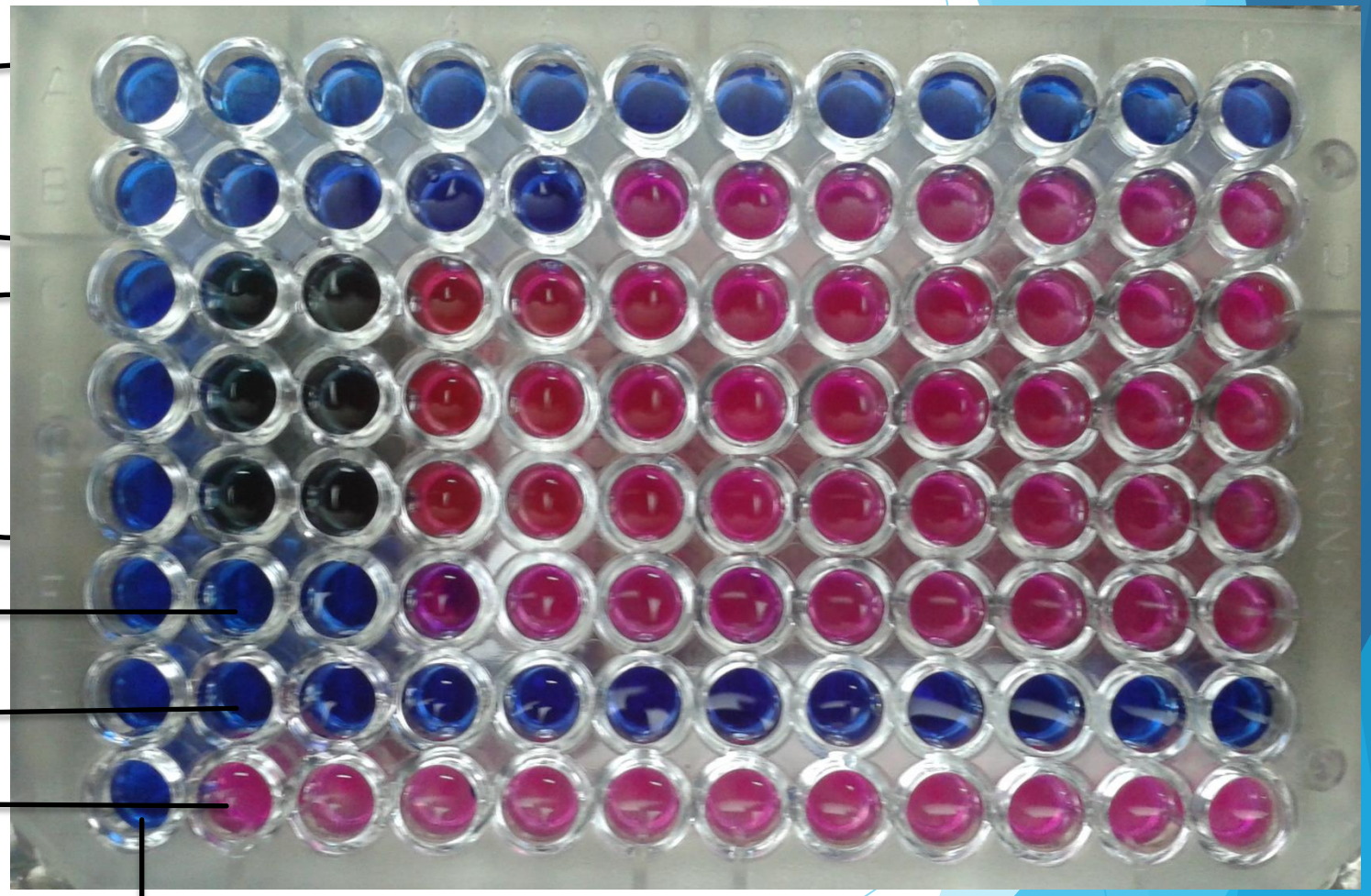
- Edge wells- Sterile water
- Row B,C & D,E- Ciprofloxacin (5mg/ml) Serial dilutions
- Row F,G – DMSO Control

RESULTS

FORMULATION I-BILAGYL LEHYA

Result Testing of Bael Methanol extract on *Escherichia coli*

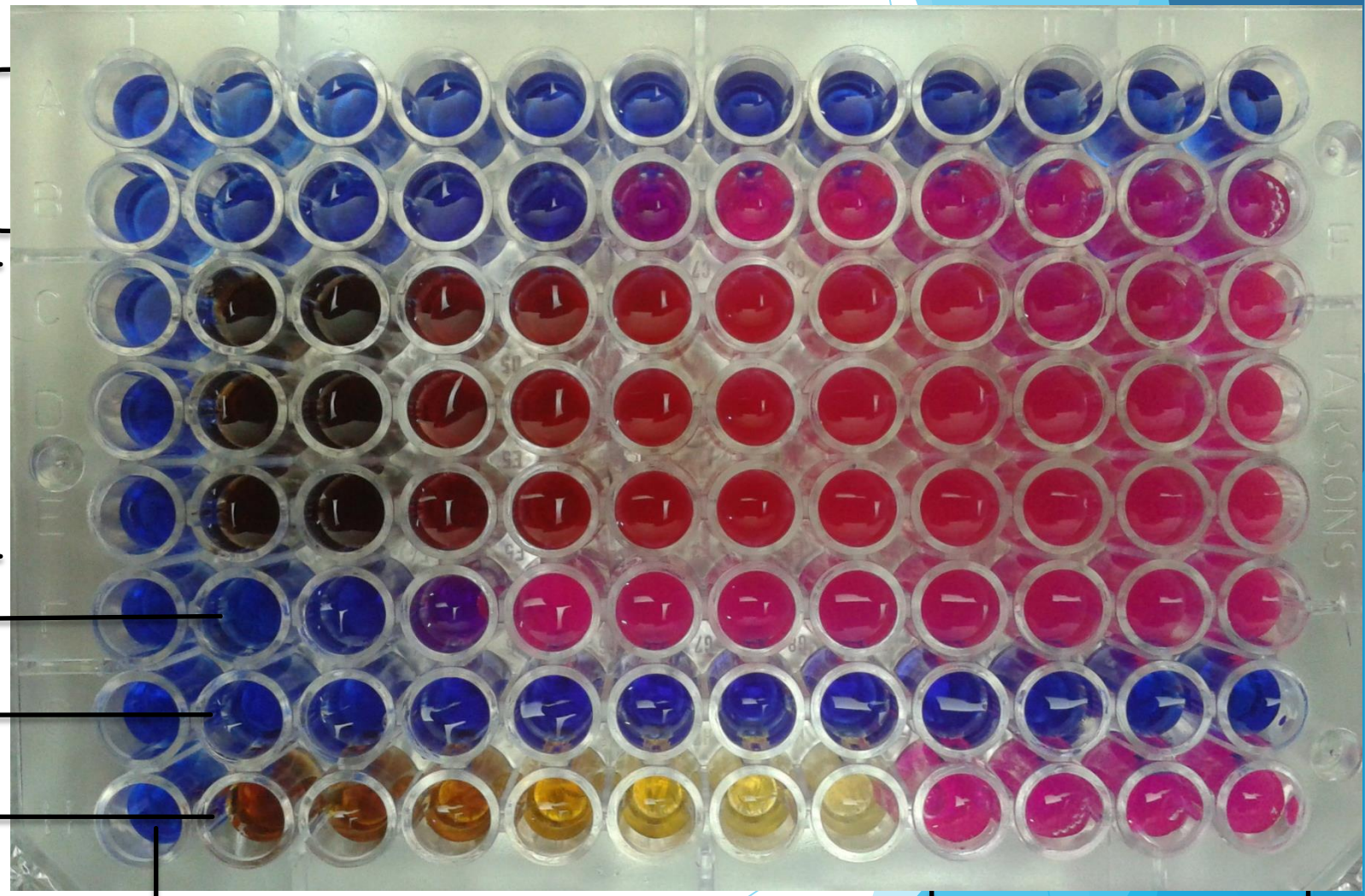
OD_{550} = 0.2003



MIC Ciprofloxacin = 0.01-0.02 µg/mL
MIC Bael Methanol extract = No activity

Result Testing of Bael aqueous extract on *Escherichia coli*

OD_{550} = 0.2208



Ciprofloxacin (1mg/mL)

Bael aqueous extract (50mg/mL)

DMSO Control (with bacteria)

Broth Control

Bael aqueous extract (50mg/mL) without resazurin

Sterile water

Growth control

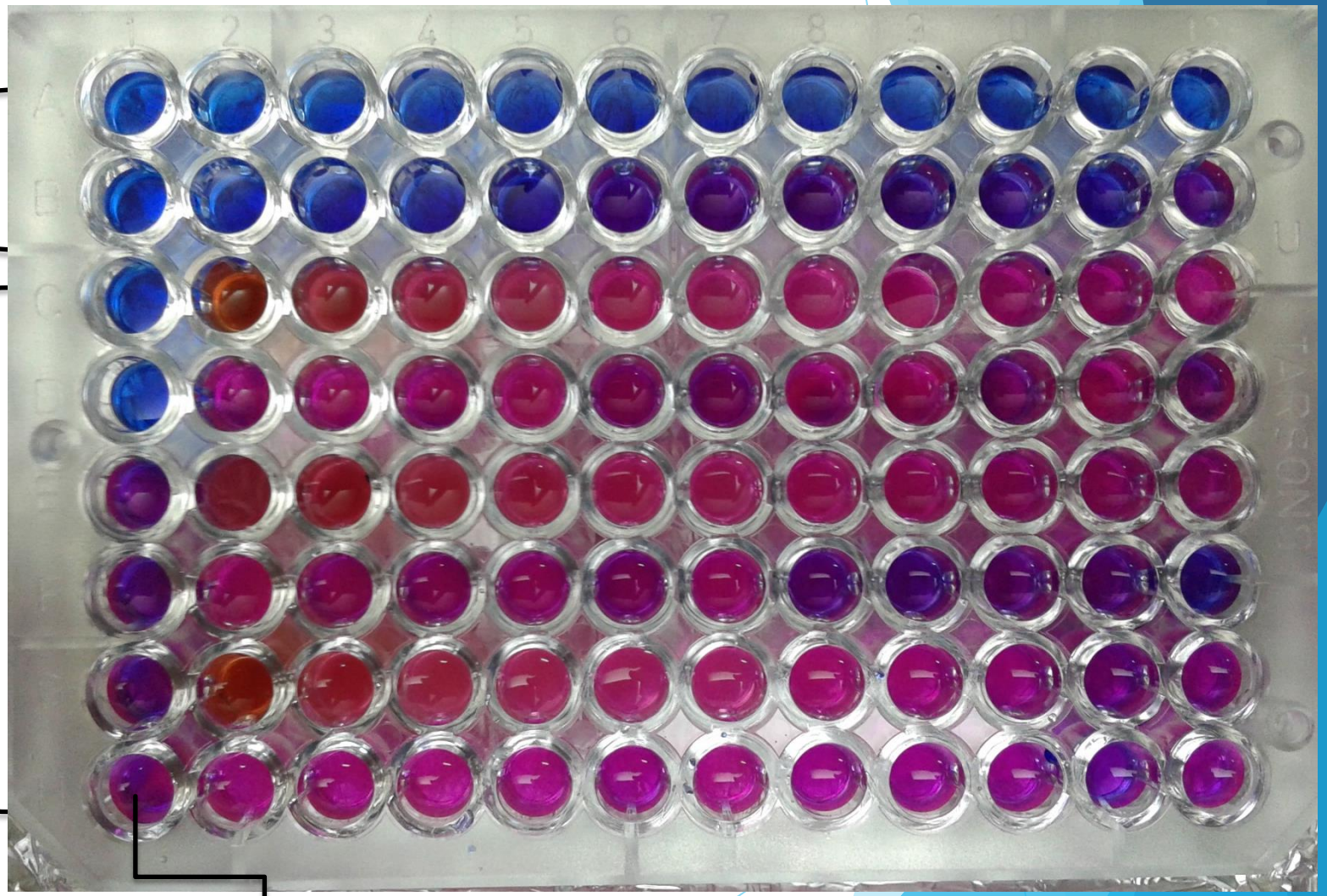
MIC Ciprofloxacin = 0.01-0.02 µg/mL
MIC Bael aqueous extract = No activity

Result Testing of Bael fruit pulp on *Escherichia coli*

OD_{550} = 0.2064

Ciprofloxacin (1mg/mL)

Bael fruit pulp (0.22g/mL) in triplicate

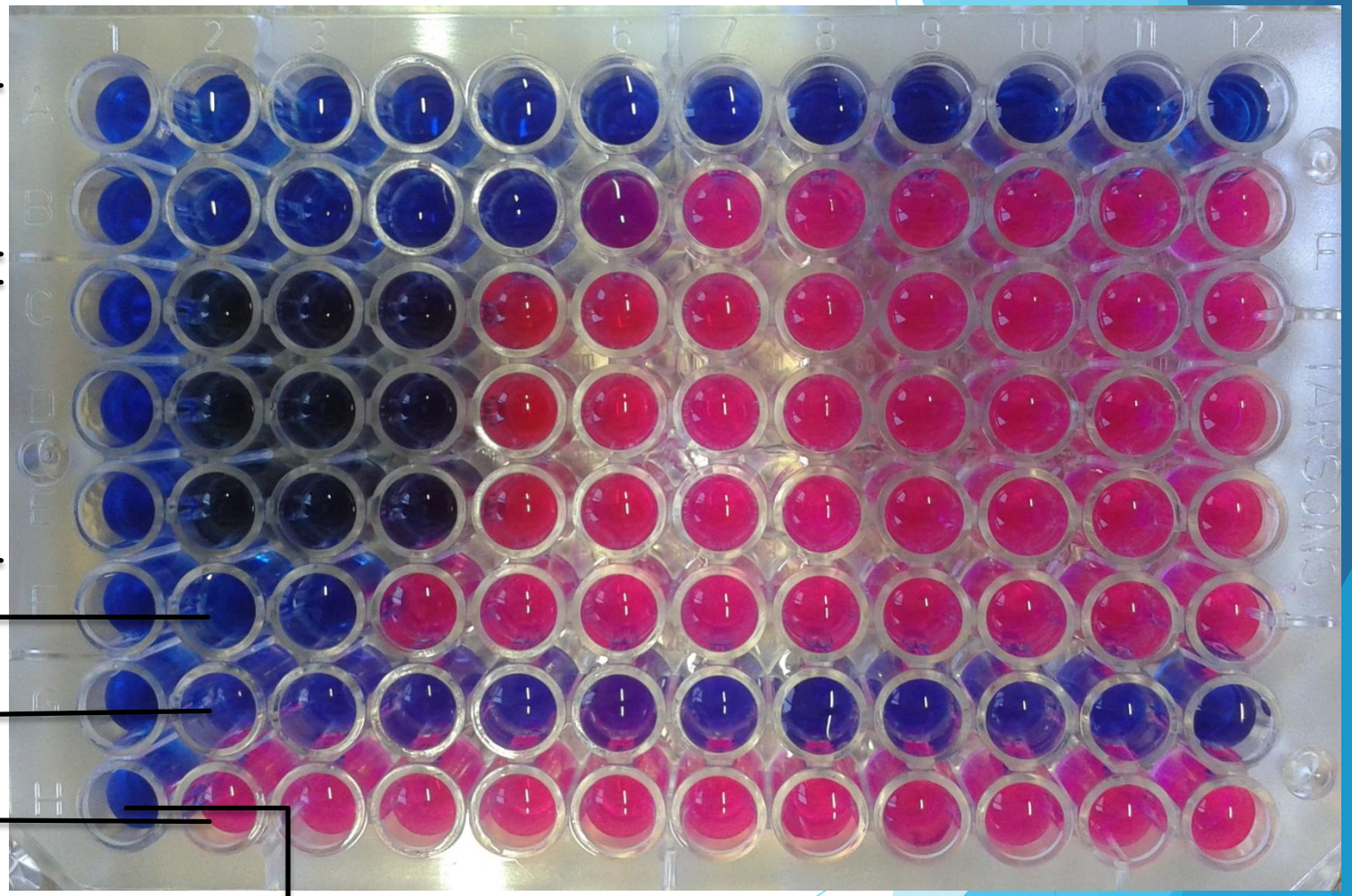


1st 4 wells-Sterile water and Last 4 wells- Growth control

MIC Ciprofloxacin = 0.01-0.02 µg/mL
MIC Bael fruit pulp = No activity

Result Testing of Bael fruit pulp n-butanol fraction on *Escherichia coli*

OD_{550} = 0.2196



MIC Ciprofloxacin = 0.01-0.02 µg/mL
MIC Bael fruit pulp n-butanol fraction = 3125-6250 µg/mL

FORMULATION II- BERBENTERONE TABLET

Result Testing of Lavang hexane extract on *Shigella flexneri*

OD_{550} = 0.2212

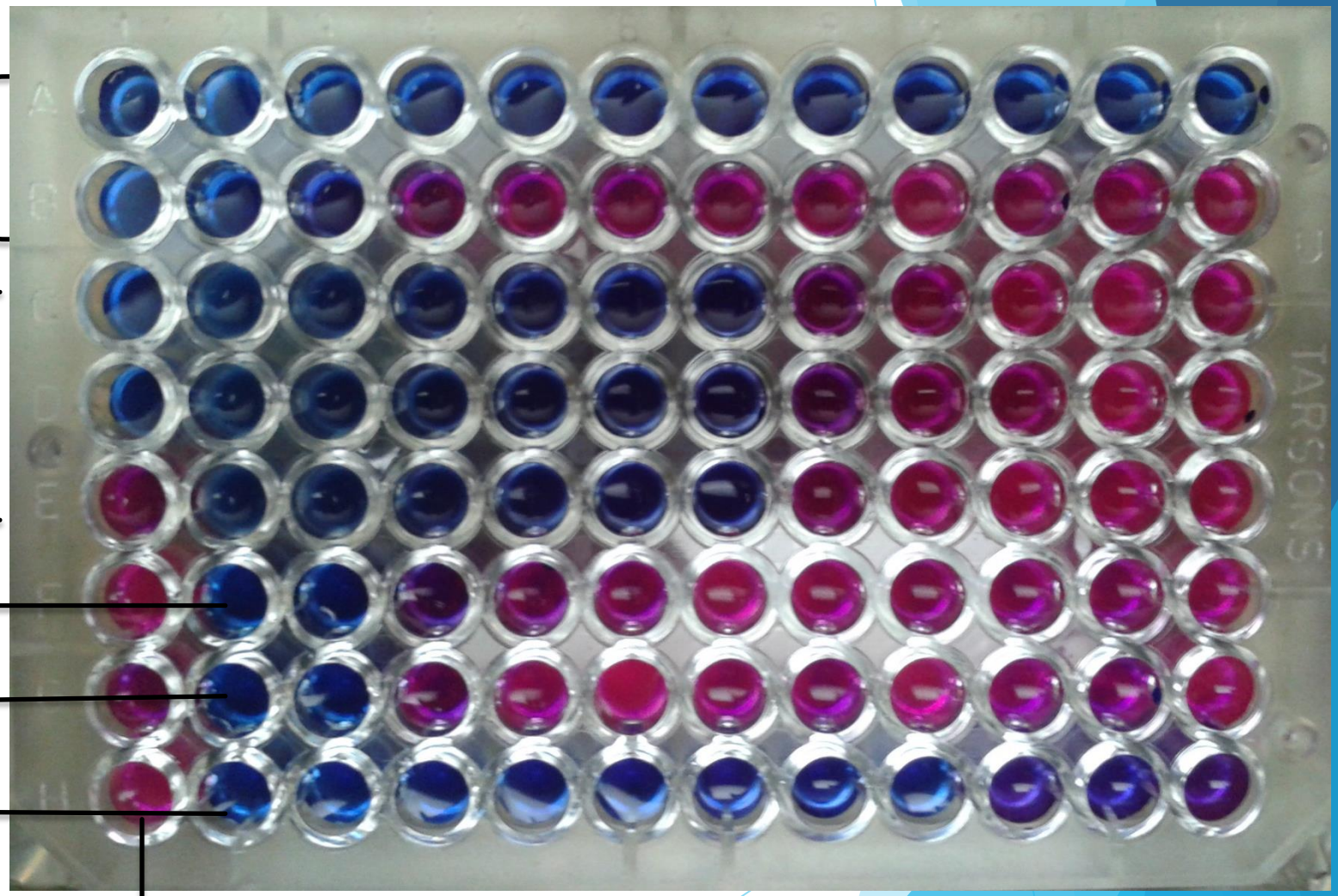
Ciprofloxacin (1mg/mL)

Lavang hexane extract (50mg/mL) in triplicate

Hexane in DMSO Control (with bacteria)

DMSO Control (with bacteria)

Broth Control



1st 4 wells-Sterile water; Last 4 wells- Growth control

MIC Ciprofloxacin = 0.04-0.08 µg/mL
MIC Lavang hexane extract = 390-780 µg/mL

Result Testing of Lavang chloroform extract on *Shigella flexneri*

OD_{550} = 0.2204

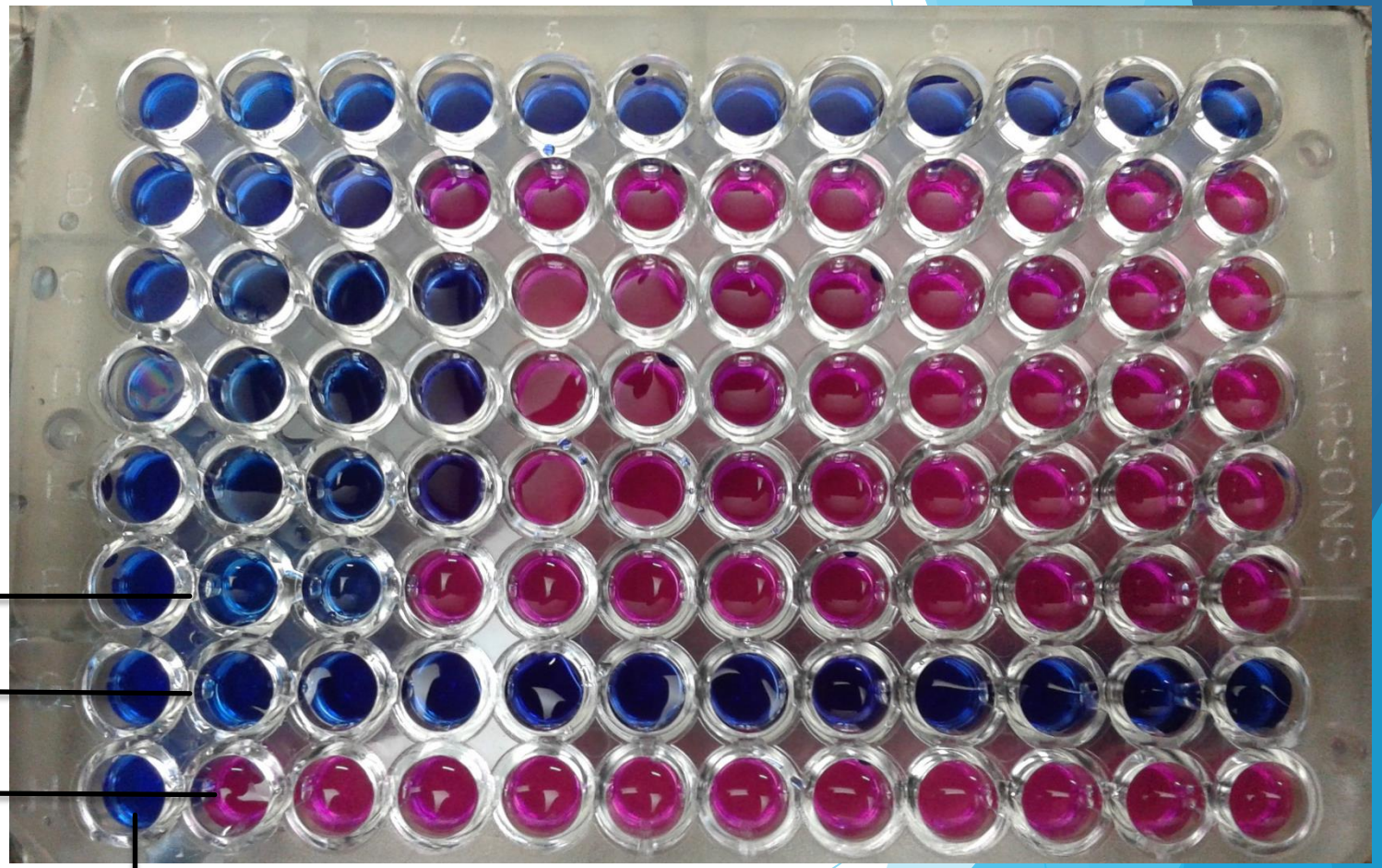
Ciprofloxacin (1mg/mL)

Lavang chloroform extract (50mg/mL) in triplicate

DMSO Control (with bacteria)

Broth Control

Growth control

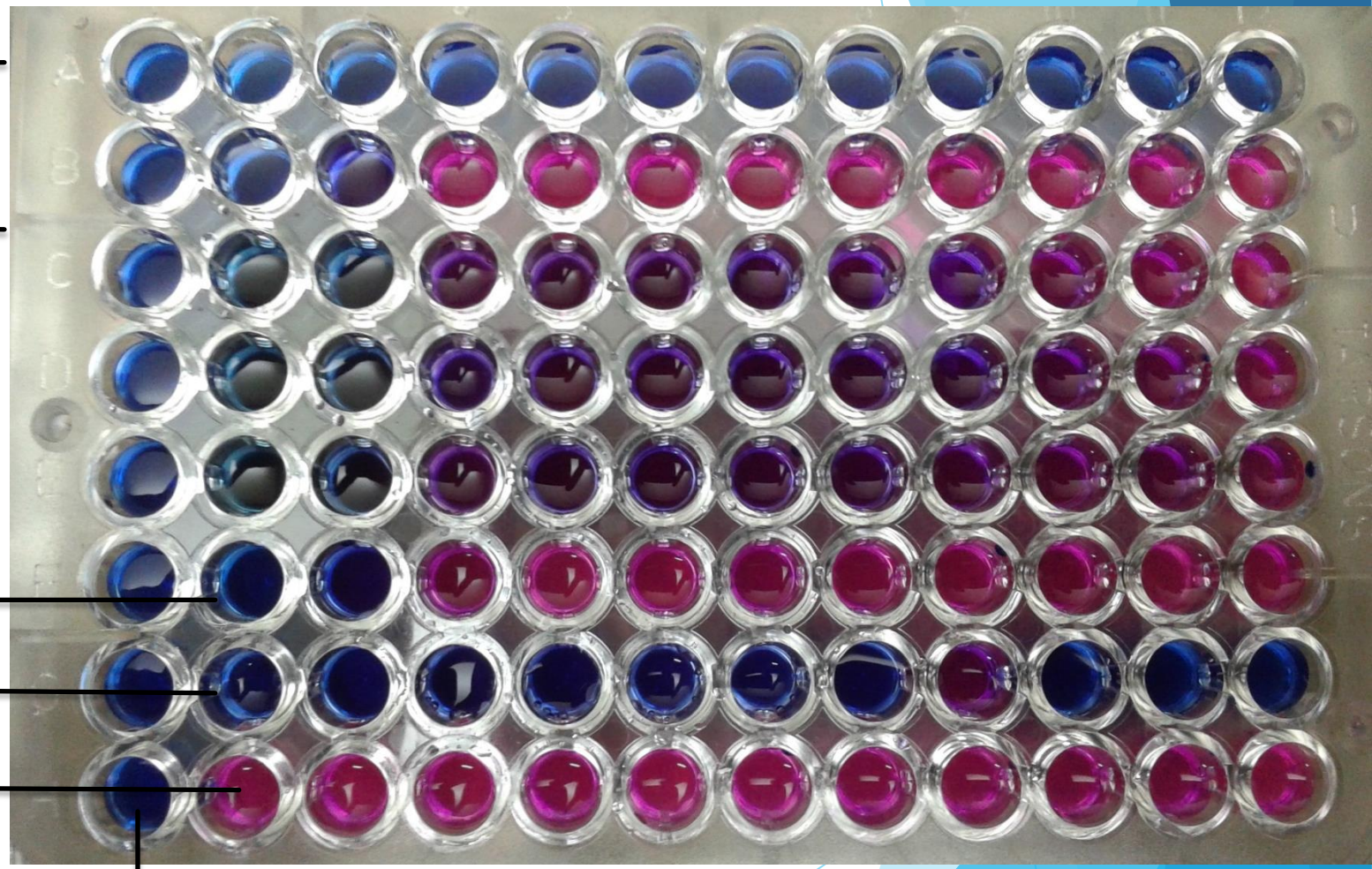


Sterile water

MIC Ciprofloxacin = 0.04-0.08 µg/mL
MIC Lavang chloroform extract = 3125-6250 µg/mL

Result Testing of Lavang methanol extract on *Shigella flexneri*

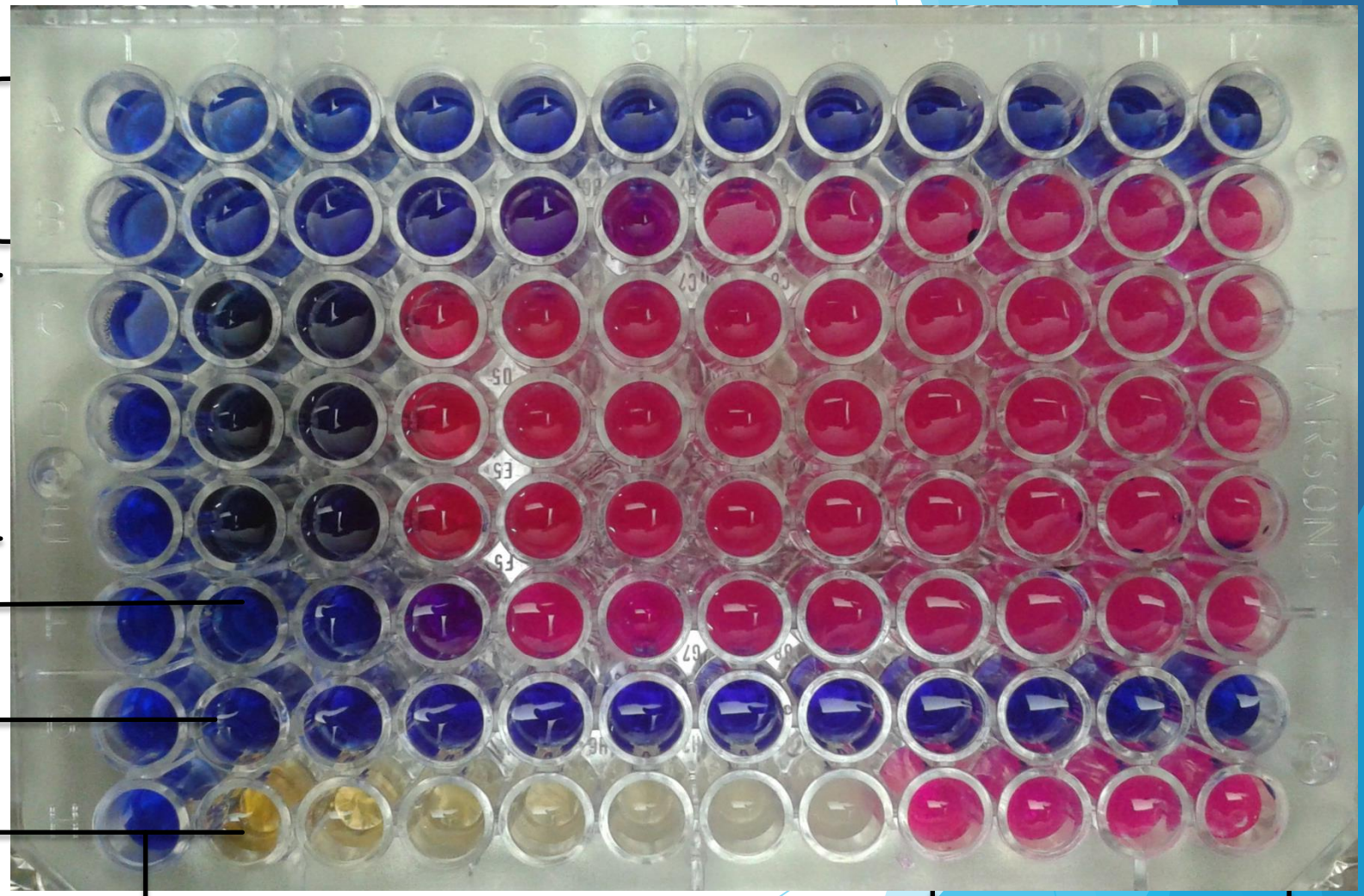
OD_{550} = 0.2204



MIC Ciprofloxacin = 0.04-0.08 µg/mL
MIC Lavang methanol extract = No activity

Result Testing of Lavang aqueous extract on *Shigella flexneri*

OD_{550} = 0.2218



Ciprofloxacin (1mg/mL)

Lavang aqueous extract (50mg/mL)

DMSO Control (with bacteria)

Broth Control

Lavang aqueous extract (50mg/mL) without resazurin

Sterile water

Growth control

MIC Ciprofloxacin = 0.02-0.04 µg/mL
MIC Lavang aqueous extract = No activity

FRACTIONATION

- Direct *in vitro* testing of formulations by REMA was not possible-

(i) formulations contain plant or plants, which in turn, contain a variety of class of compounds, and

(ii) active constituent(s) present in whole formulation may be very less in amount

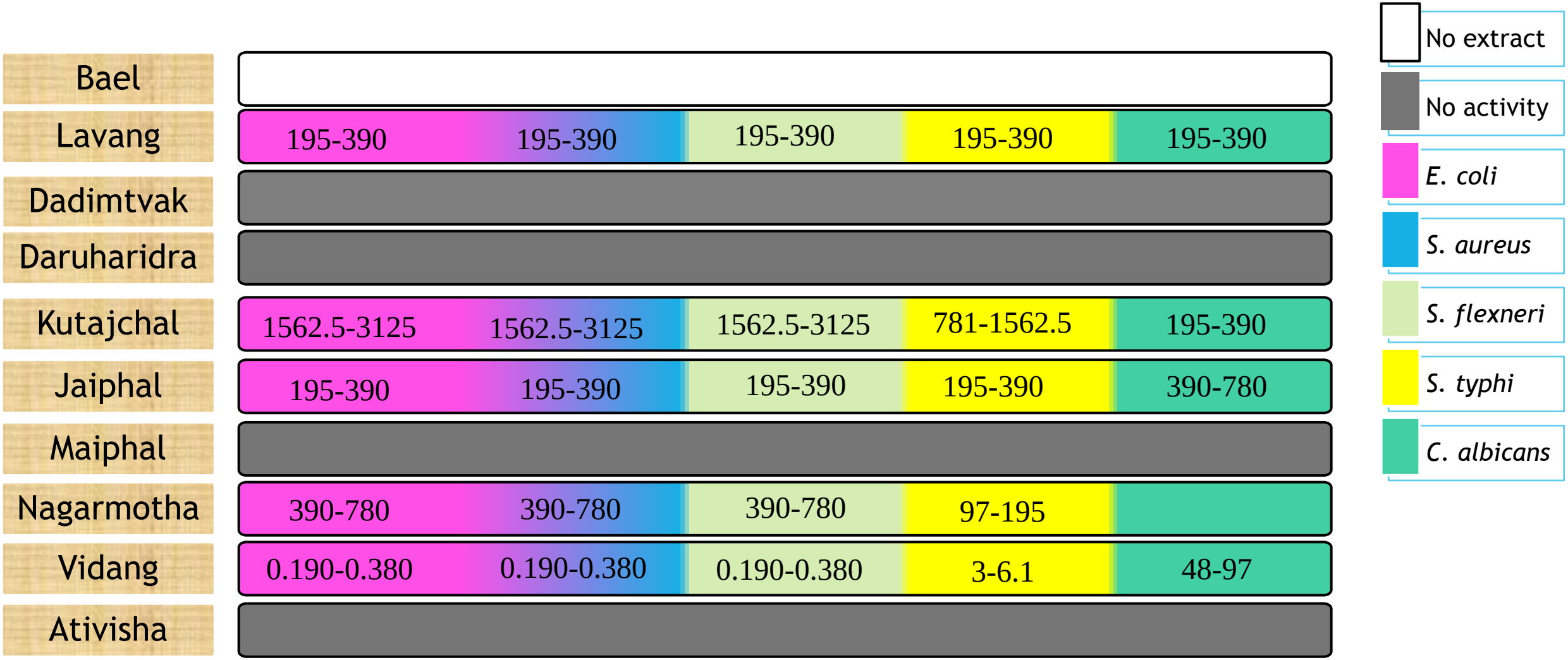
- To check anti-infective potential of these formulations, it was suggested, that their individual components need to be fractionated into solvents with varying polarity.

- Solvents selected-
  - Hexane : Non-polar
  - Chloroform : Mid polar
  - Methanol : Polar

- Aqueous extracts/fractions were also prepared.
- For successive solvent extraction, Soxhlet apparatus was used.

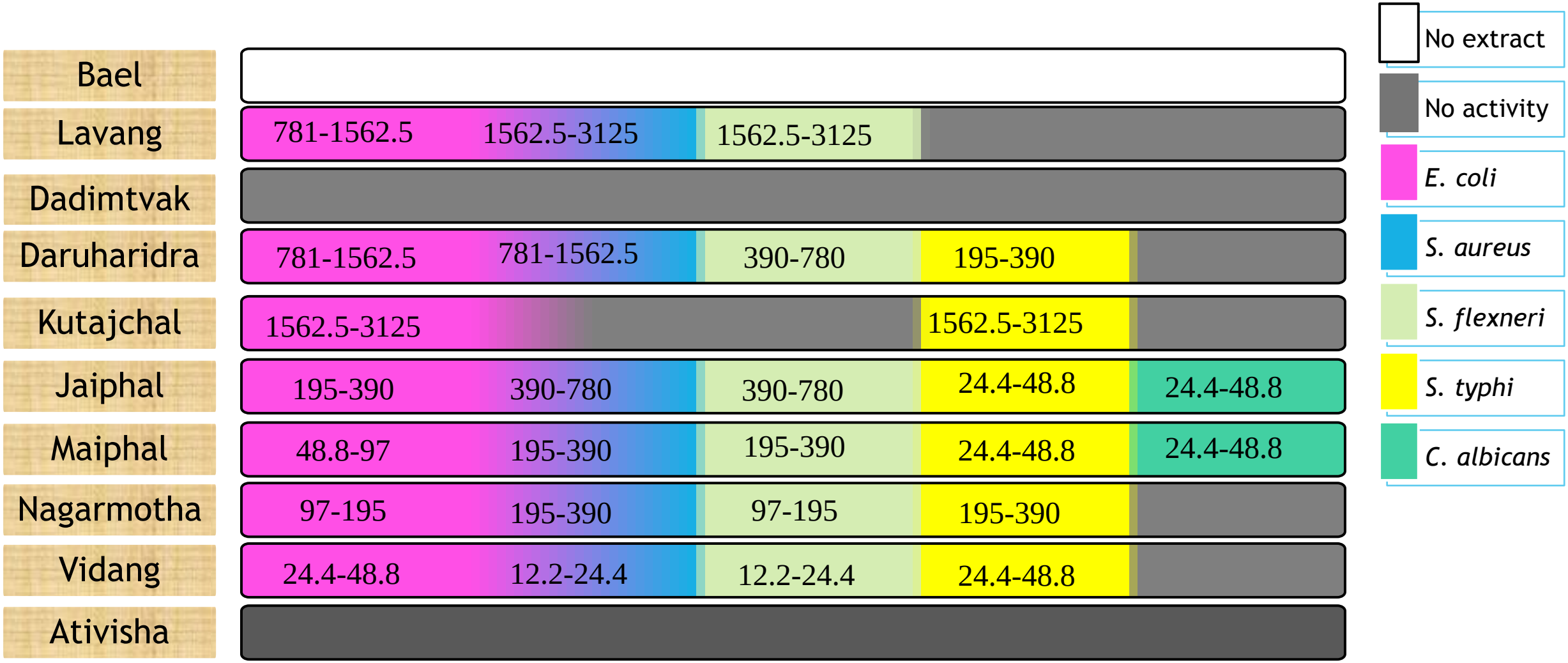
Microorganisms (MTCC No.)	Antimicrobial agent used	MIC (µg/mL)
Escherichia coli 443	Ciprofloxacin	0.01-0.02
Staphylococcus aureus 737	Ciprofloxacin	0.08-0.16
Shigella flexneri 1457	Ciprofloxacin	0.04-0.08
Salmonella typhi 98	Ciprofloxacin	0.04-0.08
Candida albicans 183	Fluconazole	4.8-9.7

Hexane extract



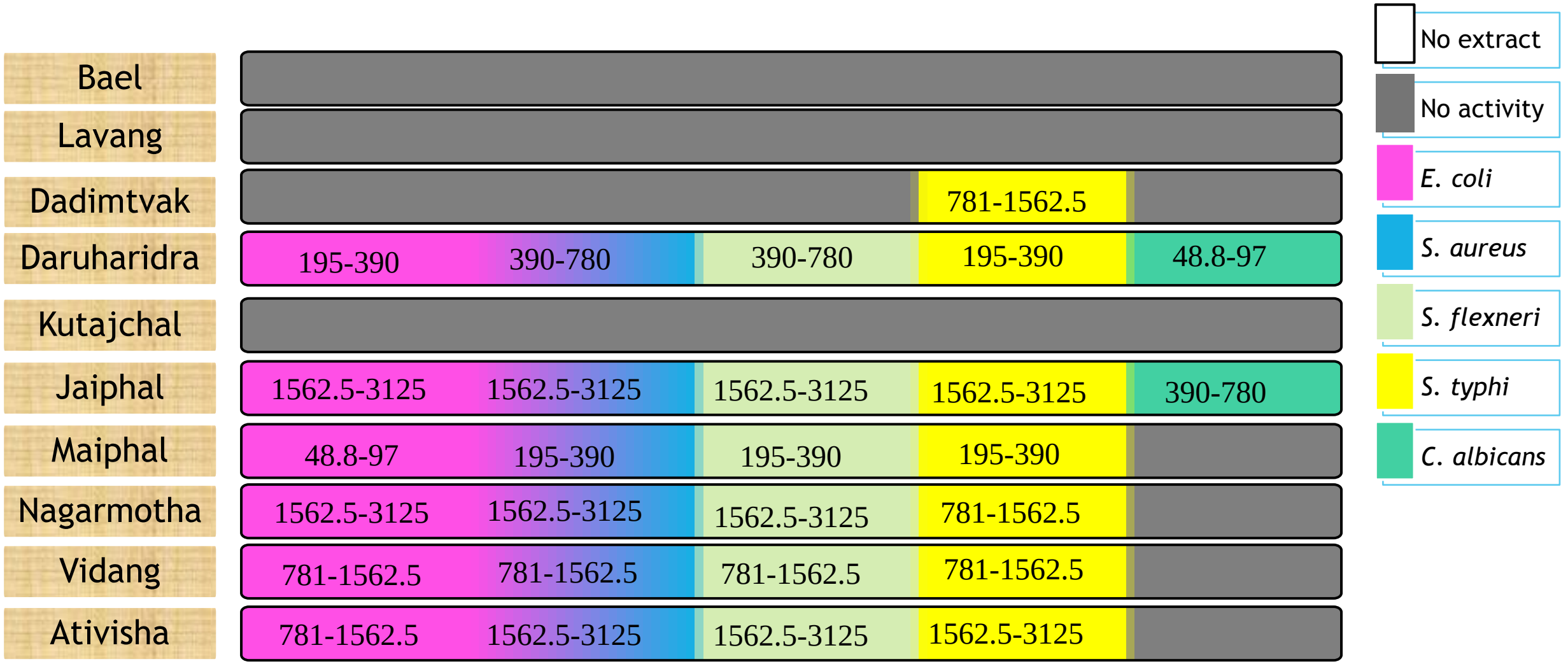
Values mentioned are of MIC range (µg/mL)

Chloroform extract



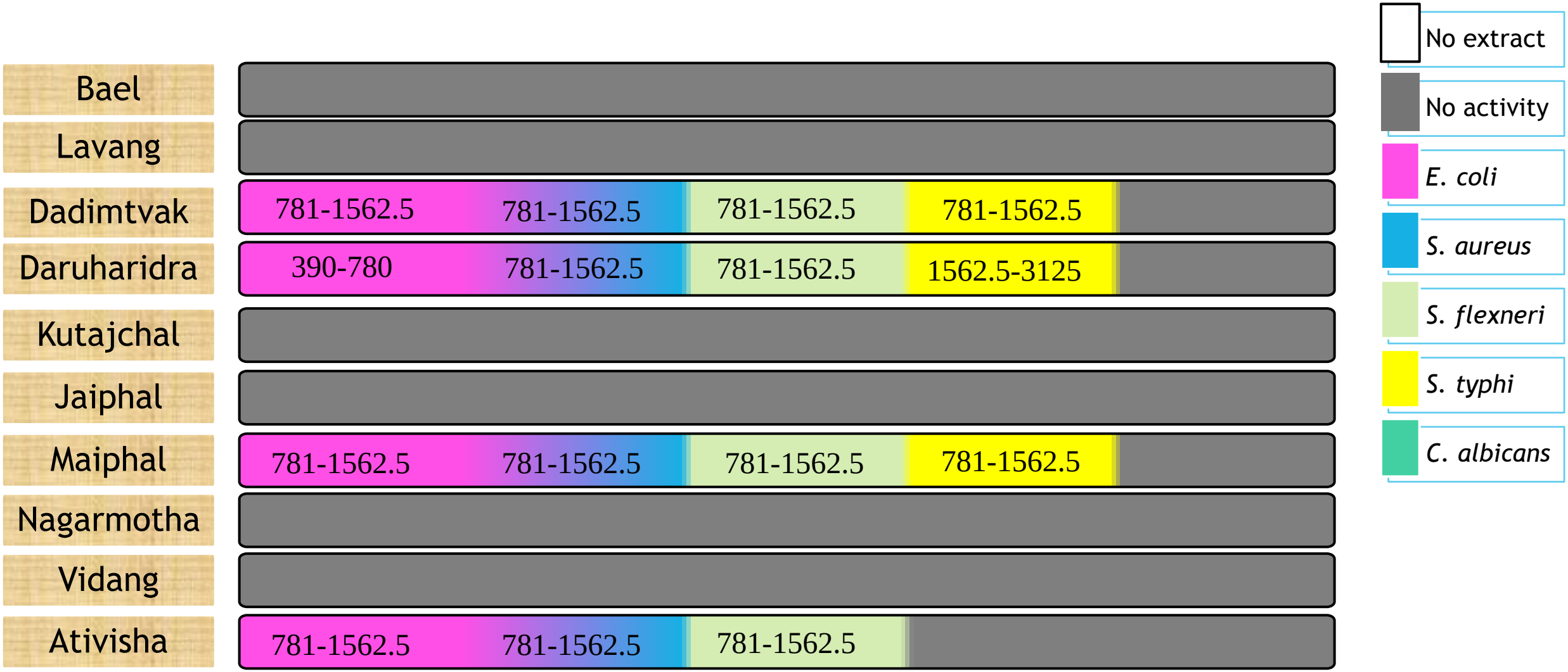
Values mentioned are of MIC range (µg/mL)

Methanol extract



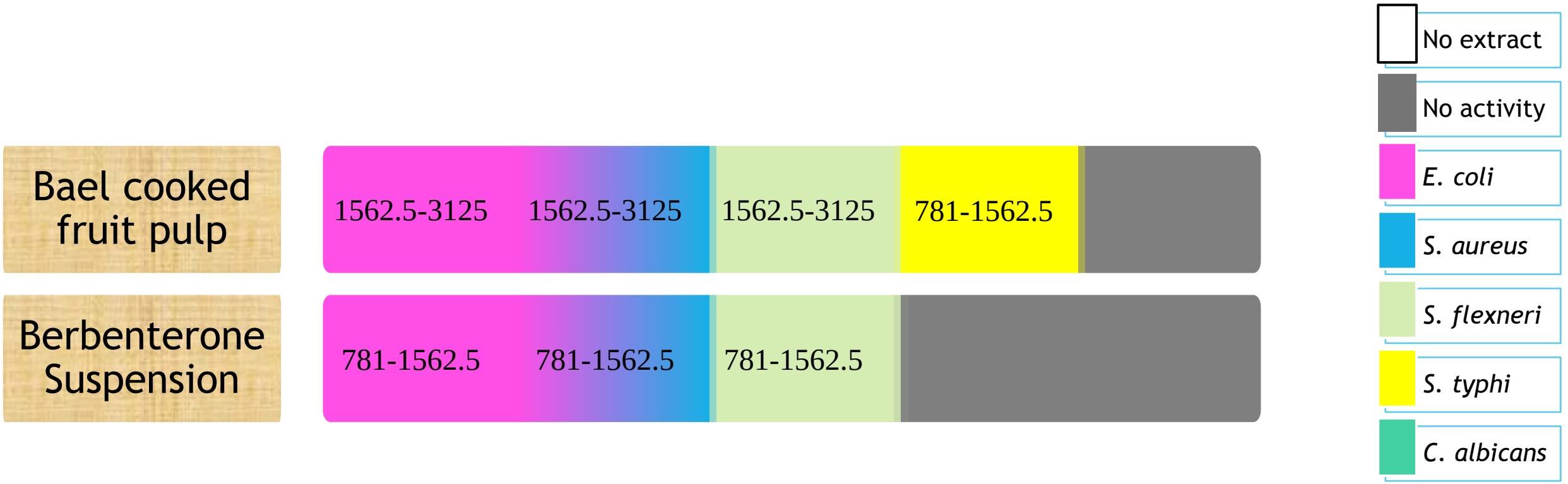
Values mentioned are of MIC range (µg/mL)

Aqueous extract



Values mentioned are of MIC range (µg/mL)

n-butanol fraction



Values mentioned are of MIC range (µg/mL)

CONCLUSION:

- Standardized REMA procedure can be successfully applied to evaluate antimicrobial activity against *E. coli*, *S.aureus*, *S.flexneri* and *S. typhi* and also to evaluate antifungal activity against *C. albicans*.

- Active plant extracts can be further fractionated to find out component(s) responsible for antibacterial activity by bioassay guided fractionation.

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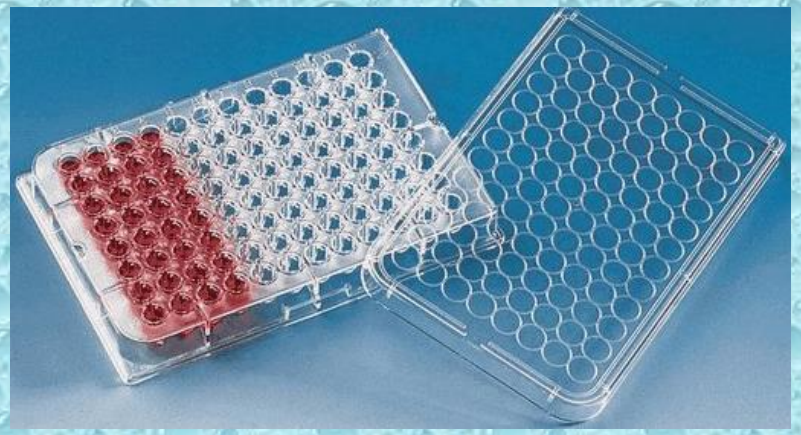
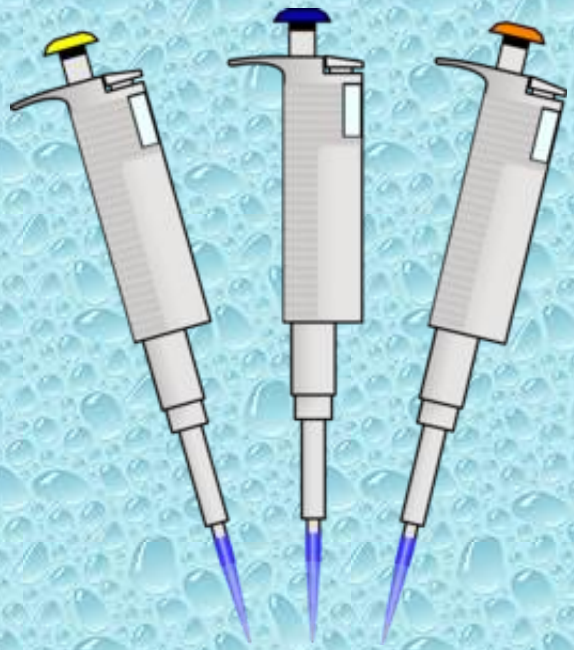


THANK YOU !!!



SANDU
OVER 100 YEARS
Estd. 1999