



Antimicrobial Activity of Essential oils from Selected Culinary herbs of *Zingiberaceae* and Detection of Antimicrobial Compounds using TLC-Bioautography

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INTRODUCTION TO ZINGIBERACEAE



- The family Zingiberaceae primarily distributed in the tropical parts of the world with about 53 genera and 1200 species
- It is one of the ten largest monocotyledonous families in India
- India is represented by 22 genera and 178 species and south India is by 9 genera and 70 species
- The members of this family cultivated for their uses as spices, medicines, condiments, flavouring agents, fresh vegetables, tuber crops and recently as cut flowers









- Generally Zingiberaceae plants are annual or perennial rhizomatous herbs and rhizome is branched and composed of distinct segments

- Many zingiberaceae members are aromatic and capable of producing essential oils with their highly specialized secretory structures known as translucent globules in cortical cells of rhizomes and glandular trichomes in leaves

- Cardamom (*Elettaria cardamomum*) seed oil is the one of the commercially important zingiberaceae essential oils, genus *Alpinia* and *Hedychium* also significant for essential oil production and these oils were popularly used in aromatherapy and preparation of soaps and perfumes etc.







OBJECTIVES



1. Collection and authentication of three selected Zingiberaceae plants (*Alpinia speciosa* (K.Schum.), *Curcuma longa* (L.) & *Zingiber officinale* (Roscoe)) plant materials
2. Extraction of essential oil by Hydro-distillation
3. Determination of antimicrobial activity by Kirby-bauer disc diffusing method & minimum inhibitory concentration (MIC) by micro-dilution assay
4. Antimicrobial identification by TLC-Bioautography and GC-MS analysis
5. Determination of antimicrobial activity of selected standard compounds to affirm the activity of essential oils








EXPERIMENTAL






PLANT MATERIALS



The basic reason for selection of three *A. speciosa*, *C. longa* & *Z. officinal* in the present study as they were commonly cultivated in Vizianagaram district, Andhra Pradesh as commercial crops and also grown in the backyards of many houses and gardens

Fresh leaf material was collected from the garden of M.R.P.G. College, Vizianagaram., and chopped into small pieces subjected for hydro-distillation for extraction of essential oil.

Plants were also authenticated by Botanical Survey of India (BSI), Deccan Regional Centre, Hyderabad, Telangana.





TEST PLANTS









Alpinia speciosa



Curcuma longa



Zingiber officinale



HYDRO-DISTILLATION



200 grams of chopped fresh leaf material was subjected for hydro-distillation in a modified Clevenger type apparatus for $3_{1/2}$ hours

Collected oil was treated with anhydrous sodium sulphate (Na_2SO_4) to remove residual water from the oil

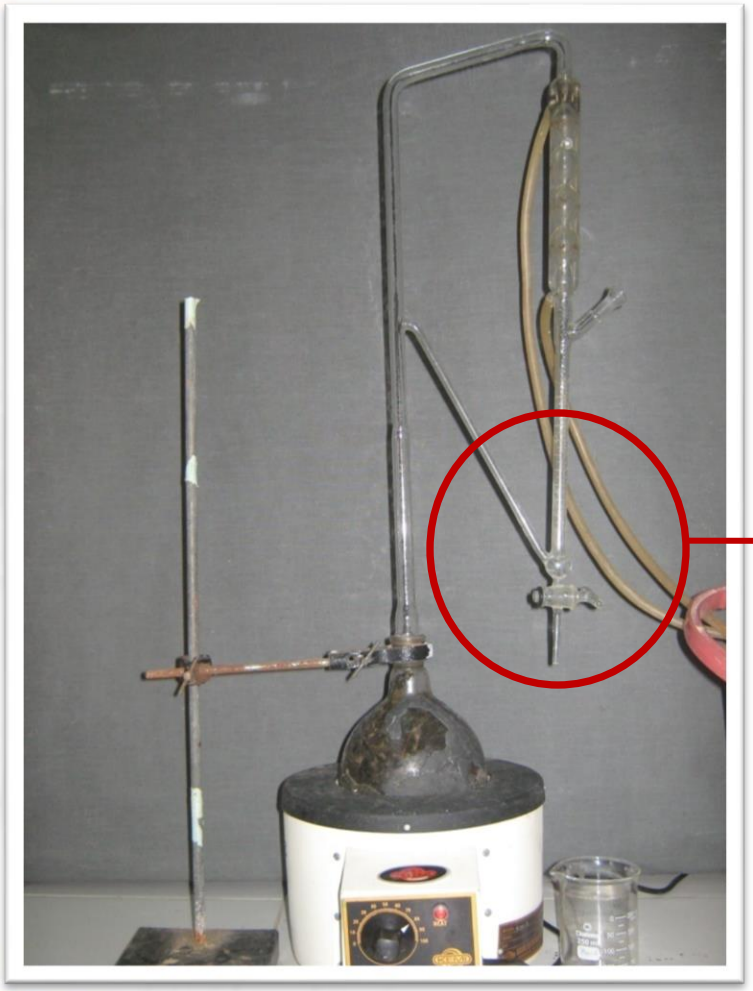
Oil yield was quantified in percentage (w/w) stored in airtight vial and kept in refrigerator at 4 °C until further experimentation

Essential oil from	Yield in %
Alpinia speciosa	0.58
Curcuma longa	0.56
Zingiber officinale	0.24





HYDRO-DISTILLATION APPARATUS







ANTIMICROBIAL ACTIVITY



Antimicrobial activity was studied with 6 Gram-positive and 10 Gram-negative bacterial strains and one fungal strains and majority of them were pathogenic in nature

All the bacterial and yeast strains were obtained from the Institute of Medical Technology, MTCC, Chandigarh, India and the test organisms were revived with media prescribed by MTCC, sub-cultured on suitable media and stored in suitable conditions.









Gram-positive bacteria:

Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis and *Streptococcus pyogenes*

Gram-negative bacteria:

Enterobacter aerogenes, Proteus mirabilis, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica ser. typhi, Shigella flexneri, Acinetobacter baumannii , Serratia marcencens and *Vibrio cholerae*

Fungi:

Candida albicans





KIRBY-BAUER DISC DIFFUSION METHOD





The agar disc diffusion (Kirby–Bauer) method that was recommended by NCCLS, 1999 (National Committee of Clinical Laboratory Standards) was used to determine antimicrobial activity

For bacteria Petri-dish with sterile Muller– Hinton agar (Hi-media, Mumbai) were spread with 0.1 mL of 24 hours active cultures of McFarland No. 0.5 (1.0×10^8 CFU/mL) and Sabouraud dextrose agar medium (Hi-media, Mumbai) was used for fungi.

Sterile 6-mm Whatman No. 1 filter paper discs impregnated with 10 µL of essential oil was placed in the center of each plate inoculated with test organism.









10 μg gentamicin and 10 μg ketoconazole were maintained as positive controls for bacteria and yeast respectively

Plates were kept for 1 hour at room temperature for proper diffusion of the sample and plates were incubated at 37°C for 24 hours (bacteria) and at 28°C for five days (fungi).

After incubation, the plates were observed for the zone of inhibition, which was measured in millimeters using the Hi Antibiotic Zone Scale (Hi Media, Mumbai). All the tests were performed in triplicate.





MIC BY BROTH DILUTION ASSAY

The evaluation of Minimum Inhibitory Concentration (MIC) was exercised by the broth dilution method using tissue culture test plates (96 wells).

Essential oils were diluted and transferred into first well, and serial dilutions were performed so that concentrations in the range of 4% to 0.03% v/v with 10% of Tween-80 in sterile distilled water.

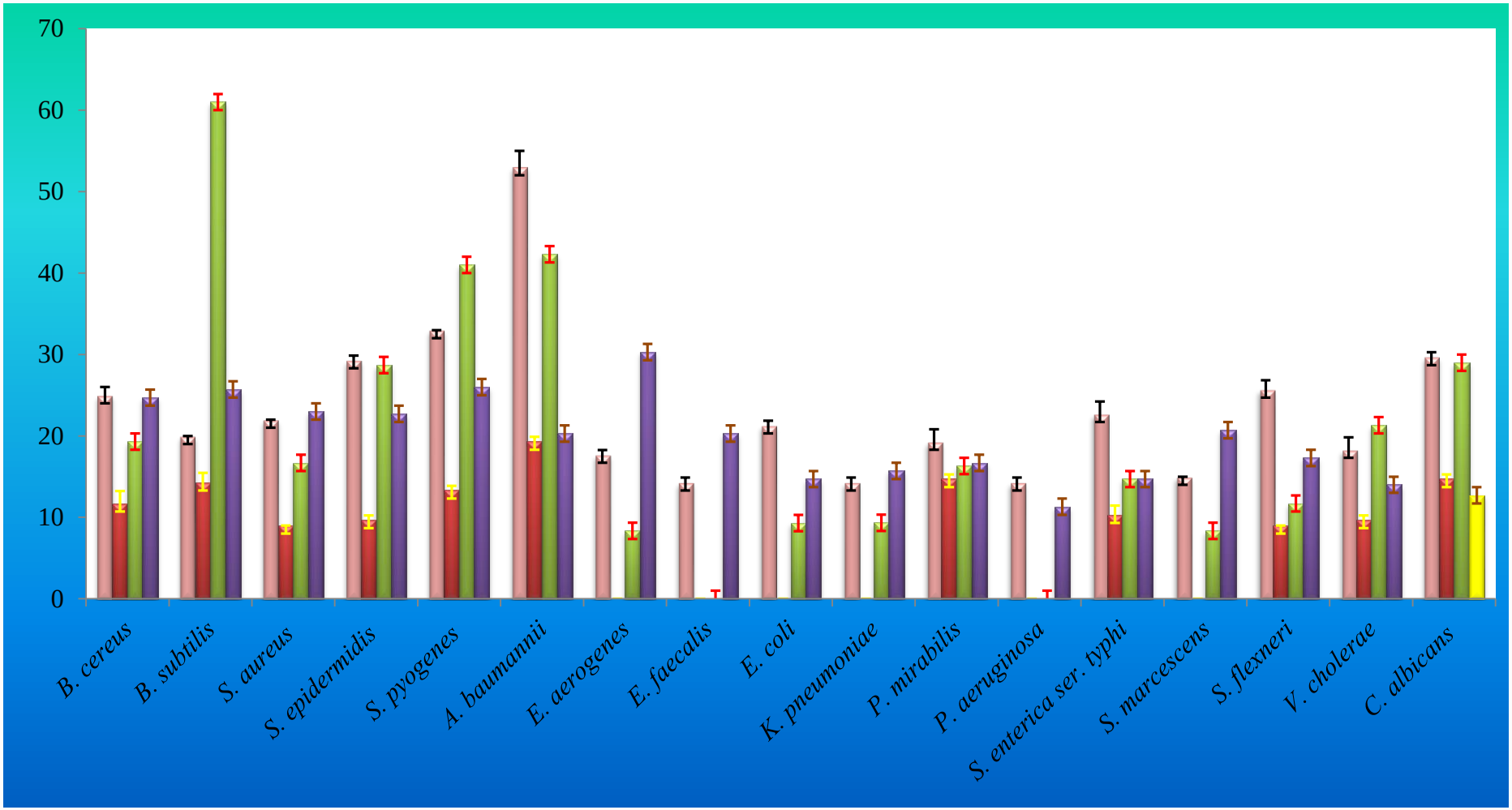
All the wells were inoculated with (24 hours active culture test) organism and incubated at 37°C for 24 hours (bacteria) and fungal plates at 28°C for 48 hours.

20 µL of 1% aqueous solution TTC was added to the each well for visualizing the growth with red colour. MIC values were taken as the lowest concentration of essential oil that prevents visible microbial growth after incubation

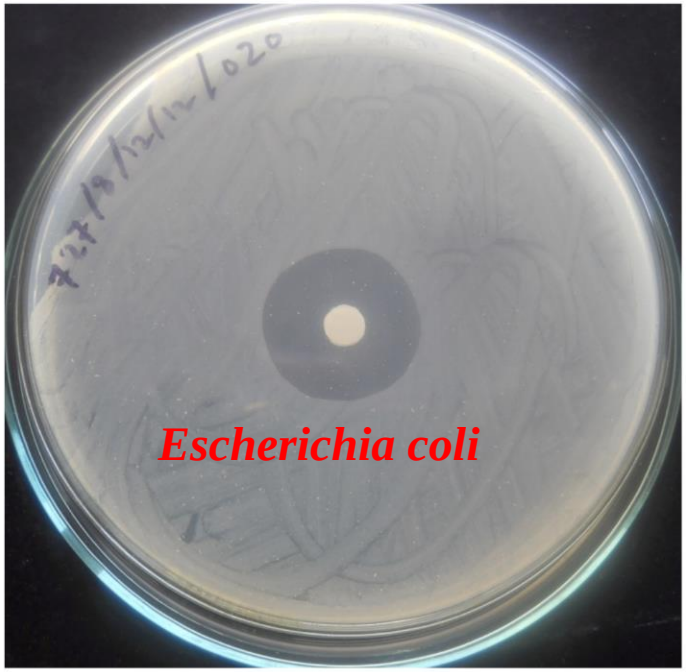
RESULTS OF ANTIMICROBIAL ACTIVITY

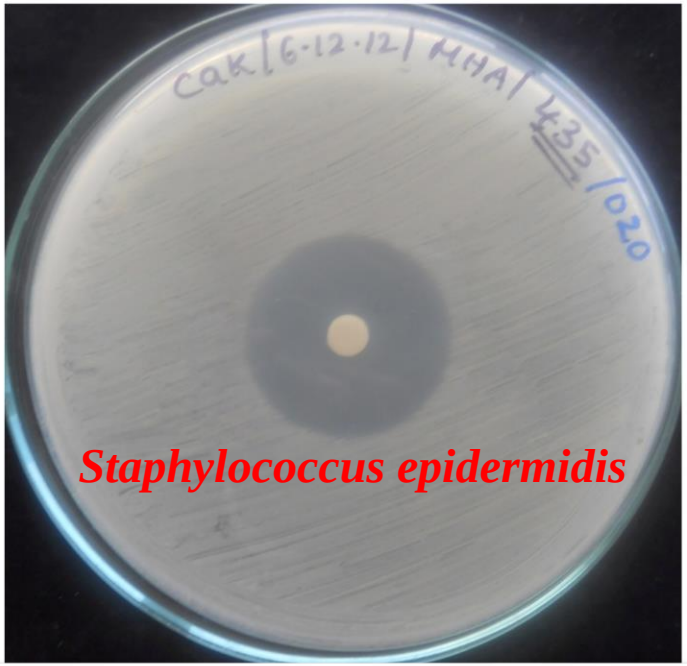
Test Organism	*A. speciosa*		*Z. officinale*		*C. longa*		+ve control gentamicin 10 mcg.	+ve control Ketoconazole 10 mcg.
	ZOI in mm	MIC	ZOI in mm	MIC	ZOI in mm	MIC		
Bacillus cereus	25±1	0.13	19.3±0.58	0.25	11.7±1.53	0.5	25.7±3.2	nt
Bacillus subtilis	20±0	0.06	61±2.65	<0.03	14.3±1.15	0.13	24.7±2.52	nt
Staphylococcus aureus	22±0	0.13	16.7±0.58	0.25	9±0	0.5	20.3±0.58	nt
Staphylococcus epidermidis	29.3±0.58	<0.03	28.7±0.58	<0.03	9.67±0.58	0.25	30.2±2.08	nt
Streptococcus pyogenes	33±0	0.06	41±2.65	2	13.3±0.58	0.13	26±2	nt
Acinetobacter baumannii	53±2	<0.03	42.3±3.21	<0.03	19.3±0.58	0.25	16.2±1.53	nt
Enterobacter aerogenes	17.7±0.58	0.25	8.33±0.58	0.5	0	1	15.7±1.53	nt
Enterococcus faecalis	14.3±0.58	0.13	00	0.25	0	0.25	20.3±0.58	nt
Escherichia coli	21.3±0.58	0.06	9.3±0.58	0.5	0	2	14.7±1.53	nt
Klebsiella pneumoniae	14.3±0.58	0.13	9.33±0.58	0.13	0	1	14±1	nt
Proteus mirabilis	19.3±1.53	0.13	16.3±1.15	0.25	14.7±0.58	0.25	23±2	nt
Pseudomonas aeruginosa	14.3±0.58	0.25	00	0.5	0	0.5	11.3±1.18	nt
S. enterica ser. typhi	22.7±1.53	0.06	14.7±0.58	0.25	10.3±1.15	0.5	22.7±2.08	nt
Serratia marcencens	15±0	0.06	8.33±0.58	0.13	00	0.25	20.7±1.53	nt
Shigella flexneri	25.7±1.15	<0.03	11.7±1.15	0.13	9±0	0.25	17.3±1.53	nt
Vibrio cholerae	18.3±1.53	0.13	21.3±0.58	0.06	9.67±0.58	0.25	14.7±1.53	nt
Candida albicans	29.7±0.58	<0.03	29±1	0.06	14.7±0.58	0.06	nt	12.7 ± 1.53

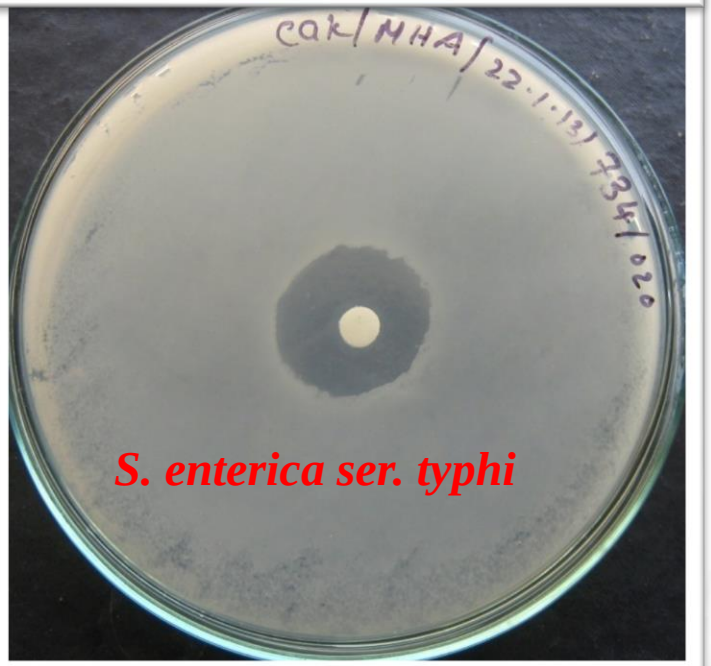
GRAPH OF ANTIMICROBIAL ACTIVITY

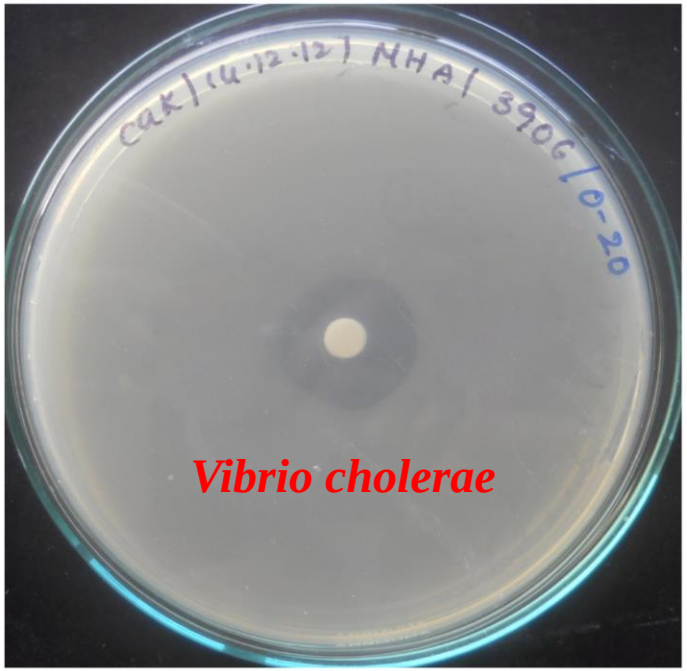


Antimicrobial activity of *Alpinia speciosa* essential oil



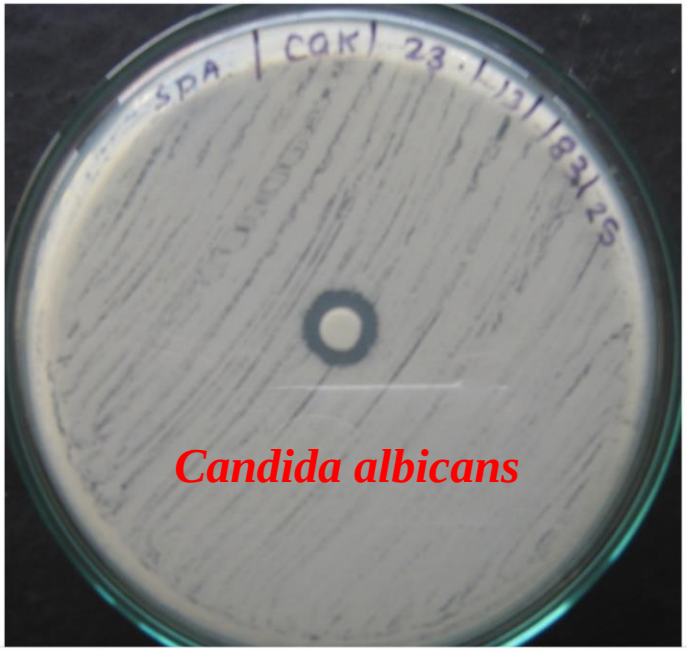


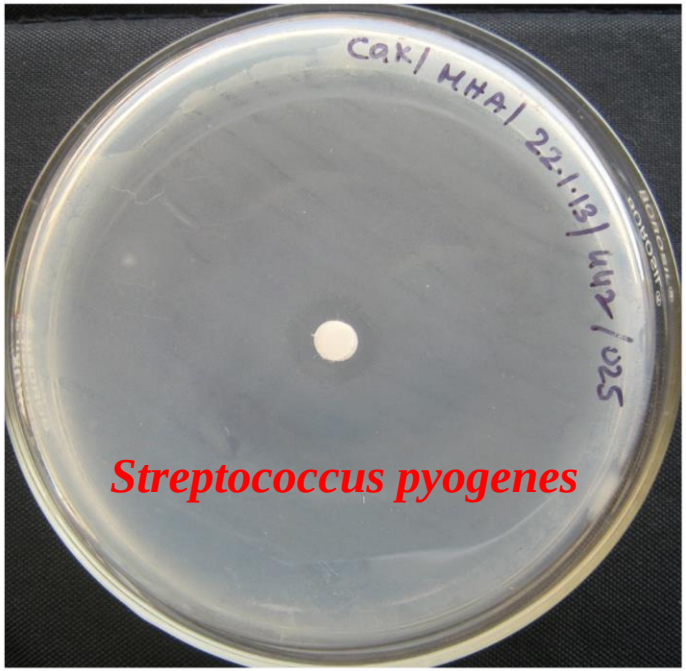


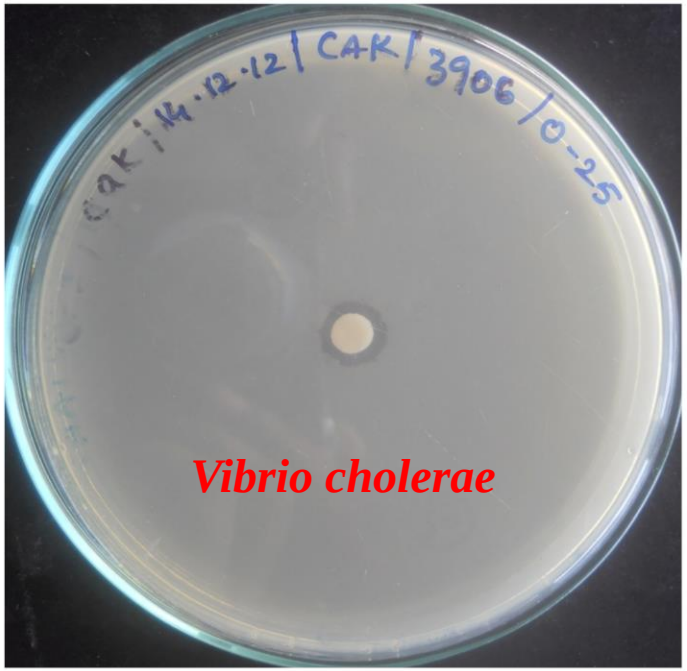


Antimicrobial activity of *Curcuma longa* essential oil

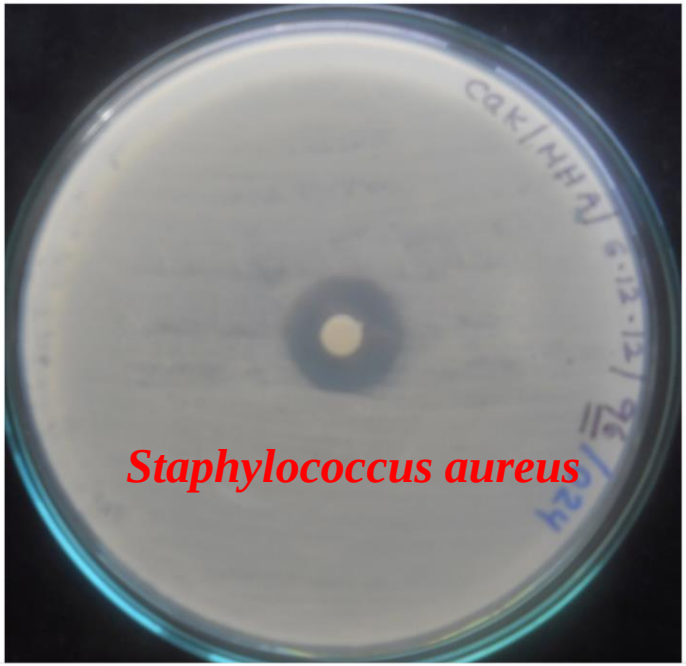


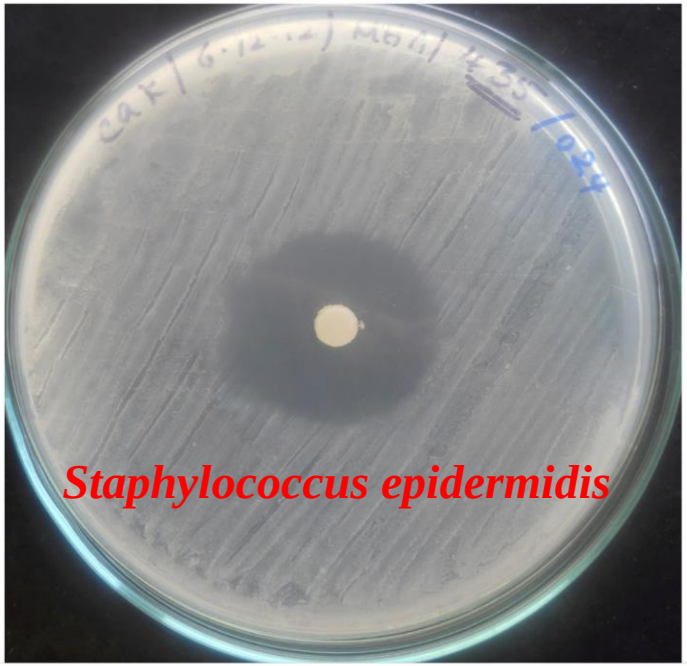


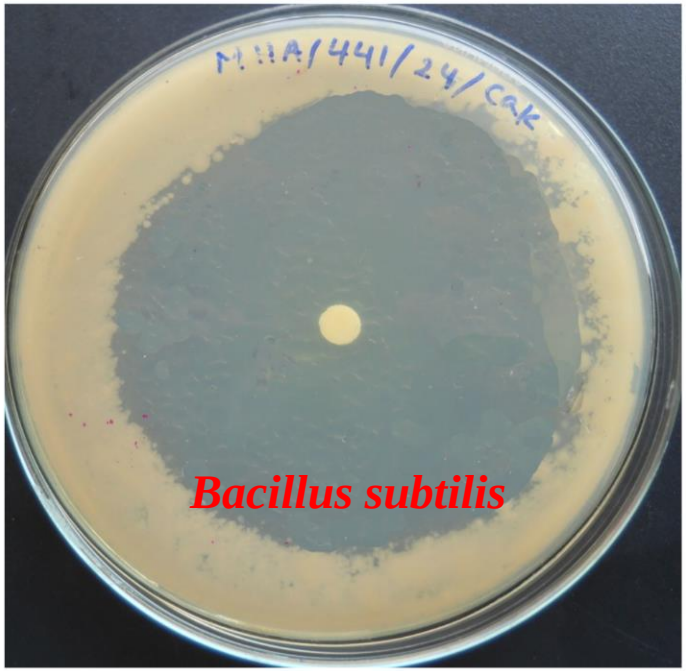


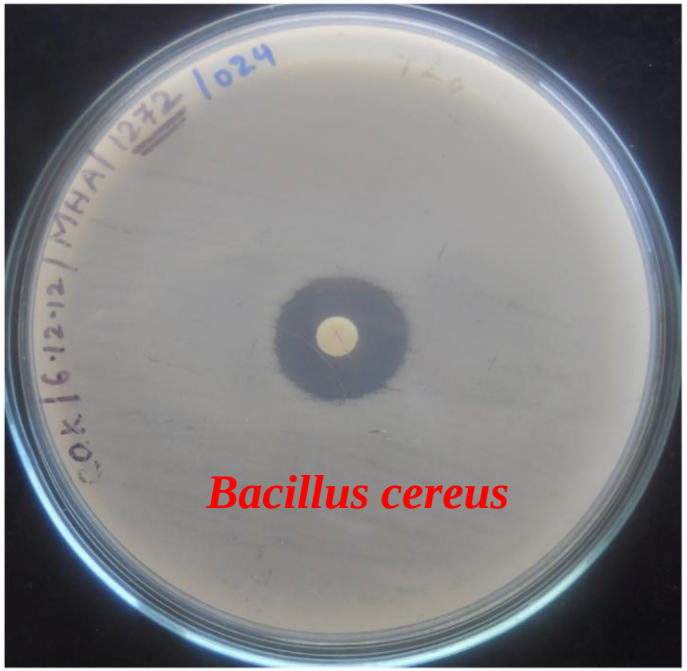


Antimicrobial activity of *Zingiber officinale* essential oil

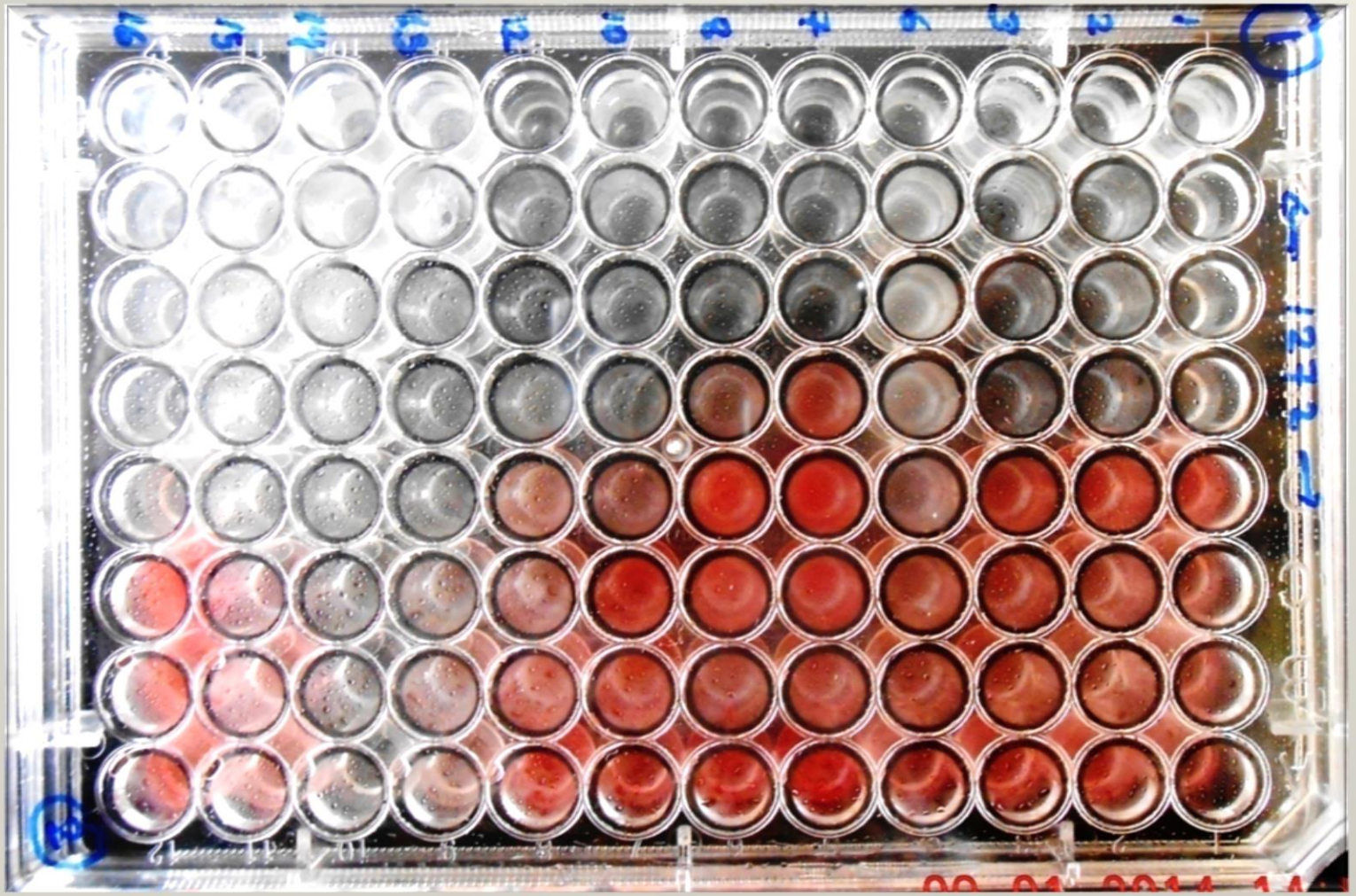








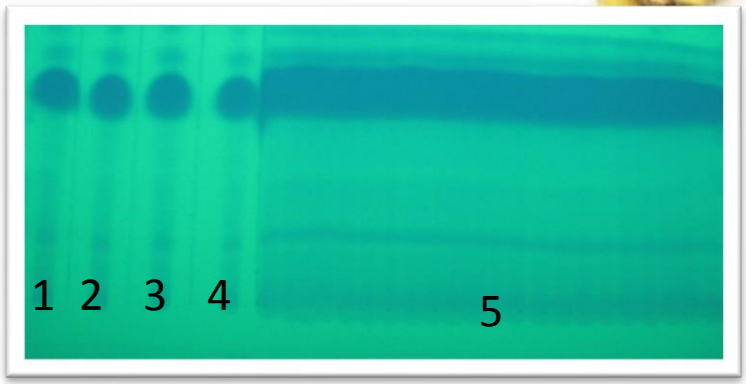
MIC IN 96 WELL PLATE



TLC- BIOAUTOGRAPHY

40 µL of essential oil was diluted with 1 µL of ethanol and sample was subjected for TLC

10 X 20 cm silica gel 60 F_{254} aluminium plate was used and 2 µL each sample was loaded, chromatogram was developed with toluene : ethyl acetate (93:7; v/v)



Out of 1 to 4 portions one portion was stained with Vanillin sulphuric acid reagent and other three portions were dipped for 10 seconds in three different organisms (each in Gram-positive, Gram-negative and fungi respectively)

Fungi dipped plates were incubated at 28°C in a water vapour chamber for 17 hours and bacterial dipped plates were incubated at 37°C for 17 hours

All the plates were dipped for 10 seconds in an aqueous solution of MTT 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (0.1 g/60 mL)





Then bacterial and fungal treated plates were incubated at 37°C for 4 hours and 28°C for 2 hours respectively and observed for appearance of yellow spots against blue background

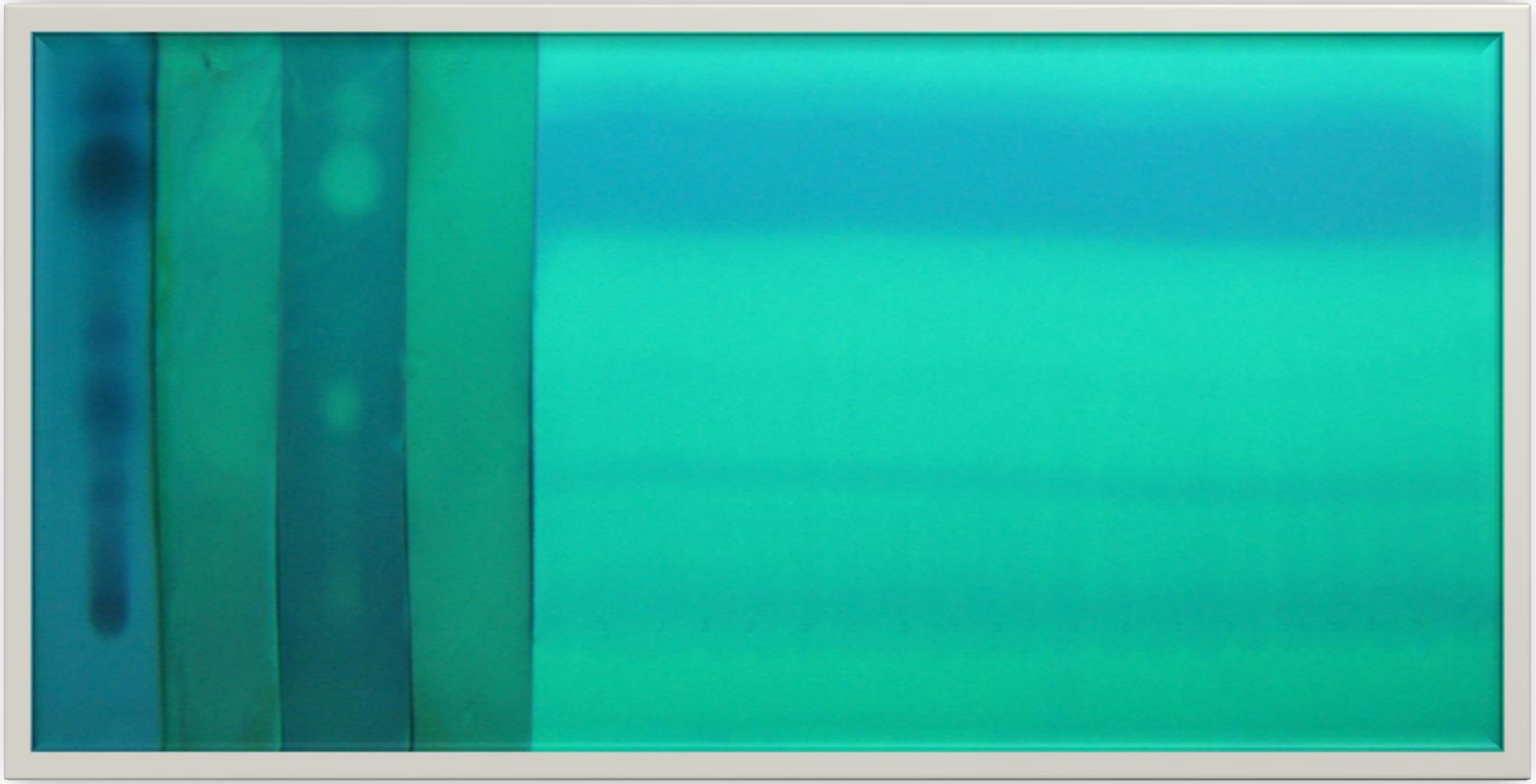
Based on the *Rf* values of bioautography antimicrobial zones were marked (under UV light) on portion -5 of TLC plate and compounds were eluted with ethyl acetate solvent and subjected for GC-MS analysis

GC-MS was performed by SAIF, IIT Mumbai and compound identification was done by comparison of retention indices and mass spectra with those of the standards included in the NIST Library.





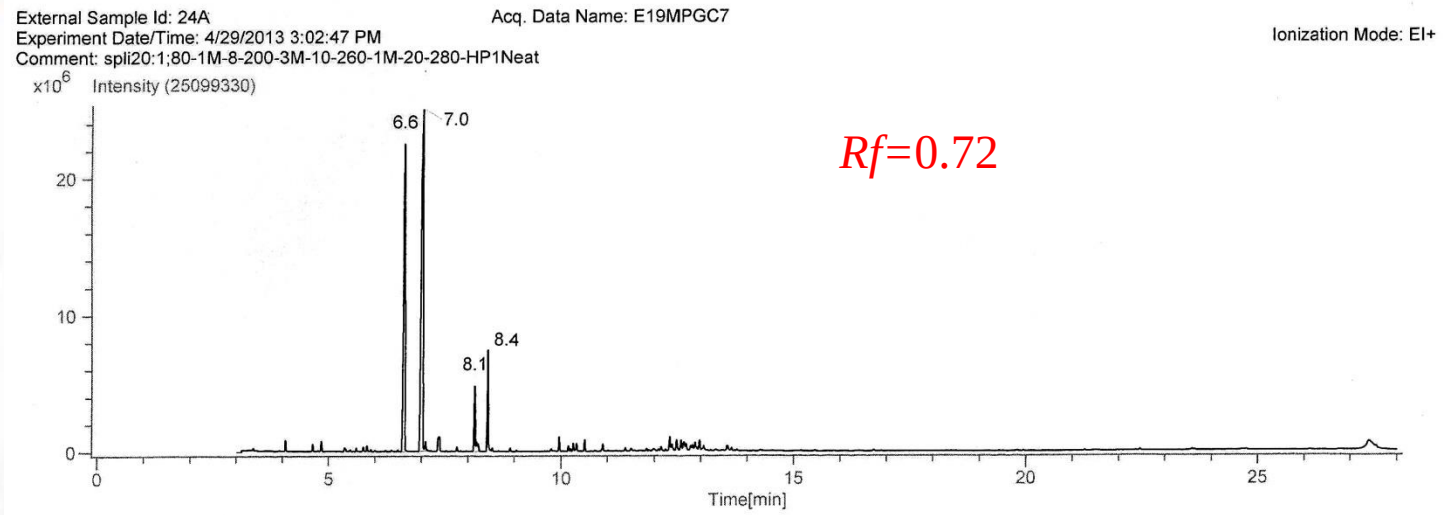
TLC PLATE AFTER BIOAUTOGRAPHY



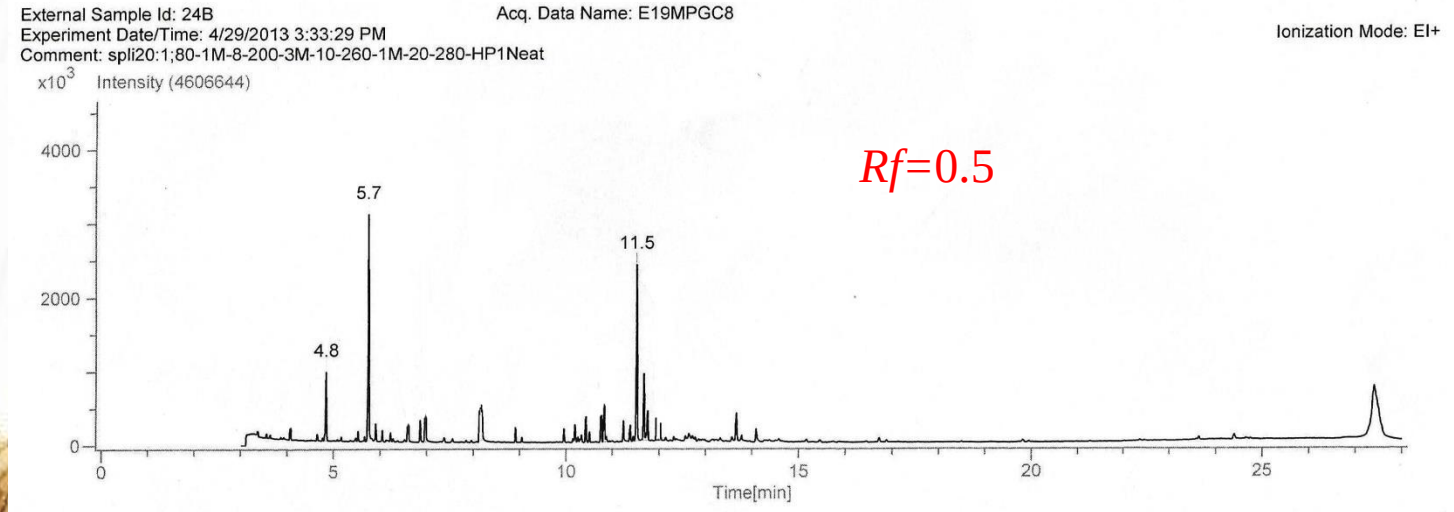
COMPOUNDS IDENTIFIED BY BIOAUTOGRAPHY & GC-MS ANALYSIS

Essential oil	No. Of spots	*Rf*	Retention time	CAS No.	Molecular formula	Common name
A. speciosa	2	*Rf*=0.68	4.2	470-82-6	$C_{10}H_{18}O$	1,8-Cineole, Eucalyptol
			5.2	78-70-6	$C_{10}H_{18}O$	Linalool
			6.8	20126-76-5	$C_{10}H_{18}O$	L-terpinen-4-ol
		Rf=0.31	5.3	98-55-5	$C_{10}H_{18}O$	α-Terpineol
			7	10482-56-1	$C_{10}H_{18}O$	L-α-Terpineol
			11.1	103-26-4	$C_{10}H_{10}O_2$	Methyl cinnamate
			19.2	544-63-8	$C_{14}H_{28}O_2$	Myristic acid
C. longa	1	*Rf*=0.5	8.8	18679-48-6	$C_{10}H_{18}O_2$	Hydroxy-1-8-Cineole
			10	78-70-6	$C_{10}H_{18}O$	Linalool
			24	98-55-5	$C_{10}H_{18}O$	α-Terpineol
			24.5	470-82-6	$C_{10}H_{18}O$	1,8-Cineole, Eucalyptol
Z. officinale	2	*Rf*=0.72	6.6	106-26-3	$C_{10}H_{16}O$	Z-Citral
			7	141-27-5	$C_{10}H_{16}O$	E-Citral
			8.1	97-53-0	$C_{10}H_{12}O_2$	Eugenol
			8.4	501-19-9	$C_{10}H_{12}O_2$	Chavibetol (m-Eugenol)
		Rf=0.5	4.8	78-70-6	$C_{10}H_{18}O$	Linalool
			5.7	464-45-9	$C_{10}H_{18}O$	Borneol
			11.5	465-28-1	$C_{15}H_{26}O$	Carotol

CHROMATOGRAM OF *Z. OFFICINALE* OIL



Rf=0.72



Rf=0.5



ANTIMICROBIAL ACTIVITY OF SELECTED STANDARD COMPOUNDS



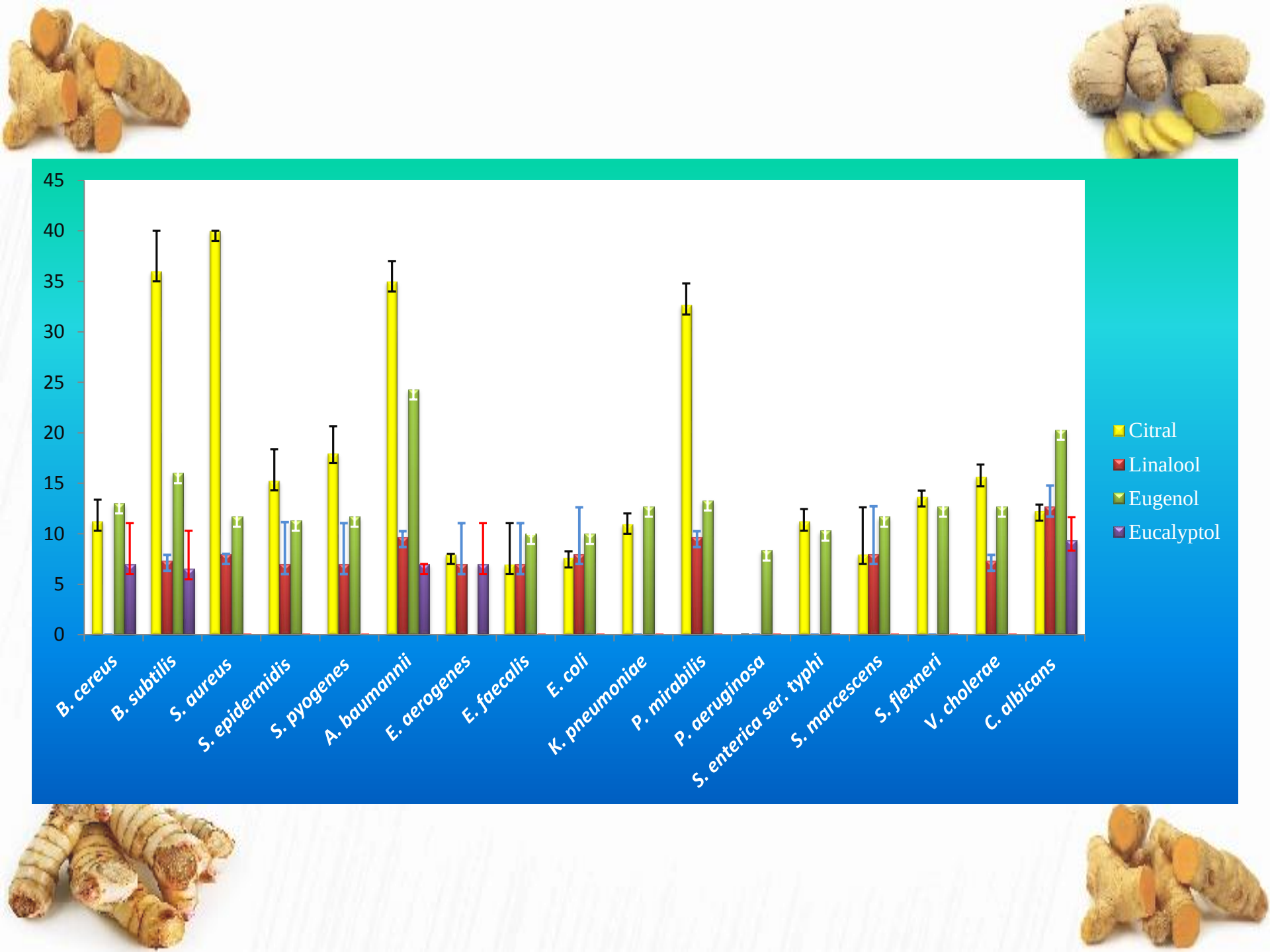
By bioautograhy studies it was observed that Linalool was commonly present in all the three oils. 1,8-Cineole (Eucalyptol) was present in *A. speciosa* & *C. longa* oil and Citral & Eugenol found to be key antimicrobial compounds in *Z. officinal* oil.

By considering the above observations antimicrobial activity of above standard compounds (procured from TCI Japan) was again tested by Kirby-bauer diffusion method individually and combinatorially

Sample was prepared by dissolving 100 µL of pure compound in 1 mL of DMSO and combinations also prepared by the same ratio



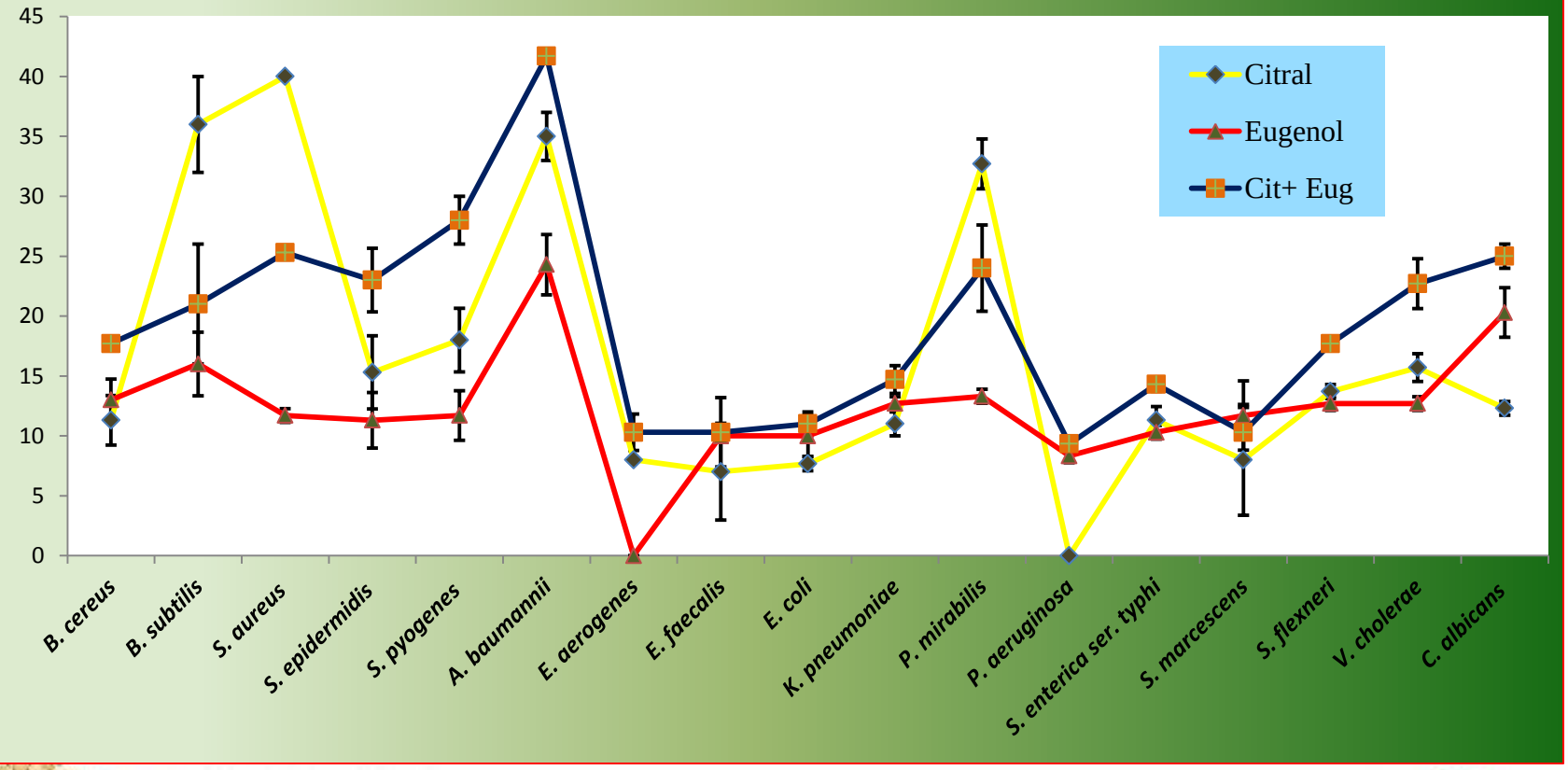



45
40
35
30
25
20
15
10
5
0
B. cereus
B. subtilis
S. aureus
S. epidermidis
S. pyogenes
A. baumannii
E. aerogenes
E. faecalis
E. coli
K. pneumoniae
P. mirabilis
P. aeruginosa
S. enterica ser. typhi
S. marcescens
S. flexneri
V. cholerae
C. albicans
Citral
Linalool
Eugenol
Eucalyptol





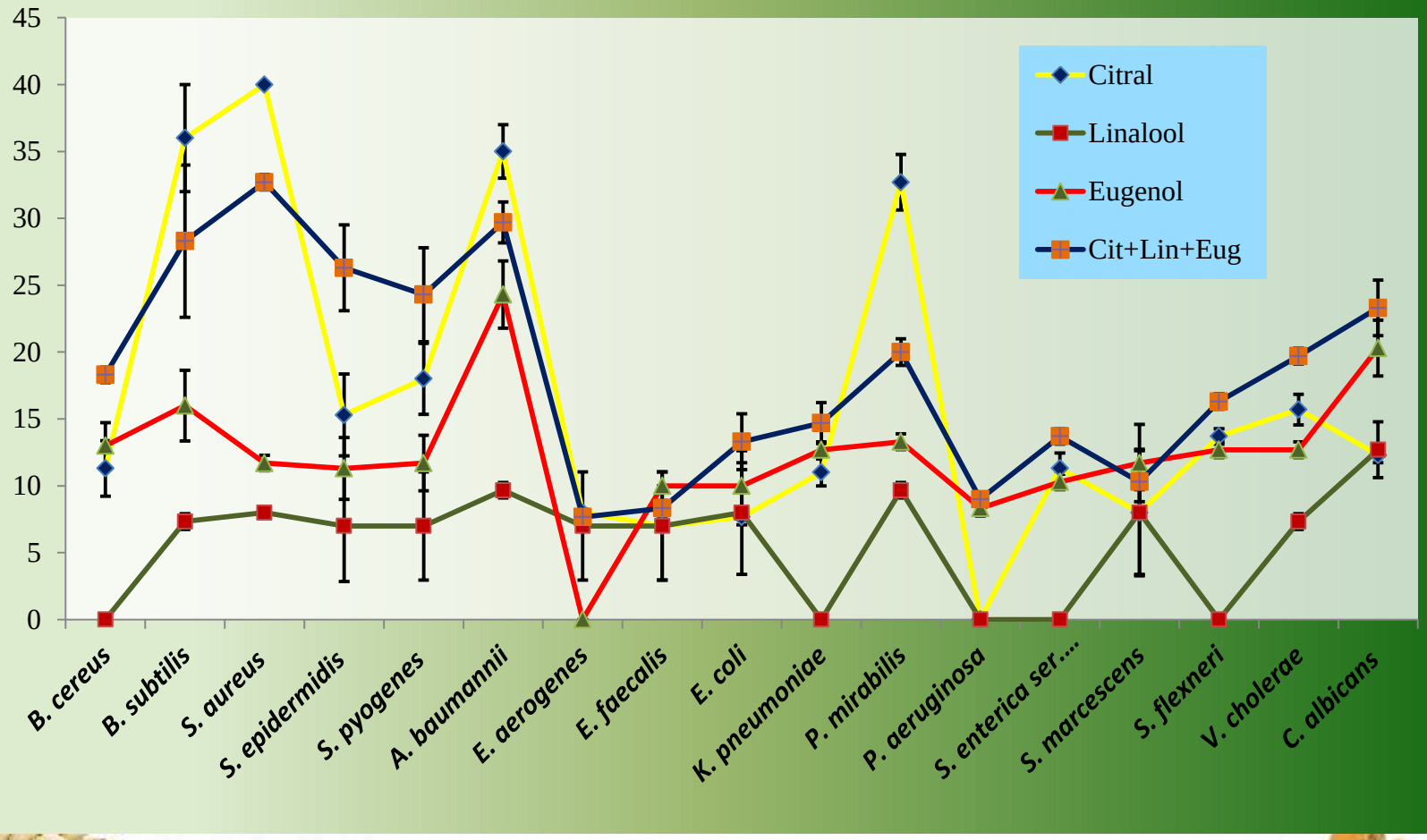
Comparitive line chart representing antimicrobial activity of Citral & Eugenol inviduallly and combinatorially







Comparitive line chart representing antimicrobial activity of Citral, Linalool & Eugenol invidually and combinatorially





CONCLUSION



As the essential oils from leaf material of selected zingiberaceae showed potential antimicrobial activity they can be used for preparation of antiseptic, surface cleaning agents and in cosmetics.

In order to make use of the foliage of test plants, agriculture practices should be slightly changed in such a way that the leaves can be collected after harvesting of rhizomes for extracting essential oils and this will help the farmers with additional financial benefits.






Thank you!