Report of the Study

The project entitled 'A Multidrug Combination (MDC) formulated on the basis of ancient old science of Ayurveda,' was **sanctioned on 23rd March, 2017** at "AIIMS" funded by **Aimil Pharmaceuticals India Ltd**. Under **DR. SARMAN SINGH**, Project Investigator, Department of Microbiology.

The Objectives of the study are as follows;

1) Determination of antibiotic susceptibility pattern of various microorganisms against the Fifatrol (MDC).
2) Determination of anti-microbial activity of Fifatrol against the various sensitive and resistant strains.
3) Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) for antibiotics.
4) In-vitro cytotoxic studies of the drug.

The experiment was performed to determine the antimicrobial pattern of some phytoextract and a multidrug combination (MDC) Fifatrol. Extracts were received on 8th March, 2019 in crude extracts (Solid or Slurry) form, to our research laboratory (Department of Microbiology, AIIMS Bhopal). Details of received samples are given below in the **Table-1**

Table-1: List of Received sample

Methanolic Extract				
S.No.	**Extract Name**	**Weight Obtained**	**Yield**	**Condition**
1	Fifatrol Tablets	28.1572 gms	5.63%	Solid
2	Tribhuvan kirti rasa	3.61465 gms	7.35%	Solid
3	Sanjeevani vati	5.958 gms	12.12%	Solid
4	Mrityunjaya Ras	5.8148 gms	11.83%	Solid
5	Sudarshan Ras	38.7159 gms	78.78%	Solid

Chloroform Extract				
S.No.	**Extract Name**	**Weight Obtained**	**Yield**	**Condition**
1	Fifatrol Tablets	18.8201 gms	3.76%	Solid
2	Tribhuvan kirti rasa	1.8721gms	1.90%	Solid
3	Sanjeevani vati	1.7642 gms	3.59%	Solid
4	Mrityunjaya Ras	6.161 gms	12.53%	Solid
5	Sudarshan Ras	12.7485 gms	25.94%	Solid

Image 1

Image 2 of Received Sample

The sensitivity of these extracts has to be performed against some fastidious, non-fastidious and anaerobic bacteria. The list of the bacterial strains used in the experiment is given below in the **Table-2**;

Table-2: List of Bacterial Strains used in the Study

S.No.	Bacterial Strains	ATCC Code	Procured from
1	*Staphylococcus aureus*	25923	Himedia
2	*Staphylococcus epidermis*	14990	Himedia
3	*Staphylococcus saprophyticus*	49453	Himedia
4	*Streptococcus pneumonia*	49619	Himedia
5	*Streptococcus pyogenes*	19615	Himedia
6	*Enterobactor species*	13048	Himedia
7	*Escherichia coli*	35218	Himedia
8	*Klebsiella species*	700603	Himedia
9	*Moraxella catarrhalis*	8176	Himedia
10	*Neisseria meningitides*	13090	Himedia
11	*Pseudomonas aeruginosa*	27853	Himedia
12	*Proteus mirabilis*	35659	Himedia
13	*Salmonella typhi*	Clinical Isolate	AIIMS Hospital
14	*Enterococcus species*	29212	Himedia
15	*Niesseria gonorrhoeae*	49226	Himedia
16	*Haemophilus influenza*	49766	Himedia

Methodology

Experiment 1- Determination of antimicrobial activity of extracts at 100mg/ml concentration.

1.1 Stock Preparation-

- Final stock concentration of extracts (100mg/ml) was prepared in DMSO (MB058-Himedia), final volume of this stock solution was 10ml.
- Extract was properly dissolved in the solution using vortex machine available in lab.
- The stock solution was stored in dark at room temperature for further work.

1.2 Media Preparation

Mueller Hinton Agar (MHA) media was used (GM391-Himedia) to determine the activity of extracts. Media plates of MHA were prepared in sterile condition and Quality control was performed using *E.coli* (ATCC-25922) as positive control. Obtained growth was pale yellow and luxuriant, uninoculated plate for sterility control.

1.3 Antimicrobial activity using Agar Well-diffusion Technique- (Balouiri *et al.*, 2016)

The agar plate surface is inoculated by spreading a volume (0.5M) of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile tip, and a volume (50µl) of the extract solution at 100mg/ml concentration is introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the test microorganism. The extract diffused in the agar medium and inhibits the growth of the microbial strain tested. This experiment was performed in triplicate against the following bacterial strains listed below in the table;

Table-1.1. List of Bacterial Strains used in the Experiment

S.No.	Bacterial Strains	ATCC No.
1	*E.coli*	25922
2	*Enterococcus feacalis*	29212
3	*P. mirabilis*	35659
4	*K. pneumonae*	700603
5	*K. aerogenes*	13048
6	*P. aeruginosa*	27853
7	*S.aureus*	25923
8	*S.epidermis*	14990
9	*S.saprophyticus*	49453

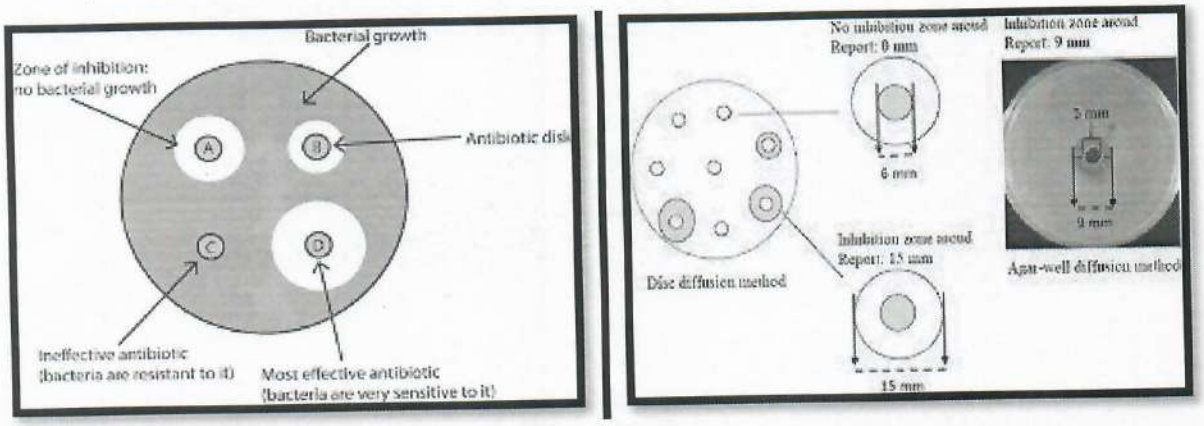


Figure: <u>Demonstration of Agar Well diffusion Method (Source-Google)</u>

1.4 Results of Antimicrobial Activity of Bacterial Strains

a) Chloroform Extract

S.No	Bacterial Strain	Fifatrol	Zone Size	Sudarshan	Zone Size	Sanjeevani	Zone Size	Tribhuvan	Zone Size	Mrityunjaya	Zone Size
1	*E.coli*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
2	*E. feacalis*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
3	*P. mirabilis*	Negative	NI	Negative	NI	Negative	NI	Positive	15.0±0.0	Negative	NI
4	*K. pneumonae*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
5	*K. aerogenes*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
6	*P. aeruginosa*	Negative	NI	Positive	12.2±0.3	Positive	13.1±0.1	Positive	13.2±0.3	Positive	13.3±0.2
7	*S.aureus*	Positive	11.8±0.2	Positive	11.2±0.2	Positive	14.5±0.3	Positive	11.6±0.1	Positive	19.5±0.3
8	*S.epidermis*	Positive	7.5±0.2	Positive	7.4±0.1	Negative	NI	Negative	NI	Positive	10±0.0
9	*S.saprophyticus*	Positive	10.6±0.2	Positive	7.4±0.2	Negative	NI	Negative	NI	Positive	12.0±0.0

b.) Methanolic Extract:

S.No	Bacterial Strain	Fifatrol	Zone Size	Sudarshan	Zone Size	Sanjeevani	Zone Size	Tribhuvan	Zone Size	Mrityunjaya	Zone Size
1	*E.coli*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
2	*E. feacalis*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
3	*P. mirabilis*	Negative	NI	Positive	10.3±0.3	Positive	12.2±0.3	Positive	12.2±0.2	Negative	NI
4	*K. pneumonae*	Negative	NI	Negative	NI	Negative	NI	Negative	NI	Negative	NI
5	*K. aerogenes*	Negative	NI	Positive	11.2±0.3	Positive	11.2±0.3	Negative	NI	Negative	NI
6	*P. aeruginosa*	Positive	15.4±0.2	Positive	14.4±0.1	Positive	14.7±0.2	Positive	13.2±0.2	Positive	15.4±0.1
7	*S.aureus*	Positive	13.3±0.2	Positive	12.7±0.3	Positive	12.6±0.1	Negative	NI	Negative	NI
8	*S.epidermis*	Positive	10.8±0.2	Positive	12.4±0.1	Positive	12.7±0.0	Negative	NI	Negative	NI
9	*S.saprophyticus*	Positive	10.8±0.3	Positive	13.0±0.0	Positive	10.7±0.3	Negative	NI	Negative	NI

***Zone of Inhibition/Zone Size presented in Mean ± SD**

Summary of the Results:

The antimicrobial potential of the experimental plants extracts and a multidrug combination Fifatrol was evaluated according to their zone of inhibition against 9 tested pathogens listed in the Table-3 and the results was quantitatively assessed on the basis of zone of inhibition.

The results revealed that the extracts are potent antimicrobials against examined *Staphylococcus* species i.e., *S.aureus, S.saprophyticus* and *S.epidermis* (Gram positive cocci) and *P.aeruginosa* (Gram negative bacilli) whereas for the species of enterobacteriacae group *E.coli ,E.faecalis, K.aerogenes* and *K. Pneumonae* are resistant for all extracts but *P.mirabilis* are sensitive for Tribhuvan extract of both solvents (Chloroform and Methanol) and the methanolic extract of Sudarshan and Sanjeevani. All three *Staphylococcus* species shows the sensitivity for methanolic extract of Fifatrol, Sudarshan and Sanjeevani whereas for the chloroform extract Fifatrol, Sudarshhan and Mrityunjaya has the potency to inhibit these strains. *P.aeruginosa* was inhibited by all extracts of both solvent, except chloroform extract of Fifatrol. The obtained value of these results are given above in Mean± SD. **(Please Refer Table 4a and 4b)**

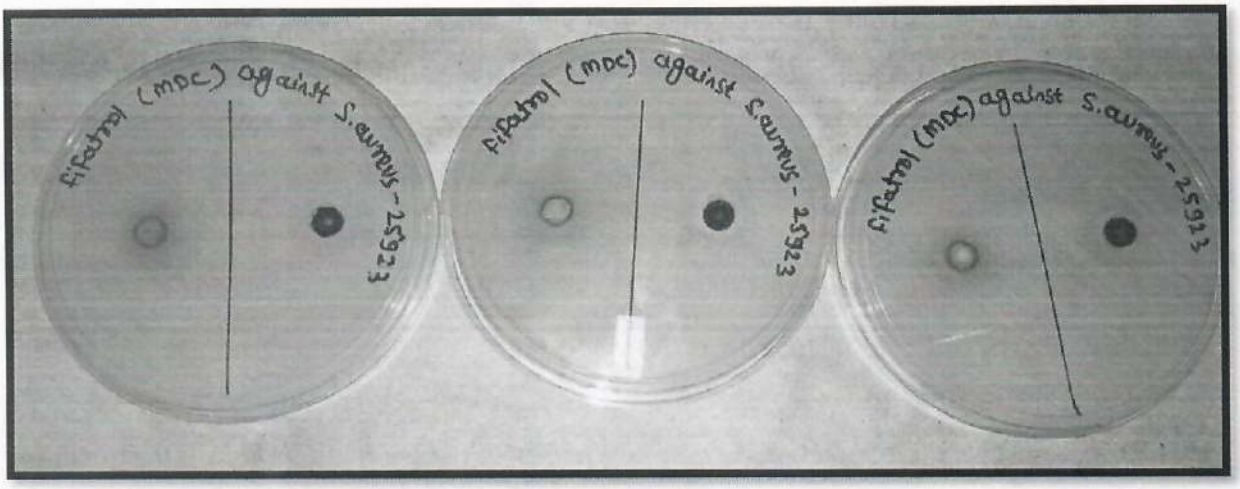


Image of Antimicrobial activity of *S.aureus* (Triplicate)

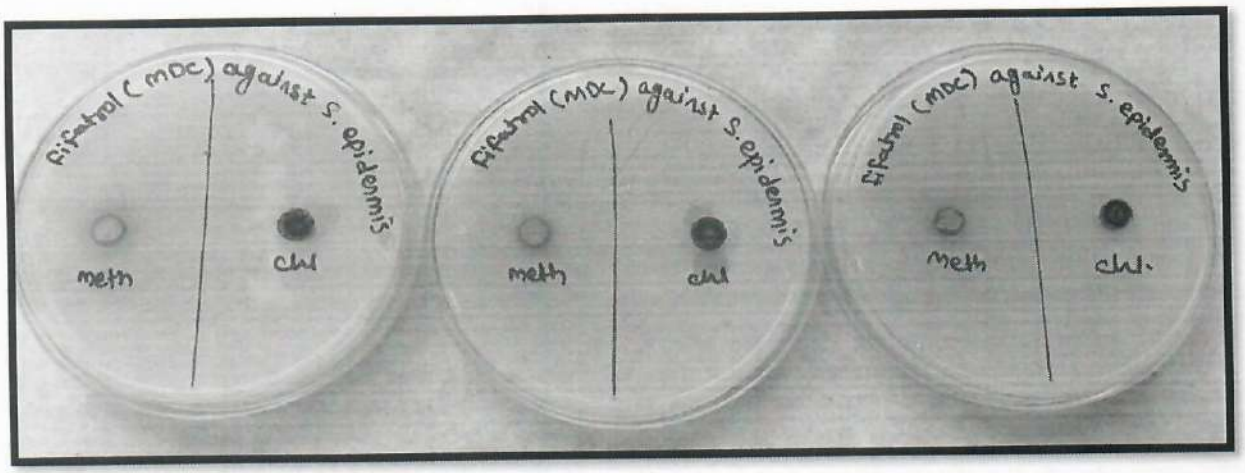
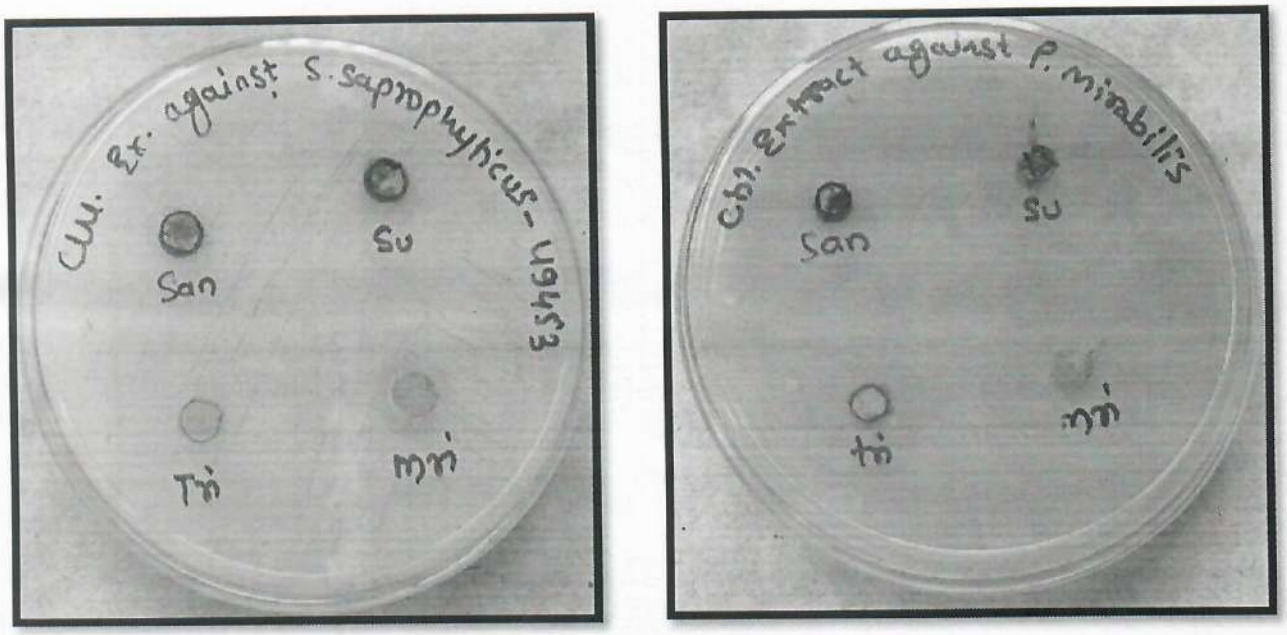


Image of Antimicrobial activity of *S.epidermis* (Triplicate)



Activity of Sanjeevani, Sudarshan, Tribhuvan and Mrityunjaya

Conclusion of the Study: Above results shows that the Fifatrol has sensitivity for *Staphylococcus spp.*(Gram positive) and *P.aeruginosa* (Gram negative) but for other tested strains, there is no inhibition at 100mg/ml concentration.

Comment: As per the discussion with Aimil team the lower concentration was used to determine the efficacy of the extracts. (1mg/ml)

The second experiment was performed to understand the efficiency of the extracts at lower concentration i.e. 1mg/ml. For this, Micro broth dilution technique was used to determine the potency and minimal inhibitory concentration (MIC) value of the extracts.

Experiment-2 Determination of efficiency and minimal inhibitory concentration of extracts.

2.1 Stock Preparation

- Final stock concentration of extracts (1mg/ml) was prepared in DMSO (MB058-Himedia), final volume of this stock solution was 10ml.
- Extract was properly dissolved in the solution using vortex machine available in lab.
- The stock solution was stored in dark at room temperature for further work.

2.2 Media Preparation

Cation-activated Mueller Hinton Broth (MHB) was prepared to perform broth micro dilution technique. 22.0gm Mueller Hinton Broth-II (90922-500G, Sigma Aldrich) was dissolved in 1000ml distilled water and autoclaved at 121^0C for 30 mins. Media was prepared in 2X concentration according to the final volume (100µl) used in the technique.

2.3 Broth micro dilution technique- (Wiegand *et al.*,2008)

For this technique 96-well sterile microtiter plate labelled with the respective extract concentrations 10 different dilutions was prepared separately to examine the inhibitory concentration. Sterility control well-11 was poured with 100µl of MHB medium. Well 12 was used for positive control. Well 1-10 was used for the dilution of extracts. 50µl cation-activated Mueller Hinton medium (2X concentration) was poured in each well then 25µl of drug (4X of 100µl) was added finally 25µl of fresh bacterial inoculum (4X of 100µl) was inoculated in each well under aseptic condition then incubated for 16-20hrs at 37 ^{0}C.The experiment was performed in triplicates and the list of bacterial strains used for the tests are given below in the table. Broth micro-dilution is performed with 10 bacterial strains given in the table below;

Table-2.1. List of Bacterial strains used in the Broth micro-dilution

S.No.	Bacterial Strains	ATCC No.
1	*E.coli*	25922
2	*Enterococcus feacalis*	29212
3	*P. mirabilis*	35659
4	*K. pneumonae*	700603
5	*K. aerogenes*	13048
6	*S.typhi*	Clinical isolate
7	*P. aeruginosa*	27853

8	*S.aureus*	25923
9	*S.epidermis*	14990
10	*S.saprophyticus*	49453

Two fold dilution technique was used to prepared extract dilutions in dimethyl sulfoxide (DMSO) solution and test was performed using 96-well microtiter plate. Prepared dilutions from the final stock concentration are given below;

Table-2.2. Dilutions of the final concentration used in the experiment

S.No.	Dilution (µg/ml)
1	1000
2	500
3	250
4	125
5	62.5
6	32.3
7	15.7
8	7.81
9	3.91
10	1.96

Observation and Result

In the experiment, the MIC is determined at the lowest concentration of the extract at which growth of the test bacteria was inhibited. The obtained results for the methanolic and chloroform extract are given in the **Table-2.3 below**;

Table-2.3. Minimal Inhibitory Concentration of the Chloroform and Methanolic extract

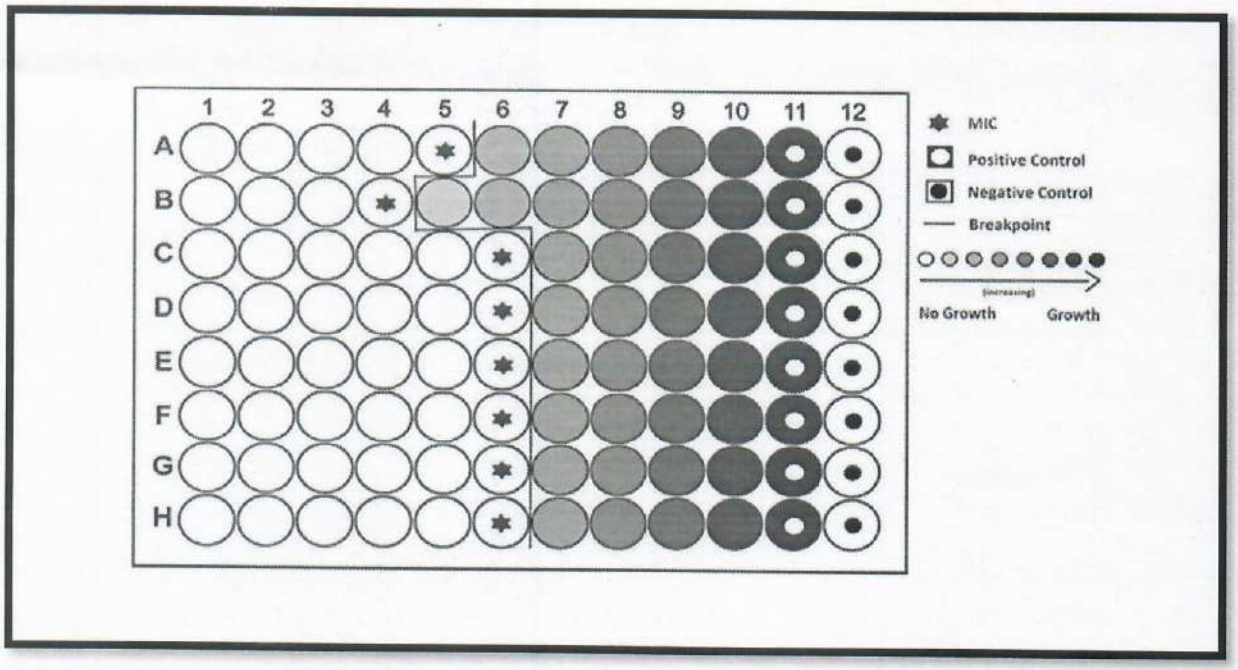
		Chloroform Extract (µg/ml)					Methanolic Extract (µg/ml)				
S.No	**Bacterial Strains**	**Fifa.**	**San.**	**Sud.**	**Mri.**	**Tri.**	**Fifa.**	**San.**	**Sud.**	**Mri.**	**Tri.**
1	***E.coli***	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
2	***E.faecalis***	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
3	***P.mirabilis***	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
4	***K.pneumonae***	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
5	***K.aerogenes***	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
6	***S.typhi***	62.5	125	NI	500	125	31.3	31.3	NI	15.7	31.3
7	***P.aeruginosa***	250	NI	NI	NI	500	NI	NI	NI	250	125
8	***S.aureus***	125	500	15.7	NI	NI	31.3	62.5	15.5	NI	NI
9	***S.epidermis***	125	31.3	250	7.81	7.81	125	62.5	31.3	31.3	3.91
10	***S.saprophyticus***	500	125	NI	500	31.3	125	500	NI	62.5	7.81

***NI- No Inhibition**

****Fifa.-Fifatrol;San-Sanjeevani;Sud-Sudarshan;Mri-Mrityunjaya;Tri-Tribhuvan**

*****Highlighted values are the MIC of the extracts against the bacteria.**

Present investigation revealed the inhibitory concentration of the extracts for respective bacterial strain. In the observation it was found that the ATCC strains of *E.coli, E.faecalis, K.aeruginosa, K.aerogenes* and *P.mirabilis* has resistant for all extracts but clinically isolated strain of *S.typhi* shows the sensitivity for each extract of chloroform and methanol except the Sudarshan extract. *S.aureus* shows the sensitivity for both chloroform and methanol extract of Fifatrol, Sanjeevani and Sudarshan and resistant for Tribhuvan and Mrityunjaya. *S.epidermis* shows the sensitivity for all extracts with high inhibitory potency i.e.,125µg/ml for Fifatrol and 250µg/ml for Sudarshan (Chloroform) extract, while *S.saprophyticus* is resistant for Sudarshan extracts but inhibited by all other extracts. For *P.aeruginosa* chloroform extract of Fifatrol, Tribhuvan and methanolic extracts of Tribhuvan and Mrityunjaya has the potency to inhibit at 250µg/ml, 500µg/ml and 15.7µg/ml and 31.3 µg/ml concentrations, sequentially. (**Please Refer Table 2.**)



Representation of Micro broth dilution on 96 well microtiter plates.

Conclusion of the study:

On the basis of above results it was concluded that the Fifatrol (MDC) extract is more susceptible for *P.aeruginosa, Salmonella typhi* and *Staphylococcus spp.* at 1mg/ml concentration, but resistant for other strains of enterobacteriacae i.e., *E.coli, E.feacalis, K.aerogenes, K.pneumonae* and *P.mirabilis.*

The strain of *Salmonella typhi* was a clinical isolate obtained from a clinical sample after standard identification methods used at AIIMS Bhopal (CLSI-2017). This strain shows encouraging results with significant growth inhibition for the Fifatrol and other extracts, except Sudarshan. Hence, we would like to repeat the susceptibility pattern of these extracts including Fifatrol against the indigenous strains (clinical isolate) of enterobacteriacae family other than *Salmonella typhi.*

References:

- Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis. 2016 Apr 1;6(2):71-9.

- Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature protocols. 2008 Feb;3(2):163.

Fund Utilization-

April 2018 to March-2019

Months	Receipts	Expenditure				
		Staff Salary	Equipment	Materials	Contingency	Prog.Total
APR-2018	0	0	0	0	0	0
MAY-2018	5,00,0000	0	0	0	50,000	50,000
JUN-2018	0	0	0	0	0	50,000
JUL-2018	0	0	0	0	0	50,000
AUG-2018	0	0	0	0	0	50,000
SEP-2018	0	98,927	0	0	0	1,48,927
OCT-2018	0	71,243	0	0	0	2,20,170
NOV-2018	0	10,934	0	0	0	2,31,104
DEC-2018	0	0	0	0	0	2,31,104
JAN-2019	0	0	0	0	0	2,31,104
FEB-2019	0	0	0	0	0	2,31,104
MAR-2019	0	0	0	0	0	2,31,104
Total	5,00,000	181104			50,000	2,31,104
Grand Total	5,00,000		Total Expenditure 2,31,104		Balance	2,68,896

April-2019 to August-2019

Months	Receipts	Expenditure				
		Staff Salary	Equipment	Materials	Contingency	Prog. Total
APR-2019	0	49,967.98	0	0	0	2,18,929
MAY-2019	0	43,413.00	0	0	0	1,75,516
JUN-2019	0	42,622.30	0	0	0	1,32,894
JUL-2019	0	14,413	0	0	0	1,18,480
AUG-2019	0	14,429	0	1,04,481	0	430
Total 6,45,819						

*Note- The material amount is still pending.

Further Work

1. Determination of antibiotic susceptibility of pattern of *Moraxella Catarrhalis is* and some fastidious strains of *Neisseria meningitides, Neisseria gonorrhoeae, Streptococcus pyogenes, Streptococcus pneumoniae and Haemophilus influenzae.*
2. Determination antibiotic susceptibility of all bacteria against fresh extracts.
3. Antibiotic susceptibility of clinical isolates of Enterobacteriacae family.
4. Determination of MIC and MBC of fresh extracts.
5. In-vitro cytotoxic study of the drugs.

Budget (already sanctioned)

Total Budget for the work in INR	***Received Amount in INR (till date)***	***Balance Amount in INR (to be recuped)***
10,19,359	**5,00,000**	**5,19,359**
No Additional Cost for further work.		

Justification for extension of project duration:

- *The project will be carried out for further work with 6 month extension.*
- *Just repeat all experiments with fresh extracts against all 16 strains including clinical isolates of enterobacteriacae other than S.typhi.*
- *One JRF will be carried out all experimental part of the project such as in-vitro antimicrobial testing (MIC & MBC), cytotoxicity studies in cell line model, maintenance of proposed 16 types of different bacterial strains.*
- *Laboratory attendant (One) is required for the preparation of culture media, reagent, washing and cleaning of glassware, autoclaving etc.*
- *If the results of in vitro studies are proving here, in-vivo study in animal will be planned but at* ***Additional cost.***

Prof. Sarman Singh
AIIMS, Bhopal
Project Investigator