ANALYSIS OF NATURAL AND SYNERGISTIC EFFECT OF PLANT MATERIAL AGAINST MULTIDRUG RESISTANT MICROORGANISMS

[1]Vaidehi Patel & [2]Sweta Patel
[1]Research scholar, [2]Research scholar
[1]Department of Microbiology,
[1]Shree K. K. Patel girls science college, Nani Kadi-382715, Gujarat

Received: April 03, 2019 Accepted: May 09, 2019

ABSTRACT: *The antimicrobial effect of four plant extracts was evaluated against medically important clinical isolates. The in-vitro antibacterial activity was performed using agar well diffusion method and the inhibitory zones were recorded in millimeters. Hydroalcoholic extract of Cinnamomum cassia, Nicotiana tabacum, Withania somnifera and Datura stramonium were used for screening of antimicrobial activity against MDR but among four tested plants, hydroalcoholic plant extract of Cinnamomum cassia was found most effective against all MDR isolates. The aim of this study was to verify the synergism between four antimicrobial drugs with Cinnamomum cassia plant extract. As compared to the normal hydroalcoholic plant extract, the combination of plant extract with antibiotics showed higher antimicrobial effect.*

Key Words: *Multi Drug Resistant (MDR), Antimicrobial activity, Plant extract, Antibiotic Susceptibility Testing(AST) and Synergistic activity*

Introduction:

Despite the existence of potent antibiotic and antifungal agents, resistant or multi-resistant strains are continuously appearing, imposing the need for a permanent search and development of new drugs[1]. As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be contained. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy[2]. There is an urgent need to systematically evaluate the plants used in traditional medicine[1].

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs[3]. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant organs[4]. A recent paper on medicinal plants and antimicrobial activity whose objective was to analyze past, present, and future of medicinal plants to suggested as fundamental research on plant extract mechanism of action, interactions with antibiotics or with other medicinal plants, and extracts pharmacokinetic profile [5]. Research on synergism is very limited and few studies have been reported[6]. Thus, in our research, we evaluated in- vitro synergism between plant extracts and antibiotic drugs against various clinical isolates.

Materials and Method

A. Collection of Plant Material

The medicinal plants used for the experiment were Bark of *Cinnamomum cassia*,leaf of *Nicotiana tabacum* , roots of *Withania somnifera,* andleaf *of Datura stramonium*. The identification of plant parts were carried out by comparing the voucher specimen with that of data available at https://plants.usda.gov/. Collected material was washed thoroughly in running tap water to remove all unwanted plant materials, air-dried, crushed and stored in an air-tight container for further use and details of collected plant recorded in Table no.:1.

Table No.1 : Information of some traditionally used Indian medicinal plant species selected for antibacterial activity

No.	Code	Scientific name	Common name	Part used	Fraction used	Solubility of Extract
1	P1	***Cinnamomum cassia***	Taj	Bark	Alcohol	Distilled water
2	P2	***Nicotiana tabacum***	Tamaku	Leaf	Alcohol	Distilled water

3	P3	***Withania somnifera***	Ashwgandha	Root	Alcohol	Distilled water
4	P4	***Datura stramonium***	Dhaturo	Leaf	Alcohol	DMSO

B. Preparation of extract

For Hydroalcoholic extraction, 10 g of air-dried powder was taken in a mixer of 50 ml of ethanol and 50 ml sterile distilled water in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 hours the mixture was filtered using a whatman filter paper no. 1. The solvent was evaporated in hot air oven at 45^0C and dried extracts were collected and used for the further experiment.

C. Collection of bacterial strains: Isolation of clinical strain

Isolation of clinical strain was done from clinical samples using N-agar supplemented with 100µg/ml of ampicillin as a selective media. The clinical strains were selected based on distinct colony morphology and growth in media. For preservation strains were transferred to the slant (N-agar medium containing 100µg/ml of ampicillin) and stored at 4°C. The organisms sub cultured by at 15 days intervals and details recorded in Table no.: 02.

Table No. 2: Information of Clinical strains. (Means: SP- Sputum, ST- Stool, U- Urine, P- Pus)

No.	Sample	Collection site	Observation	Code
1	SP-1-DH	Dharpur Hospital, Patan	Big, Mucoid, Opaque	Code-1
2	SP-2-SAL	SAL Hospital, Ahmedabad	No growth	
3	SP-3-SAL	SAL Hospital, Ahmedabad	Big, Mucoid, Opaque	Code-2
4	ST-1-SAL	SAL Hospital, Ahmedabad	No growth	
5	ST-2-DH	Dharpur Hospital, Patan	Pin point transluant colonies	Code-5
6	ST-3-SAL	SAL Hospital, Ahmedabad	Pin point transluant colonies	Code-4
7	U-1-DH	Dharpur Hospital, Patan	No growth	
8	U-2-DH	Dharpur Hospital, Patan	No growth	
9	P-1-SAL	SAL Hospital, Ahmedabad	Small mucoid with bluish green pigment	Code-3
10	P-2-SAL	SAL Hospital, Ahmedabad	No growth	

D. Morphological and biochemical characterization of clinical strain

Morphological characterization carried out by routine bacteriological tests i.e., by colony morphology and gram staining. Thebiochemical tests were carried out by using Transasia Biochemical Test Kits such as ENTEROtest 24N, OXItest, VPtest and INDOLtest.

E. Antimicrobial susceptibility testing (AST) of clinical isolates

Antimicrobial susceptibility testing of clinical strains was performed by agar disk diffusion method using HiMedia Dodeca Enterobacteriaceae-1 (HiMedia DE053) and Dodeca G-II-minus (HiMedia DE010) disc.

F. Antimicrobial assay:

a. ***Effect of hydroalcoholic plant extract on multidrug resistant isolates***

Antibacterial activity of the different extracts was determined by cup diffusion method on nutrient agar medium against multi drug resistance strains. Wells made in nutrient agar plate using cork borer (6 mm diameter) and desired concentration of inoculums containing of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension and hundred micro-liters of the working solution of different medicinal plant extract and same volume of extraction solvent for control was filled in the wells with the help of micropipette. Plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h [7]. After overnight incubation the plates were observed for the zone of inhibition and the diameter of the inhibition zone were measured.

b. Effect of commercially available antibiotics on multidrug resistant isolates.

Anti MDR activity testing for commercially available antibiotics (Table no. 3) Ciprofloxacin-CP (2mg/ml), Levofloxacin-LV (5mg/ml), Amikacin-AK (125mg/ml), and Kanamycin KN (333.3 mg/ml) was determined by cup diffusion method on nutrient agar medium . Wells are made in nutrient agar plate using cork borer (6 mm diameter) and desired concentration of inoculums containing of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension and hundred micro-liters of the different antibiotics solution was filled in the wells with the help of micropipette. Plates were left for some time till

the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h [7]. After overnight incubation the plates were observed for the zone of inhibition and the diameter of the inhibition zone were measured.

Table no. 3: List of commercially available antibiotics use in this study

Commercial antibiotics	
Antibiotic	**Company name**
Ciprofloxacin (2mg/ml)	Eurolife healthcare Pvt.Ltd.
Levofloxacin (5mg/ml)	AKUMS Drugs and Pharmaceuticals Ltd.
Amikacin (125 mg/ml)	Abbott Healthcare Pvt. Ltd.
Kanamycin (333.3 mg/ml)	MACLEODS Pharmaceuticals Ltd.

c. Antimicrobial activity testing for combination of lower and higher classes of commercially available antibiotics on multidrug resistant isolates.

Anti MDR activity testing carried out for commercially available antibiotics (50% each) Levofloxacin-(LV) in combination with Ciprofloxacin-(CP) and Amikacin-(AK) in combination with Ciprofloxacin-(CP), Levofloxacin-(LV), Kanamycin-(KN) and Kanamycin-(KN) in combination with Ciprofloxacin-(CP), Levofloxacin-(LV) using agar well diffusion method. Incubated plates were observed for the zone of inhibition and the diameter of the inhibition zone were measured.

d. Antimicrobial activity testing for combination of commercially available antibiotics and plant extract on multidrug resistant isolates.

Synergistic antibacterial activity of the *Cinnamomum cassia* extract in combination (50% each) with ciprofloxacin, Amikacin, Kanamycin, and Levofloxacin was determined by cup diffusion method on nutrient agar medium against multi drug resistance strains. Wells made in nutrient agar plate using cork borer (6 mm diameter) and desired concentration of inoculums containing of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension and hundred micro-liters of the mixer of *Cinnamomum cassia* extract and antibiotic was filled in the wells with the help of micropipette. Plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h. After overnight incubation the plates were observed for the zone of inhibition and the diameter of the inhibition zone were measured.

Results and Discussion

A. Morphological and biochemical characterization of clinical strain

Morphological characterization carried out by routine bacteriological tests i.e., by colony morphology and gram staining(Table no:4). Thebiochemical tests were carried out by using Transasia Biochemical Test Kits such as ENTEROtest 24N, OXItest, VPtest and INDOLtest. Further identification done by GIDEON online software and results were recorded in Table no.: 5.

Table: 4 Colony characteristics on N.agar and Gram's reaction

Isolate	Size	Shape	Margin	Elevation	Surface texture	Opacity	Pigmentation	Gram's reaction
Code-1	Big	Circular	Even	Convex	Smooth	Opaque	Colorless	Gram Negative
Code-2	Big	Circular	Even	Convex	Smooth	Opaque	Colorless	Gram Negative
Code-3	Small	Circular	Undulate	Convex	Smooth	Translucent	Green	Gram Negative
Code-4	Small	Circular	Even	Low convex	Smooth	Translucent	Colorless	Gram Negative
Code-5	Small	Circular	Even	Low convex	Smooth	Translucent	Colorless	Gram Negative

Table no. 5: Biochemical characterizations of clinical strains

Biochemical Results						
Test		**Code-1**	**Code-2**	**code -3**	**Code-4**	**Code-5**
Urease	URE	-	-	-	-	-

Arginine	ARG	-	-	-	+	+
Ornithine	ARN	-	-	-	-	-
Lysine	LYS	+	+	-	-	-
Hydrogen sulphide	H2S	-	-	-	-	-
Simmone citrate	SCI	+	+	-	-	-
Salicine	SAL	+	+	-	-	-
Sorbitol	SOR	+	+	-	-	-
Malibiose	MAL	+	+	-	-	-
Cellobiose	CEL	+	+	-	-	-
Lactose	LAC	+	+	-	-	-
Trehalose	TRE	+	+	-	-	-
Duleitol	DUL	-	-	-	-	-
Adonitol	ADO	+		-	-	-
Arabitol	ART	+	+	-	-	-
Sucrose	SUC	+		-	-	-
Inositol	INO	+	+	-	-	-
Raffinose	RAF	+	+	-	-	-
Malonate	MAL	+	+	-	-	-
ß-galactosidase	ONP	+	+	-	+	+
ß-glukuronidase	GLR	-	-	-	-	+
Mannitol	MAN	+	+	-	-	-
Ensculine	ESL	+	+	-	-	-
ß-xylocidase	BXY	+	-	-	-	-
Indole	IND	-	-	-	-	-
V-P	VP	+	+	-	-	-
Oxidase	OXI	-	-	+	-	-
GIDON RESULTS		*Klebsiella pneumonia*	*Klebsiella pneumoniae*	*Pseudomonas aeroginosa*	*Shigella sonnie*	*Shigella boydii*

B. Antimicrobial susceptibility testing (AST) of clinical isolates

Antimicrobial susceptibility testing of clinical strains was performed by agar disk diffusion method using

HiMedia Dodeca Enterobacteriaceae-1 (HiMedia DE053) and Dodeca G-II-minus (HiMedia DE010) disc. All five clinical isolates have multidrug resistant towards tested antibiotics and results were recorded in Table no.:6.

Table no. 6: Antimicrobial susceptibility testing (AST) of clinical isolates

			Zone of inhibition(mm)				
Antibiotic	**symbol**	**conc.**	**code-1**	**code-2**	**code-3**	**code-4**	**code-5**
Amikacin	AK	30 mcg	00	00	00	30	30
Ciprofloxacin	CIP	5 mcg	10	00	00	12	00
Cephotaxime	CTX	30 mcg	00	00	00	20	22
Cefuroxime	CXM	30 mcg	00	00	00	00	00
Augmentin	AMC	30 mcg	00	00	00	00	00
Lomefloxacin	LOM	30 mcg	14	00	20	10	14
Ceftazidime	CAZ	30 mcg	00	00	00	00	00
Cefaperazone	CPZ	75 mcg	00	00	00	18	00
Gentamicin	GEN	19 mcg	00	00	00	14	28
Netillin	NET	30 mcg	00	00	00	24	30
Pefloxacin	PF	5 mcg	04	00	14	00	00
Ofloxacin	OF	5 mcg	06	00	12	00	6
Amphicilin	AMP	10 mcg	00	00	00	20	26
Co-trimoxazole	COT	25 mcg	10	00	00	26	30
Ceftazidime	CAC	30 mcg	12	00	00	24	28
Cefepime	CPM	30 mcg	12	00	10	28	30
Imipenem	Imp	10 mcg	24	10	16	32	30

The antimicrobial activity of the plant extracts were studied against different five Gram negative pathogenic bacterial strains-*Pseudomonas aeruginosa, Shigella sonnie, Shigella boydii and Klebsiella pneumoniae(Cl-1), Klebsiella pneumoniae(Cl-2)*and based on AST result all five isolates were multidrug resistance strains. Four different types of test samples were prepared including hydroalcoholic extracts, commercially available antibiotics, combined lower and higher classes of antibiotics and hydroalcoholic extract in combination with antibiotics. Antibacterial potential of test samples were assessed in terms of zone of inhibition of bacterial growth.

C. Effect of hydroalcoholic plant extract on multidrug resistant isolates

In comparison to *Nicotiana tabacum, Withania somnifera* and *Datura stramonium* plant extracts, *Cinnamomum cassia* (P1) given maximum zone of inhibition against all five isolates. The results presented in Figure: 1.

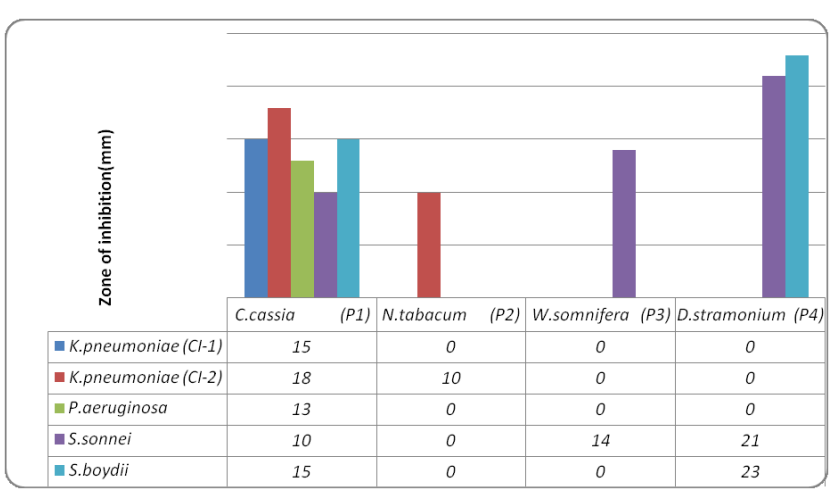
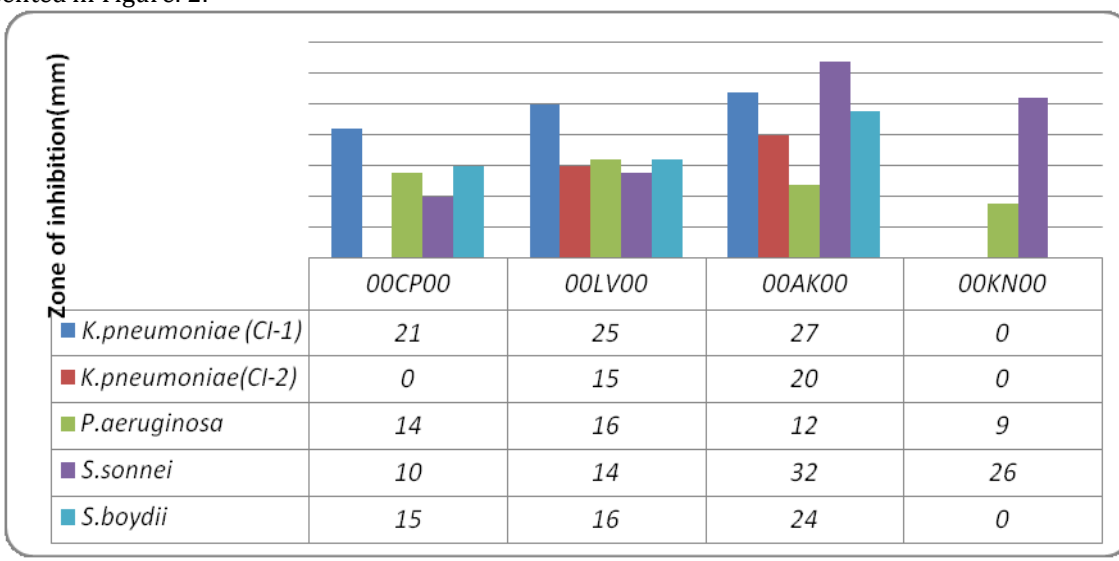


Figure 1: Antimicrobial activity of plant extracts against clinical isolates

D. Effect of commercially available antibiotics on multidrug resistant isolates

Antimicrobial activity of antibiotics against clinical isolates was carried out. In which, Amikacin and Levofloxacin gave maximum inhibition against all five clinical isolates.Here, Kanamycin gave inhibition against only two isolates *P. aeruginosa* and *S. sonnei.* Data of antimicrobial activity of various antibiotics presented in Figure: 2.

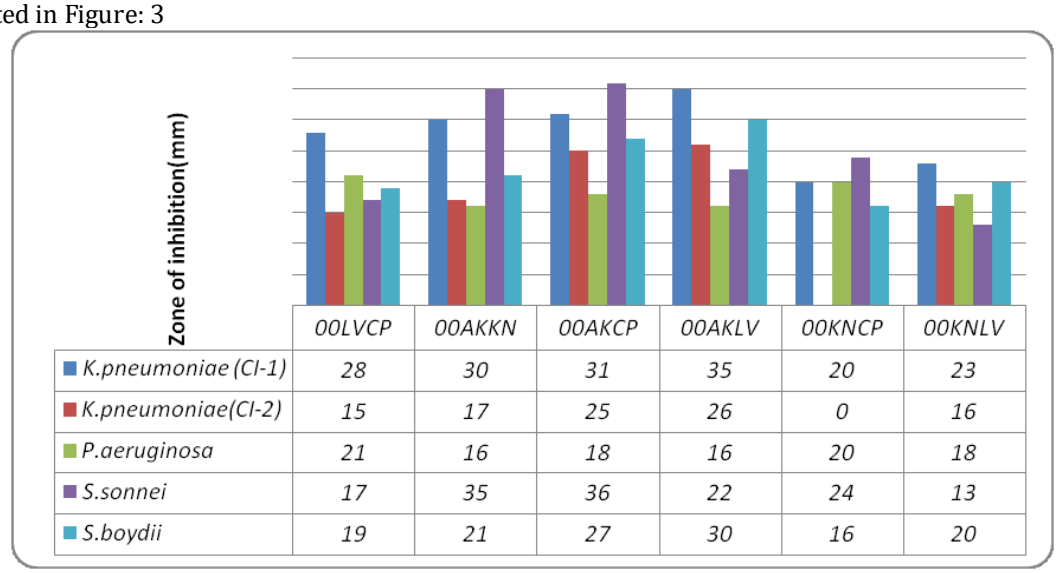


	00CP00	00LV00	00AK00	00KN00
K.pneumoniae (CI-1)	21	25	27	0
K.pneumoniae(CI-2)	0	15	20	0
P.aeruginosa	14	16	12	9
S.sonnei	10	14	32	26
S.boydii	15	16	24	0

Figure 2: Antimicrobial activities of Antibiotics against clinical isolates

E. Effect of combination of lower and higher classes of antibiotics on multidrug resistant isolates.

The combined activity of antibiotics gave higher inhibition as compared to individual antibiotic. The results of the antibacterial activities for various combination mixers of commercial available antibiotics are presented in Figure: 3

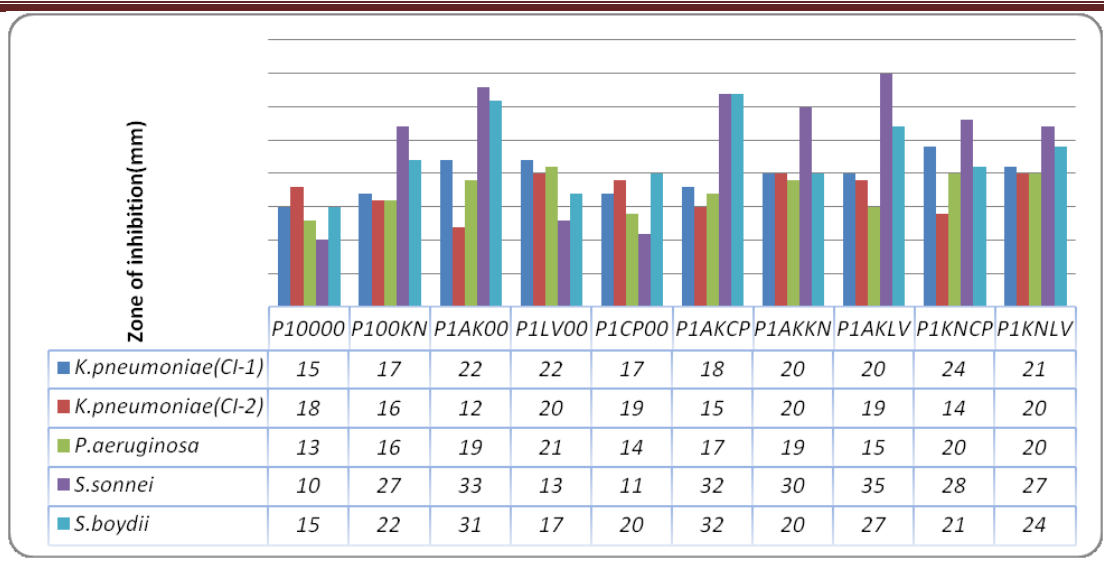


	00LVCP	00AKKN	00AKCP	00AKLV	00KNCP	00KNLV
K.pneumoniae (CI-1)	28	30	31	35	20	23
K.pneumoniae(CI-2)	15	17	25	26	0	16
P.aeruginosa	21	16	18	16	20	18
S.sonnei	17	35	36	22	24	13
S.boydii	19	21	27	30	16	20

Figure 3: Antimicrobial activities of antibiotic combinations against clinical isolates

F. Antimicrobial activity testing for combination of commercially available antibiotics and plant extract on multidrug resistant isolates

In our study, it was interested to note that among all four plant extract*Cinnamomum cassia* gave highest antimicrobial activity against all MDR isolates. On bases of that data synergistic interaction between antibiotics like Ciprofloxacin, Amikacin, Kanamycin, and Levofloxacin with crude plant extract of *Cinnamomum cassia* (P1) was determined by agar well diffusion method. As compared to individual plant extract the combination of plant extract with antibiotics gave higher inhibition of MDR isolates. (Figure:4)



	P10000	P100KN	P1AK00	P1LV00	P1CP00	P1AKCP	P1AKKN	P1AKLV	P1KNCP	P1KNLV
K.pneumoniae(Cl-1)	15	17	22	22	17	18	20	20	24	21
K.pneumoniae(Cl-2)	18	16	12	20	19	15	20	19	14	20
P.aeruginosa	13	16	19	21	14	17	19	15	20	20
S.sonnei	10	27	33	13	11	32	30	35	28	27
S.boydii	15	22	31	17	20	32	20	27	21	24

Figure 4: Antimicrobial activities of plant extract in combination with antibiotics against clinical isolates

Conclusion:

Plant extracts have potential as an antimicrobial compounds against multidrug resistant microorganisms. Thus, they can be used as an antimicrobial agent in infectious diseases caused by resistant microbes. In the present study, the antimicrobial activity of plant extracts on MDR strains was confirmed and synergism with four antimicrobial drugs tested. The synergistic effect from the combination of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. As many medicinal plants still remain unexplored, there are enormous opportunities for the discovery of novel resistance modifying compounds or isolation of pharmacologically active compounds from the plant origins and also, combination of plant extract with available antibiotics to improve action of antibiotics. This could in future be followed by *in vivo* assessments to determine the clinical relevance of such compounds. This denotes a latent area of future investigation.

References:

1. J. Parekh and S. Chanda, In-vitro Antimicrobial Activities of Extracts of Launaea procumbens Roxb . (Labiateae), Vitis vinifera L . (Vitaceae) and Cyperus rotundus L . (Cyperaceae), African Journal of Biomedical Research, 9 (2006), pp. 89 -93 https://www.ajol.info/index.php/ajbr/article/view/48780/35130
2. T. Sibanda and A. I. Okoh, The challenges of overcoming antibiotic resistance : Plant extracts as potential sources of antimicrobial and resistance modifying agents,African Journal of Biotechnology, 6(25) (2007), pp. 2886-2896
3. N. S. Ncube, A. J. Afolayan, and A. I. Okoh, Assessment techniques of antimicrobial properties of natural compounds of plant origin : current methods and future trends, African Journal of Biotechnology, 7 (12) (2008), pp. 1797-1806 https://www.ajol.info/index.php/ajb/article/viewFile/58804/47130
4. B. Mahesh and S. Satish, Antimicrobial Activity of Some Important Medicinal Plant Against Plant and Human Pathogens,World Journal of Agricultural Sciences, 4 (S) (2008), pp. 839-843
5. J. L. Ríos and M. C. Recio, Medicinal plants and antimicrobial activity, Journal of Ethnopharmacol, 100(2005), pp. 80-84 https://doi.org/10.1016/j.jep.2005.04.025
6. J. E. C. Betoni, R. P. Mantovani, L. N. Barbosa, L. C. Di Stasi, and A. Fernandes, Synergism between plant extract and antimicrobial drugs used on Staphylococcus aureus diseases, Mem. Inst. Oswaldo Cruz, 101(2006), pp. 387–390
7. B. Joshi, G. P. Sah, B. B. Basnet, M. R. Bhatt, and D. Sharma, Phytochemical extraction and antimicrobial properties of different medicinal plants: Ocimum sanctum (Tulsi), Eugenia caryophyllata (Clove), Achyranthes bidentata (Datiwan) and Azadirachta indica (Neem), Journal of Microbiology and Antimicrobials, 3(2011), pp. 1-7