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Screening for Antimicrobial Synergism between Phytochemicals and Antibiotics against Methicillin-Resistant *Staphylococcus aureus* (MRSA) using a Microplate Method

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Abstract

Agar diffusion techniques have been widely used over time to assay plant extracts for antimicrobial activity, but some problems have been identified with this technique over time. A microdilution technique was developed using 96-well microplates to indicate bacterial growth to determine their assay. The intent behind this study is to appraise the antimicrobial synergism of plant extracts and antibiotics; Antimicrobial synergism between aqueous extracts of *Hunteria umbellata*, *Moringa oleifera*, *Azadirachta indica,* and existing antibiotics (Azithromycin, Clindamycin, and Vancomycin) was examined on Methicillin-Resistant *Staphylococcus aureus* (ATCC 33591) using a Microplate method. The Microdilution technique, using a Microplate photometer was used to determine the Minimal Inhibitory Concentration (MIC) of both the plant extracts and antibiotics. The MICs of all antibiotics ranged between 0.39 and 100 µg/mL while those of plant extracts varied between 1.56 and 100 mg/ml. Combination studies were cross-examined using the microbroth dilution by characterizing all the expected effects as synergistic, additive, and antagonistic between various plant extracts, and the antibiotics. Synergy was observed more frequently with Azithromycin at a significant level of P>0.05. The microplate method showed synergistic effects between the combination of antibiotics and plant extracts with a significant reduction in the MICs of the test antibiotics against strains of MRSA (ATCC 33591) justifying their use during antibiotic treatment. The synergistic interactions indicated that the inhibitory potentials of the plant extracts increased; combining natural products derived from Phytochemicals and antibiotics could be another way to mitigate and fight against antibiotics resistant infectious bacteria

Keywords: Microplate Method, MRSA, Minimum inhibitory concentration, Synergism, Antibiotics, Phytochemicals

1 Introduction

The resurgent of resistant bacterial strains majorly in communities i.e. schools, hospitals, warehouses, etc [1] has led to the increase in the number of research papers on antibacterial activity of phytochemicals (plant extracts) over the years [2, 3], most common methods for screening and assaying antibacterial potentials of plant extracts/phytochemicals are the disc diffusion test and the agar diffusion assay. These two methods do not differentiate between bactericidal and bacteriostatic effects of these phytochemicals, hence making Minimal Inhibitory Concentration (MIC) difficult to be determined or quantified [4]. Hewitt and Vincent, [5] through their research work postulate that plant extracts contain unsubstantiated and unknown components which might lead to a problem of the false positive and false negative during the screening of antimicrobial compounds of plant extracts, hence the Microplate method was developed as a technique and methods for determining and quantifying MICs of large numbers of test samples requiring small amounts of substances [6] just to address the problem of the limitations of agar diffusion assays. Serial dilution of the extracts in some test tubes has been deployed as an alternate technique for general microbial assay followed by the addition of the test organisms to evaluate the MIC for the test organism using turbidity as an indicator for growth. This method requires large quantities of extracts and is therefore not useful in bioassay-guided isolation of antimicrobial compounds. This method can also be used for various microorganisms (both Plate-cultured and broth-cultured), this technique is not that expensive and presents reproducible results. The aim of this study is poised toward screening for synergism of antimicrobial activities of plant extracts and conventional antibiotics using the Microplate method; in this paper, scaling down of the serial dilution technique using 96-well microplates to evaluate plant extracts and antibiotics were carried out.

2 Materials & Methods

2.1 Antibiotics and Preparation of stock solution

Antimicrobials were purchased from their respective manufacturers, such as Azithromycin and Vancomycinfrom Ciron drugs & pharmaceutical Pvt, Ltd (N-118/119, M.I.D.C, Tarapur, Bolsar; India) and whereas Clindamycin were purchased from Arco Life sciences Pvt Ltd (C-86, M.I.D.C.,

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Hingna, Nagpur-16, India). Stock solutions were prepared using the formula:

$$W = \frac{1000 \times V \times C}{P} \qquad (Eq.1)$$

where P is potency given by the manufacturer (µg/mg), V is the volume required (mL), C is the final concentration of the solution (multiples of 1000) (mg/L), and W is the weight of antibiotic in mg to be liquefied in volume V (mL). For example, 1000 x 20 x 10 = 204.08 mg. The stock solutions from the dry powders were prepared at a concentration of 5.120 mg/L for the antibiotics according to Clinical and Laboratory Standards Institute (CLSI) recommendations [7-9].

2.2 Confirmation of MRSA strain and Growth conditions

A control reference Methicillin Resistant *Staphylococcus aureus* ATCC 33591 (clinical isolate) and environmental isolates of Methicillin-Resistant *Staphylococcus aureus* from a study conducted by Akinrotoye *et al*., [10] were used for this research which was confirmed and verified according to CLSI guidelines using BBL™ Oxacillin agar screen test (Müller Hinton Agar with 6 µg/mL Oxacillin and 4 % NaCl) whereby it was spot inoculated on a 0.5 McFarland standard suspension. The plate was then incubated at 37°C for 24h. If any growth (colony) was detected, the isolate will be classified as Oxacillin or Methicillin-resistant [9, 11]. The culture was grown aerobically for 20 h and was continuously shaken at 100 rpm at 37 °C, for antibacterial activity assays 1 mL of each culture was diluted with Müller Hinton Broth medium to 10^5–10^6 CFU/mL

2.3 Minimal inhibitory concentration (MIC) determination

The MICs for *Hunteria umbellata*, *Moringa oleifera*, and *Azadirachta indica* extracts, Azithromycin, Clindamycin, and Vancomycin were evaluated using a Spectrophotometric Microdilution method (SMM) against Methicillin-Resistant *Staphylococcus aureus* ATCC 33591 (clinical isolate) and five other environmental isolates of MRSA from a previous study [10]. The antibiotics were prepared as stock solutions of 5.12 mg/ml in nutrient broth containing glucose 1.0, yeast extracts and tryptone). The wells of a 96-well ELISA tray were filled with 100 µl of exponentially growing culture of MRSA ATCC 33591(about 10^5–10^6 CFU/mL) and added with 100 µl of diluted (stock solution) drugs which include Azithromycin, Clindamycin, and Vancomycin; and also *Hunteria umbellata*, *Moringa oleifera* and *Azadirachta indica* extracts (seeds, stem barks and leaves) in two-fold serial dilution. Control wells were prepared with culture medium, bacterial suspension only, plant extracts only and antibiotics only and then stirred mildly for even distribution [6]. Absorbance level was measured using a Biotek Microplate reader at 630 nm at 0 hours of incubation and 24 hours after incubation, calculating percentage inhibition (95% confidence interval). The plate was incubated at 37 ºC for 24 hours, agitated, and an absorbance reading was taken again at the same wavelength. The absorbance values obtained were subtracted from those taken before incubation. This procedure eradicated the interference of the tested substance and all tests were carried out in triplicate. The MICs value for the antibiotics/plant extracts was expressed as the lowest concentration that inhibits bacterial growth [12, 13]. Calculate the Percentage % of inhibition at a 95% confidence interval

$$Inhibition\ (\%) = 1 = \left[\frac{t24 - to}{C24 - C0}\right] \times 100 \qquad (Eq.2)$$

where t_{24} is the absorbance level of test wells at 24-hour incubation, $\mathbf{t_0}$ is the absorbance level of test wells at 0-hour incubation, C_{24} is the absorbance level of control wells at 24-hour incubation, and C_o is the absorbance level of control wells at 0-hour incubation.

2.4 Evaluation of synergy

MIC of *Hunteria umbellata*, *Moringa oleifera*, and *Azadirachta indica* (aqueous) such as leaves, root, stem bark, and seed extracts alone or in combination with the various antibiotics (Clindamycin, Vancomycin, and Azithromycin) was determined by Microdilution/Microplate technique in line with CLSI standards [14] as stipulated above. Data were analyzed using Statistical Package for Social Sciences (SPSS) version 17.0 for Windows (SPSS, Chicago IL, and the U.S.A). The Mean Standard Deviation (SD), Standard Error (SE), median, and ranges were calculated for continuous variables whereas proportions and frequency tables were used to summarize categorical variables. The levels of significance were considered, and data obtained were inputted using SPSS (version 17.0). Significant differences between means ($p < 0.05$) were separated using Duncan multiple range test.

3 Results and Discussion

The MICs for Azithromycin, Clindamycin, and Vancomycin were evaluated using a Spectrophotometric microdilution method (SMM) by comparison with the microdilution method (MM) against Methicillin Resistant *Staphylococcus aureus* and MRSA (ATCC 33591). The Minimum Inhibitory Concentrations (MIC) of Azithromycin ranged between 1.56 and 12.5µg/mL on all tested MRSA, Clindamycin MIC was 100µg/mL on all test bacteria while MIC of Vancomycin ranged between 50 – 100 µg/mL on all test bacteria while that of the plant extracts (*M.oleifera*, *H.umbellata*, and *A.indica*) varied between 0.39 and 100 mg/mL as shown in Table 1.

TEST WELL	CONTROL WELL	BLANK WELL
Extracts/antibiotics+ Organisms + Nutrient Broth	Organisms + Nutrient Broth	Extracts/antibiotics + Nutrient Broth
→Increase in absorbance level will indicate no inhibition →Decrease in absorbance level will indicate inhibition	→Absorbance level is expected to increase	→No change in absorbance level if extracts/antibiotics is sterile

Figure 1: Showing diagrammatic formula or representation for inhibition percentage

The absorbance of each well was determined using an automatic spectrophotometer tray reader adjusted at 630 nm, the plate was incubated at 37ºC for 18 h, agitated, and absorbance reading was taken at the same wavelength. These absorbance reading values is then subtracted from those obtained before incubation. This procedure helps to eradicate the clumsiness and interference of the tested substance. All tests were performed in triplicate. The MICs value for antibiotics/extracts was expressed as the lowest concentration that inhibits bacterial growth. The MIC determined by the Spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value. MICs obtained with Spectrophotometric microdilution and Macrodilution methods for the antibiotics and plant extracts against Methicillin-Resistant *S. aureus* and the control organisms are shown in Table 2. The results showed that synergism effects between antibiotics and plant extracts occur in 50% of MRSA tested (Table 2). The microdilution method showed synergistic effects between combinations of antibiotics and plant extracts with a significant reduction in the MIC of all the test antibiotics against five strains of MRSA (environmental isolates) and a control organism (ATCC 33591). Table 3 shows the summary of the comparison of the Minimum Inhibitory Concentration of *H. umbellata*, *M. oleifera*, and *A.indica* extracts against all the antibiotics, in which only Azithromycin showed a significant value at $P > 0.05$**.**

These results were surprising because the number of cells inoculated has an effect on the MIC in larger volume serial dilution, the difference in MIC was observed in some plant extracts against test antibiotics inclusive of those, which showed weak antibacterial activity. These results were in agreement with a previous report who mentioned a synergetic effect even though the extracts did not show any activity by themselves [15]. In addition, it was revealed that synergistic effects occurred in about 75 % of resistant strains in which the minimum fold inhibition decreases [11, 16]. Some plant extracts (leaves, roots, bark) showed a decrease in MIC to test antimicrobial agents and this could be referred to that these aqueous extracts possess many different phytochemicals [17], which might inhibit bacteria by different mechanisms and action as exemplified by the double attack of both agents on different target sites of the bacteria; this has been proven to either be an additive or a synergistic effect [18].

Table 1: Minimum Inhibitory Concentration (MIC) of Antibiotics against *Methicillin-Resistant S. aureus*

Antibiotics	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5	Control Organisms
	The minimum inhibitory concentration of antibiotics µg/Ml					
Azithromycin	6.25	3.125	3.125	3.125	1.56	12.5
Clindamycin	>100	>100	>100	>100	>100	>100
Vancomycin	>100	>100	>100	>100	>100	50

KEYS: MRSA = Methicillin Resistant *Staphylococcus aureus*, Control organisms: MRSA (ATCC 33591)

Table 2: Continued: Minimum Inhibitory Concentration (MIC) of Plant extracts against *Methicillin Resistant S. aureus* (mg/ml)

S/N	PLANT EXTRACTS	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5	CONTROL ORGANISMS
		The minimum inhibitory concentration of Plant extracts (mg/mL)					
1	*Azadirachta indica(leaves)*	>100	>100	>100	>100	25	>100
2	*Azadirachta indica(root part)*	>100	>100	>100	>100	>100	>100
3	*Azadirachta indica(stem bark)*	>100	>100	>100	>100	>100	>100
4	*Hunteria umbellate (leaves)*	>100	>100	>100	>100	>100	>100
5	*Hunteria umbellate (seed)*	50	50	>100	>100	>100	>100
6	*Hunteria umbellate (stem bark)*	>100	>100	>100	>100	>100	>100
7	*Moringa oleifera (stem bark)*	>100	>100	0.39	>100	>100	>100
8	*Moringa oleifera(leaves)*	50	>100	>100	>100	50	>100
9	*Moringa oleifera(root)*	>100	>100	>100	>100	>100	>100

KEYS: MRSA = Methicillin Resistant *Staphylococcus aureus*, Control organisms: MRSA (ATCC 33591)

Table 3: Synergistic activity of different Plant extracts and antibiotics [Azithromycin]

PLANT EXTRACTS	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5	CONTROL ORGANISM
	The minimum Inhibitory Concentration (mg/mL)					
(*Azadirachta indica* root part)	>100	>100	>100	>100	>100	>100
Azadirachta indica (leaves)	50	25	12.5	50	50	50
Hunteria umbellate (leaves)	>100	50	50	>100	50	50
Hunteria umbellate (seed)	>100	>100	>100	>100	>100	>100
Hunteria umbellate (stem bark)	25	>100	>100	>100	>100	>100
Moringa oleifera (stem bark)	>100	>100	6.25	>100	>100	>100
Moringa oleifera (leaves)	>100	50	>100	>100	>100	>100

KEYS: MRSA = Methicillin Resistant *Staphylococcus aureus*, Control organisms: MRSA (ATCC 33591)

Table 4: Continued: Synergistic activity of different Plant extracts and antibiotics [Clindamycin]

PLANT EXTRACTS	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5	CONTROL ORGANISMS
	The minimum Inhibitory Concentration (mg/mL)					
Azadirachta indica(leaves)	50	25	12.5	>100	>100	25
Azadirachta indica(root part)	>100	>100	>100	>100	>100	>100
Hunteria umbellate (leaves)	50	12.5	6.25	50	>100	>100
Hunteria umbellate (seed)	25	12.5	50	>100	>100	>100
Hunteria umbellate (stem bark)	>100	>100	>100	>100	>100	>100
Moringa oleifera (stem bark)	>100	>100	>100	>100	>100	>100
Moringa oleifera(leaves)	25	50	>100	>100	>100	>100

KEYS: MRSA = Methicillin Resistant *Staphylococcus aureus*, Control organisms: MRSA (ATCC 33591)

Table 5: Continued: Synergistic activity of different Plant extracts and antibiotics [Vancomycin]

PLANT EXTRACTS	**MRSA1**	**MRSA2**	**MRSA3**	**MRSA4**	**MRSA5**	**CONTROL ORGANISMS**
	The m**inimum Inhibitory Concentration (mg/mL)**					
Azadirachta indica(leaves)	>100	25	50	25	50	25
Azadirachta indica(root part)	>100	>100	50	>100	>100	>100
Hunteria umbellate (leaves)	>100	>100	>100	25	6.25	>100
Hunteria umbellate (seed)	>100	>100	50	>100	50	>100
Hunteria umbellate (stem bark)	>100	>100	>100	>100	25	>100
Moringa oleifera (stem bark)	>100	>100	>100	>100	25	>100
Moringa oleifera(leaves)	>100	>100	>100	>100	>100	>100

KEYS: MRSA = Methicillin Resistant *Staphylococcus aureus*, Control organisms: MRSA (ATCC 33591)

Table 6: Summary of the comparison of the Minimum Inhibitory Concentration of *H. Umbellata* extracts against all antibiotics

EXTRACTS	**ANTIBIOTICS**	**MEAN**	**S.E**	**t-test**	**P-value**
Hunteria Seed	Azithromycin	78.39	10.59	7.405	0.001*
	Clindamycin	-16.67	10.54	-1.581	0.175
	Vancomycin	-8.33	15.36	-0.542	0.611
Hunteria stem	Azithromycin	95.05	1.63	58.14	0.001*
	Clindamycin	100	0.0	0.0	0.0
	Vancomycin	8.33	8.33	1.00	0.363
Hunteria Leaves	Azithromycin	95.05	1.63	58.14	0.001*
	Clindamycin	100	0.0	0.0	0.0
	Vancomycin	8.33	8.33	1.00	0.363

Key: * indicate significant value at P > 0.05, P = 0.05 by Duncan's multiple range test, S.E = Standard Error of mean

Table 7: Continued: Summary of the comparison of the Minimum Inhibitory Concentration of *M. oleifera* against all antibiotics

EXTRACTS	**ANTIBIOTICS**	**MEAN**	**S.E**	**t-test**	**P-value**
Moringa Stem	Azithromycin	78.45	16.31	4.808	0.005*
	Clindamycin	-16.60	16.60	-1.00	0.363
	Vancomycin	-8.27	20.01	-0.413	0.697
Moringa Leaves	Azithromycin	78.39	10.34	7.584	0.001*
	Clindamycin	-16.67	10.54	-1.581	0.175
	Vancomycin	-8.33	15.37	-0.542	0.611
Moringa Root	Azithromycin	95.05	1.63	58.14	0.001*
	Clindamycin	100	0.0	0.0	0.0
	Vancomycin	8.33	8.33	1.00	0.363

Key: * indicate significant value at P>0.05, P=0.05 by Duncan's multiple range test, S.E = Standard Error of mean

Table 8: Continued: Summary of the comparison of the Minimum Inhibitory Concentration of *A. Indica* against all antibiotics

EXTRACTS	ANTIBIOTICS	MEAN	S.E	t-test	P-value
Neem Leaves	Azithromycin	82.55	11.91	6.928	0.001*
	Clindamycin	-12.50	12.50	-1.00	0.363
	Vancomycin	-4.17	16.35	-0.255	0.809
Moringa Stem	Azithromycin	95.05	1.63	58.14	0.001*
	Clindamycin	100	0.0	0.0	0.0
	Vancomycin	8.33	8.33	1.00	0.363
Moringa Root	Azithromycin	95.05	1.63	58.14	0.002*
	Clindamycin	100	0.0	0.0	0.0
	Vancomycin	8.33	8.33	1.00	0.363

Key: * indicate significant value at P>0.05, P=0.05 by Duncan's multiple range test, S.E = Standard Error of mean

For Azithromycin, a good correlation was presented when MIC visual determination was obtained by the Microplate Method and the endpoint of the optical density was measured using the Spectrophotometric method. The MICs obtained by these two contrasting methods were similar for the MRSA but a little discrepancy was observed also; this fact corresponds with the investigation reported by Turner *et al*., [19] in which visual endpoints for Ampicillin against gram-negative rods were more difficult to detect than for others antibiotics. The methodology that has been devised to investigate the antimicrobial potential of plant extracts (phytochemicals) is diverse and many; thereby making the comparison of the obtained MICs very difficult. Standardized *in-vitro* tests are needed for screening trials, the Microplate technique worked even better with reference microorganisms because no precipitate is not going to be formed [20]. Inoculums' size has a large effect on MIC values and some organisms are also affected by external concentrations. In screening for antibacterial compounds or using bioassay-guided fractionation relative values are used, the advantage of using large inoculums and working under non-sterile conditions outweighs the difficulties caused by subsequent inaccuracies. By using agar diffusion techniques [21] found that the MIC of galangin, an antimicrobial compound isolated from *Helichrysum aureoniterls* for *S. aureus* was between 0.5 and 0.1 mg/ml.

4 Conclusion

This method is robust, is less expensive, gives reproducible results, is 30 times more sensitive than other methods used in the literature, and requires a small number of samples, can be used for higher numbers of samples, does not require high levels of skill, leaves a permanent record, and requires little time. One or two of the series of wells should be used with a known antibiotic to provide reference MIC values for the test organism.

Ethical issue

Authors are aware of and comply with, best practices in publication ethics specifically about authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics. Authors adhere to publication requirements that the submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that no conflict of interest would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution to data collection, data analyses, and manuscript writing.

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