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Research Article

Synergistic Interaction of Different Extracts of *Cassia javanica* L. against Multi Drug Resistant Clinical Isolates of *Staphylococcus aureus*

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ABSTRACT
The original prospective of this research is to find out the antibacterial activity of *Cassia javanica* L. in combination with Vancomycin against some multi drug resistant *Staphylococcus aureus* hospital isolates. Minimum inhibitory concentration for each extract of *Cassia javanica* L. was determined by agar dilution and broth macrodilution method. Antimicrobial combination therapy is the most effective future drug treatment with potential bioactivity to treat multi drug resistant bacteria. Methanol extract of bark, flower, leaves and ethyl acetate extract of bark showed excellent synergism with Vancomycin at a concentration of 0.25mg/ml and 0.51mg/ml. The best synergistic activity was observed between methanol extracts of bark and Vancomycin with four time fold reduction from the original MIC values and found very much promising for further novel antibacterial drug discovery research.

Keywords: antimicrobial agent, synergism, natural product, antibiotics and multi drug resistant.

INTRODUCTION

Cassia javanica L. is a medicinally less explored plant in North East India. This is very hard to state about its native because of the widespread cultivation of this plant in tropical regions of Asia, China, Indonesia, and Philippines[1]. *Cassia javanica* L. is very fast growing semi deciduous tree. This tree is commonly known as apple blossom tree or pink and white shower tree. It has a height of about 25-30 meter and breadth 35 cm. *Cassia javanica* L. has a strong traditional belief as an antibacterial within indigenous communities[2]. Young leaves are already proved as hypoglycemic agent with anti-HSV activity[3,4]. Phytochemical screening of different parts of *Cassia javanica* L. testifies the presence alkaloid, saponin, tannin, steroid, flavonoid, anthraquinones etc. and their sensitivity tests proved it to be a potent bactericidal antimicrobial agent[5,6,7]. To prevent recent bacterial infection, different extracts of *Cassia javanica* L. were tested against multi drug resistant clinical isolates. Moreover, their combined effects with Vancomycin were also tested. Antibacterial combination therapy is not a new approach. Nowadays, synergistic effect of glycopeptides and beta-lactam combinations are used to treat Vancomycin resistant *Staphylococcus aureus* (VRSA) strains[8] but synergistic interaction of plant active phytoconstituents in combination with marketed antibiotics always increases their bactericidal ability to treat multi drug resistant strains**.** Recently, it has been reported that plant derived phytochemicals have a very complex chemical structure for which bacteria fails to recognize the active site for mutation or any kind of bacterial resistance. Because of this advantage of natural compounds, our present research is based on to find out the synergistic activity of synthetic drug in combination with plant phytochemicals[9]. Hence, traditionally used medicinal plants are verified for their pharmacological properties to isolate new bioactive leads which are less toxic in nature and can act as a future natural substitute in place of synthetic drugs[10,11,12].

Staphylococcus aureus is the main causative agent in almost all human infectious diseases. The evolution and development of multidrug resistant *Staphylococcus aureus* against all therapeutic antibiotics causes human pyogenic infections with high morbidity rate[13]. During the time of World War II, Penicillin was regarded as the incredible drug to open a new era in the treatment of bacterial diseases[14]. This breakthrough almost convinced people that bacterial infections could be cured permanently. However, at the same time in 1941, Skinner and Keefer first reported the death caused by *Staphylococcus aureus* in Boston city hospital[15]. In 1942, again Penicillin resistant

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Table 1

Biochemical Tests for confirmation	Bacterial strain	Collection source	Drug resistance
Catalase test Coagulase test Citrate test Indole test Oxidase test Gram stainig	*Staphylococcus aureus* ATCC 25923	HiMedia culture	Sensitive
	Staphylococcus aureus (PN1)	Oral swab culture	Ciprofloxacin, Penicillin G, Erythromycin and Oxacillin resistant
	Staphylococcus aureus (PN2)	Pus culture	Penicillin G, Erythromycin and Oxacillin resistant
	Staphylococcus aureus (PN3)	Oral swab culture	Penicillin G, Erythromycin and Oxacillin resistant
	Staphylococcus aureus (PN4)	Pus culture	Penicillin G resistant

Table 2: MIC and MBC* in both agar dilution and broth macrodilution method

No	Bacterial Strains		Different extracts of *Cassia javanica* L. (mg/ml)				Antibiotic (µg/ml)
			MB	EB	ML	MF	Vancomycin
1	*Staphylococcus aureus* ATCC 25923	MIC	1.02	1.02	1.02	2.04	1
		MBC	2.04	2.04	>8.19	8.19	2
2	PN 1	MIC	2.04	2.04	2.04	2.04	1
		MBC	2.04	8.19	>8.19	8.19	8
3	PN 2	MIC	2.04	2.04	2.04	2.04	1
		MBC	4.09	4.09	>8.19	8.19	2
4	PN 3	MIC	2.04	2.04	2.04	2.04	1
		MBC	8.19	4.09	>8.19	4.09	2
5	PN 4	MIC	2.04	2.04	2.04	2.04	1
		MBC	8.19	8.19	>8.19	8.19	8

**MBC in broth macrodilution method*

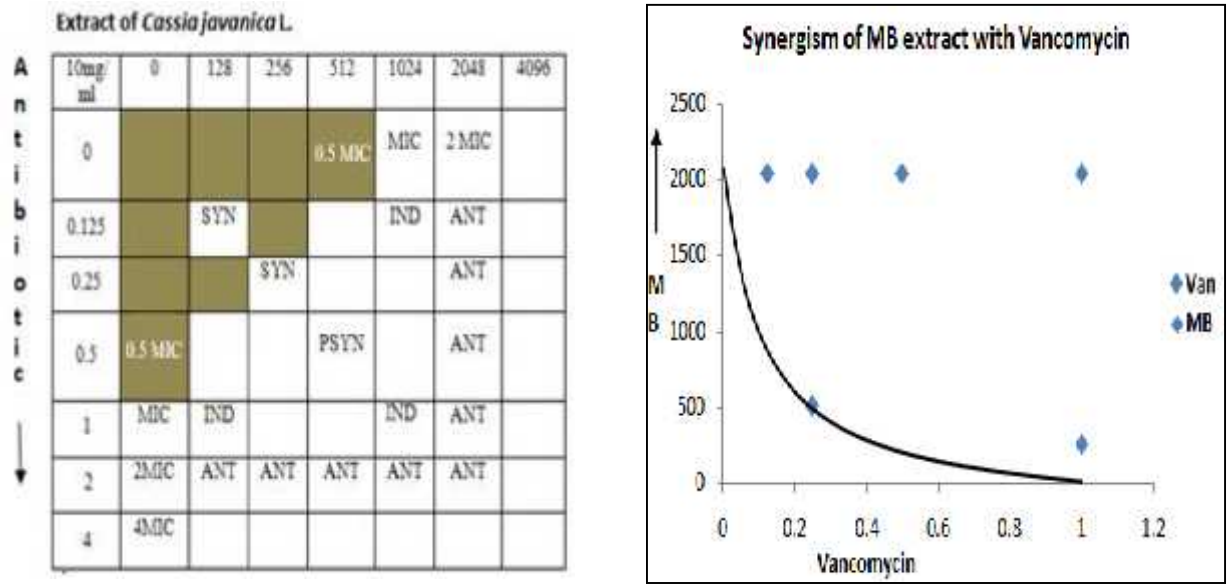

Fig. 1: Checkerboard table for synergy test and isobologram graph plotted from the combined effects of MB and Vancomycin against (A) Staphylococcus aureus (PN1).

Staphylococcus aureus (PRSA) was reported in hospitals[16,17]. Later, such continuous and vigorous use of beta-lactam antibiotics leads to the identification of Methicillin resistant *Staphylococcus aureus* (MRSA) strain. In recent time, Vancomycin was the only drug to treat MRSA strains. However, vigorous use of Vancomycin now result two different kinds of multi drug resistant (MDR) strains viz. Vancomycin intermediate *Staphylococcus aureus* (VISA) and Vancomycin resistant *Staphylococcus aureus* (VRSA). In addition, uncontrollable use of antibiotics gradually creates a continuous pressure over bacteria to acquire resistance mechanism and became more virulent and unvanquished[18]. Based on the above study, our research was carried out to find out antibacterial activity of all parts of *Cassia javanica* L. and evaluates its synergistic activity in

Table 3: Fractional inhibitory concentration (FIC) and FIC index (∑FIC) of MB, EB, ML and MF of *Cassia javanica* L. in combination with Vancomycin. Different points of synergism, partial synergism and indifferent results. MB: methanol extract of bark, EB: ethyl acetate extract of bark, ML: methanol extract of leaves and MF: methanol extract of flower. In results SYN: synergism, PSYN: partial synergism and IND: indifferent

	MB				EB				ML				MF			
	FIC-VAN	FIC-MB	∑FIC	RESULT	FIC-VAN	FIC-EB	∑FIC	RESULT	FIC-VAN	FIC-ML	∑FIC	RESULT	FIC-VAN	FIC-MF	∑FIC	RESULT
Staphylococcus aureus ATCC 25923	0.125 0.25 0.25	0.25 0.125 0.5	0.37 0.37 0.75	SYN SYN PSYN	0.125 0.25 0.5	1 0.12 0.12	1.12 0.37 0.62	IND SYN PSYN	0.25 0.5 0.5	0.25 0.125 0.25	0.50 0.625 0.725	PSYN PSYN PSYN	0.125 0.25 0.5	0.5 0.5 1	0.62 0.75 1.5	PSYN PSYN IND
PN 1	0.25 0.125 0.25	0.25 1 1	0.5 1.12 1.25	SYN IND IND	0.25 0.5 0.125	0.5 0.12 1	0.75 0.62 1.125	PSYN PSYN IND	0.5 0.5 0.25	0.125 0.25 1	0.625 0.75 1.25	PSYN PSYN IND	0.125 0.25 0.125	0.125 0.5 1	0.5 0.75 1.12	SYN PSYN IND
PN 2	0.125 0.25 0.25	0.5 0.25 1	0.62 0.5 1.25	PSYN SYN IND	0.125 0.25 0.5	0.5 0.5 0.12	0.62 0.75 0.62	PSYN PSYN PSYN	0.5 0.5 0.25	0.125 0.25 1	0.625 0.75 1.25	PSYN PSYN IND	0.12 0.25 0.25	0.5 0.5 2	0.625 0.75 2.25	PSYN PSYN IND
PN 3	0.125 0.25 0.25	0.5 0.25 1	0.62 0.5 0.75	PSYN SYN IND	0.125 0.25 0.5	1 1 0.5	1.12 1.25 1	IND IND IND	0.5 0.5 0.125	0.125 0.25 2	0.625 0.75 2.125	PSYN PSYN IND	0.25 0.5 0.125	0.5 0.125 1	0.75 0.625 1.125	PSYN PSYN IND
PN 4	0.125 0.125 0.25	0.125 0.5 0.25	0.25 0.62 0.5	SYN PSYN SYN	0.125 0.25 0.5	0.5 1 0.5	0.62 1.25 1	PSYN IND IND	0.125 0.25 0.5	2 2 2	2.125 2.25 2.5	IND IND IND	0.125 0.25 0.5	0.5 0.5 0.125	0.62 0.75 0.625	PSYN PSYN PSYN

combination with synthetic drug Vancomycin.

MATERIALS AND METHODS

Collection of plant material and preparation: Bark, leaves and flower of *Cassia javanica* L. were collected from Assam University campus. Voucher specimen of the plants no. 2505 was deposited in Department of Life science and Bioinformatics, Assam University, Silchar.

Collected plant materials were air dried, grinded and dipped in different solvents with an increasing polarity gradient. Hexane, ethyl acetate and methanol extracts were prepared by maceration technique at room temperature[19]. 160g powder was dipped in 500ml solvent for 3 days. Each day the dipped bottles were shacked and stirred at an interval of 24 hour. Filtered extracts were dried and stored in a refrigerator at 4˚C for further use[20].

Test organism collection and biochemical used: *Staphylococcus aureus* ATCC 25923, four different MDR pathogenic *Staphylococcus aureus* (PN 1, PN 2, PN 3 and PN 4) strains were collected from Silchar Medical College, Silchar. They were biochemically tested; cultured and isolated pure cultures were preserved in -20˚C deep freezer by mineral oil preservation process[21]. Nutrient agar, Muller Hinton agar, Mannitol Salt agar and Muller Hinton broth were purchased from HiMedia and used for different culture and biochemical identifications.

MIC and MBC determination

Agar dilution and broth macrodilution method: Muller Hinton broth bacterial stock solution was prepared from 3-4 fresh isolates of *Staphylococcus aureus* and its optical turbidity was adjusted with 0.5 McFarland standard[22]. According to the Nature's protocol 2008, Muller Hinton agar plates were prepared with desired antimicrobial concentrations and 2µl of standardized bacterial solution was spotted over it and kept in an incubator at 37˚C for 24 hour[23]. Next day, MIC was determined by the presence of visible growth of *Staphylococcu aureus* in each plate.

Broth macrodilution method is a serial two-fold dilution technique to verify the MIC of each extracts. Six test tubes were selected for each test with different antimicrobial concentration ranges from 0.25mg/ml, 0.51mg/ml, 1.02mg/ml, 2.04mg/ml, 4.09mg/ml and 8.19mg/ml. 10µl of standardized bacterial suspension was added in each antimicrobial broth containing tubes and incubated for 24 hour at 37˚C temperature[23]. MIC for all extracts of *Cassia javanica* L. were found similar in agar dilution method. Minimum bactericidal concentrations (MBC) were determined by sub culture from all the tubes where visible growth was not seen.

Synergism testing

Checkerboard method by agar dilution: Checkerboard method is most frequently used to know the best combination between two antimicrobials that gives the most effective and less toxic result. Here, agar dilution checkerboard method was considered with five different concentrations of both plant extract and Vancomycin combination based on their MIC results.

Usually, the concentrations were - $\frac{1}{8}$MIC, $\frac{1}{4}$MIC, $\frac{1}{2}$MIC, MIC and 2MIC of both tested antimicrobials. In each test, 25 different concentrations of antimicrobial combination

plates were prepared for both extracts and Vancomycin. 2µl bacterial strains were spotted over it and incubated for 24 hour at 37˚C. Next day, bacterial growths were noted and sum of $\sum FIC_{index}$ was calculated by adding both Fractional Inhibitory Concentrations (FIC) of individual antimicrobials. If calculated $FIC_{index} \leq 0.5$, signify synergism. $0.5 < FIC_{index} < 1$ for partial synergism, $1 < FIC_{index} \leq 4$ for indifference and $FIC_{index} > 4$ for antagonism properties. FIC index was calculated on the base of the following equation $\sum FIC_{index} = FIC_{extract} + FIC_{Vancomycin}$.

RESULT AND DISCUSSION

The MIC for all methanol extract of bark (MB), ethyl acetate extract of bark (EB), methanol extract of leaves (ML) and methanol extract of flower (MF) were same against all clinical isolates of *Staphylococcus aureus i.e.* 2.04mg/ml. MIC of all extracts against *Staphylococcus aureus* ATCC 25923 is 1.02mg/ml in both agar dilution and broth macrodilution method. All clinical isolates were found as Vancomycin sensitive MIC at 1µ/ml. Different MIC and MBC values for all extracts were given in the following Table 2. All parts of *Cassia javanica* L. were found as equally active to inhibit bacterial growth. MB was found most active plant extract against *Staphylococcus aureus* strains. Minimum bactericidal concentration for MB was 2.04mg/ml for PN 1 and *Staphylococcus aureus* ATCC 25923, 4.09mg/ml for PN 2 and 8.19mg/ml for PN 3 and PN 4. EB was another potent inhibitor with an MBC at 2.04mg/ml for *Staphylococcus aurous* ATCC 25923, 4.09mg/ml for PN 2 and PN 3 and 8.19mg/ml for PN 1 and PN 4. ML has more than 8.19mg/ml MBC value for all isolates. MF has MBC at 4.09mg/ml for PN 3 and 8.19mg/ml for PN 4. Vancomycin was used as positive control that exhibited strong antibacterial activity with MIC 1µg/ml.

With the extension of our study, all extracts of *Cassia javanica* L. in combination with Vancomycin were tested against all resistant verities and a complete inhibition of bacterial growth was noticed rather than single antibiotic effect.

Synergistic activity of *Cassia javanica* L. in combination with Vancomycin illustrated significant bactericidal ability. Different breakpoints of synergism, partial synergism, indifference activity are listed below in Table 2. Vancomycin was found to be more synergistic with methanol extract of bark. The MIC of the MB and Vancomycin decreases upto the lowest FIC index ($\sum FIC=0.25$) against PN 4. 0.25mg/ml of MB extract with Vancomycin (0.125µg/ml) showed best synergism with a four time fold reduction from their original MIC values (Figure 1). Another best synergism is 0.51mg/ml of MF with Vancomycin 0.125µg/ml against PN 1. 0.12mg/ml of EB showed synergism with Vancomycin at 0.125 µg/ml against *Staphylococcus aureus* ATCC 25923. This combination therapy resulted complete inhibition of bacterial growth after 24 hour of incubation. This test was repeated for all extracts in combination with Vancomycin and significant results were observed. Calculated FIC values of different extracts are listed below in accordance to their individual clinical isolates.

In our present investigation, all parts of *Cassia javanica* L. has found to have potential bactericidal ability to treat MDR *Staphylococcus aureus* strains. However, Vancomycin was regarded as a quality antibiotic but it has some high neurotoxic effect. Therefore, an effective and promising drug or drug combination therapy is urgently required. This drug to drug interaction with plant natural products increases the antibiotic activity with lower toxic effects. Some earlier researches have reported the synergistic activity Vancomycin with Erybraedin A isolated from the roots of *Erythrina zeyheri,* Butylated hydroxyanisole from (BHA) green tea and stilbenoids from stilbene to treat VRE and MRSA strains[24,25,26]. However, their findings were limited in partial synergistic state with a ΣFIC value ranges between 0.562-1.0. Recently, johorenol A in combination with Vancomycin has reported to have synergistic activity at ΣFIC=0.32 against *Staphylococcus aureus* strains[26]. Our finding has replaced these earlier results with the lowest FIC index *i.e.*, ΣFIC=0.25 which was considered as the best synergism of Vancomycin in combination with the crude extracts from *Cassia javanica* L.

CONCLUSION

Cassia javanica L. is widely distributed in North East India. Bark, leaves and flower of *Cassia javanica L.* are found to have potent antimicrobial activity against multi drug resistant *Staphylococcus aureus* strains. Methanol extract of bark and methanol extract of flower of *Cassia javanica* L. in combination with Vancomycin showed significant activity by inhibiting the bacterial growth. Antimicrobial combination therapy is the most effective future drug treatment to treat multi drug resistant bacteria. It was seen that combination of natural product and marketed antibiotics dramatically increased their bactericidal efficacy with low toxicity. Drug to drug interaction in synergism is the main key role to treat MDR strain and help us to create a healthy environment. Like *Cassia javanica* L. different unidentified traditional medicinal plants are there which has not been explored till date but modern natural product drug discovery system can carry an evolution to find out some native drug to treat emergent MDR bacterial strain.

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REFERENCES

1. Shu JM. *Cassia Linnaeus. Flora of China*. 2010; 10(1): 27–28.
2. Chittam KP, Deore SL. *Cassia Javanica Linn*: A Review on Its Phytochemical and Pharmacological Profile. *Journal of Biomedical and Pharmaceutical Research*. 2013; 2 (1): 33-35.
3. Ganesan S. Traditional oral care medicinal plants survey of Tamil Nadu. *Natural product radiance.* 2008; 7(2): 166-172.

4. Kumavat UC, Shimpi SN and Jagdale SP. Hypoglycemic activity of *Cassia javanica* Linn. in normal and streptozotocin-induced diabetic rats. *J. of Adv. Pharm. Tech. and Res.* 2012; 3(1): 47–51.
5. Bauer, RW, Kirby MDK, Sherris JC and Turck M. Antibiotic susceptibility testing by standard single disc diffusion method. *Amer. J. of Clin. Pathol*. 1966; 45(4): 493-496.
6. Iqbal PJ. Phytochemical screening of certain plant species of Agra city. *J. of Drug Deliv. and Therapeu.*2012; 2(4): 135-138.
7. Neog P, Choudhury S, Bhattacharjee A, Chetia P, Laskar MA and Choudhury MD. *Cassia javanica* Linnaeus [Caesalpiniaceae]: Phytochemical analysis and antimicrobial activity against multi-drug resistant hospital isolates of *Staphylococcus aureus*. *East Himalayan Society for Spermatophyte Taxonomy, Pleione.* 2013; 7(2), 406 - 412.
8. Perichon B and Courvalin P. VanA-Type Vancomycin-Resistant *Staphylococcus aureus*. *Anti. Agent. Chemothe*. 2009; 53(11): 4580-4587
9. Buhner SH. Herbal Antibiotics: Natural Alternatives for Treating Drug-Resistant Bacteria. Published January 8th. Storey Publishing, United States, 1999.
10. Pandian MR, Banu GS and Kumar G. A study of the antimicrobial activity of *Alangium salviifolium*. *Indi. J. Pharm*. 2006; 38(3): 203-204.
11. Ushimaru PI, da Silva MTN, Stasi LCD, Barbosa L and Junior AF. Antibacterial activity of medicinal plant extracts. *Braz. J. of Micro*. 2007; 38(4): 717-719
12. Khazir J, Mirc BA, Mird SA and Cowanc D. Natural products as lead compounds in drug discovery. *J. of Asian Natural Products Research*. 2013; 5(7): 764-788
13. Miles F, Voss L, Segedin E and Anderson BJ. Review of *Staphylococcus aureus* infections requiring admission to a paediatric intensive care unit. *Archives of Disease in Childhood*. 2005; 90: 1274–1278.
14. Wright GD. Q &A: Antibiotic resistance: where does it come from and what can we do about it? *BMC Biology*. 2010; 8: 123.
15. Archer GL. *Staphylococcus aureus*: A Well-Armed Pathogen. *Clinical Infectious Diseases*. 1998; 26: 1179–81.
16. Rammelkamp CH and Maxon T. Resistance of *Staphylococcus aureus* to the Action of Penicillin. *Exp Biol Med*. 1942; 51(3): 386-389.
17. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest*. 2003; 111(9): 1265–1273.
18. Saderi H, Owlia P, Shahrbanooie R. Vancomycin resistance among clinical isolates of *staphylococcus aureus. Archives of Iranian medicine,*2005; 8 (2): 100 –103.
19. Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction Technologies for Medicinal and Aromatic Plants. International centre for science and high technology, Trieste, 2008.
20. Ahmad I and Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of Ethnopharmacology.*2001; 74: 113–123.
21. Benson. Microbiological Applications Lab Manual, 8th Edition. The McGraw−Hill Companies. 2001
22. Isenberg HD. Clinical microbiology procedures handbook. Washington, DC. 2007.
23. Wiegand I, Hilpert K and Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature protocols*, 2008; 3: 163-175.
24. Sato M, Tanaka H, Oh-Uchi T, Fukai T, Etoh H and Yamaguchi R. Antibacterial activity of phytochemicals isolated from *Erythrina zeyheri* against Vancomycin resistant enterococci and their combinations with vancomycin. *Phytother*. 2004; 18(11): 906–910.
25. Simonetti G, Simonetti N and Villa A. Increased microbicidal activity of green tea (*Camellia sinensis*) in combination with butylated hydroxyanisole. *J. of Chemother*. 2004; 16(2): 122–127.
26. Basri DF, Luoi CK, Azim AM and Latip J. Evaluation of the Combined Effects of Stilbenoid from *Shorea gibbosa* and Vancomycin against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Pharmaceuticals*. 2012; 5(9): 1032-1043.