International Journal of Advanced Scientific Research
www.allscientificjournal.com
E-ISSN: 2456-0421
Received: 22-09-2026, Accepted: 22-10-2026, Published: 06-11-2026
Volume 11, Issue 4, 2026, Page No. 53-56

Phytochemical, antimicrobial and synergistic interaction of *Cassia javanica* linn. plant extracts against clinical isolates of *Staphylococcus aureus* multi drug resistant

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DOI: https://doi.org/10.66856/ijasr.2026.11.4.11082

Abstract
The original prospective of this research is to find out the antibacterial activity of *Cassia javanica* Linn. in combination with Vancomycin against some multi drug resistant *Staphylococcus aureus* hospital isolates. Minimum inhibitory concentration for each extract of *Cassia javanica* Linn. 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 0.25 mg/ml and 0.51 mg/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, *Cassia javanica*

Introduction

Cassia javanica Linn. is a medicinally less explored plant in Northern 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* Linn. is a 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* Linn. 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]. Phytochemical screening of different parts of *Cassia javanica* Linn. testifies the presence of alkaloid, saponin, tannin, steroid, flavonoid, anthraquinones etc. and their sensitivity tests proved it to be a potent bactericidal antimicrobial agent [4,5].

To prevent recent bacterial infection, different extracts of *Cassia javanica* Linn. 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 [6] 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 finding out the synergistic activity of synthetic drug in combination with plant phytochemicals. 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 [7,8].

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 [9]. 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 [10]. 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 [11]. In 1942, again Penicillin resistant.

Table 1: Biochemical Tests for Confirmation of *Cassia javanica* Linn. Plant

Biochemical Tests for Confirmation	Bacterial Strain	Collection Source	Drug Resistance
Catalase test Coagulase test Citrate test Indole test Oxidase test	*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 Strain		Different extracts of *Cassia javanica* Linn. (µg/ml)				Antibiotic (µg/ml)
			MB	EB	ML	MF	Vancomycin (µg/ml)
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	PN1	MIC	2.04	2.04	2.04	2.04	1
		MBC	2.04	8.19	8.19	8.19	8
3	PN2	MIC	2.04	2.04	2.04	2.04	1
		MBC	4.09	4.09	8.19	8.19	2
4	PN3	MIC	2.04	2.04	2.04	2.04	1
		MBC	8.19	4.09	8.19	4.09	2
5	PN4	MIC	2.04	2.04	2.04	2.04	1
		MBC	8.19	8.19	8.19	8.19	8

Staphylococcus aureus (PRSA) was reported in hospitals [12,13]. 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 results 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 [14]. Based on the above study, our research was carried out to find out antibacterial activity of all parts of *Cassia javanica* Linn. and evaluates its synergistic activity.

Table 3: Fractional inhibitory concentration (FIC) and FIC index (ΣFIC) of MB, EB, ML and MF of *Cassia javanica* Linn. in combination with Vancomycin. Different points of synergism, partial synergism0, 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.

Bacterial Strain	MB (FIC-VAN / FIC-MB / ΣFIC / Result)	EB (FIC-VAN / FIC-EB / ΣFIC / Result)	ML (FIC-VAN / FIC-ML / ΣFIC / Result)	MF (FIC-VAN / FIC-MF / ΣFIC / Result)
Staphylococcus aureus ATCC 25923	0.125 / 0.25 / 0.37 / SYN0.25 / 0.125 / 0.37 / SYN0.25 / 0.5 / 0.75 / PSYN	0.125 / 1 / 1.12 / IND0.25 / 0.12 / 0.37 / SYN0.5 / 0.12 / 0.62 / PSYN	0.25 / 0.25 / 0.50 / PSYN0.5 / 0.125 / 0.625 / PSYN0.5 / 0.25 / 0.725 / PSYN	0.125 / 0.5 / 0.62 / PSYN0.25 / 0.5 / 0.75 / PSYN0.5 / 1 / 1.5 / IND
PN1	0.25 / 0.25 / 0.50 / SYN0.125 / 1 / 1.12 / IND0.25 / 1 / 1.25 / IND	0.25 / 0.5 / 0.75 / PSYN0.5 / 0.12 / 0.62 / PSYN0.125 / 1 / 1.125 / IND	0.5 / 0.125 / 0.625 / PSYN0.5 / 0.25 / 0.75 / PSYN0.25 / 1 / 1.25 / IND	0.125 / 0.125 / 0.50 / SYN0.25 / 0.5 / 0.75 / PSYN0.125 / 1 / 1.12 / IND
PN2	0.125 / 0.5 / 0.62 / PSYN0.25 / 0.25 / 0.50 / SYN0.25 / 1 / 1.25 / IND	0.125 / 0.5 / 0.62 / PSYN0.25 / 0.5 / 0.75 / PSYN0.5 / 0.12 / 0.62 / PSYN	0.5 / 0.125 / 0.625 / PSYN0.5 / 0.25 / 0.75 / PSYN0.25 / 1 / 1.25 / IND	0.12 / 0.5 / 0.625 / PSYN0.25 / 0.5 / 0.75 / PSYN0.25 / 2 / 2.25 / IND
PN3	0.125 / 0.5 / 0.62 / PSYN0.25 / 0.25 / 0.50 / SYN0.25 / 1 / 0.75 / IND	0.125 / 1 / 1.12 / IND0.25 / 1 / 1.25 / IND0.5 / 0.5 / 1 / IND	0.5 / 0.125 / 0.625 / PSYN0.5 / 0.25 / 0.75 / PSYN0.125 / 2 / 2.125 / IND	0.25 / 0.5 / 0.75 / PSYN0.5 / 0.125 / 0.625 / PSYN0.125 / 1 / 1.125 / IND
PN4	0.125 / 0.125 / 0.25 / SYN0.125 / 0.5 / 0.62 / PSYN0.25 / 0.25 / 0.50 / SYN	0.125 / 0.5 / 0.62 / PSYN0.25 / 1 / 1.25 / IND0.5 / 0.5 / 1 / IND	0.125 / 2 / 2.125 / IND0.25 / 2 / 2.25 / IND0.5 / 2 / 2.5 / IND	0.125 / 0.5 / 0.62 / PSYN0.25 / 0.5 / 0.75 / PSYN0.5 / 0.125 / 0.625 / PSYN

Materials and Method

Collection of plant material and preparation: Bark, leaves and flower of *Cassia javanica* Linn. were collected from FRI, Dehradun (Uttarakhand). Voucher specimen of the plants no. 2505 was deposited in School of Life Science Dr. Bhimrao Ambedkar University, Agra. 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 [15]. 160 g powder was dipped in 500 ml solvent for 3 days. Each day the dipped bottles were shaken and stirred at an interval of 24 hours. Filtered extracts were dried and stored in a refrigerator at 4°C for further use [16].

Test organism collection and biochemical used: *Staphylococcus aureus* ATCC 25923 and four different MDR pathogenic *Staphylococcus aureus* (PN1, PN2, PN3 and PN4) strains were collected from FRI, Dehradun, Uttarakhand. They were biochemically tested, cultured and isolated. Pure cultures were preserved in −20°C deep freezer by mineral oil preservation process. Nutrient agar, Muller Hinton agar, Mannitol Salt agar and Muller Hinton broth were purchased from HiMedia and used for different culture and biochemical identifications.

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 [17]. According to the Nature's Protocol (2008), Muller Hinton agar plates were prepared with desired antimicrobial concentrations and 2 µl of standardized bacterial solution was spotted over them and incubated at 37°C for 24 hours. The next day, MIC was determined by the presence of visible growth of *Staphylococcus aureus* on each plate. Broth macrodilution method is a serial two-fold dilution

technique to verify the MIC of each extract. Six test tubes were selected for each test with antimicrobial concentrations of 0.25, 0.51, 1.02, 2.04, 4.09 and 8.19 mg/ml. A 10 µl standardized bacterial suspension was added into each antimicrobial broth tube and incubated for 24 hours at 37°C [18]. MIC values for all extracts of *Cassia javanica* Linn. were found to be similar in the agar dilution method. Minimum bactericidal concentrations (MBC) were determined by subculture from all tubes where visible growth was not seen.

Checkerboard method by agar dilution: Checkerboard method is most frequently used to determine the best combination between two antimicrobials that gives the most effective and least toxic result. In this study, agar dilution checkerboard method was carried out using five different concentrations of both plant extract and Vancomycin based on their MIC results. Usually, the concentrations were ⅛ MIC, ¼ MIC, ½ MIC, MIC and 2 MIC of both tested antimicrobials. In each test, 25 different concentrations of antimicrobial combinations were prepared.

Plates were prepared for both extracts and Vancomycin: 2 µl bacterial strains were spotted over it and incubated for 24 hours at 37°C. Next day, bacterial growths were noted and sum of ΣFIC index was calculated by adding both Fractional Inhibitory Concentrations (FIC) of individual antimicrobials. If calculated FIC index ≤0.5, signify synergism. 0.5 < FIC index < 1 for partial synergism, 1 < FIC index ≤ 4 for indifference and FIC index > 4 for antagonism properties. FIC index was calculated on the base of the following equation:

$$\Sigma FIC_{index} = FIC_{extract} + FIC_{Vancomycin}$$

Results 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. 2.04 mg/ml. MIC of all extracts against *Staphylococcus aureus* ATCC 25923 is 1.02 mg/ml in both agar dilution and broth macrodilution method. All clinical isolates were found as Vancomycin sensitive MIC at **1** µg/ml. Different MIC and MBC values for all extracts were given in the following Table-1,2. All parts of *Cassia javanica* Linn. 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 2.04 mg/ml for PN1 and *Staphylococcus aureus* ATCC 25923, 4.09 mg/ml for PN2 and 8.19 mg/ml for PN3 and PN4. EB was another potent inhibitor with an MBC at 2.04 mg/ml for *Staphylococcus aureus* ATCC 25923, 4.09 mg/ml for PN2 and PN3 and 8.19 mg/ml for PN1 and PN4. ML has more than 8.19 mg/ml MBC value for all isolates. MF has MBC at 4.09 mg/ml for PN3 and 8.19 mg/ml for PN4. Vancomycin was used as positive control that exhibited strong antibacterial activity with MIC 1 µg/ml with the extension of our study, all extracts of *Cassia javanica* Linn. 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* Linn. in combination with Vancomycin illustrated significant bactericidal ability. Different breakpoints of synergism, partial synergism, indifference activity is listed below in Table-3. Vancomycin was found to be more synergistic with methanol extract of bark. The MIC of the MB and Vancomycin decreases up to the lowest FIC index (ΣFIC = 0.25) against PN4. 0.25 mg/ml of MB extract with Vancomycin (0.125 µg/ml) showed best synergism with a four-time fold reduction from their original MIC values. Another best synergism is 0.51 mg/ml of MF with Vancomycin **(**0.125 µg/ml**)** against PN1. 0.12 mg/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 hours 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* Linn. 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 of Vancomycin with Erybraedin A isolated from the roots of *Erythrina zeyheri*, Butylated hydroxyanisole (BHA) from green tea and stilbenoids from stilbene to treat VRE and MRSA strains [19,20]. However, their findings were limited in partial synergistic state with a ΣFIC value between 0.562–1.0. Recently, johorenol A in combination with Vancomycin has been reported to have synergistic activity at ΣFIC = 0.32 against *Staphylococcus aureus* strains [21]. Our findings have 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* Linn.

Conclusion

Cassia javanica Linn. is widely distributed in North East India. Bark, leaves and flower of *Cassia javanica* Linn. are found to have potent antimicrobial activity against multidrug resistant *Staphylococcus aureus* strains. Methanol extract of bark and methanol extract of flower of *Cassia javanica* Linn. in combination with Vancomycin showed significant activity by inhibiting the bacterial growth. Antimicrobial combination therapy is the most effective future drug treatment to treat multidrug 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* Linn., different unidentified traditional medicinal plants are there which have 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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