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ANTI-MICROBIAL PROPERTIES OF FLAVONOIDS FROM ABUTILON INDICUM AGAINST PSEUDOMONAS AERUGINOSA AND STAPHYLOCOCCUS AUREUS

Medical Microbiology

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ABSTRACT

Developing new antibiotics, it is becoming increasingly difficult, with greater time-consuming and more expensive to develop new antibiotics, especially because emerging strains are becoming increasingly resistant to existing antibiotics. Phytochemically, flavonoids are plant secondary metabolites with powerful therapeutic effects and there are several scientific evidences that plant flavonoids are potent pharmacological agents. In this study, Effect of Flavonoids derived from the leaves of Abutilon indicum on bacterial growth and efflux pump activity were tested on Pseudomonas aeruginosa and Staphylococcus aureus. A standard antibiotic ampicillin was comparable to the inhibitory effects of flavonoids at 1.80 mg/mL of concentration on bacterial growth. The flavonoids from Abutilon indicum had showed a MIC of 0.0036 mg/mL and an MBC of 0.96 mg/mL, making them the most effective against S. aureus and P.aeruginosa. It was proven that plant flavonoids had bacteriostatic effects on P.aeruginosa and S.aureus. Abutilon indicum flavonoids inhibited the drug efflux pump susceptible in P.aeruginosa, which resulted in a 132% increase in Rhodamine-6G accumulations compared to the control. As a result, Leaf flavonoids from Abutilon indicum exhibited antibacterial activity as well as the ability to inhibit the ATP-dependent transport of compounds across cell membranes, and these flavonoids can be used as potential lead compounds for the development of plant-based antibacterial agents and their adjunct compounds.

KEYWORDS

Abutilon indicum, Colony forming units (CFU), Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC).

INTRODUCTION

One of the main factors contributing to causes of infectious diseases in India is prokaryotic microbial infections. On worldwide, immune compromised convalescents in the emerging nations are dying from life-threatening illnesses caused by pathogenic bacterial species. A concerted effort to fight infectious diseases is made more challenging by the fact that bacteria are continually acquiring resistance to a wide variety of medicines, despite their availability. Since from the advent of antimicrobials/antibiotics were used to treat bacterial illnesses, bacteria have retorted by developing a variety of resistance mechanisms. Treatment failures may be caused by the level and complexity of bacterial infections' resistance mechanisms, which are evolving with time and becoming more sophisticated.

Gram-positive and Gram-negative microorganisms that are characteristically comprehended in community and hospital acquired infections, respectively; Staphylococcus aureus and Pseudomonas aeruginosa [1]. More critically, P. aeruginosa and Staphylococcus aureus infections are potentially fatal because the exotoxins and endotoxins they release continue to induce inflammation and injury even after the bacteremia has been treated with antibiotics [2].

The global escalation in each community and hospital acquired antimicrobial microbes is intimidating the potent remedy of patients, underscoring the necessary for sustained reconnaissance, prudent disease control, and innovative treatment options [3]. New antimicrobials are required to control the increasing number of antibiotic-resistant infections, yet resistance development is inevitable because it is a fundamental key element of microbial evolution [4].

Hence, there is an exigent necessity for progression of novel unique anti- bacterial agents that targets and combat bacterial resistance mechanisms. Herbal remedy use, whether in traditional medicine or complementary and alternative medicine (CAM) practices, is common in Asia and is claimed to have slight undesirable side effects [5]. Moreover, with concerns of escalating cost of drug discovery, plants have proven to be some of the greatest cost-effective and cheap alternative sources of medicine [6].

The Abutilon indicum has belongs to Malvaceae family, which is widely founded throughout in all parts of India's tropical and subtropical regions. It is well known that the plant have been medicinal potentials. The Abutilon indicum plant commonly named to as kanghai in Hindi. The plant has historically been used as an anti-inflammatory, an anthelmintic, and is useful in the treatment for jaundice, ulcers, leprosy, piles, and lumbago [7]. The leaves of Abutilon indicum are used to treat liver diseases, toothaches, lumbago, piles, and anti-fertility [8]. Root and bark are used as diuretics, nerving tonics, aphrodisiacs, and anti-diabetics [9]. Higher plants may be a source of novel antibiotic prototypes, as evidenced by the antibacterial activity of plant extracts and their derivatives [10]. The major goal was to assess the efficacy of Abutilon indicum against S. aureus and P. aeruginosa.

MATERIALS AND METHODS

Microorganisms and Plant Provenance

The plant material was collected from different areas in Telangana and it was authenticated by a taxonomist at the Botanical Department of Osmania University Telangana, India (Voucher. No: OU-4026). The test microorganisms were obtained from Department of Microbiology, Nizam Medical College, Hyderabad, and Telangana, India.

Ethanolic Extract Preparation

The leaves of Abutilon indicum were used for the extraction. Before use, leaves were dried and powdered after being cleaned with sterile distilled water. The resulting powder was extracted with ethanol (ethanol and water in a ratio of 70:30) in a Soxhlet Extractor for 72 hr at 60°C. By evaporating the solvent on a rotavapor, the solvent was evaporated while taking care to keep the temperature at 50°C. The result was a light brown powder that was retrieved using sterile distilled water and extract were reserved at 20°C in shade for further use.

Flavonoids' Extraction and Identification

The types of the compounds contained in the ethanolic extract were separated on the basis of compound nature and structure by partitioning between solvents. The principal separation was performed with petroleum ether to remove non-phenolic compounds. This phase was then aborted. Followed by principle separation subsequent partition was performed with diethyl ether to extract aglycones and

phenolic acids. The 3rd separation was performed with ethyl acetate, which extracted the remaining monogly cosylated aglycones and flavonoids. Thus, the remaining aqueous phase contained only flavonoids bound to two or more glycosides.

Microbial Susceptibility Test:
Flavonoids extract of Abutilon indicum, prepared at a concentration of 25 mg/ml, and were distinctly added to 96-well plates in a volume of 20 µl in order to pervade the tested cell cultures with 500 µg each 1.67 mg /ml extract in every well. Pseudomonas aeruginosa and Staphylococcus aureus at $1x10^6$ cfu/ml and wells of microtiter plate were diluted with broth to made each well contain a total volume of 300 □L. A progressive (+ve) control containing ampicillin was also prepared, with 500 µg added to each well containing bacterial culture medium and broth. Appropriate destructive (-ve) control containing medium only/medium with extract were also prepared. Absorbance measurements at 600nm were determined before incubation using a microplate reader. Post-incubation After incubation in a Lab Companion incubator for 24 hours at 37°C cell density was determined.

Determination of MIC and MBCs:
Abutilon indicum Flavonoids extracts MICs and MBCs were also measured. In order to create 10 different dilution concentrations, the Flavonoids extracts were sequentially serially diluted twofold in a 96-well polystyrene microplate from 1.80mg/ml to 0.0045 mg/ml. 20□l of each dilution were successively transferred in triplicate into the wells of a separate 96-well microplate. Each species in its own microplate, 100 □l of P. aeruginosa and S. aureus broth cultures ($1x10^6$cfu/mL) were added to the wells. Then, in each well 180 □l of broth was added to the well to build a total volume of 300 □l. As a positive control, rows of wells with media containing 100 □l of cell cultures as well as extract-only and extract-free controls were employed and broth medium without extract were also used as negative controls for each well. Using a microplate reader, pre incubation absorbance values were recorded. Following a 24-hour incubation period at 37°C in a Lab-Companion incubator, absorbance values were read and recorded. The MIC values in the well with the least noticeable purple coloration in each test row were found using MTT. The experiment was carried out three times.

Rhoda mine 6G: Drug Efflux and Accumulation
In an incubator, P.aeruginosa and S.aureus were incubated in separate culture flasks for an overnight at 37°C with proper agitation at 120rpm. Subsequently, the bacterial cultures were transferred into centrifuge tubes (50 mL), centrifuged for 10min at 3000 rpm and the supernatant were discarded. The pellet was re-suspended in the buffer after being washed twice with phosphate buffered saline. The revived cells were poured into centrifuge tubes that had been pre weighed and spun over again for 5min at 4,000rpm. After decanting the supernatant, the cells were again washed in PBS centrifuged once more for five minutes at 4,000rpm. The pellet was weighed and the supernatant was decanted. P.aeruginosa and S.aureus pellets were suspended in PBS containing 10mM NaN3 at a concentration of 40 mg/mL. In a Lab-Companion incubator for 1 hour stirred at a speed of 90 rpm agitation with a final concentration of 10M of rhodamine-6G added immediately. After that, they were divided into two centrifuge tubes in the following ratio of 1: 3, to be responsible for tubes A and B for each species.

The tubes were spun for 5 min at 4000 rpm in a Centromix-BLT. Cells from tube A of the each bacterial species were resuspended in PBS unaided at a concentration of 40 mg/mL after the supernatant was discarded. In PBS with 1M glucose, cells from tube B of the two species were re-suspended. The cells from tube B of both bacteria were separated into two 5 mL quantities and placed in six distinct falcon tubes, Two of which were used for glucose alone, Two of for reserpine + glucose and two of for Flavonoids derived from the leaves of Abutilon indicum extracts + glucose, and cells from tube A.

This was carried out for each of the S.aureus and P.aeruginosa pellets. The Flavonoids were added to their respective tubes at an absolute concentration of 61□g /mL, and reserpine was added to tubes labeled reserpine + glucose at an absolute concentration of 61□g/mL. The tubes were shaken on a Vortex mixer to proper mixing them, and put in a shaking incubator at 37°C with proper agitation at 90 rpm for 30 minutes. To determine how much R6G was pumped out of the cell after 30 minutes, tubes were spun in a centrifuge-5425 (Sigmaaldrich) for 10 minutes at 4000 rpm. The supernatant was then collected to quantitate the amount R6G pumped. Each tube's pellet was lysed by being reconstituted in 5 mL of 3M glycine pH 3.

The tubes were shaken together on a vortex mixer before being incubated at 37°C for overnight. Subsequent being spun at 4000 rpm for 10 minutes, the amount of R6G that had accumulated in the cells was measured in the supernatant. After evaluating the absorbance in 96-well microplates at 527 nm using a microplate reader, R6G was calculated from the samples using a standard R6G calibration curve.

Statistical Analysis:
Graph Pad Instant software was used for data analysis. ANOVA with Dunnett's posttest was used to establish levels of significance when all columns of treatments were compared to the control. All data were presented in the form of mean standard deviation. $P \leq 0.05$ or less was regarded to be a statistically significant difference.

RESULTS

Microbial Susceptibility Test:
The effects of Abutilon indicum Flavonoids extract on bacterial species were assessed by measuring absorbance values at 600nm before and after incubation. Abutilon indicum Flavonoids extract significantly reduced bacterial growth at an initial screening concentration of 1.80 mg/mL (P=0.001, Figure 1). The mean absorbance for the cells exposed to the Abutilon indicum Flavonoids extract had 0.028 AU, making it the most effective at slowing the growth of P. aeruginosa. As the recorded mean absorbance was 0.0062 AU, ampicillin as expected nearly killed all of the cells in the positive controls. In comparison to S.aureus, P.aeruginosa was generally more resistant to the antibacterial effects of the flavonoids and ampicillin.

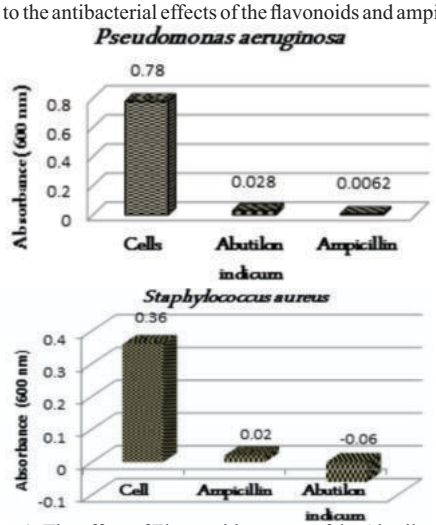

Figure.no.1: The effect of Flavonoids extract of the Abutilon indicum, ampicillin on growth of S. aureus & P. aeruginosa. Concentrations of 1.80 mg/mL & 1 × 106 cfu/mL of the extract and bacteria, Values are expressed as mean absorbance at 600nm wavelength.

Determination of MIC and MBCs:
The MIC was determined for the well with least discernible MTT color. The Abutilon indicum Flavonoids extract was most effective, with MIC as low as 0.0036 mg/mL against S.aureus and 0.32 mg/mL against P.aeruginosa. The Abutilon indicum Flavonoids extract demonstrated bactericidal activity against S.aureus (Table.no1), with an MBC of 0.96 mg/mL, whereas ampicillin had an MBC of 0.008mg/mL [25]. Flavonoids extract of the Abutilon indicum were bacteriostatic against both bacterial species.

Table-1: Determination of Minimum inhibitory concentration and Minimum bactericidal concentration of Abutilon indicum against Staphylococcus aureus and Pseudomonas aeruginosa.

Table.no1: Assay of MIC and MBC of Abutilon indicum against Staphylococcus aureus and Pseudomonas aeruginosa.

Name of Micro-Organism	G^{+ve}/G^{-ve}	Extract	MBC (mg/mL)	MIC (mg/mL)
Pseudomonas aeruginosa	G^{-ve}	Abutilon indicum	-	0.32
Staphylococcus aureus	G^{+ve}	Abutilon indicum	0.96	0.0036

Rhodamine-6G: Drug Efflux and Accumulation

Fluorescent dye Rhodamine-6G, dynamically pumped out by the ATP-dependent efflux pumps of both species in this investigation, was used to measure the effects of the extracts on drug accumulation [26]. With a 132% the glucose increase above only in control, Abutilon indicum Flavonoids extract showed the highest accumulation of Rhodamine-6G against P.aeruginosa. Due to a 116% increase in accumulation, S.aureus was found to be less vulnerable to efflux pump inhibition. Abutilon indicum Flavonoids extract blocked efflux pumps to a greater extent. The conventional inhibitor reserpine's efficacy was more perceptible against P.aeruginosa than against S. aureus, which is a notable finding because it showed that P. aeruginosa was more vulnerable to efflux pump inhibition than S. aureus (Figure.no.2 and Table.no.2).

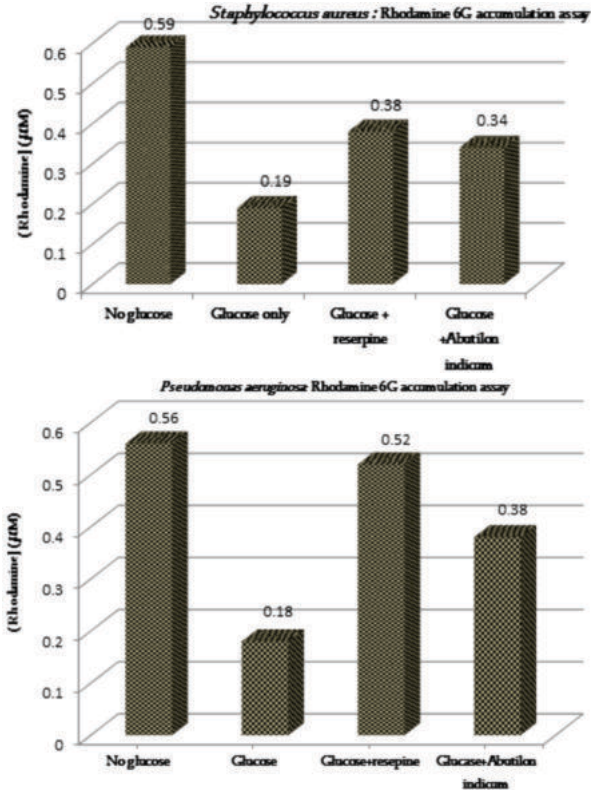

Figure. no. 2: The effect of flavonoids extract of the Abutilon indicum on accumulation of Rhodamine-6G over time and ATP-dependent efflux pumps.

Table no. 2: Determination of the R-6G concentration that accumulated in the bacterial cells after exposure to flavonoids extract of the Abutilon indicum.

S.No	Name of Micro-Organism	Glucose	Reserpine	Abutilon indicum
0.1	Pseudomonas aeruginosa	0.19 ± 1.36	0.64 ± 1.09 (268%)	0.42 ± 2.28 (132%)
0.2	Staphylococcus aureus	0.19 ± 0.42	0.45 ± 1.21 (140%)	0.40 ± 1.56 (116%)

DISCUSSION

The intrinsic potential of pathogens to generate and adopt antibiotic resistance mechanisms necessitates the search for antimicrobial substances for the benefit of humans. The emphasis is gradually turning to plant-derived phytochemicals with therapeutic value as a result of potentially harmful side effects connected with the use of new chemical entities created artificially and the unacceptably high costs of drug development [11]. An innovative strategy for combating antibiotic resistance is the co-formulation of naturally derived antimicrobial adjuvants, such as efflux pump inhibitors, with both old and new generation antibiotics remains a novel antimicrobial resistance mechanism. Antibacterial susceptibility tests, minimum inhibitory concentration (MIC) analyses, and minimum bactericidal concentration (MBC) determinations were used to investigate the effects of flavonoids extract of Abutilon indicum. The Abutilon indicum extract exhibited strong antibacterial activity, as conforming according to the results of antibacterial susceptibility tests.

This study's findings revealed that S. aureus was more sensitive to flavonoids extract than P. aeruginosa, which was consistent with earlier research that indicated Gram^{+ve} strains are more vulnerable to harmful xenobiotics than Gram^{-ve} microorganisms [12]. In this regard, the flavonoids extract showed efficacy against Gram^{+ve} and Gram^{-ve} bacteria, implying broad range activity.

Flavonoids from Abutilon indicum were more potent against S.aureus, which had a 0.0036mg/mL value of MIC and 0.96 mg/mL of MBC, according to the MIC and MBC assay. The common conventional antibiotic ampicillin's MBC was 0.008mg/mL [13], which means that activity of the flavonoids is much outweighed by ampicillin. Flavonoids from Abutilon indicum exhibited bactericidal activity only against S.aureus. All plant flavonoids extracts may have a bacteriostatic impact on P.aeruginosa because of the pathogen's thick, highly hydrophobic outer membrane, which may have a permeability barrier to extract [14].

One of the primary mechanisms for stopping the accretion of an effective concentration of antibiotics at molecular target locations within the bacterial cell is the use of ATP-dependent efflux pumps. Mex(XY)-Opr(M) or Mex(CD)-Opr(J) are the main efflux pump systems investigated in Gram-^{ve} organism like P.aeruginosa and have been linked to acquire multidrug resistance [15]. MFS transporters Qac-A, Qac-B, Nor-A, and Nor-B have been discovered in strains of Gram+^{ve} bacteria like S.aureus that cause hospital-acquired infections and confer resistance to puromycin and fluoroquinolones [16]. One feasible strategy that can be used to successfully battle the effects of resistance is to inhibit efflux pumps.

Additionally, it was discovered that inhibiting efflux pumps was also reported to reduces the MICs for bacteria that were both susceptible to and resistant to antibiotics, as well as reversed acquired resistance in certain strains of P. aeruginosa [17]. Rhodamine-6G could accumulate in both as a result of plants' flavonoids extract able to cause accumulation. Hence Flavonoids from Abutilon indicum were powerful inhibitor and increases in R6G accumulation in S. aureus and P. aeruginosa of 116% and 132%, respectively. According to the findings of this investigation, cells exposed to Abutilon indicum induced the greatest accumulation of R6G in P.aeruginosa. As a result, less sensitive to growth suppression compare to P.aeruginosa was more responsive to phytoconstituent induced efflux pump inhibition than Gram+^{ve} bacteria. The study detection that was made when crude extracts showed a strong suppression of Rhodamine-6G drug accumulation in P. aeruginosa cells by 64–100% [18].

It is significant to recognize the substances that prevent bacteria from extruding medications, as these substances could serve as sources of adjuvants in formulations that combine them with traditional antibiotics [19]. An effective co-formulation of amoxicillin, a traditional beta-lactam antibiotic, and clavulanic acid, a penicillinase inhibitor, in the coamoxiclav generic serves as a clinical illustration. In vitro studies have showed considerable synergistic effects when an MDR pump inhibitor is used with combinations of antimicrobial phytoconstituents and conventional antibiotics [20]. The efficiency of Abutilon indicum extract for in vitro antibacterial activity has already been established by prior studies [21-23].

It is critical to identify the substances that prevent drug efflux from within bacteria because they are potential sources of adjuvants in co-formulated antibiotics [24]. In the coamoxiclav generic, clavulanic acid, a penicillinase inhibitor, was successfully coformulated with amoxicillin, an old traditional beta-lactam antibiotic. In vitro studies of the synergistic effects of MDR pump inhibitors combination with other antimicrobial phytoconstituents as well as conventional antibiotics have yielded remarkable findings [8]. Previous research has demonstrated the efficacy of Abutilon indicum extract for in vitro antibacterial activity [25-27].

In conclusion, S.aureus and P.aeruginosa have exhibited potent antibacterial efficacy when exposed to the flavonoids extract from Abutilon indicum. The flavonoids extract from Abutilon indicum exhibited more growth-inhibitory action. However, compared to ampicillin, the extract's activity was much, much lower. The efflux pump was most susceptible in P.aeruginosa by Abutilon indicum flavonoids, which also induced significant accumulation in S. aureus. For the reason that P. aeruginosa has been shown to be particularly sensitive to efflux pump inhibition, pharmaceutical formulations

containing EPI activity may be more effective than expected against this particular bacterial species. To identify the precise compounds that have antibacterial activity, more research must be done.

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