Research Article

In-vitro Antioxidant and Anti-microbial Properties of Anthraquinones from Rhizomes of *Rheum emodi* against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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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 as emerging strains are becoming increasingly resistant to existing antibiotics. Phytochemically, anthraquinones are plant secondary metabolites with powerful therapeutic effects and there are several scientific evidences that plant anthraquinones are potent pharmacological agents. In this study, Effect of anthraquinones derived from the *rhizomes of Rheum emodi* on prevalent bacterial pathogens growth and efflux-pump activity were tested on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. An antibiotic standard ampicillin was comparable to the inhibitory effects of anthraquinones at 1.80 mg/mL of concentration on bacterial growth. The anthraquinones from *Rheum emodi* 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 anthraquinones had bacteriostatic effects on *P. aeruginosa* and *S. aureus*. *Rheum emodi* anthraquinones 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, Anthraquinones from *rhizomes of Rheum emodi* exhibited antibacterial activity as well as the ability to inhibit the ATP-dependent transport of compounds across cell membranes, and these anthraquinones can be used as potential principal composites for the development of herbal based anti-pathogenic bacterial agents and their adjunct composites.

Keywords: *Rheum emodi*, Colony forming units-(CFU), Minimum inhibitory concentration-(MIC), Minimum bactericidal concentration-(MBC).

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INTRODUCTION

One of the main factors contributing to causes of communicable diseases in 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 infectious 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; *S.aureus* and *Pseudomonas aeruginosa*[1]. More critically, *P. aeruginosa* and *S. 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 as claimed to have slight undesirable side effects[5]. Moreover, with concerns of escalating cost of drug discovery, globally majority of plants have proven to be some of the greatest cost-effective and cheap alternative sources of medicine[6].

The *Rheum emodi* belongs to Polygonaceae family, which is widely available throughout in all parts of India's tropical and subtropical regions. It is well known that the plant has medicinal potentials. The *Rheum emodi* commonly known as rewand chini 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 *rhizomes of Rheum emodi* 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 *Rheum emodi* against *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Microorganisms and Plant Provenance

The plant material was collected from different regions of India and was authenticated by a taxonomist at the Botanical Department of K.K. University Nalanda, India (Voucher. No: K.K.U-4026). The test microorganisms were obtained from Department of Microbiology, K.K. University, Nalanda, India.

Extraction of the *Rheum emodi* anthraquinones

One kilogram (1kg) of dried coarsely powdered *Rheum emodi* rhizome was extracted with 3x3L of 70% ethanol by nonstop hot extraction method for three days using a soxhlet apparatus. At the 4th day ethanolic extract was concentrated by removing solvent under reduced pressure and temperature with vacuum distillation. The residue was freeze-dried and stored at -10^{0C} in airtight container. The crude ethanol extract 75 g was re extracted with 170 ml of water and 30 ml of 70% ethanol. Fallowed by re extraction 10.5% w/v ferric chloride hexa hydrate solution ($FeCl_3 \cdot 6H_2O$) 50 ml was added, the whole mixture was refluxed for thirty minutes before addition of 20 ml concentrated hydrochloric acid (HCl) and refluxed for another half one hour and permit the mixture to cool. Mixture was filtered and filtrate was extracted with chloroform (5 x 200 ml). The obtained extract from chloroform layer was evaporated and add aqueous layer with 0.1gm of sodium bicarbonate ($NaHCO_3$) to adjust to pH 7.0. The whole solution was then centrifuged at 4000 rpm for 15-20 min and supernatant was collected. The supernatant was subjected to evaporation which contained anthraquinone glycosides with 2.25% of yield. It showed positive results for Borntragers test, indicating the presence of Anthraquinone glycosides.

Microbial Susceptibility Test

Extract of Anthraquinones from *rhizomes of Rheum emodi*, 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. *P. aeruginosa* and *S. aureus* at 1x10^6 cfu/ml and wells of microtiter plate were diluted with broth to made each well contain a total volume of 300μL. A progressive (+ve) control containing ampicillin was also prepared, with 500µg 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 in a Lab Companion incubator for 24 hours at 37°C cell density was determined.

Determination of MIC and MBCs

MICs and MBCs of *Rhizomes of Rheum emodi* Anthraquinones extracts were measured. In order to create 10 different dilution concentrations, the Anthraquinones extracts were sequentially serially diluted twofold in a 96-well polystyrene microplate from 1.80 mg/ml to 0.0032 mg/ml. 20μl of each dilution were successively transferred in triplicate into the wells of a separate 96-well microplate. Each species in its own microplate, 100μl of *P. aeruginosa* and *S. aureus* broth cultures (1x10^6 cfu/mL) were added to the wells. Then, in each well 180μl of broth was added to the well to build a total volume of 300 μl. As a positive control, rows of wells with media containing 100 μl 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, preincubation absorbance values were recorded. Following a 24hr 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.

In vitro antioxidant properties of *Rheum emodi*

The DPPH radical assay is a suitable model for estimating the total antioxidant potential of antioxidants (Huang *et al*., 2005). The assay was carried out in 96 well microtitre plate. DPPH solution of 200µl, 10µl of each of the test sample or the rutin standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/ml, respectively. The plates were incubated at 37oC for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader.

Rhodamine-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 (50mL), 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 10 mM NaN_3 at a concentration of 40 mg/mL. Companion Incubator, pellets were stirred and at a speed of 90 rpm and 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. Cells from tube B of the two species were re-suspended in 1M glucose. 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 for reserpine + glucose and two o for Anthraquinones derived from the *rhizomes of Rheum emodi* extracts + glucose, and cells from tube A.

This was carried out for each of the *S. aureus* and *P. aeruginosa* pellets. The Anthraquinones were added to their respective tubes at an absolute concentration of 61μg /mL, and reserpine was added to tubes labelled reserpine + glucose at an absolute concentration of 61µg/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 90rpm for 30minutes. To determine how much R6G was pumped out of the cell after 30 minutes, tubes were spun in a centrifuge-5425 (Sigmaaldrich) for 10minutes at 4000rpm. 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 527nm 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 ≤ 0.05 or less was regarded to be statistically significant difference.

RESULTS

Microbial Susceptibility Test

The effects of *rhizomes of Rheum emodi* Anthraquinones extract on bacterial species were assessed by measuring absorbance values at 600nm before and after incubation. *Rhizomes of Rheum emodi* Anthraquinones 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 *rhizomes of Rheum emodi* Anthraquinones 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 anthraquinones and ampicillin.

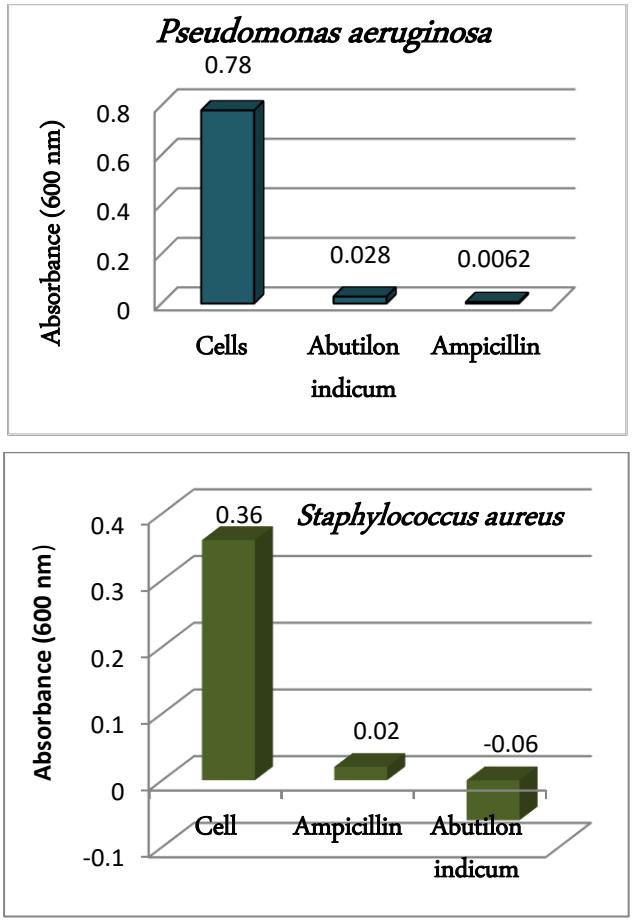


Figure 1: The effect of Anthraquinones extract of the *rhizomes of Rheum emodi,* ampicillin on growth of *S. aureus* & *P. aeruginosa*. Concentrations of 1.80 mg/mL & 1 × 106 cfu/mL of the extract and bacteria, Values are expressed as mean absorbance at 600nm wavelength.

Determination of MIC and MBCs

The MIC was determined for the well with least discernible MTT color. The *rhizomes of Rheum emodi* Anthraquinones 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 *rhizomes of Rheum emodi*

Anthraquinones 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. Anthraquinones extract of the *rhizomes of Rheum emodi* were bacteriostatic against both bacterial species.

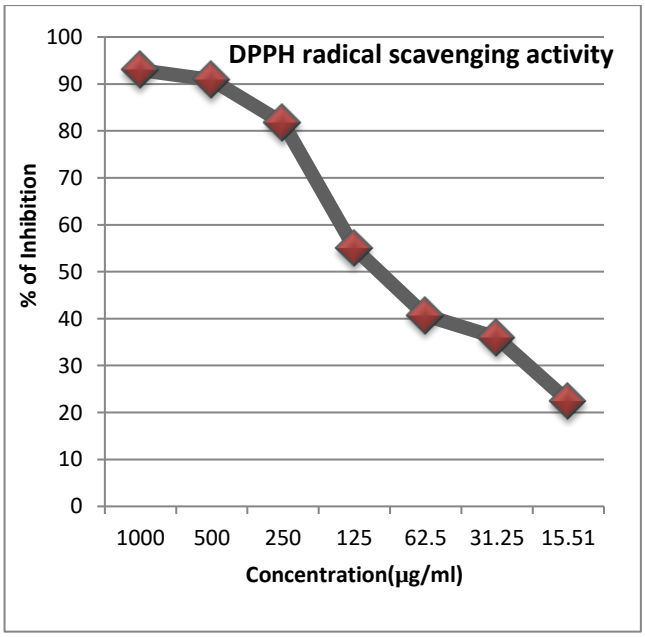
Table 1 illustrate the determination of Minimum inhibitory concentration and Minimum bactericidal concentration of *rhizomes of Rheum emodi* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Table 1: Assay of MIC and MBC of *rhizomes of Rheum emodi* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Name of Micro-Organism	G^{+ve}/G^{-ve}	Extract	MBC (mg/mL)	MIC (mg/mL)
Pseudomonas aeruginosa	$Gram^{-ve}$	*Rheum emodi*	-	0.32
Staphylococcus aureus	$Gram^{+ve}$	*Rheum emodi*	0.96	0.0036

In vitro antioxidant activities of *Rheum emodi*

DPPH radical scavenging activity has been widely used to evaluate the antioxidant activity of plant extracts and foods. The presence of antioxidant in the sample extract reacts with DPPH, which is a stable free radical, and convert it to 1,1 diphenyl 2 (2,4,6 trinitrophenyl) hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant compounds which can be detected spectrophotometrically at 517 nm. Figure no 2 shows the DPPH radicals scavenging capacity of extracts *R. emodi* with reference to rutin. Concentration of the sample necessary to decrease initial concentration of DPPH by 50% (IC50) under the experimental condition was calculated. Therefore, lower value indicates a higher antioxidant activity. The experimental data indicated that extracts of *R.emodi* displayed the highest DPPH scavenging effect 96%.

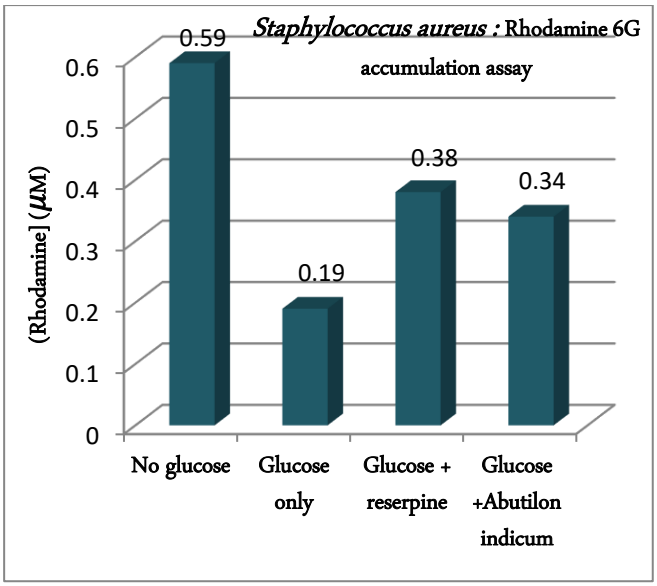


Sample	DPPH
Extract	*Rheum emodi*
	30.68±1.26
Standard	Rutin
	3.89±1.08

Figure 2: DPPH radicals scavenging capacity of extracts *R. emodi* with reference to rutin

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, *rhizomes of Rheum emodi* Anthraquinones 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. *Rhizomes of Rheum emodi* Anthraquinones 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 3 and Table 2).



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].

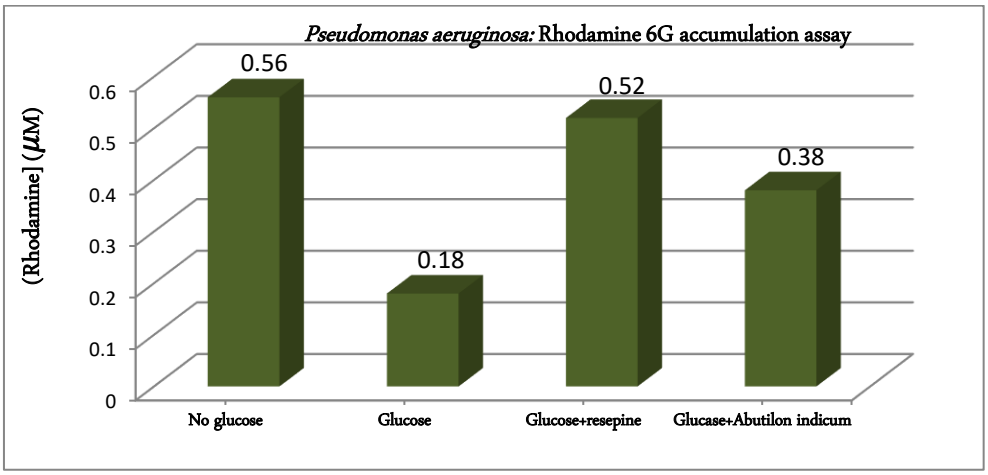


Figure 3: The effect of anthraquinones extract of the *rhizomes of Rheum emodi* on accumulation of Rhodamine-6G over time and ATP-dependent efflux pumps.

Table 2: Determination of the R-6G concentration that accumulated in the bacterial cells after exposure to anthraquinones extract of the *rhizomes of Rheum emodi*.

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%)

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 anthraquinones extract of *rhizomes of Rheum emodi*. The *rhizomes of Rheum emodi* 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 anthraquinones 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 anthraquinones extract showed efficacy against Gram+ve and Gram-ve bacteria, implying broad range activity.

Anthraquinones 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 anthraquinones is much outweighed by ampicillin. Anthraquinones from *rhizomes of Rheum emodi* exhibited bactericidal activity only against *S.aureus*. All plant anthraquinones 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 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' anthraquinones extract able to cause accumulation. Hence, Anthraquinones from *rhizomes of Rheum emodi* 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 *rhizomes of Rheum emodi* 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 *rhizomes of Rheum emodi 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 *rhizomes of Rheum emodi* extract for in vitro antibacterial activity[25-27].

In conclusion, *S.aureus* and *P.aeruginosa* have exhibited potent antibacterial efficacy when exposed to the anthraquinones extract from *rhizomes of Rheum emodi*. The anthraquinones extract from *rhizomes of Rheum emodi* exhibited more growth-inhibitory action. However, compared to ampicillin, the extract's activity was much, much lower. The action of drug efflux pump was more susceptible in *P. areuginosa* by the treatment with *rhizomes of Rheum emodi* anthraquinones, 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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