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Antibacterial activity of *Hibiscus sabdariffa* L. calyces against hospital isolates of multidrug resistant *Acinetobacter baumannii*

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ABSTRACT

Objective: To evaluate the antibacterial activity of methanol extract of *Hibiscus sabdariffa* (*H. sabdariffa*) calyces employed in Sudanese folk medicine against five hospital isolates of multidrug resistant *Acinetobacter baumannii* (MDR *A. baumannii*).

Methods: The antibacterial activity of 80% methanol extract (v/v) of *H. sabdariffa* calyces was evaluated by agar disc diffusion, minimum inhibitory concentration and minimum bactericidal concentration methods. Antibiotic susceptibility of selected *A. baumannii* strains was tested.

Results: In the present investigation, the methanol extract from the calyces of *H. sabdariffa* exhibited significant antibacterial properties against the non-MDR *A. baumannii* as well as the MDR *A. baumannii* strains with a zone of inhibition ranging from (11.3 ± 0.3) to (13.6 ± 0.3) mm. The relative percentage inhibition of *H. sabdariffa* extract (10 mg/disc) with respect to gentamicin (10 μg/disc) had potent antibacterial properties and was much more effective than gentamicin. Values of minimum inhibitory concentration and minimum bactericidal concentration ranged from 25 to 50 and 50 to 100 mg/mL, respectively, revealing the potential bactericidal properties of the extract.

Conclusions: According to the present study, the calyces of *H. sabdariffa* can be used as a substitute source of the current ineffective synthetic antibiotics used against MDR *A. baumannii*.

1. Introduction

In the 21st century, the infectious diseases remain the leading cause of death classified by the World Health Organization, where around 15 million people (accounting for >25% annual world death) die every year from infectious diseases worldwide[1]. Antibiotics, the most effective drugs against microbial infections in the 1950s, are recently losing their efficacies as most microorganisms have an acquired resistance[2]. Despite the negative side effects of antibiotics on human organs, the intensive use of antibiotics has led to emerging of what is called multidrug resistant (MDR) bacteria which are now raising remarkably all over the world and become an international public health threat[3]. Moreover, such pathogens have negative impacts on economy that infections have costed the United States 21–34 billion dollars annually[4]. *Acinetobacter baumannii* (*A. baumannii*) is a short Gram-negative plump rod (coccobacilli) belonging to genus *Acinetobacter*. It was earlier believed that this bacterium is ubiquitous in nature. But recently, it emerged as one of the most dangerous nosocomial pathogens worldwide, since it showed resistance to all known antibiotics[5]. *A. baumannii* can frequently cause pneumonia, bacteraemia, meningitis, wound infections and urinary tract infections[6]. The MDR *A. baumannii* is defined as resistants to more than two antibiotics classes, especially ampicillin-sulbactam, fluoroquinolones (ciprofloxacin or levofloxacin), aminoglycosides (gentamicin, tobramycin or amikacin), antipseudomonal cephalosporins (ceftazidime or cefepime) and antipseudomonal carbapenems (imipenem or meropenem)[5]. MDR *A. baumannii* is widely prevalent which has been reported and isolated from hospitals in many countries and areas, such as India, Turkey, Taiwan, Argentina, Korea, Japan, Iran, Saudi

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Arabia, Latin America, North America, Europe, Asia–Pacific, Brazil, Australia, Spain, US and UK[7].

Plants are the main source of medications for human, since they appear on earth and have abilities to synthesize endless secondary metabolites known as phytochemical compounds which serve as plant defense mechanism against macro and micro-organisms[8]. Alkaloids, flavonoids, phenolics and tannins are among the most important phytochemicals used in phytotherapy[9]. The World Health Organization estimated that, during the past decade, a large proportion of the population depended on traditional medicinal plants for treatment of different illnesses and preferred the modern medication, and even in developed countries, many people have begun to use medicinal plants as an alternative therapy[10]. Therefore, it is a logical approach to search for new antibacterial agents from natural sources like plants, since most of the recent drugs are initially obtained or semi-synthesized from these sources, particularly from those which are prescribed in traditional medicine[11]. Numerous studies are published showing a potent antibacterial activity of many medicinal plants[8]. However, little is known about the antibacterial activity of plants against MDR *A. baumannii*.

Roselle [*Hibiscus sabdariffa* L. (*H. sabdariffa*)] is a well known multipurpose medicinal plant, which belongs to family Malvaceae. It is an annual tropical short shrub and distributed in many tropical and sub-tropical regions in the world[12]. It is used traditionally for many purposes, such as hot and cold beverage, flavoring agent, food industry and traded as herbal medicine[13]. It also holds a plentiful potential of phytochemical compounds and has antioxidant, hypotensive, hypocholesterolemic, immune-modulated, hepatoprotective, renoprotective, diuretic, anti-obesity, antiurolithic, antidiabetic, antimicrobial and anti-cancer properties without any significant genotoxic effects[14]. Calyces of roselle is a famous public beverage in Sudan employed traditionally for the treatment of many ailments, such as respiratory tract infections, colds, fevers, hypertensions and malaria[15]. The previous study showed that it has significant antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella enterica*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris* and *Bacillus cereus*, *etc*.[16]. The aim of the present study is to evaluate the antibacterial activity of the Sudanese roselle calyces (*H. sabdariffa*) against some hospital isolates of MDR *A. baumannii*.

2. Materials and methods

2.1. Plant material and extraction

The dried flowers of *H. sabdariffa* were purchased from local herbal markets in 2015 from Khartoum, Sudan. The plant materials were taxonomically identified by a botanist at College of Sciences and Arts, Al-Rass, Qassim University. Voucher sample specimen was deposited. Calyces of *H. sabdariffa* were separated from the dried flowers and ground to a fine powder. About 100 g dried powder was taken and put in a sterile dark glass container and 500 mL of 80% v/v methanol (Sigma–Aldrich, St. Louis, USA) was loaded gradually into the container, soaked and subjected to frequent shaking and macerated for 3 days at room temperature [(37 ± 2) °C] in a dark cabinet. Then, the suspension was filtered using Whatman filter paper No. 1 (Sigma–Aldrich, St. Louis, USA). The methanolic filtrate was allowed to evaporate in the incubator (BINDER GmbH, Neckarsulm, Germany) at 45 °C for up to 10 days till a sticky extract obtained. Several hours before the antibacterial testing, 1 g of the extract was reconstituted in 2 mL of absolute methanol to get 500 mg/mL[16].

2.2. Isolation and identification of bacteria and antibiotic sensitivity test

A. baumannii was isolated from different clinical samples like pus, wound and sputum by Dr. Fiaz Ahmed (pathologist) from Department of Pathology and Laboratory Medicine, Al-Rass General Hospital (Table 1). The samples were identified by subculturing onto blood agar and MacConkey agar for 24 h at 37 °C. The growing colonies were examined with Gram-staining, which were Gram-negative coccobacilli under the microscope confirmed as *A. baumannii* by further biochemical tests and then classified as either MDR or non-MDR strain by the antibiotics susceptibility testing according to Kirby–Bauer disk diffusion method recommended by Clinical and Laboratory Standards Institute guidelines[17]. *A. baumannii* isolates were tested against the following antibiotics: amikacin (30 μg/disc), ceftazidime (30 μg/disc), aztreonam (30 μg/disc), piperacillin (100 μg/disc), imipenem (10 μg/disc), ciprofloxacin (5 μg/disc) and cefotaxime (30 μg/disc) purchased from Oxoid Limited, Basingstoke, UK. Isolates showing resistance to at least three antibiotics were considered as MDR *A. baumannii*.

2.3. Antibacterial assay

The modified Kirby–Bauer disc diffusion method was used to evaluate the antibacterial activity of the *Hibiscus* extract[16]. Prior to the experiment, *A. baumannii* strains were subcultured in nutrient broth (Watin–Biolife, Riyadh, Saudi Arabia) and incubated for 18 h at 37 °C in order to reach the exponential phase, then adjusted by adding normal saline to be equivalent to 0.5 McFarland standard, which comprised 1.0×10^8 CFU/mL. About 100 μL from each adjusted strain was loaded separately in 90 mm sterile disposable Petri dishes (Jalil Medicals, Manama, Bahrain), and 20 mL sterile warm Mueller–Hinton agar (Watin–Biolife, Riyadh, Saudi Arabia) was poured into each plate and left until solidified at room temperature (35–37 °C). About 6 mm blank discs were cut off from Whatman filter paper No. 1 and autoclaved. Then, 20 μL *H. sabdariffa* methanol extract at a concentration of 500 mg/mL (10 mg/disc) was loaded to the sterile disc and put over the seeded Mueller–Hinton agar plate. About 10 μg/disc gentamicin (Oxoid Limited, Basingstoke, UK) served as a positive control and 6 mm disc saturated with methanol served as negative control, which were also loaded. The plate was incubated for 24 h at 37 °C. The experiment was repeated thrice and the mean zone of inhibition around the discs was recorded.

Table 1
Sources of *A. baumannii*.

Strain	Source
Ab1	Wound
Ab2	Sputum
Ab3	Pus
Ab4	Wound
Ab5	Wound

Ab: *A. baumannii*.

2.4. Determination of relative percentage inhibition (RPI)

RPI of *H. sabdariffa* methanol extract (10 mg/disc) and the methanol (20 μL/disc) as a negative control, with respect to gentamicin (10 μg/disc) as a positive control against MDR *A. baumannii* were calculated using the following formula[18].

$$\text{RPI}\ (\%) = \frac{100 \times (X - Y)}{Z - Y}$$

where X is total area of inhibition of the tested extract, Y is total area of inhibition of the solvent, Z is total area of inhibition of the antibiotic.

The total area of the inhibition was calculated according to the following equation:

$$\text{Area} = \pi r^2$$

where r is radius of the zone of inhibition, $\pi = 3.14$.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC of the calyces of *H. sabdariffa* methanol extract against MDR *A. baumannii* strains which showed sensitivity towards the extract was estimated by adding 1.0 mL of the methanol extract at a concentration of 400 mg/mL to a tube containing 1.0 mL Mueller–Hinton broth (Watin–Biolife, Riyadh, Saudi Arabia). Then, two-fold serial dilutions were used to get the concentrations 400, 200, 100, 50, 25, 12.5 and 6.25 mg/mL. While a tube containing 1.0 mL sterile distilled water and 1.0 mL broth was served as positive control. All tubes were seeded with 10 μL tested bacterial strains adjusted to 0.5 Mac-Farland. Another tube containing only 1.0 mL absolute methanol and 1.0 mL broth was used as negative control. All tubes were incubated for 18 h at 37 °C. After incubation, the tube of the least concentration of *H. sabdariffa* methanol extract that did not show any visible growth was considered as MIC[19].

MBC of the calyces of *H. sabdariffa* methanol extract against MDR *A. baumannii* strains were evaluated as the following: 100 μL from each of the MIC tubes that did not show any growth was subcultured on Mueller–Hinton agar plates and incubated at 37 °C for 24 h and inspected after incubation for growth. The least concentration that showed no single colony of bacteria was taken as MBC[20].

2.6. Statistical analysis

Data were analyzed using the statistical package program SPSS 15.0. The experiments were repeated thrice. Values were expressed as mean ± SEM and $P < 0.05$ was considered as statistically significant. One-way ANOVA was carried out by Tukey honestly significant difference test.

3. Results

As shown in Table 2, non-MDR *A. baumannii* strain (Ab1) was susceptible to all tested antibiotics. Ab2 and Ab4 strains were resistant to six types of antibiotics and sensitive only to imipenem (10 μg/disc). Ab3 strain was resistant to five types of antibiotics and sensitive to piperacillin (100 μg/disc) and imipenem (10 μg/disc). Ab5 strain was resistant to five types of antibiotics and sensitive to amikacin (30 μg/disc) and imipenem (10 μg/disc). The antibacterial activity of the Sudanese *H. sabdariffa* calyces against the above-mentioned hospital isolates of *A. baumannii* has been evaluated in the present research work. The results revealed that the tested extract exhibited significant antibacterial activity against these pathogens. Table 3 shows the mean zone of inhibition of *A. baumannii* strains (Ab1–Ab5). The non-MDR *A. baumannii* (Ab1) showed sensitivity against the extract (11.3 ± 0.3) mm, but still less than gentamicin (22.5 ± 0.2) mm. Interestingly, other MDR *A. baumannii* strains (Ab2–Ab5) revealed significant sensitivity to the extract and were higher than gentamicin, particularly Ab3 which was totally resistant to gentamicin (0.0 ± 0.0) and sensitive to the extract. RPI of the methanol extract of *H. sabdariffa* calyces with respect to gentamicin was obtained. The methanol extract of the calyces of *H. sabdariffa* showed the maximum RPI against *A. baumannii* strain (Ab2) which was 223.3%, followed by Ab4 (191.8%), Ab5 (131.4%) and Ab1 (63.5%). The *A. baumannii* strain (Ab3) was omitted, since the effect of gentamicin on it was zero. The antibacterial efficacy of the calyces of *H. sabdariffa* was also evaluated using MIC and MBC assays (Table 4). The results showed that the MIC values ranged between 25 and 50 mg/mL, while the MBC values ranged between 50 and 100 mg/mL with MBC/MIC ratio between 1 and 2.

Table 2
Antibiotic sensitivity patterns of the tested organisms.

Acinetobacter isolates	Antibiotics						
	AK	CAZ	ATM	PRL	IMI	CIP	CTX
Ab1	S	S	S	S	S	S	S
Ab2	R	R	R	R	S	R	R
Ab3	R	R	R	S	S	R	R
Ab4	R	R	R	R	S	R	R
Ab5	S	R	R	R	S	R	R

S: Sensitive; R: Resistant; AK: Amikacin; CAZ: Ceftazidime; ATM: Aztreonam; PRL: Piperacillin; IMI: Imipenem; CIP: Ciprofloxacin; CTX: Cefotaxime.

Table 3
Antibacterial activity of the methanol extract of *H. sabdariffa* against different strains of *A. baumannii* compared to gentamicin.

Treatments	Mean zone of inhibition of *A. baumannii* (mm)				
	Ab1	Ab2	Ab3	Ab4	Ab5
Methanol extract (10 mg/disc)	11.3 ± 0.3	13.6 ± 0.3	11.6 ± 0.3	13.3 ± 0.3	13.3 ± 0.3
Gentamicin (10 μg/mL)	22.5 ± 0.2	9.1 ± 0.2	0.0 ± 0.0	9.6 ± 0.2	11.6 ± 0.2

Table 4
MIC, MBC and MBC/MIC rations of calyces of the methanol extract of *H. sabdariffa* against different *A. baumannii* strains.

Bacterial strains	Methanol extract of *H. sabdariffa* (mg/mL)		
	MIC	MBC	MBC/MIC
Ab1	50	50	1
Ab2	25	50	2
Ab3	25	50	2
Ab4	25	50	2
Ab5	50	100	2

4. Discussion

In the present study, the *Acinetobacter* strains used in this investigation were four representing MDRs and one representing non-MDR in order to compare the susceptibility of these microorganisms towards the tested plant extract of *H. sabdariffa*. As shown in Table 2, most of the tested strains revealed resistance against many antibiotics. Most of these seven antibiotics were recently launched, which revealed that *A. baumannii* is a highly virulent pathogen and able to acquire resistance rapidly against antibiotics. This observation was in agreement with Lowings *et al.*[21] who stated that *A. baumannii* evolves resistance to all known antibiotics and has rapid developing mechanisms and no effective treatment options seen in the near future. As represented in Table 3, the MDR *A. baumannii* strains (Ab2–Ab5) showed significant sensitivity to the methanol extract of *H. sabdariffa* and were higher than gentamicin. That is, the methanol extract of the *H. sabdariffa* calyces has potent antibacterial activity against MDR *A. baumannii*. It was previously reported that calyces of *H. sabdariffa* have antibacterial properties against different pathogens, foodborne pathogens, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *A. baumannii*[16,22,23]. These potent antibacterial properties of the calyces of *H. sabdariffa* could be attributed to its richness in secondary phytochemical metabolites. The previous study on Sudanese roselle revealed the presence of alkaloids, phenolic compounds, flavonoids and saponins[16]. These compounds are considered to be the major groups of antimicrobial compounds in plants[24].

The results of the antibacterial activity of the extract were compared with the positive control (gentamicin) for evaluating their RPI, which indicated that calyces of *H. sabdariffa* have potent antibacterial properties and are much effective than gentamicin. Recently, *A. baumannii* strains were reported developed high resistance to gentamicin[25]. Table 4 represents MIC, MBC and MBC/MIC rations of calyces of the *H. sabdariffa* methanol extract against different *A. baumannii* strains. The extract is considered to be bactericidal when the ratio of MBC/MIC $\leq$4, while considered bacteriostatic when MBC/MIC >4[26]. Accordingly, the calyces of *H. sabdariffa* possess effective bacteriocidal activity against all tested strains of *A. baumannii*. These findings are in agreement with Liu *et al.*[23] who mentioned that, based on MIC values, *A. baumannii* was inhibited effectively. Several previous studies on medicinal plants were reported antibacterial activity against MDR clinical strains of *A. baumannii* including *Magnolia officinalis*, *Mahonia bealei*, *Rabdosia rubescens*, *Rosa rugosa*, *Rubus chingii*, *Scutellaria baicalensis* and *Terminalia chebula*[27]. Accordingly, findings from the present study showed that the crude extract (80% methanol) of the calyces of *H. sabdariffa* exhibited potent antibacterial activity against MDR strains of *A. baumannii* competitor to antibiotics and this activity tended to be bactericidal. Further future studies are recommended for the fraction the compound(s) responsible for their antibacterial activity in addition to other pharmacological studies, which may lead to the discovery of new natural antibiotic from roselle.

Given the lack of new antibiotics and the spread of MDR *A. baumannii*, there will be an essential need for new antibacterial drugs with a different mode of action. This study suggested that Sudanese roselle calyces (*H. sabdariffa*) possess potent antibacterial properties against MDR *A. baumannii* which could be used as an antibacterial drug. It is worthy for the research scholars and pharmaceutical companies to carry out further investigations on natural products and medicinal plants for isolation and identification of new antibacterial compounds which can be formulated as effective antibiotics.

Conflict of interest statement

The author reports no conflict of interest.

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