

THE ANTIBIOFILM ACTIVITY OF *HIBISCUS SABDRIFFA L.* AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

The current study was aimed to investigate the antibacterial and antibiofilm activity of ethanolic extract of *Hibiscus sabdariffa L.* calyx against locally isolated methicillin-resistant *Staphylococcus aureus* (MRSA) at different concentrations were started from 0.078 to 40 mg/ml. The extract was prepared by soaked calyces powder of *Hibiscus sabdariffa L.* with 80% ethanol in the Soxhlet extraction unit, and then it was aseptically filtered. The antibacterial activity was tested by agar diffusion method and broth microdilution method, this method was used to determine the minimum inhibitory concentration of extract, while the antibiofilm activity was determined by using 96-well polystyrene microtiter plates. The results revealed that the ethanolic extract has antibacterial activity in a concentration-dependent manner, the average diameter zone of inhibition observed against MRSA isolates ranged from 14±0.5 mm to 20±0.5 mm. Moreover, at sub-inhibitory concentration, this extract developed an isolate-specific antibiofilm effect and presented highly significant ($P < 0.05$) variability in biofilm formation before and after addition of ethanolic extract, Mr1, and Mr7 isolates were gave the lowest and highest antibiofilm activity, respectively. In conclusion, the ethanolic extract of *H. sabdariffa L.* calyx is a promising alternative medication that can be used to treat the infection caused by MRS.

Keywords: MRSA, antibacterial, antibiofilm formation.

جدوع وغرب

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الفعالية المضادة لتكوين الغشاء الحيوي لمستخلص *Hibiscus sabdariffa L.* ضد العنقوديات الذهبية المقاومة للمثيسلين

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المستخلص

هدفت الدراسة الحالية الى التحقق من الفعالية المضادة للبكتريا والمضادة لتكوين الغشاء الحيوي للمستخلص الكحولي لنبات الشاي كجرات ضد بكتريا العنقوديات الذهبية المقاومة للمثيسلين المعزولة محليا وبتركيز مختلفة بدءا من 0.078 ولغاية 40 ملغم/مل. حضر المستخلص بنقع أوراق الكأس لنبات الشاي كجرات بالكحول الايثيلي بتركيز 80% باستعمال جهاز الاستخلاص الخاص للمركبات الفعالة للنبات ثم عقم بالفلتر، فحصت الفعالية المضادة للبكتريا بطريقة الانتشار بالاكار وطريقة التخفيف للوسط السائل واستعملت هذه الأخيرة لتحديد قيمة التركيز المثبط الأدنى للمستخلص، بينما حددت الفعالية المضادة لتكوين الغشاء الحيوي باستعمال أطباق المعايرة ذات الـ 96 حفرة. وأظهرت النتائج ان للمستخلص فعالية مضادة للبكتريا وتعتمد هذه الفعالية على التركيز المستخدم، وان معدل قطر التثبيط ضد البكتريا تراوح بين 14±0.5 ملم الى 20±0.5 ملم، كما وأظهر المستخلص فعالية ضد تكوين الغشاء الحيوي بالتركيز المثبط الأدنى وبدرجات تثبيط متفاوتة من عزلة الى أخرى. وأظهرت نتائج التحليل الاحصائي وجود فروق معنوية عالية عند مستوى احتمالية ($P < 0.05$) في تكوين الغشاء الحيوي قبل وبعد إضافة المستخلص، كما بينت النتائج ان العزلات Mr1 و Mr7 أعطت اقل واعلى قيمة لتثبيط الغشاء الحيوي على التوالي. ويمكن الاستنتاج أن المستخلص الكحولي لنبات الشاي كجرات يمكن أستعماله كعلاج بديل واعد ضد الإصابة ببكتريا المكورات العنقودية المقاومة للمثيسلين.

الكلمات المفتاحية: مرسا، المضاد البكتيري، المضاد لتكوين الغشاء الحيوي

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INTRODUCTION

The appearance of resistance of pathogenic bacteria to multiple antimicrobial agents has become a threat to public health. Besides, the available antimicrobial agents, sometimes, do not affect infections caused by either Gram-positive or Gram-negative bacteria (4,17) At this time, the main therapy for bacterial infections is using antimicrobial agents. Nonetheless, the misuse and overuse of antibiotics have become the key factor for the rise of drug-resistant strains of numerous groups of microorganisms (29). Almost all synthetic or semi-synthetic antimicrobials presented with many side effects. Most of these antimicrobials triggered resistance mechanisms (7). To resolve such a problematic issue, scrutinizing various plant-therapeutic and -antimicrobial compounds have the most attention. Especially when these substances have no or minimal side effects, easily biodegradable and are easily accessible at low prices (16). World Health Organization reported that medicinal plants served as the safest source among others. Given that, the plants produce a wide range of bioactive molecules (21). Antimicrobials agents derived from the plants have enormous therapeutic potential (27). Plants have the ability to synthesize secondary metabolites known as phytochemical compounds which serve as a plant defense mechanism against macro and micro-organisms (9). Alkaloids, flavonoids, phenolics, and tannins are among the most important phytochemicals used in phytotherapy (24). *Hibiscus sabdariffa* is a medicinal plant that belongs to the Malvaceae family, commonly found in the tropics and subtropics. It is known as roselle and sorrel (30). The *H. sabdariffa* plant is consumed as food and also useful in herbal medicine (8). It is used traditionally for many purposes, such as hot and cold beverage, flavoring agent, food industry and used as herbal medicine (10). It also holds an abundant potential of phytochemical compounds and medicinally used as a laxative, an anticarcinogenic, an antihypertensive, and hypocholesterolemic immune -modulator, hepatoprotective, renoprotective, diuretic, anti-obesity, antiurolithic, antidiabetic, antimicrobial properties without any significant genotoxic

effects (23). Many studies are published viewing a powerful antibacterial activity of many therapeutic plants (9). Nonetheless, little is known about the antibiofilm activity of plants against multi-drug resistant such as methicillin-resistant *staphylococcus aureus* (MRSA). For the above-stated reasons, this study aims to evaluate the antibacterial and antibiofilm efficacy of ethanolic extract of *H. sabdariffa* against clinical isolates of MRSA as an alternative treatment.

MATERIALS AND METHODS

Preparation of plant extract

The dried flowers of *H. sabdariffa* were purchased from the local herbal market. The plant was taxonomically identified by a botanist at the College of Science / University of Baghdad. Calyces of *H. sabdariffa* were powdered to a fine powder. The ethanolic extract obtained by placing 50 g of *Hibiscus sabdariffa* L.calyces powder in Soxhlet extraction unit with 550 ml of 80% ethanol. Extraction continued for 6 hours at 40°C (15). Thereafter, the plant extracts were filtered via a Whatman filter paper No. 42 (125 mm), concentrated by Vacuum Rotary Evaporator at 35°C and kept at 4°C in sterile universal tubes. Before the antibacterial testing, 1 g of the dried extract was mixed with 10 ml of Dimethyl sulfoxide (DMSO) and sterilization was accomplished using membrane filter (0.22 µm).

Microorganisms (Isolation and identification)

Ten MRSA isolates were obtained from Microbiology laboratory at Department of biology, College of Science, University of Baghdad. Originally these isolates were collected from different specimens samples. Identification was previously performed using *16SrRNA* (20); while Methicillin resistance was tested phenotypically via cefoxitin disk method (18) and molecularly via PCR technique (11). These isolates were stored at deep freeze in glycerol vials by lab staff.

Antibiotics sensitivity test

MRSA isolates were tested against the following antibiotics: Oxacillin (5 µg/disk), Vancomycin (20 µg/disk), Imipenem (10 µg/disk), Gentamicin (10 µg/disk) and Erythromycin (15 µg/disk)

Bacterial suspension preparation

Inocula were prepared as follows; few colonies were picked from overnight bacterial cultures were transferred into 5 ml of normal saline. Subsequently, it was adjusted to be compatible with a 0.5 McFarland tube (1.5×10^8 CFU/ml).

Antibacterial assay

Agar diffusion method: The antibacterial activity of the ethanolic extract of *H. sabdariffa* was studied by agar diffusion well-variant method as described by AL-Gbouri and Hamzah (2) with slight modifications. In brief, four serial dilutions (40, 20, 10, and 5 mg/ml) were achieved using DMSO as a diluent. By aid of sterile cotton swab, the inoculum of bacteria (prepared as described previously) was uniformly spread on a surface of Mueller Hinton plate. 50 μ l of each dilution was added to each of the four wells (6 mm in diameter holes were cut in the agar). DMSO alone was reflected as control. Finally, all plates were incubated, aerobically, overnight at 37°C. The resultant inhibition zones were measured in mm. Assays were carried out in triplicate.

Microdilution method: For determining the minimal inhibitory concentration (MIC) of ethanolic extract of *H. sabdariffa* that inhibits the visible growth of MRSA isolates, the protocol mentioned by Silva *et al.* (5) was followed. The double serial dilutions of ethanolic extract were started from 0.078 to 40 mg/ml which prepared from a stock solution (100 mg/ml) using Mueller Hinton broth as a diluent. Further, an aliquot of 100 μ l of each dilution was distributed in microtiter plates. Control wells contained bacteria-free growth media plus DMSO. All wells were inoculated with 10 μ l of a bacterial suspension (1.5×10^8 CFU/ml). All trials were repeated in triplicate. Afterward, the microtiter plates were incubated, aerobically, for 24 hrs at 37°C.

Biofilm quantification assay: Quantification of biofilm formation was assessed as described by Atshan *et al.* (6) In brief; each isolate was propagated in tryptic soy broth containing 1% glucose at 37°C for 24h; thereafter, bacterial culture was adjusted to McFarland standard no. 0.5. A volume (200 μ l) of an isolated culture was added to three wells of sterile 96-well polystyrene microplates. All plates were covered with their lids to avoid evaporation and incubated under aerobic conditions at

37°C for 24h. Three wells filled with bacteria-free tryptic soy broth were considered as a negative control. After incubation growth medium was carefully removed from the biofilm plate, gently washed thrice with distilled water, dried, and fixed at 60°C for 1 hr. Afterward, an aliquot of methanol (200 μ l) was added to each well for 15 min. at room temperature. Subsequently, the plates were washed thrice and covered with 0.1% crystal violet for 15 min at room temperature. Subsequently, plates were washed thrice with tap water and dried at 37°C for approximately 30 min. The adherent cells were resolubilized by the addition of 96% ethanol for 10 min. The absorbance of stained wells was determined at 630 nm with a microtiter reader (BioTek, USA). The classification of biofilm intensity is summarized in Table 1.

Table 1. Classification of biofilm intensity (6)

OD ₆₃₀	Biofilm
OD \leq OD _c *	Non-producer
2OD _c > OD > OD _c	Weak
4 OD _c > OD > 2OD _c	Moderate
OD > 4 OD _c	Strong

*Cut off value (OD_c) = average OD of negative control + (3 *Standard Deviation of negative control).

Antibiofilm activity of *Hibiscus sabdariffa* at MIC concentration : The same protocol was used for the biofilm formation assay, which previously mentioned. However, tryptic soy broth contained *H. sabdariffa* ethanolic extract at MIC was added after biofilm formation. The microtiter plates were incubated at 37°C for 24h. After that, all wells washed, stained, and read at 630 nm. Positive controls were performed as well by adding 200 μ l of *H. sabdariffa* extract-free fresh bacterial suspension (compatible to 0.5 McFarland standard). The antibiofilm activity of samples was given as the percentage of inhibition is expressed as:

%inhibition = (OD₆₃₀ of control (without extract) – (OD₆₃₀ value in the presence of extract) / OD₆₃₀ control \times 100 (32)

Statistical analysis

Data were presented as mean \pm standard deviation. T-test was employed for the evaluation of the efficacy of plant extract. P-

value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Susceptibility of bacteria to antibiotics : The results revealed all tested isolates were resistant to four types of antibiotics Oxacillin, Imipenem, Gentamicin, and Erythromycin. Whilst, sensitive only to vancomycin

Determination the effect of *Hibiscus sabdariffa* ethanolic extract on MRSA

The results have shown that the ethanolic extract of *H. sabdariffa* has good antimicrobial efficacy against MRSA isolates in a concentration-dependent manner. The different concentrations of ethanolic extract revealed inhibitory properties against all tested isolates. Relatively large zones of inhibition were seen; 20 ± 0.5 , 16 ± 0.5 , 15 ± 0.5 , and 14 ± 0.5 mm at 40, 20, 10, and 5 mg/ml, respectively.

Estimation of minimal inhibitory concentration of ethanolic extract: The antibacterial efficacy of the ethanolic extract of *H. sabdariffa* calyces was evaluated using MIC assays, the results revealed that the MIC value was 10 mg/ml for all ten MRSA isolates. Such potent antibacterial properties of the calyces of *H. sabdariffa* could be due to its productivity of secondary phytochemical metabolites such as alkaloids, phenolic compounds, flavonoids, and saponins (1). These compounds are considered as the main groups of antimicrobial compounds in plants (12). These substance may effect on ion leakage from the bacterial cells by inhibit the electron transport, protein translocation, phosphorylation steps, and other enzyme-dependent reactions, subsequently an increase permeability of plasma membrane (31).

Antibiofilm activity of ethanolic extract of *H. sabdariffa*

Biofilm production is considered as a marker of virulence. Numerous new approaches have been suggested for studying biofilm in terms of biofilm physiology and structure. In this study, the ability of biofilm-producing MRSA isolates was evaluated using pre-sterilized 96-well polystyrene microtiter plates, which considered as a standard test for the detection of biofilm biomass (3, 6, 14). Due to the crystal violet stained only the cells, not the slime materials and the cell, which is not in the

biofilm structure is rinsed –off by washing steps (28). To estimate biofilm intensity, absorbance was determined at 630_{nm} by a microplate reader. Given that, absorbance values represented the intensity of the biofilm thickness that formed by the studied isolates on the surface of the microtiter well The results of the current study summarized in Table 2 revealed that all bacterial isolates were biofilm producer but the biofilm intensity varies from one isolate to another. Approximately, 60% of isolates formed strong biofilm, whereas only 40% developed moderate biofilms according to OD 630 Limits (0.122 to 0.244 represented moderate biofilm; while $\text{OD } 630 > 0.244$ exemplified the strong biofilm. To determine the effect of MIC of ethanolic extract of *H. sabdariffa* on biofilm formation, ten isolates were incubated with 10 mg/ml of the extract; the results clarified that the MIC level was effective against all bacterial isolates. Yet, the effectiveness differs from one isolate to another and presented highly significant ($P < 0.05$) variability in biofilm formation either with or without the addition of ethanolic extract, the lowest antibiofilm activity was exhibited in Mr1 isolate with (11%) inhibition while the Mr7 gave the strongest antibiofilm activity, percentage of inhibition (74%). These results indicated that the extract at MIC level inhibits the biofilm formation of MRSA isolates. *H. sabdariffa* calyx is rich in secondary metabolites of medicinal characteristics. For example; flavonoids such as gossypetin, hibiscetin, sabdaretine, alkaloids, and saponins (22). In addition, the extract contains hibiscus acid, hydroxybenzoic acids, anthocyanins, flavonols, and other polyphenolic compounds (26). The antibiofilm activity of ethanolic extracts is due to the presence of these bioactive compounds, the herbal extracts have glycoside hydrolase enzyme that aids in degrading Glycosidic linkages within the polysaccharide chain of biofilm, by breaking them into smaller subunits or monomers which in turn will help in inhibiting the biofilm (13). Proanthocyanidins resist the formation of biofilms by preventing intercellular adhesion; thence, decreasing cell attachment (19). Tannins are the groups of water-soluble polyphenolic compounds that serve as defense

mechanisms for plants, act as antibiofilm as it is a putative lytic transglycosylase that acts as a breakdown the β -1,4 glycosidic bond

between N-acetylglucosamine and N-acetylmuramic acid (25).

Table 2. Biofilm formation by MRSA isolates before and after treatment with an ethanolic extract of *H. sabdariffa* and percentage of inhibition.

isolates	Before treatment		After treatment		T-test
	O.D630±SD*	Biofilm intensity	O.D630±SD	Percentage of inhibition	
mr1	0.186	moderate	0.165	11%	7.82E-03
mr2	0.134	moderate	0.102	24%	2.87E-06
mr3	0.450	strong	0.246	45%	7.72E-05
mr4	0.203	moderate	0.107	47%	3.25E-07
mr5	0.234	moderate	0.113	52%	1.24E-08
mr6	0.328	strong	0.020	63%	3.04E-10
mr7	0.390	strong	0.101	74%	9.75E-09
mr8	0.280	strong	0.090	68%	2.05E-09
mr9	0.293	strong	0.085	71%	2.84E-08
mr10	0.320	strong	0.108	66%	1.30E-09

*SD denotes to standard deviation

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