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Antimicrobial activity of *Hibiscus sabdariffa* extract against uropathogenic strains isolated from recurrent urinary tract infections

Issam Alshami*, Ahmed Eid Alharbi

Department of Medical Microbiology and Immunology, College of Medicine, Taibah University, Al Madinah, Saudi Arabia

PEER REVIEW

ABSTRACT

Peer reviewer

Dr Mohammed Aljeldah, Department of Medical Laboratory Sciences, College of Applied Medical Sciences, University of Dammam, Dammam, Saudi Arabia.

Tel: 00966567665555

E-mail: mmaljeldah@ud.edu.sa

Comments

This is a good paper where authors evaluated the antibacterial activity of *H. sabdariffa* against strains isolated from recurrent UTIs. The results are interesting where it has been found that the extract inhibits the biofilm forming capacity of *E. coli* and *K. pneumoniae* which could prevent UTIs. Details on Page 321

Objective: To report the antimicrobial effect and biofilm forming capacity of the uropathogenic strains that have been isolated from recurrent urinary tract infections (UTIs) in the presence of *Hibiscus sabdariffa* (*H. sabdariffa*) extract.

Methods: Six *Escherichia coli* and two *Klebsiella pneumoniae* isolates were collected from patients with recurrent UTIs. The susceptibility of bacterial isolates to *H. sabdariffa* extracts were tested by determining their minimum inhibitory concentrations (MICs), and minimum bactericidal concentration (MBC) by using the broth microdilution method in accordance to Clinical and Laboratory Standards Institute guidelines. Time–kill curves were plotted against the eight isolates based on the MIC results. The biofilm forming capacity of the isolates were evaluated using the microtiter plate assay. Detection of biofilms was done using the crystal violet staining method.

Results: Various levels of the extracts MIC were observed against all the uropathogenic isolates. MIC values ranged from 0.5 to 4 mg/mL, and MBC values ranged from 8 to 64 mg/mL. Both the time–kill experiment and MBC–MIC ratio demonstrated that the extracts' effect was in general, bacteriostatic. The biofilm capacity inhibition assay results showed that extracts inhibited biofilm production of all the isolates. The level of biofilm inhibition however, had varied among the bacterial strains and ranged from 8%–60% reduction in optical density.

Conclusions: The results of the study support the effective potential of *H. sabdariffa* extract to prevent recurrent UTIs and to emphasize the significance of the plant extract, in order to approach it as a potential antimicrobial agent.

KEYWORDS

Hibiscus sabdariffa, Biofilm, *Escherichia coli*, *Klebsiella pneumoniae*, Urinary tract infections**1. Introduction**

Urinary tract infections (UTIs) are regarded as amongst the most common serious health problems that affect millions of people each year. It is estimated that there are more than 150 million UTIs in the world reported per year and it bears an economic and medical burden worldwide. The term “UTI”

refers to the presence of clinical signs and symptoms arising from the genitourinary tract as well as the presence of a certain threshold number of bacteria in the urine (usually higher than 10^5 CFU/mL)^[1,2].

Most infections are localized to the lower urinary tract (bladder) and are called bacterial cystitis. Although single cystitis episodes are very common in both men and

*Corresponding author: Dr Issam Alshami, Department of Medical Microbiology and Immunology, College of Medicine, Taibah University, Almadinah, Saudi Arabia.

Tel: +966 581322214

E-mail: ishami@taibahu.edu.sa

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women, women are more prone to infection with a 50–fold predominance compared to men and infections occur in up to 30% of all women at some stage during their lives. In addition, recurrent cystitis is also very common, occurring in up to one–third of women with an average of two to three episodes per year^[3]. In recurrent cystitis, unplanned long–term antibiotics are administered to the patients and this can generally be unfavourable from the point of medical cost views and also increases chances of any potential development of bacterial resistance^[4].

Most UTIs result from the ascending route and represent classical biofilm disease problems. The first step is colonisation of periurethral cells with uropathogenic bacteria, which is then followed by the passage of bacteria through the urethra. The second step is adherence of the bacteria biofilm to the bladder wall^[5]. Therefore, the prevention of recurrent UTIs using a natural non–antimicrobial based approach is the most desirable. Also the potential effects of natural options for the long–term prevention of UTIs which include probiotics, cranberry and mannose have been discussed^[6–8].

Hence, in this study we examined the *in vitro* antimicrobial activities of *H. sabdariffa* extract against uropathogenic bacteria isolated from recurrent UTIs. *H. sabdariffa* is a common herbal drink consumed both hot and cold by people around the world and is used in traditional medicine for the treatment of hypertension and UTIs^[9–11]. The infusion is, usually known as karkade or red tea, made from the calyces of the *H. sabdariffa*. The red calyces are the part of the plant with commercial interest and are rich in organic acids, minerals, anthocyanins, and other phenolic compounds^[12]. The calyces are potentially a good source for antioxidant agents such as anthocyanins and ascorbic acid^[13]. In animal models, extracts of this flower have demonstrated hypocholesterolemic and antihypertensive properties^[9]. Relatively few studies have been carried out to evaluate the antimicrobial activities of this plant against some sort of common human pathogens^[13–16]. However, there have been no studies that concern the effect the plant has reported in the biofilm forming capacity of uropathogenic bacteria. The aim of this study was to assess the effectiveness of *H. sabdariffa* extract in inhibiting the biofilm forming capacity of uropathogenic bacteria in order to achieve a better understanding of the therapeutic properties of the extract.

2. Materials and methods

2.1. Plant extracts

Air dried hibiscus calyces were purchased from the local market. The identity of the plant was confirmed by comparing collected voucher specimens with those of a known identity located in the Herbarium of the College of Science, Taibah University. A voucher specimen was

deposited at the herbarium under the reference number 1433/14. The extracts were prepared according to a previously described protocol^[14,17]. Using 1 L of 80% aqueous methanol (BDH, UK), the phenolics were extracted from 100 g of the grinded calyces. The mixture was then sonicated for 20 min and then filtered through Whatman No. 2 filter paper. The solvents were then evaporated using a rotary evaporator at 40 °C under reduced pressure and the extracts were stored in tightly sealed glass vials and considered to be *H. sabdariffa* extract, which was then used for the antibacterial assays. Dried plant extracts were then dissolved in methanol to give a stock solution of 500 mg/mL, then filter sterilized before use.

2.2. Bacterial strains

In this particular study, six *Escherichia coli* (*E. coli*) strains (EC1, EC2, EC3, EC4, EC5, and EC6), and two *Klebsiella pneumoniae* (*K. pneumoniae*) strains (KP1 and KP2) were studied; all UTIs are from patients with recurrent UTIs isolated from Ohad Hospital, Al Madinah, Saudi Arabia. The overall susceptibility patterns of the isolates are displayed in Table 1. *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) used in this study as control strains were obtained from Dr Arif Al–Hamad, Al–Qatif Central Hospital Laboratory. Since the bacterial strains were obtained during routine microbiology examinations, there was no ethical requirement to take informed consent from individual patients for their subsequent use. The study was approved by the Clinical Ethical Committee of College of Medicine, Taibah University. Bacterial identities were confirmed using Vitek II (bioMérieux, France) and standard biochemical tests.

Table 1

Antimicrobial susceptibility pattern of the uropathogenic strains isolated from recurrent UTIs.

Isolates	Susceptibility pattern									
	AMP	AMC	NIT	COT	CAZ	CIP	GM	AK	NOR	IMP
EC1	R	S	R	R	S	S	S	S	R	S
EC2	R	R	S	R	R	S	R	S	R	S
EC3	R	R	S	S	R	R	S	S	R	S
EC4	R	S	R	R	S	R	R	S	S	S
EC5	R	R	R	S	R	S	R	S	R	S
EC6	R	S	S	R	S	R	S	S	S	S
KP1	R	R	R	R	R	S	R	S	R	S
KP2	R	R	R	R	S	R	S	S	S	S

AMP: ampicillin; AMC: amoxicillin/clavulanic acid; NIT: nitrofurantoin; COT: co–trimoxazole; CAZ: ceftazidime; CIP: ciprofloxacin; GM: gentamicin; AK: amikacin; NOR: norfloxacin; IMP: imipenem. R: resistant; S: susceptible. EC: *E. coli*. KP: *K. pneumoniae*.

2.3. Antimicrobial activity

Different methods were used on all the isolates in order to assess their susceptibility to the hibiscus extract. Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and time–kill curves were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. All susceptibility testing were performed

using Mueller–Hinton broth and stock solutions were prepared in accordance with CLSI guidelines[18]. A solvent control containing the highest concentration of solvent present was also run.

2.4. Biofilm formation

The ability to form a bacterial biofilm was tested on polyvinyl chloride 96–well flat–bottomed plates and the biofilm was quantified by measuring the optical density (OD) after dyeing it with crystal violet (Sigma–Aldrich, UK) followed according to a previously described protocol[19]. This method measures the bacterial cells which adhere to the bottom of the microtiter plate wells even after repetitive cycles of washing. Strains from an overnight culture were inoculated with the tested organism in the presence and the absence of sub–MIC concentrations of the extract. Briefly, fresh bacterial suspensions were prepared in tryptone soya broth from overnight cultures and adjusted to OD₆₀₀. Individual wells were filled with 0.1 mL aliquots of the diluted culture. Following overnight incubation at 37 °C without agitation, plates were gently washed with phosphate buffered saline (pH 7.4) and stained with 100 µL of 0.1% crystal violet for 30 min at room temperature. Excess crystal violet was removed by washing, and biofilm was then quantified by measuring the corresponding OD₅₇₀ nm of the supernatant following the solubilisation of crystal violet in 0.15 mL of 95% ethanol. For each clinical strain tested, biofilm assays were performed in triplicate and the mean biofilm absorbance value was determined.

2.5. Statistical analysis

All the experimental results were performed in triplicate and the results were expressed as means±SD. Statistical significance between treated groups were analyzed using

the Kruskal–Wallis test (SPSS) followed by paired *t*–test to compare the control with each treated group. *P* values < 0.05 were considered significant.

3. Results

3.1. Antimicrobial activities

The antimicrobial activity of the *H. sabdariffa* extract was evaluated on eight different uropathogenic bacteria. Significant antimicrobial effects, expressed as various concentrations of the extract, were observed against all the uropathogenic isolates and the control strains. MIC values ranged from 0.5 to 4±0.00 (SD) mg/mL, and MBC values ranged from 8 to 64±0.00 (SD) mg/mL. In general, *E. coli* strains were more sensitive than *K. pneumoniae* against the extract. The results of the antimicrobial activities are presented in Table 2.

Table 2

MIC and MBC±0.00 (SD) results of *H. sabdariffa* extract against the control strains and the common uropathogenic strains isolated from recurrent UTIs.

Isolates	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC ratio	Mode of action*
EC1	1	16	16	Bacteriostatic
EC2	0.5	16	32	Bacteriostatic
EC3	2	8	4	Bactericidal
EC4	0.5	8	16	Bacteriostatic
EC5	2	16	8	Bacteriostatic
EC6	4	32	8	Bacteriostatic
KP1	4	64	16	Bacteriostatic
KP2	4	64	16	Bacteriostatic
EC (ATCC 25922)	1	8	8	Bacteriostatic
KP (ATCC 700603)	4	32	8	Bacteriostatic

EC: *E. coli*; KP: *K. pneumoniae*; MIC: Minimum inhibition concentration; MBC: Minimum bactericidal concentration. * Based in time–kill experiment and MBC–MIC ratio.

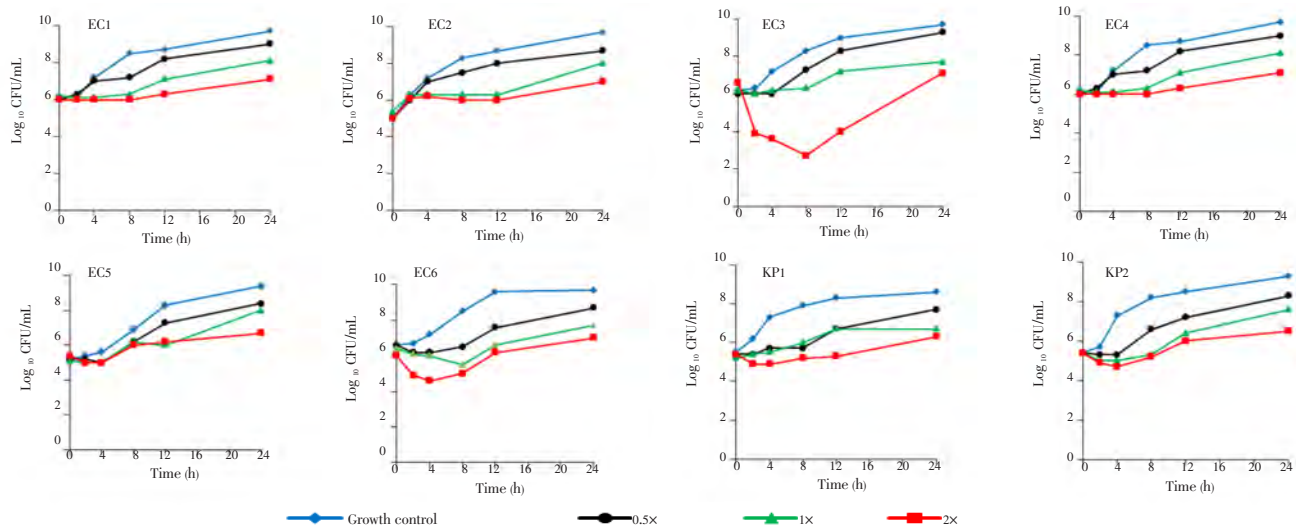


Figure 1. Time kill experiments evaluating the activity of *H. sabdariffa* extract against *E. coli* (EC) and *K. pneumoniae* (KP) isolates. Bacteriostatic activity was defined as a reduction in the numbers of viable bacteria of <3 log₁₀ CFU/mL at any of the incubation time tested.

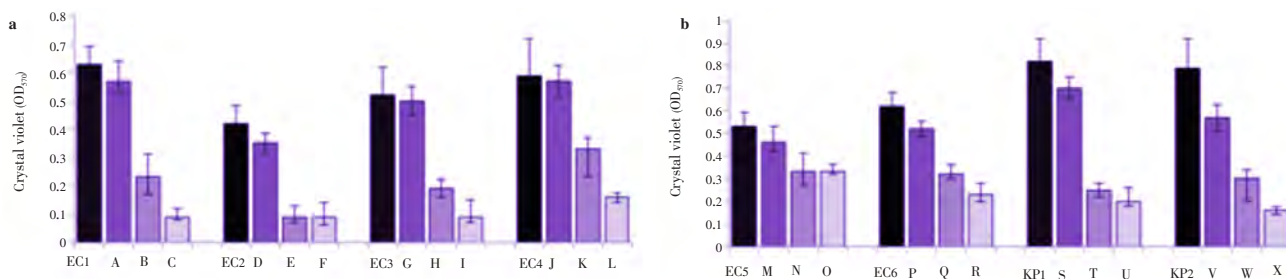


Figure 2. (a–b) Inhibition of biofilm formation in microtiter plates of *E. coli* (EC) and *K. pneumoniae* (KP) isolates by *H. sabdariffa* extract. (a) Inhibition of biofilm formation for four isolates of *E. coli*. (b) Inhibition of biofilm formation for four isolates of *E. coli* and *K. pneumoniae*.

All strains showed reduced biofilm formation in the presence of different sub-MIC concentrations of *H. sabdariffa* extract. Biofilm formation was determined by crystal violet staining. A: EC1+0.125×MIC; B: EC1+0.25×MIC; C: EC1+0.5×MIC; D: EC2+0.125×MIC; E: EC2+0.25×MIC; F: EC2+0.5×MIC; G: EC3+0.125×MIC; H: EC3+0.25×MIC; I: EC3+0.5×MIC; J: EC4+0.125×MIC; K: EC4+0.25×MIC; L: EC4+0.5×MIC; M: EC5+0.125×MIC; N: EC5+0.25×MIC; O: EC5+0.5×MIC; P: EC6+0.125×MIC; Q: EC6+0.25×MIC; R: EC6+0.5×MIC; S: KP1+0.125×MIC; T: KP1+0.25×MIC; U: KP1+0.5×MIC; V: KP2+0.125×MIC; W: KP2+0.25×MIC; X: KP2+0.5×MIC.

3.2. Mode of action

Time–kill curves were performed for the isolates using different concentrations of the extract ranging from 0.5–2 × the MIC value. The results obtained for the time–kill curves are summarized in Figure 1. For five *E. coli* strains and two *K. pneumoniae* strains, the extract effects were bacteriostatic. The extract showed moderate bactericidal activity against one of the studied *E. coli* strains within 8 h at a concentration of 2× the MIC value. Both the time–kill experiment and MBC–MIC ratio demonstrated that the extract was in general bacteriostatic. Bacteriostatic activity has been defined as a ratio of MBC to MIC of >4 or < 3 log₁₀ reduction in CFU/mL using time–kill^[20].

3.3. Inhibition of biofilm formation

The results showed that the *H. sabdariffa* extract inhibited biofilm production on all of the isolates. The biofilm inhibition varies between the different isolates and ranges from 8%–60% reduction in OD. The results obtained for the biofilm formation assays are summarized in Figure 2.

4. Discussion

Similar reports of antimicrobial activities of *H. sabdariffa* show various inhibitory effects against Gram positive and negative bacteria. Gram positive bacteria were less sensitive to the extract than Gram negative bacteria. It was suggested that this may be due to the differences in cell wall composition^[16]. *H. sabdariffa* was found to be effective at all levels in inhibiting *E. coli* O157:H7 (non uropathogenic *E. coli*) isolates from food, veterinary, and clinical samples. *H. sabdariffa* produced zones of inhibition against *E. coli* O157:H7 isolates and showed that the most effective concentration was at 10%, whereas the least effective was at 2.5%^[14]. Another study has already evaluated the effect of *H. sabdariffa* in combination with other antimicrobial agents against *E. coli*. Extracts of the plant significantly enhanced the inhibitory effects of neomycin, doxycycline,

chloramphenicol, cephalexin and nalidixic acid against both the standard strain and the resistant strain of *E. coli*^[16]. All previous studies have shown this effect in terms of inhibition zones or agar dilution method. In our study, we chose the most appropriate and reliable method for antimicrobial susceptibility testing (*ie*, the MIC using the microdilution method). The advantage of this technique was that it generated quantitative results in which further analysis provided a clearer interpretation of the results in terms of MIC and MBC.

The present study confirms the antimicrobial effect of the extract. Nevertheless, when comparing our results with that of the aforementioned study, the susceptibility of *E. coli* and *K. pneumoniae* against the extract was better in our study, with MIC values ranging from 0.5–4 mg/mL. Moreover, the present study allows us to establish, for the first time the actual effect of the extract against uropathogenic bacteria isolated from recurrent UTIs. The high potency of *H. sabdariffa* against these bacteria, gives a scientific basis for its use in folk medicine to treat and prevent UTIs^[10,11].

The mechanism of the action is not fully understood but it has been suggested that *H. sabdariffa* products contain phenolic compounds including flavonoids and cyanidin^[21,22]. Phenolic compounds have been reported to exhibit antimicrobial activities^[23,24]. The antimicrobial effect of the phenolic compounds may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes^[25].

The bacteriostatic effect of other plant extracts has been well documented. Cranberry extracts have been shown to eliminate recurrent UTI incidence in women and children with chronic UTIs. The bacteriostatic effects of cranberries prevent uropathogenic bacteria from attaching to the mucous membrane of the bladder and urethra and colonizing the urinary tract. Instead, they are flushed away during the natural voiding process^[26,27]. Because both *H. sabdariffa* and cranberry extracts contain flavonoids and cyanidin, it is reasonable to assume that a similar effect would be seen when using *H. sabdariffa*^[21,22,25]. The present study allows us to establish for the first time that *H. sabdariffa* has a bacteriostatic effect against the uropathogenic isolates.

There is no previous data that suggests *H. sabdariffa* extracts may inhibit biofilm formation capacity against uropathogenic isolates. Thus, the current study was initiated to evaluate *H. sabdariffa* extracts on these clinically relevant pathogens. The results showed that the *H. sabdariffa* extract inhibited biofilm production on all of the isolates.

Nearly all uropathogenic *E. coli* strains possess different ranges of virulence factors, including adhesins P and type 1 fimbriae. It is believed that the attachments of these bacteria are critical in their pathogenicity and virulence. However, both are associated with pyelonephritis and bladder colonization^[28,29]. Type 1 fimbriae *E. coli* binds to mannose-like receptors and P-fimbriated *E. coli* adheres to the oligosaccharide receptor sequence projected on the surface of uroepithelial cells. Biofilm forming uropathogens are often associated with long term persistence and display a dramatically increased resistance to antibiotics^[30]. *H. sabdariffa* contains compounds known as proanthocyanidins. Research shows that proanthocyanidins from cranberry extracts inhibit the adhesion of P-fimbriated *E. coli* to the uroepithelium^[31]. The present study suggests that proanthocyanidins from *H. sabdariffa* have the same anti-adhesins effect against uropathogenic isolates, which inhibit formation of biofilm *in vitro*.

The use of plant extracts and phytochemicals with known antimicrobial properties is becoming a very common practice worldwide and is of great significance in therapeutic use. Although the extensive use of antibiotics leads to the selection of clinical isolates that show multidrug resistance. On the other hand, fewer synthetic antibiotics are being developed and even fewer are launched each year. The identification of plant extracts with antimicrobial properties would be of an immense practical use as a safe and effective means of controlling clinical infections^[32–34].

In conclusion, the current study shows that *H. sabdariffa* extract significantly inhibits the uropathogenic growth and prevents the formation of *in vitro* biofilm. The antimicrobial activities of the extract observed, provide basic evidence for the potential antimicrobial effects of this plant for preventing recurrent UTIs. It also emphasizes a significant approach of the plants extract as an antimicrobial agent. Although a preventive effect of *H. sabdariffa* has been shown *in vitro*, further studies with more robust methodology would be needed in order to determine the *in vivo* clinical efficacy of the extract to prevent recurrent UTIs.

Conflict of interest statement

We declare that we have no conflict of interest.

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funding for this research (Grant No. 433/768).

Comments

Background

The development of antimicrobial resistance among pathogenic bacteria prompted the application of natural plant-derived as a possible antibacterial approach against uropathogenic strains isolated from recurrent UTIs.

Research frontiers

This study was conducted to find alternative compounds that can be used to develop a natural antibacterial agent. The study was carried out to investigate the antibacterial activity of *H. sabdariffa* extract against uropathogenic bacteria.

Related reports

There are different reports on the separate issues of antimicrobial effects on *H. sabdariffa* extract (Rukayadi *et al.*, 2008; Fullerton *et al.*, 2011). However, this is the first report on the extract effect in biofilm forming capacity. The finding fills this research gap and may help base information for further *in vivo* research.

Innovations & breakthroughs

The finding of the study indicates that the extract is a potential source for an antimicrobial agent. The present study provides scientific evidence for using this plant in treatment and prevention of recurrent UTIs.

Applications

The results from this study can be used for isolation and characterization of new antimicrobial agents. As the extracts are edible and consumed for a long period of time without any noticeable unwanted effects, the isolated compounds are safe as well.

Peer review

This is a good paper where authors evaluated the antibacterial activity of *H. sabdariffa* against strains isolated from recurrent UTIs. The results are interesting where it has been found that the extract inhibits the biofilm forming capacity of *E. coli* and *K. pneumoniae* which could prevent UTIs.

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