Detection of potential AcrAB-TolC multidrug efflux pump inhibitor in calyces extract of Hibiscus sabdariffa

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ABSTRACT

Aim: The aim of this study is to investigate the occurrence of potential efflux pump inhibitor (EPI) against AcrAB-TolC efflux pump in the methanol extract of Hibiscus sabdariffa. Materials and Methods: Calyces of H. sabdariffa were purchased from the local market in April 2014, used in methanol extraction. The methanol extract of H. sabdariffa was subjected to agar plate diffusion against Escherichia coli TG1 and its ΔacrB-ΔtolC followed by a thin layer chromatography (TLC) bioassay. The fraction corresponding to EPI fraction was eluted from the silica gel by methanol. The synergistic effect of antimicrobials and EPI fraction was measured by minimum inhibitory concentration (MIC) determination for E. coli and Erwinia amylovora strains. The ability of EPI fraction to enhance ethidium bromide (EtBr) accumulation was conducted. Results: E. coli TG1 was more sensitive to the methanol extracts of H. sabdariffa than E. coli ΔacrB-ΔtolC. Inhibition zone corresponding to flavones on TLC bioassay plate has been formed which might be related to the fraction of potential EPI. The MIC values revealed that EPI fraction enhanced the activity of the used antimicrobials by 4-8 folds in E. coli TG1 and by 4-10 folds in E. amylovora 1189. Addition of EPI fraction in a dose-dependent manner increased the intercellular accumulation of EtBr in the wild type stains of E. coli TG1 and E. amylovora 1189. Conclusion: An EPI fraction behaves like a multidrug EPI, and further investigation should be conducted for determination the structure of chemical constituents in EPI fraction.

INTRODUCTION

Resistance-nodulation-cell division (RND) multidrug efflux transport proteins deserve special attention; they are the most dominant system and span the entire Gram-negative cell envelope [1,2]. The well-studied RND system is AcrAB-TolC of Escherichia coli composed of three proteins; an inner membrane protein (AcrB) located in the cytoplasmic membrane, a membrane fusion protein (AcrA) in the periplasmic space and outer membrane factor (TolC) in the outer membrane [3,4].

E. coli and Erwinia amylovora are Gram-negative bacteria, belong to the Enterobacteriaceae family. E. coli species include both harmless strains that commonly found in human and animal intestines, and pathogenic strains causing various infections, while E. amylovora is a plant pathogen causing fire blight disease on Rosaceae. In E. coli, AcrAB-TolC mediate resistance toward metabolic byproducts such as bile salt, environmental antimicrobials, toxins, dyes, and detergents [5]. AcrAB-TolC of E. amylovora plays an important role in resistance toward phytoalexins, as virulence and fitness factors that are required for successful colonization of a host plant [6,7].

Due to the emergence of multidrug resistance (MDR) phenotypes, a new approach to overcome the efflux-mediated drug resistance is blocking the activity of drug efflux pumps via so-called EPI [8,9]. Combination of EPIs with an antibiotic is a promising therapeutic agent, which is expected to increase intracellular accumulation of antibiotics [8].

Few compounds were identified as EPIs for AcrAB-TolC, for example: Arylpiperazines were suggested as MDR reversal agent for RND efflux pumps [10], quinolone derivatives were promising EPIs for AcrAB-TolC in Enterobacter aerogenes [11], artesunate enhances the activity of β-lactam antibiotics through inhibition of AcrAB-TolC of E. coli [12], pimozide inhibits the AcrAB-TolC of E. coli [13], and benzothiazoles were identified as potential AcrAB-TolC efflux pump inhibitors (EPIs) in E. coli [14].
Very few plant-borne EPIs were identified such as 5'-methoxycynarin, *Staphylococcus aureus* NorA inhibitor, which was extracted from *Berberis* species [12,15,16], 2,6-dimethyl-4-phenyl- pyridine-3,5-dicarboxylic acid diethyl ester which was extracted from *Jatropha elliptica* was a resistance-modifying agent for MsrA and NorA *S. aureus* [17].

*Hibiscus sabdariffa* has been used in traditional medicine in treatment for several diseases such as hypertension, hepatic disease, cardiovascular disease, atherosclerosis, and diabetes [18-22].

The antimicrobial efficiency of *H. sabdariffa* L. against *E. coli* and some other Gram-negative bacteria has been demonstrated [23-25]. Combination of methanol extracts of *H. sabdariffa* L. with antibiotics enhances the activity of some antibiotics against resistant strain and standard strain of *E. coli* [26].

Many antioxidant compounds have been reported in *H. sabdariffa* such as cyanidin 3-rutinoside, delphinidin 3-glucoside [18,19,27,28].

Therefore, the question of this research is there a potential compound(s) with EPI activity for AcrAB-TolC of *E. coli* and *E. amylovora* in *H. sabdariffa* L. calyces extract.

MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions**

Bacterial strains used in this study are listed in Table 1, routinely maintained on Luria-Bertani (LB) medium at 37°C or 28°C for *E. coli* and *E. amylovora*, respectively. Medium was supplemented with 50 µg/ml ampicillin (Ap), 25 µg/ml chloramphenicol (Cm), 2 µg/ml gentamycin, and 25 µg/ml kanamycin when necessary (Sigma-Aldrich).

**Plant Material and Extraction**

Calyces of *H. sabdariffa* were purchased from the local market in April 2014 and compared with the collected voucher specimen number 1018 at the Biological Sciences Department at The University of Jordan. Finely powdered and extracted in a Soxhlet with 2 L of methanol for 48 h. The methanol was evaporated by vacuum pressure, the ×10 concentrated extract 10 mg/ml was used in further experiments [23,25,31].

**Agar Plate Diffusion Assay**

In screening for the presence of inhibitory compound(s) for the multidrug efflux pump AcrAB-TolC system, *E. coli* TG1 and its ∆acrB-∆tolC mutant were used in agar plate diffusion assay. Where 100 µl of OD_{600} ~1.0 (~10^7 CFU/ml) bacterial suspension were inoculated separately on LB medium and LB medium supplemented with sub-lethal concentration of crystal violet (CV), as known substrate for AcrAB-TolC efflux pump to trigger assembly of the tripartite system for recruitment in the efflux function, at concentration of 0.78 µg/ml and 0.078 µg/ml for the TG1 and ∆acrB-∆tolC mutant, respectively. 10 µl of the ×10 concentrated extracts were applied in wells; 10 µl of 12.5 mg/ml of Cm and 10 µl of 80% methanol were used for positive and negative control, respectively. The plates were incubated at 37°C for 24 h and monitored for formation of inhibition zone.

**Thin Layer Chromatography (TLC) - Bioassay and Elution of EPI Fraction**

The ×10 concentrated extract was subjected to separation by TLC, 50 µl were loaded on 20 cm width TLC plates (Macherey-Nagel, Germany), separation was conducted by ethyl acetate/acetic acid/formic acid/water mixture (100:11:11:26) [32]. After drying, two plates were overlaid, separately; one with *E. coli* TG1-LB suspension (500 µl of OD_{600} ~1.0 in 50 ml LB - 7.5% agar) supplemented with a sub-lethal concentration of CV 0.78 µg/ml, the other plate was overlaid with *E. coli* TG1-LB. The plates were incubated at 37°C for 24 h. Visualization inhibition zones were conducted by spraying the plates with a p-iodonitrotetrazolium solution (2 mg/ml) (Sigma-Aldrich). Formation of pinkish color indicates bacterial growth while clear zone indicates inhibition of bacterial growth [33,34].
The fraction corresponding to the inhibitory compound was eluted from the silica gel, concentrated to 1mg/ml and subjected to antagonistic assay against E. coli TG1 and its ΔacrB-ΔtolC.

Determination of Minimum Inhibitory Concentration (MIC) for Antimicrobials ± EPI Fraction

The MIC of different antimicrobials compounds, listed in Tables 2 and 3, were determined in the absence and presence of EPI fraction by a two-fold dilution assay in Mueller-Hinton broth (MHB) medium (Mast Group Ltd., UK). All tests were done in triplicate in accordance with the National Center for Clinical Laboratory Standards recommendations [35]. In the synergistic wells, the final concentration of EPI fraction was 25 µg/ml. E. coli strains were incubated at 37°C and E. amylovora strains were incubated at 28°C. MHB was used as a blank and MHB inoculated with test strains was used as a growth control. Bacterial growth was examined visually after 24 h of incubation. In general, differences in MIC values were only considered significant if they were at least four-fold. This cutoff is consistent with the previous publications [7,12].

**Table 2: Synergetic effect of EPI fraction from H. sabdariffa with selected antimicrobial compounds in E. coli strains**

| Compounds          | MIC (µg/ml) |
|--------------------|-------------|
|                    | TG1         | ΔacrB       | ΔtolC       | ΔacrB/ΔtolC | ΔacrB (acrAB) | ΔtolC (tolC) |
| Bile salt          | 1000±250    | 31.25±31.25 | 31.25±15.62 | 31.25±31.25 | 1000±125     | 1000±125     |
| Phloretin          | 1000±125    | 250±250     | 250±125     | 250±125     | 1000±250     | 1000±250     |
| Berberine          | 1000±125    | 31.25±31.25 | 62.50±31.25 | 31.25±31.25 | 1000±125     | 1000±125     |
| Acriflavine        | 31.25±6.25  | 3.13±3.13   | 3.13±1.56   | 3.13±1.56   | 31.25±3.16   | 31.25±6.25   |
| Novobiocin         | 500±62.5    | 50±25       | 50±25       | 50±25       | 500±62.5     | 500±62.5     |
| Ampicillin         | 15.62±3.13  | 0.31±0.17   | 0.31±0.31   | 0.31±0.31   | 6.25±1.56    | 12.5±1.56    |
| Tetracycline       | 12.5±1.56   | 0.31±0.17   | 0.31±0.31   | 0.31±0.31   | 6.25±1.56    | 12.5±1.56    |
| Nalidixic acid     | 10±2.5      | 0.5±0.5     | 0.5±0.25    | 0.5±0.25    | 10±2.5       | 5±1.25       |
| Ciprofloxacin      | 0.16±0.03   | 0.06±0.06   | 0.06±0.06   | 0.06±0.06   | 0.16±0.03    | 0.16±0.03    |
| SDS                | 500±62.5    | 50±25       | 50±25       | 50±25       | 500±31.25    | 500±62.5     |
| Ethidium bromide   | 125±15.62   | 25±12.5     | 50±50       | 50±25       | 125±7.81     | 125±15.62    |
| Crystal violet     | 25±3.13     | 1.25±1.25   | 1.25±1.25   | 1.25±1.25   | 25±3.13      | 25±3.13      |

**Table 3: Synergetic effect of EPI fraction from H. sabdariffa with selected antimicrobial compounds in E. amylovora strains**

| Compounds          | MIC (µg/ml) |
|--------------------|-------------|
|                    | 1189        | ΔacrB       | ΔtolC       | ΔacrB/ΔtolC | ΔacrB (acrAB) | ΔtolC (tolC) |
| Bile salt          | 1000±250    | 125±125     | 125±125     | 125±125     | 1000±125     | 1000±125     |
| Phloretin          | 1000±125    | 125±125     | 125±125     | 125±125     | 1000±125     | 1000±125     |
| Berberine          | 1000±125    | 62.5±62.5   | 62.5±62.5   | 62.5±62.5   | 1000±125     | 1000±125     |
| Acriflavine        | 15.6±3.125  | 1.56±1.56   | 3.12±3.12   | 3.12±3.12   | 31.2±3.125   | 31.2±3.125   |
| Novobiocin         | 62.5±6.25   | 12.5±12.5   | 12.5±12.5   | 12.5±12.5   | 62.5±6.25    | 31.2±12.5    |
| Ampicillin         | 62.5±15.63  | 6.25±6.25   | 6.25±6.25   | 6.25±6.25   | 62.5±7.81    | 62.5±15.63   |
| Tetracycline       | 6.25±1.25   | 0.63±0.63   | 0.63±0.63   | 0.63±0.63   | 6.25±1.25    | 12.5±1.25    |
| Nalidixic acid     | 0.63±0.16   | 0.13±0.13   | 0.13±0.13   | 0.13±0.13   | 1.25±0.16    | 0.63±0.08    |
| Ciprofloxacin      | 0.63±0.08   | 0.06±0.06   | 0.06±0.06   | 0.06±0.06   | 0.63±0.16    | 1.25±0.16    |
| SDS                | 1000±125    | 100±100     | 100±100     | 100±100     | 1000±125     | 1000±125     |
| Ethidium bromide   | 31.25±3.13  | 3.13±3.13   | 3.13±3.13   | 3.13±3.13   | 62.5±6.25    | 62.5±6.25    |
| Crystal violet     | 3.13±0.78   | 0.63±0.63   | 0.63±0.63   | 0.63±0.63   | 3.13±0.78    | 6.25±1.56    |

**Intercellular Accumulation of Ethidium Bromide (EtBr)**

E. coli TG1 and its ΔacrB-ΔtolC mutant, E. amylovora and its ΔacrB-ΔtolC mutant were used in intercellular EtBr accumulation assay according to Coldham et al. [36]. Bacterial strains were grown in LB medium, 250 rpm until it reaches to an OD of 1, and centrifuged at 4000 rpm for 30min. The bacterial pellets were re-suspended in phosphate buffer saline (PBS) supplemented with 0.4% glucose (pH 7.4), and the optical density was re-adjusted to OD of 1. The EPI fraction was added at increasing concentrations. Samples were placed into a 96-well plate (flat-bottomed, black supplied by Santa Cruz Biotechnology, Inc.). EtBr was added at a final concentration of 1.0 µg/ml. Fluorescence was measured from the top of the wells in Synergy HTX Multi-mode Reader, BioTek at excitation and emission filters of 528/2 and 590/2 nm, respectively.
Inhibition zones have been formed on plates inoculated separately with E. coli TG1 and E. coli ΔacrB-ΔtolC mutant [Table 4]. The inhibition zone on agar plate inoculated with E. coli TG1 (~11 mm in diameter), was slightly larger than those of E. coli ΔacrB-ΔtolC mutant (~8 mm in diameter). In contrary, to the used antibiotic as a positive control, the E. coli ΔacrB-ΔtolC was more sensitive than E. coli TG1 to Cm.

Separation of H. sabdariffa extract on TLC showed similar profile to that been reported by Sarr et al., which was interpreted as following; blue zone: Phenolic acids, yellow-orange: Flavonols, and yellow-green: Flavones [32]. TLC-bioassay plate overlaid with bacterial medium agar suspension only one inhibition zone, corresponding to phenolic acids, was formed which is related to the antimicrobial compound. While on plate overlaid with bacterial medium agar suspension supplemented with a sub-lethal concentration of CV resulted in formation of two inhibition zones, one corresponding to flavones and the other corresponding to phenolic acids. These results indicate that the inhibition zone corresponding to flavones might be related to the fraction of potential EPI.

To confirm the ability of the flavones fraction to enhance the activity of AcrAB-TolC, the flavones fraction has been eluted from the silica gel, and antagonistic assay was conducted against E. coli TG1 and its ΔacrB-ΔtolC mutant on LB medium supplemented with a sub-lethal concentration of CV. The result showed that the flavones eluted fraction formed inhibition zone on plate inoculated with the E. coli TG1 but not on plate inoculated with the mutants [Figure 1].

Determination of MIC values of different antimicrobial compounds alone and in a combination of EPI fraction was used to examine the susceptibility of E. coli strains, and E. amylovora strains in MHB medium [Tables 2 and 3]. In E. coli TG1, the synergic effect between the antimicrobials and EPI fraction decreased MIC values by four-fold for bile salt, Ap, nalidixic acid, and CV, five-fold for acriflavine and tetracycline, eight-fold for phloretin, berberine, ciprofloxacin, and SDS, and 10-fold for novobiocin and EtBr.

These results revealed that EPI fraction has an inhibitory effect on AcrAB-TolC efflux system in both tested organisms. A comparison between the MIC values for antimicrobials alone and the synergetic effect with EPI fraction shows that there was no significant influence for the combination of antimicrobials and EPI fraction on the MIC values of ΔacrB and ΔtolC single mutant nor ΔacrB-ΔtolC double mutant in both tested organisms. It is remarkable to mention that there were no significant differences between the complemented mutants and the mother cells of E. coli TG1 and E. amylovora 1189 in MIC values for both cases antimicrobials alone and combination of antimicrobials with EPI fraction [Tables 2 and 3].

In addition to the EPI fraction, in a dose-dependent, increases the relative fluorescence intensity, which indicates the increase in EtBr intercellular accumulation in E. coli and E. amylovora cells [Figure 2].

### DISCUSSION

Formation of inhibition zone on plate inoculated with E. coli ΔacrB-ΔtolC indicates accumulation of antimicrobial compound in the mutant cells which are most likely transported by AcrAB-TolC efflux system. The antimicrobial activity of H. sabdariffa has been reported against foodborne and food spoilage microorganisms, like E. coli O157:H7, is a major foodborne pathogen [23,37], also against Gram-negative bacteria such as Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Salmonella enterica and some multidrug resistant Salmonella strains, in addition to some Gram-positive bacteria such as Bacillus subtilis, S. aureus, Staphylococcus epidermis, and Staphylococcus cereus [24,25,37,38].

Although the AcrAB-TolC efflux system in E. coli TG1 was recruited in efflux of the CV, the methanol extract of H. sabdariffa was able to inhibit E. coli TG1 growth more than the E. coli ΔacrB-ΔtolC mutant, which might indicate the occurrence of another microbial compound in the extract, or occurrence of a certain compound that increases accumulation of the antimicrobial compound, that is found in the methanol extract of H. sabdariffa, in E. coli TG1 cells due to blocking of the AcrAB-TolC efflux system. Formation of inhibition zone by the eluted fraction on agar plate inoculated with the E. coli TG1 but not on plate inoculated with the mutants demonstrates that the eluted fraction has no antimicrobial activity alone, but it enhances the accumulation of CV in E. coli TG1. These observations fulfill the main characteristics of EPI suggested by Lomovskaya et al. [39], where EPI fraction enhances activities of CV in E. coli TG1 that containing functioning pump, and does not potentiate the activities of CV in ΔacrB-ΔtolC mutants that lack efflux pump.

There was no significant influence for the combination of antimicrobials and EPI fraction on the MIC values of ΔacrB

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**Table 4: Susceptibility of E. coli strains to H. sabdariffa methanol extract**

| Bacterial strains | H. sabdariffa | Cm | 80% methanol |
|------------------|--------------|----|--------------|
| E. coli TG1      | 10.9±0.5*    | 12.3±0.5* | 0.0          |
| E. coli ΔacrB-ΔtolC | 8.3±0.5     | 16.3±0.5* | 0.0          |

Diameter of measured inhibition zones resulted from 10 µl of ×10 H. sabdariffa methanol extracts loaded in 5 mm in diameters well, 10 µl of 12.5 mg/ml of Cm and 10 µl of 80% methanol were used as a positive and negative control, respectively. Assay was repeated 3 times, and the average of three replicates was recorded ± standard errors of means. Similar experiments were conducted with E. amylovora 1189 and its ΔacrB-ΔtolC, (data not shown), *Analysis of ANOVA and Fisher’s least significant differences at P=0.05 with a significant value of 2.0 have been conducted by IBM SPSS Statistics 24.. Cm: Chloramphenicol, H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli.
and ΔtolC single mutant nor ΔacrB-ΔtolC double mutant in both tested organisms. No significant differences between the complemented mutants and the mother cells of *E. coli* TG1 and *E. amylovora* 1189 were observed in MIC values for both cases; antimicrobials alone and combination of antimicrobials with EPI fraction. These results are suggesting that both proteins AcrB and TolC should be assembled in both tested organisms to enable the EPI fraction to accomplish its activity. These results might explain former results of Darwish and Aburjai [26], where combinations of *H. sabdariffa* extract with nalidixic acid reduced the growth percentage of *E. coli* by 20%, combinations of *H. sabdariffa* extract with nalidixic acid, and tetracycline reduced the growth percentage of *P. aeruginosa* by 17% and 55%, respectively [40], which might be related to inhibition of MexAB-OprM, AcrAB-TolC homolog, and *P. aeruginosa* [9].

Increase the EtBr intercellular accumulation in *E. coli* and *E. amylovora* cells fulfill another characteristic of EPI suggested by Lomovskaya *et al.* [39], where EPI fraction increased the level of accumulation and decreased the level of extrusion of efflux pump specific substrate. These observations suggest that EPI faction may act as multidrug EPI primarily through inhibition of AcrAB-TolC.

It can be concluded that the eluted fraction act as an EPI, it triggered the activity of a wide range of antimicrobial

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**Figure 1:** Antagonistic assay against *Escherichia coli* TG1 by the putative efflux pump inhibitor (EPI) (eluted fraction); (a) *E. coli* TG1, (b) *E. coli* its ΔacrB-ΔtolC. Bacterial suspension (100 µl of OD₆₀₀ ~1.0) was spread on Luria-Bertani medium, after drying, 10 µl of 25 µg/ml putative EPI fraction was loaded in 5 mm in diameters well (right wells), 10 µl of 12.5 mg/ml of chloramphenicol was used as positive control (upper wells), and 10 µl of 80% methanol was used as negative control (left wells). The arrow in part A shows the inhibition zone formed by the putative EPI fraction. Similar experiments were conducted with *Erwinia amylovora* 1189 and its ΔacrB-ΔtolC (data not shown).
compounds and reduced the MIC values, and increase accumulation of EtBr in the tested organisms cells. Further investigation will be putative to determine the chemical structure of the putative EPI.

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