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Original Research

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.

KEY WORDS: AcrAB-TolC, efflux pump inhibitor, *Hibiscus sabdariffa*

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'-methoxyhydnocarpin, *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-sambubioside, cyanidin 3-sambubioside, cyanidin 3-glucoside, and 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 Δ*acrB*-Δ*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 Δ*acrB*-Δ*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].

Table 1: Bacterial strains used in this study

Strain	Relevant characteristics	Source
E. coli		
TG1	*subE hsd*Δ5 *thi* Δ(*lac-proAB*) F`(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	[29]
KAM3 (Δ*acrB*)	*acrB* mutant of TG1	[30]
TG1-1 (Δ*tolC*)	Gm^r, *tolC* mutant of TG1	[5]
KAM3-1 (Δ*acrB*- Δ*tolC*)	Gm^r, *tolC* mutant of KAM3	[5]
KAM3-1 (pBBR-Ec *tolC*)	Cm^r, complemented *acrB* mutant carrying pNK18	[5]
TG1-1-1 (pBBR-Ec *acrAB*)	Gm^r, Cm^r complemented *tolC* mutant carrying pNK17	[5]
E. amylovora		
1189	Ap^r, wild type	GSPB[a]
1189-3 (Δ*acrB*)	Km^r, *acrB* mutant carrying Kmr cassette in the *acrB* gene	[6]
1189-25 (Δ*tolC*)	Gm^r, *tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene	[7]
1189-3-3 (Δ*acrB*-Δ*tolC*)	Km^r, Gm^r, *acrB/tolC* mutant carrying GFP-Gm^r cassette in the *tolC* gene and Km^r cassette in *acrB* gene	[7]
1189-25-1 (pBBR-Ea *tolC*)	Gm^r, Cm^r, complemented *tolC* mutant carrying pNK7	[7]
1189-3-1 (pBBR-Ea *acrAB*)	Km^r, Cm^r, complemented *acrB* mutant carrying pNK8	[7]

[a]GSPB: Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany, *E. coli: Escherichia coli, E. amylovora: Erwinia amylovora,* Ap: Ampicillin, Cm: Chloramphenicol, Gm: Gentamycin, Km: Kanamycin

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 Δ*acrB*-Δ*tolC*.

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

The MIC of different antimicrobial 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 synergetic 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].

Intercellular Accumulation of Ethidium Bromide (EtBr)

E. coli TG1 and its Δ*acrB*-Δ*tolC* mutant, *E. amylovora* and its Δ*acrB*-Δ*tolC* 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_{600} 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_{600} 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.

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

Compounds	MIC[a] (μg/ml)					
	TG1	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	ΔtolC (*tolC*)
	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction
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±125
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±50	500±62.5	500±62.5
Ampicillin	15.62±3.13	1.57±0.78	1.56±1.56	1.56±1.56	15.62±1.56	15.62±3.13
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±50	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

[a]MIC determination in MHB medium by the dilution assay was repeated 3 times in each case thereby confirming consistencies of MIC values. Differences in MIC values were only considered significant if they were at least four-fold. SDS: Sodium dodecyl sulfate, *H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli*, MIC: Minimum inhibitory concentration, MHB: Mueller-Hinton broth, EPI: Efflux pump inhibitor

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

Compounds	MIC[a] (μg/ml)					
	1189	Δ*acrB*	Δ*tolC*	Δ*acrB*/Δ*tolC*	Δ*acrB* (*acrAB*)	Δ*tolC* (*tolC*)
	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction	No EPI±EPI fraction
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±62.5
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	6.25±6.25	62.5±6.25	31.2±12.5
Ampicillin	62.5±15.63	6.25±6.25	6.25±6.25	12.5±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

[a]MIC determination in MHB medium by the dilution assay was repeated 3 times in each case thereby confirming consistencies of MIC values. Differences in MIC values were only considered significant if they were at least four-fold, *H. sabdariffa: Hibiscus sabdariffa, E. coli: Escherichia coli*, MIC: Minimum inhibitory concentration, SDS: Sodium dodecyl sulfate, MHB: Mueller-Hinton broth, EPI: Efflux pump inhibitor

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

Inhibition zones (mm in diameter)			
Bacterial strains	*H. sabdariffa*	Cm	80% methanol
E. coli TG1	10.9±0.5*	12.3±0.5[b]	0.0
E. coli ΔacrB-ΔtolC	8.3±0.5	16.3±0.5[a]	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*

RESULTS

Inhibition zones have been formed on plates inoculated separately with *E. coli* TG1 and *E. coli* Δ*acrB*-Δ*tolC* 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* Δ*acrB*-Δ*tolC* mutant (~8 mm in diameter). In contrary, to the used antibiotic as a positive control, the *E. coli* Δ*acrB*-Δ*tolC* 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 Δ*acrB*-Δ*tolC* 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 synergetic effect between the antimicrobials and EPI fraction decreased MIC values by four-fold for bile salt and nalidixic acid, five-fold for Ap, acriflavine and ciprofloxacin, and eight-fold for other antimicrobials; phloretin, berberine, novobiocin, tetracycline, SDS, EtBr, and CV. While in *E. amylovora* 1189 the synergetic 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 Δ*acrB* and Δ*tolC* single mutant nor Δ*acrB*-Δ*tolC* 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* Δ*acrB*-Δ*tolC* 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* Δ*acrB*-Δ*tolC* 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 Δ*acrB*-Δ*tolC* mutants that lack efflux pump.

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

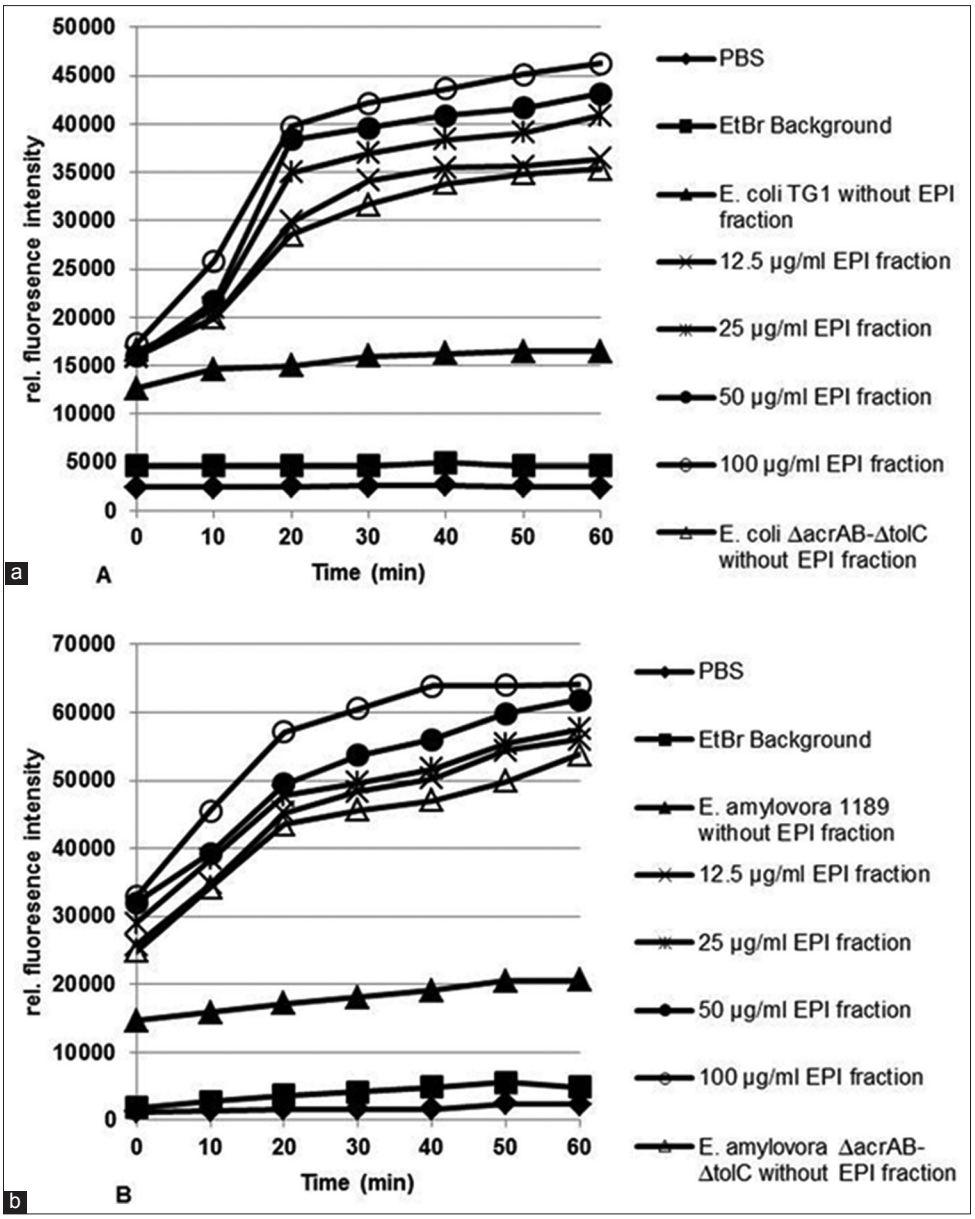

Figure 1: Antagonistic assay against *Escherichia coli* TG1 by the putative efflux pump inhibitor (EPI) (eluted fraction); (a) *E. coli* TG1, (b) *E. coli* its Δ*acrB*-Δ*tolC*. Bacterial suspension (100 µl of OD_{600} ~1.0) was spread on Luria-Bertani medium, after drying, 10 µl of 25 µg/ml putative 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 Δ*acrB*-Δ*tolC* (data not shown)

and Δ*tolC* single mutant nor Δ*acrB*-Δ*tolC* 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

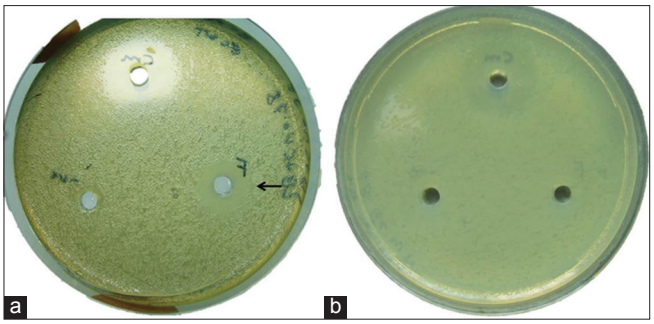

Figure 2: Effect of addition increasing concentrations of efflux pump inhibitor (EPI) fraction on ethidium bromide (EtBr) accumulation in *Escherichia coli* TG1 (a); effect of addition of increasing concentrations of EPI fraction on EtBr accumulation in *Erwinia amylovora* 1189 (b). Cells grown in Luria-Bertani until reaches to OD_{600} 1, centrifuged at 4000 rpm for 30 min, re-suspended in PBS supplemented with 0.4% glucose, and EPI fraction was added at increasing concentrations. EtBr was added (1.0 μg/ml) at time point 0, and fluorescence was measured with 10 min interval for 1 h by fluorospectrometer. *E. coli* Δ*acrAB*-Δ*tolC* and *E. amylovora* Δ*acrAB*-Δ*tolC* were used for comparison

compounds and reduced the MIC values, and increase accumulation of EtBr in the tested organisms cells. Further investigation will be conducted to determine the chemical structure of the putative EPI.

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