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Research Article

Evaluation of silibinin as an efflux pump inhibitor in *Bacillus subtilis*

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Abstract: Antibiotic resistance has become a global health problem for humankind. Improper use of antibiotics resulted in the increasing evolved bacterial resistance to them. There are different types of bacterial resistance mechanisms including efflux pumps. To overcome the efflux pump activity on the drugs, combinatorial therapy of the existing antimicrobials with natural products is a promising insight to prevent increasing multidrug resistance. In this study, the inhibitory action of a plant-derived molecule silibinin on efflux pumps of *Bacillus subtilis* was investigated. The cellular effect of silibinin was investigated using minimum inhibitory concentration and growth studies. In addition, the efflux pump action of silibinin was monitored by ethidium bromide accumulation assay on the organism. According to results, silibinin has a MIC value between 100-200 μgmL^{-1} on microplate assay and 100 μgmL^{-1} of silibinin inhibited the cell growth. Ethidium bromide accumulation assays were performed at a safe silibinin range (25 and 50 μgmL^{-1}) for eliminating the cell death, and ethidium bromide accumulation was increased with the increasing silibinin concentration. Ethidium bromide accumulation and growth results proved that silibinin has significant efflux pump inhibitor activity on *Bacillus subtilis* cells and silibinin is a promising inhibitor candidate to eliminate bacterial resistance mechanism.

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1. INTRODUCTION

Increased pressure imposed by improper and reckless use of antimicrobial agents has triggered the pace in development and transmission of bacterial resistance (Schwarz and Chaslus-Dancla, 2001). The non-susceptibility developed by bacteria to different classes of antimicrobials has led to the emergence of multidrug resistance (MDR) which is one of the most important global health threats (Magiorakos *et al.*, 2012). The basic types of resistance mechanisms are known as enzymatic inactivation of the antimicrobials, modification of the target sites, reducing the intracellular accumulation of antimicrobials by arranging influx/efflux mechanisms (Van Duijkeren *et al.*, 2018). Among these, efflux systems are considered as the major mechanisms

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that lead to MDR. These systems which are commonly comprised of transmembrane proteins enable the organisms to keep the concentrations of a wide range of different compounds at non-toxic levels by preventing their intracellular accumulation.

Efflux pumps are classified into six families based on their structure. These are (i) major facilitator superfamily (MFS), (ii) multidrug and toxic compound extrusion (MATE) family, (iii) small multidrug resistance (SMR) family, (iv) resistance-nodulation-cell division (RND) family, (v) ATP-binding cassette (ABC) superfamily, and (vi) proteobacterial antimicrobial compound efflux (PACE) superfamily. The latest one has been recently identified therefore its transport mechanism has not been clarified yet. Among the rest, only the ABC-type utilizes ATP while the other four families use proton motive force (PMF) for efflux (Du *et al.*, 2015; Lamut *et al.*, 2019).

The loss in available drug efficacies and the decrease in new antimicrobial discovery rates have increased the search on alternative strategies including combinatorial therapies. Combinatorial therapies of existing antimicrobials with natural products emerge as attractive approaches in the fight with increasing MDR. To this end, utilization of efflux pump inhibitors (EPIs) to interfere with efflux is one of the major strategies. Plants are immense sources of natural compounds, some of which are potential EPIs, so they are of utmost importance in the discovery of new antimicrobial agents. Isolation and identification of new EPIs will bring antimicrobials with lost efficacies back into the clinic. To date, many plant-based EPIs have been identified as reserpine (Gibbons *et al.*, 2003; Neyfakh *et al.*, 1991), piperine (Kumar *et al.*, 2008), roemerine (Avci *et al.*, 2019), baicalein (Chan *et al.*, 2011), 5'-methoxy-hydnocarpin (Stermitz *et al.*, 2000), and catechin gallates (Gibbons *et al.*, 2004).

Silibinin (or silybin) is the major component of silymarin extract from the seeds of *Silybum marianum* (or milk thistle, Asteraceae) and a member of flavonolignans. (Dobiasová *et al.*, 2020). It receives wide attention due to its anticancer, antioxidant, antibacterial, antifungal, anti-inflammatory, cardioprotective, neuroprotective, and hepatoprotective activities (de Oliveira *et al.*, 2015; Shen *et al.*, 2018; Wlcek *et al.*, 2013). More recently, silibinin has also been associated with an EPI property as it inhibited the efflux of norfloxacin through the NorA efflux pump protein in *Staphylococcus aureus* (Mahmood *et al.*, 2016).

In the present study, the ability of silibinin to inhibit the efflux pumps of *Bacillus subtilis* was evaluated using minimum inhibitory concentration determination, growth studies, and ethidium bromide accumulation assay. Berberine was used in growth studies since it is the substrate of many efflux pumps and its activity is weakened due to the activity of efflux pumps (Avci *et al.*, 2019). The clinical importance of *B. subtilis* is limited, it constitutes one of the model organisms for low G+C Gram-positives with a significant genomic abundance of multidrug transporters. *B. subtilis* is also the first microorganism in which the bacterial MDR phenomenon was discovered. Furthermore, it possesses a mechanism analogous to the mammalian multidrug transporter, P-glycoprotein (Lorca *et al.*, 2007; Neyfakh *et al.*, 1991)

2. MATERIAL and METHODS

2.1. Bacterial Strains and Chemicals

The efflux pump inhibitor (EPI) property of silibinin was tested in wild-type *Bacillus subtilis* 168 (DSM 402). Silibinin (CAS No. 22888-70-6) and berberine chloride hydrate (CAS No. 141433-60-5) were obtained from Sigma-Aldrich. Silibinin and berberine solutions were prepared in dimethyl sulfoxide (DMSO) (Duchefa, Netherlands).

2.2. Minimum Inhibitory Concentration (MIC) Determination for Silibinin

Minimum inhibitory concentration (MIC) of silibinin was determined via broth micro-dilution assay (Amsterdam, 1997). Two-fold serial dilutions of silibinin from 200 μgmL^{-1} to 0.097

μgmL^{-1} were prepared with Nutrient Broth (NB, Merck, Germany) in sterile 96-well U-bottom plates and a single line of the test plate was prepared with serial dilutions of the solvent DMSO as control. Each well was inoculated with 10^5 $CFUmL^{-1}$ cells. After a 24-hour incubation at 37°C, 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, Germany) dye reduction test was utilized to determine the wells with no visible cell growth. TTC solution at a final concentration of 0.5% (w/v) was added into each well. Plates were incubated at 37ºC for 1 hour. MIC was determined based on the color change in wells. Viable cells were recognized with the development of red color in the wells.

2.3. Growth Conditions

B. subtilis 168 cells were grown in NB medium at 37°C and 180 rpm and treated with silibinin, berberine, or silibinin-berberine combination as OD_{600} reached 0.45-0.55. Control cells were only treated with equal volume of DMSO. The growth of the cells was monitorized spectrophotometrically by measuring OD_{600} values in 1-hour intervals.

2.4. Ethidium Bromide Accumulation Assay

A modified version of the previously reported method was used to perform ethidium bromide (EtBr) accumulation assay (Jin *et al.*, 2011; Steinfels *et al.*, 2004).

After overnight growth, *B. subtilis* 168 cells were inoculated into a tube containing 5 mL fresh NB and grown at 37ºC and 180 rpm until OD_{600} reached 0.5. Cells were then centrifuged for 4 min at 2,000 *g* and 4ºC. Cell pellets were suspended in 2 mL of 0.35 M sodium chloride (NaCl). 180 µL of the cell suspension was mixed with 50 mM KP_i, 5 mM magnesium sulfate ($MgSO_4$), and 25 mM glucose. 10 µM of EtBr (Invitrogen, California, USA) was added to the mixture immediately after the addition of glucose. Silibinin treatment (25 and 50 μgmL^{-1}) was made prior to the addition of glucose. Control samples were supplied with an equal volume of DMSO. Fluorescence intensities were monitored for 20 min via Synergy HTX Multi-Mode Reader Reader (BioTek Instruments, Inc., Winooski, VT, USA) with excitation at 540 nm and emission at 590 nm (Serçinoğlu *et al.*, 2020).

3. RESULTS and DISCUSSION

Antibiotic resistance development against available antimicrobials has reached alarming rates which causes a bottleneck during the fight against bacterial infections. Since the novel antibiotics with different targets are limited, the discovery of new antimicrobial classes has a high priority. Plant-derived molecules may be considered as potential alternatives to existing drugs with their various biological activities and multi-target properties. In spite of that, the limited information about their mechanisms restricts their use.

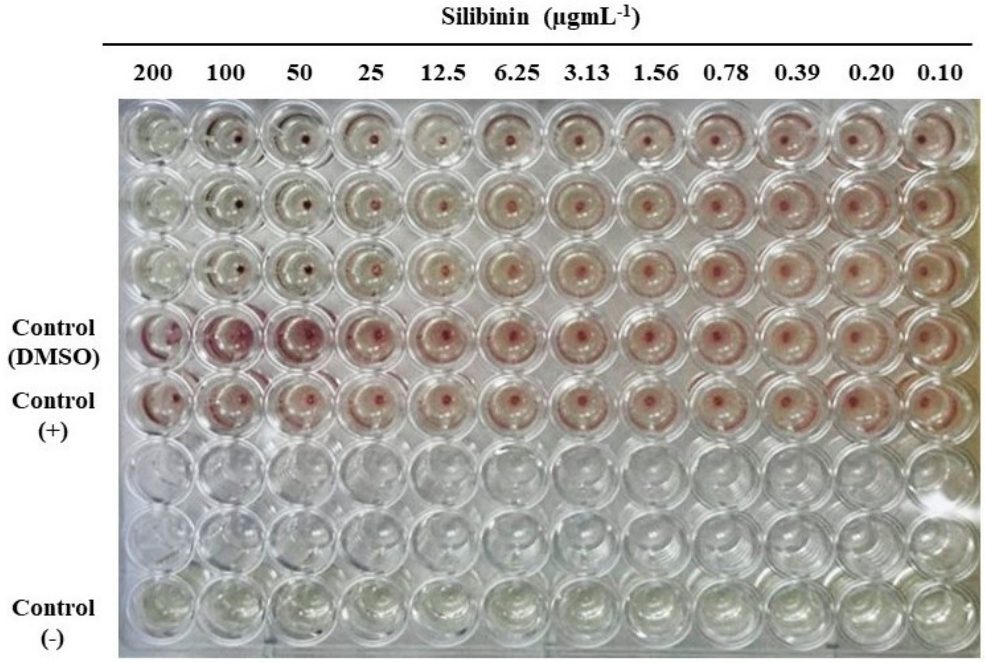
Silibinin has been reported to display diverse biological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, free radical scavenging, and membrane stabilizing properties under *in vitro* and *in vivo* conditions (Cai *et al.*, 2017; de Oliveira *et al.*, 2015). It has also been reported that silibinin inhibits P-glycoprotein-mediated cellular efflux (Zhou *et al.*, 2004) and is involved in bacterial resistance through drug efflux (Wang *et al.*, 2018). In the light of these recent findings, EPI property of silibinin was evaluated on the Gram-positive model organism *B. subtilis* 168. Following the analysis of cellular growth in the presence of silibinin alone and in a combinatorial treatment, the contribution of silibinin to intracellular EtBr accumulation was monitored.

3.1. Berberine and Silibinin Combinatorial Treatments

Broth micro-dilution assay was carried out to find that the MIC of silibinin lied between 100-200 μgmL^{-1} (Figure 1). In order to study the EPI property of silibinin, a working concentration

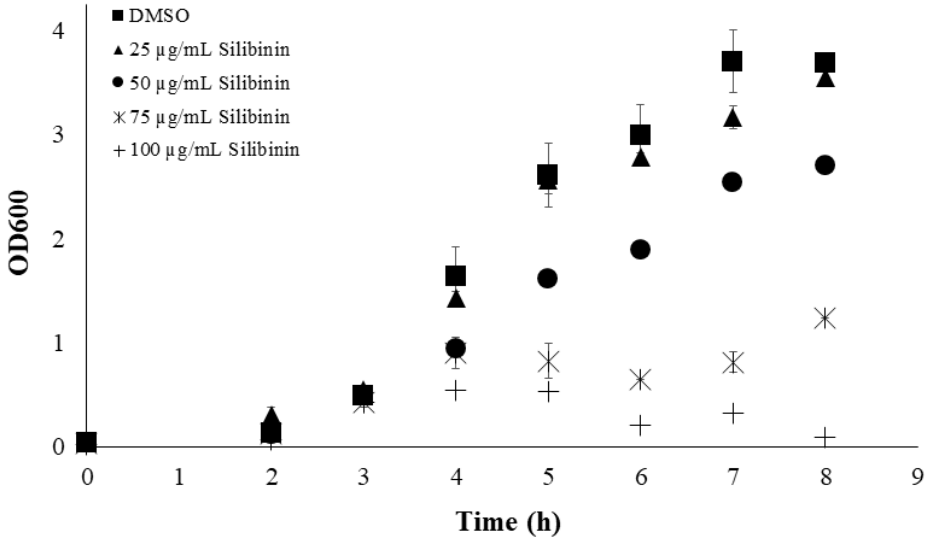
that is well below its MIC value was selected. Thus, microbial growth studies were carried out in the presence of increasing silibinin concentrations from 25 to 100 µgmL^{-1} (Figure 2).

Figure 1. Minimum inhibitory concentration (MIC) determination for silibinin.



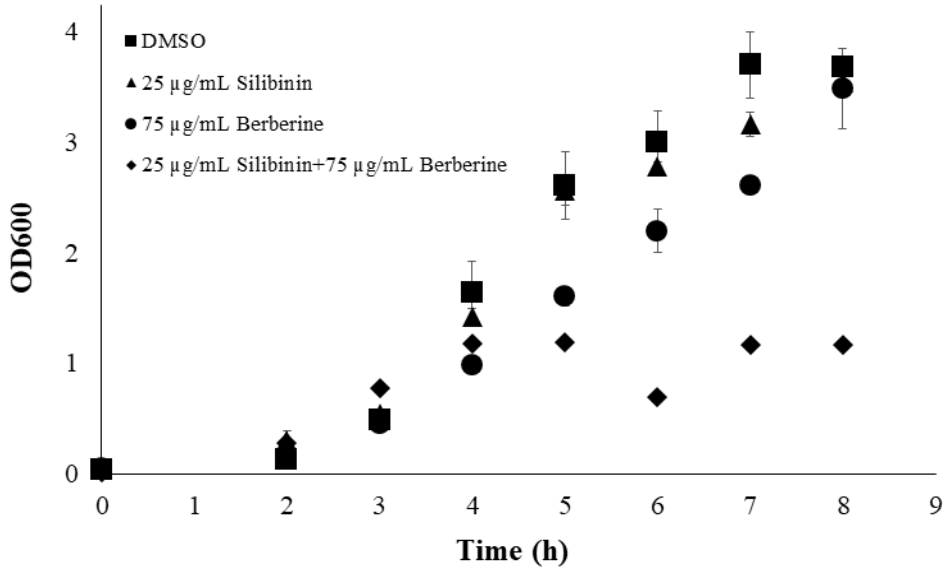
Treatment of the cells with 25 µgmL^{-1} silibinin barely altered the growth profile of *B. subtilis* 168 cells whereas treatment with 50 µgmL^{-1} silibinin led to a slight retardation in growth. When silibinin concentration was raised to 75 µgmL^{-1}, growth was severely affected. With 100 µgmL^{-1} there was no growth at all. Growth curves obtained with increasing silibinin concentrations have shown that 25 µgmL^{-1} silibinin is suitable for testing its EPI feature since it did not alter growth.

Figure 2. Microbial growth under increasing silibinin concentrations.



The antimicrobial berberine is widely known to be a substrate of a number of drug efflux pumps therefore its combination with different natural pump inhibitors may offer a way to enhance its efficacy (Avci *et al.*, 2019; Stermitz *et al.*, 2000). In order to evaluate the EPI property of silibinin, its combination with berberine has been tested on *B. subtilis* cells. Berberine working concentration was determined based on previous work (Avci *et al.*, 2019). In our previous work, 75 μgmL^{-1} berberine has been shown to only slightly affect *B. subtilis* 168 growth. Thus, 25 μgmL^{-1} silibinin was combined with 75 μgmL^{-1} berberine (Figure 3). Although either 25 μgmL^{-1} silibinin or 75 μgmL^{-1} berberine has no significant effect on cell growth alone, their combination killed the cells. Since plant-derived natural products are known to have multiple targets to cause death, EtBr accumulation test was further carried out to verify that silibinin's EPI property was responsible for the observed behavior.

Figure 3. Effect of the berberine and silibinin combination on microbial growth.



3.2. Effect of Silibinin on Efflux Pumps of *B. subtilis* 168

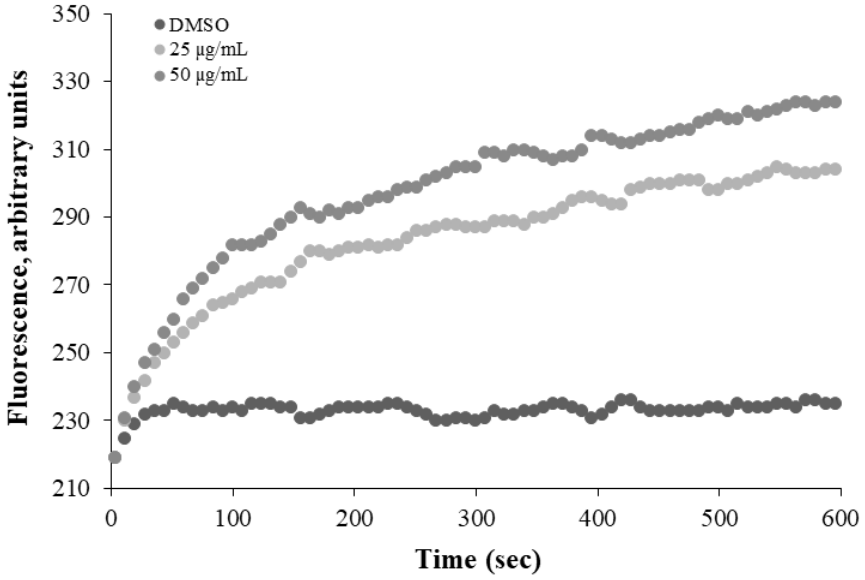
EtBr accumulation is a commonly used assay to monitor the activity of efflux pumps. EtBr passes the cell membrane and binds by intercalating between DNA base pairs. Therefore, its intracellular presence can be detected by fluorometry (540 nm as excitation and 590 nm as emission) (Steinfels *et al.*, 2004). However, free EtBr is also continuously effluxed through efflux pumps. Thus, in the presence of an EPI, the increase in fluorescence would be expected to be higher.

To this end, EtBr accumulation in *B. subtilis* 168 cells in the presence of silibinin was investigated to assess its EPI potential. The selected concentrations were believed to facilitate EtBr accumulation, while not causing cell death. Based on the growth results obtained in the presence of increasing concentrations of silibinin (Figure 2), this assay was carried out with 25 and 50 μgmL^{-1} silibinin.

In the control cell sample, which was treated with DMSO (the solvent of silibinin), fluorescence intensity was pretty constant after approximately 50 s. This showed that EtBr was inside the cells but with the action of the pumps, its concentration remained at its steady state value. The increase in fluorescence intensity due to EtBr accumulation was highest in the presence of 50 μgmL^{-1} silibinin. Since this concentration had a considerable effect on cell growth (Figure 2), this observed behavior in fluorescence could as well be a result of released genetic material due to cell death. When silibinin concentration was dropped to 25 μgmL^{-1}, the

recorded fluorescence was only slightly affected which demonstrated that silibinin displayed an EPI character (Figure 4).

Figure 4. EtBr accumulation in *B. subtilis* in the presence of silibinin.



Due to the significant genomic abundance compared to other Gram-positives, *B. subtilis* transporters have been extensively studied (Lorca *et al.*, 2007; Neyfakh *et al.*, 1991). Blt and Bmr (Ahmed *et al.*, 1995; Baranova *et al.*, 1999; Woolridge *et al.*, 1997) of MFS, YerP (Tsuge *et al.*, 2001) of RND superfamily, EbrAB (Masaoka *et al.*, 2000) of SMR family, and BmrA (Steinfels *et al.*, 2004) of ABC superfamily are some of the well-characterized efflux pumps in *B. subtilis*. Among these pumps, EtBr has been reported to be a substrate for BmrA, Blt, Bmr, and EbrAB (Ahmed *et al.*, 1995; Masaoka *et al.*, 2000; Neyfakh *et al.*, 1991; Steinfels *et al.*, 2004). The results strongly suggest that the silibinin binds and inhibits the efflux pump(s) of *B. subtilis* 168 so that EtBr uptake rate is higher than its efflux rate in the presence of silibinin. This leads to continuous EtBr accumulation in the cells.

Silibinin has been reported to inhibit the efflux through the mammalian P-glycoprotein (Zhou *et al.*, 2004) and since BmrA of *B. subtilis* cells is a homologue of P-glycoprotein (Steinfels *et al.*, 2004), it could be proposed that the ABC transporter BmrA could be a target of silibinin. However, plant derived molecules are commonly known as multi-target molecules: thus, silibinin could be binding the other pumps in *B. subtilis* for which EtBr is a substrate.

4. CONCLUSION

Within the scope of this study, silibinin was assessed as a candidate for inhibiting efflux mechanisms in *B. subtilis* 168 cells. Here, it demonstrated a significant efflux pump inhibitor activity in the EtBr accumulation test and enhanced the activity of berberine. Because of the multi-target properties of the plant-derived molecules, it was not possible to determine the exact target of the silibinin. Further studies that will involve the purified efflux pump proteins are necessary. Although *B. subtilis* 168 is not a pathogenic microorganism, it is a well-known model organism for low G+C Gram-positives including many pathogenic bacteria, which means that silibinin could have comparable effects on these pathogenic bacteria. For future work, the synergistic antimicrobial effect of silibinin with available drugs or antibiotics could be assessed to overcome the bacterial defense mechanisms.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fatma Ece Altinisik Kaya: Design of the study, performing the experiments, writing. **Basak Atas**: Design of the study, performing the experiments, writing. **Fatma Gizem Avci:** Design of the study, writing, editing, validation. Fatma Ece Altinisik Kaya and Basak Atas contributed equally to this work.

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