Developing resistance to several antibiotics has a crucial importance. For this reason, researchers are trying to discover alternative methods or agents. More recent studies focused on the potential antimicrobial properties of hypericin. In this study, antibacterial activity of hypericin was tested against *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis ATCC* 19433, *Escherichia coli ATCC* 25922, *Staphylococcus aureus ATCC* 29213, *Staphylococcus epidermidis ATCC* 12228, and *Klebsiella pneumoniae ATCC* 700603 by broth macro-dilution method. The study was designed in two different conditions: exposure to daylight and non-exposure to daylight (in the dark). 64 mg/mL concentration of hypericin inhibited the growth of *S. aureus ATCC* 29213 and *S. epidermidis ATCC 12228*, without light. Conversely, there was no inhibition of hypericin in the range of concentration investigated for other bacteria. Positive or negative effect of daylight has not been observed. Perhaps, the usage of a different wavelength of light or the investigation of antibacterial activity at higher concentrations of hypericin may lead to different results. From our study, hypericin causes inhibition of growth of some bacteria through mechanisms different from photo-oxidation. Mechanisms between hypericin and bacterial enzymes, receptors and wall components should be investigated in further studies.

**Key words:** Hypericin, *Hypericum perforatum* L, broth macrodilution method, antibacterial activity.

**INTRODUCTION**

The development of antibiotics drugs has played a key role in reducing morbidity and mortality rates of many infectious diseases. However, developing resistance to antibiotics of several bacteria has produced a situation. Thus, antimicrobial agents have lost their effectiveness. As a result, persistence of drug-resistant organisms occurred. Therefore, more and more antibiotics with increased therapeutic and prophylactic action will need to be developed (WHO, 1983).

For this reason, researchers are in constant search for alternative methods or agents. So, researchers are interested in several folk remedy or ethnotropical methods as a source of inspiration. In relation to this, there are a lot of researches on *Hypericum perforatum* L (Hypericaceae) commonly known as St. John's Wort. These studies focused on antidepressant and antimicrobial effects of the plant (Saddiqe et al., 2010). *H. perforatum* L contains major product groups such as: phenylpropanes, flavonol derivatives, bi flavones, proanthocyanidins, xanthones, phloroglucinols, some amino acids, naphthodianthrones and essential oil (Nahrstedt and Butterweck, 1997). To date, studies conducted on antibacterial-activity of *H. perforatum* L focused on antibacterial creams, ointments and plant extracts. However, some constituents of *H. perforatum* such as hyperforin, proanthocyanidins and xanthones were investigated as pure molecule (Reichling et al., 2001; Nahrstedt and Butterweck, 1997). More recent studies focused on the potential antimicrobial properties of hyperforin and hypericin (Avato et al., 2004).

Hypericin is typical of the genus, Hypericum. It has an intense red color and phototoxic properties. It is insoluble
in water at ambient temperature (Durán and Song, 1986). There are many studies showing anti-depressant activity of this molecule as a monoamine oxidase inhibitor (Lawvere and Mahoney, 2005). Hypericins have been also explored for a variety of therapeutic (anticancer and antiviral) and diagnostic applications (fluorescence detection of bladder cancer) (Diwu, 1995). A study of “photodynamic therapy (PDT) and cancer” suggested that blocking such cell redox systems could enhance the efficacy of hypericin photodynamic therapy (Karioti, 2010). Many studies tried various antibacterial preparations of *H. perforatum* L; however, there are questions to be answered about antibacterial activity of hypericin (Reichling et al., 2001).

In this study, antibacterial efficacy of hypericin was investigated by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values via the broth macro-dilution method in a group of pathogenic bacteria.

**MATERIALS AND METHODS**

In this study, antibacterial activity of the hypericin was tested with *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Klebsiella pneumoniae* ATCC 700603 by broth macro-dilution method according to the Clinical and Laboratory Standards Institute (CLSI) performance and interpretive guidelines (CLSI, 2003). These bacteria were preferred, because they are important infectious pathogens. The strains were supplied by Laboratory of Clinical Microbiology Department of Meram Medical School. Hypericin was supplied commercially (56690 Sigma, St. Louis).

**Preparation of microbial inocula**

The inocula of bacteria were prepared in 24 h at Cation-Adjusted Mueller-Hinton Broth (CAMHB) (Becton, Dickinson and Company, USA). The absorbance was read at 600 nm and adjusted with sterile physiological solution to match the value of 0.5 McFarland standard solution (10^5 colony forming units). The solutions were prepared to give a final concentration of 5 × 10^5 colony forming units (CFU) per milliliter for bacteria.

**Preparation of hypericin**

Hypericin stock solution was prepared by dissolving Hypericin powder (56690 Sigma, St. Louis) in Dimethyl sulfoxide (DMSO) (Karioti, 2010). The concentration was adjusted to 512 µg / mL. The stock solution was stored in the dark before usage.

**The broth macrodilution test**

The study was designed in two different conditions: exposure to daylight and non-exposure to light. The same procedures were performed for both of them. First group tubes were exposed to light of 18 Watt capacity (550 to 600 nm) bulb with a distance of 30 cm. We intended to evaluate normal daylight conditions. The second group tubes were preserved from light by covering with foil. 10 series tubes were used to test for each bacteria. 0.5 ml medium (CAMHB) was added to all tubes except the first tube in the series. 0.5 ml hypericin stock solution (512 µg / mL) was added to the first and second tubes and serial two-fold dilutions were performed for two to nine tubes (0.5 ml from each test tube was transferred into next tube filled). 0.5 mL 10^5 CFU bacteria solution was added in two to 10 tubes. Thus, concentrations of hypericin reached respectively 128, 64, 32, 16, 8, 4, 2 and 1 µg / mL in two to nine tubes. The first tube was used as a negative control and the last tube was used as a positive control in this process. All the tubes in both conditions were incubated at 35°C for 24 h. Protective foil was removed immediately before the assessment was made. Then, the tubes were evaluated for MIC/MBC of hypericin. The antibacterial activity was defined after 24 h determination of the lowest concentration of hypericin that evidenced a complete inhibition of visible growth in the broth culture (MIC). The minimum bactericidal concentration (MBC) of hypericin is the lowest concentration in µg/mL that results in more than 99.9% killing of the bacteria being tested. To determine the MBC, cultivation was made to plaques from tubes which have not got a visible growth.

**RESULTS**

A total of 120 tubes were evaluated for six bacteria species. Growth was evaluated based on turbidity of tubes. Growth was not observed in all negative control tubes; while growth was determined in all positive control tubes. In addition, cultivation was made from all turbid tubes to plates, because of detection of contamination. MIC value was defined by the lowest concentration of hypericin that evidenced a complete inhibition of visible growth in the broth culture. Hypericin MIC value could be observed for only *S. aureus ATCC 29213* and *S. epidermidis ATCC 12228*. The visible inhibition was determined in the third tube as a result of macro-dilutions for both bacteria. This tube has a concentration of 64 µg/mL hypericin. To determine the MBC, cultivation was made to plaques from the tube (3rd tube) where MIC was observed, and also from the 2nd and 4th tubes. The growth on these plaques was observed. So, MBC value was not determined for *S. aureus ATCC 29213* and *S. epidermidis ATCC 12228* in these concentrations of hypericin.

On the contrary, turbidity was observed in other tubes where the antibacterial effect of hypericin against to *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 was investigated. For this reason, MIC and MBC values were not determined for these bacteria in these concentrations of Hypericin.

On the other hand, there were same results for all bacteria obtained from tubes protected from light and tubes exposed to daylight. There was no difference between light and dark conditions. The results of the MIC / MBC of hypericin are shown in Table 1.

**DISCUSSION**

Although there are various studies on the effectiveness of
antimicrobial extracts of *H. perforatum* L, there are a few researches on the effectiveness of hypericin (Conforti et al., 2005; Cecchini et al., 2007). The study found that hypericin is effective after irradiation against propionibacterium acnes (Kusari et al., 2008). Nowadays, the antibacterial effects of hypericin are believed to arise from its ability to photo-oxidize cells. However, there are limited studies on the effect of hypericin on potential pathogens. Therefore, we aim to investigate hypericin antimicrobial activity by determining MIC and MBC values.

When planning the research, we considered both phototoxic and lack of connection with light properties of hypericin. We looked for an answer whether antibacterial activity of hypericin has an effect and if it has, how this effect is associated with daylight. Our findings show that daylight has no effect on the result. However, there are some studies suggesting photosensitization of hypericin being effective in some types of bacteria (Engelhardt et al., 2010; Kairyte et al., 2012; Yow et al., 2012). These reports have described remarkable effect against gram-positive bacteria such as *S. aureus* and *L. monocytogenes*. In contrast, it was mentioned that its impact was low against Gram-negative bacteria such as *E. coli* and *Salmonella enterica*. In this research, the antibiotic effect has been associated with the formation of singlet oxygen by photosensitization of hypericin. Lower wavelength of light such as ultraviolet (UV) has been used in this study. At the end, they have concluded that gram-negative and-positive cell wall structure makes a difference in the effect. However, our results show that daylight does not affect the antibacterial activity of hypericin in all tested bacteria. On the other hand, in a study, it was shown that hypericin damaged the human lens in the presence of both UV and visible light; but damage did not occur in the dark (Schey et al., 2000). In this study, it was indicated that specific oxidation of methionine, tryptophan and histidine residues increased with irradiation time. We used daylight of about 550 nm (400 to 700) wavelength. There are studies which suggest that it is necessary to use a lower wavelength for a more efficient photo-oxidation. Indeed, various studies that tested different molecules confirm these data (Zhenfeng et al., 1996; Xingzhou Hu, 1998). We used mostly daylight, because we aimed to find the relationship between daylight and antibacterial activity of hypericin. At the end of the study, we thought that visible light may be sufficient for photo-oxidative damage of hypericin in human tissues such as the lens, but lower wavelength of light may be required to damage the bacterial cell. Bacteria have antioxidants such as mannitol, glutathione, catalase or superoxide dismutase that were effective against photo-oxidative stress (Ziegelhoffer and Donohue, 2009). Methionine sulfoxide reductases in the Enterococcus species and catalase produced by *S. aureus*, *S. epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are important enzymes that protect from oxidative stress (Zhao et al., 2010). Therefore, wavelength which we used may not have created an oxidative stress that exceeds the capacity of antioxidants in tested bacteria.

64 mg/mL concentration of hypericin inhibited the growth of *S. aureus ATCC 29213* and *S. epidermidis ATCC 12228*, without light effect. Conversely, there was no inhibition of hypericin in concentration range found in *Enterococcus faecalis ATCC 19433*, *Escherichia coli ATCC 25922*, *Klebsiella pneumoniae ATCC 700603*, and *Pseudomonas aeruginosa ATCC 27853*. This situation cannot be explained with the difference of cell wall structure between Gram positive and negative bacteria. In fact, Enterococcus is gram-positive but it has no inhibitory effect on Hypericin, in contrast to other tested gram positive bacteria like staphylococci in our study. Perhaps, the inhibition could be achieved in higher concentrations of hypericin for these bacteria. However, we believe that there are mechanisms different from photo-oxidation. We thought that different bacterial species may have different target molecules or different uptake mechanisms. *S. aureus ATCC 29213* and *S. epidermidis ATCC 12228* were affected the same way. Their structures of cell and enzymes were similar. This situation supports our opinion. Hydroxyl groups of hypericin may be linked with some of the target molecules in the cell, or Hypericin may cause the

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus ATCC 29213</em></td>
<td>64</td>
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<tr>
<td><em>Staphylococcus epidermidis ATCC 12228</em></td>
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<td>*</td>
<td>64</td>
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<tr>
<td><em>Enterococcus faecalis ATCC 19433</em></td>
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<td><em>Escherichia coli ATCC 25922</em></td>
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<td><em>Klebsiella pneumoniae ATCC 700603</em></td>
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<td><em>Pseudomonas aeruginosa ATCC 27853</em></td>
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*MBC was only investigated for Staphylococcus aureus ATCC 29213 and Staphylococcus epidermidis ATCC 12228, because they were growing in all tubes for other bacteria.*
inhibition of re-uptake of certain mediators just in the re-uptake of certain neurotransmitters.

Conclusion

Hypericin inhibited growth of S. aureus ATCC 29213 and S. epidermidis ATCC 12228, without light mechanism. However, it was not effective for the other tested bacteria in both cases. The positive or negative effect of daylight has not been observed. The use of a different wavelength of light or the investigation of antibacterial activity at the higher concentrations of Hypericin may lead to different results. Our study suggests that hypericin causes inhibition of growth of some bacteria through mechanisms different from photo-oxidation. This situation is not looked into till date. New studies which can explain possible mechanisms between hypericin and bacterial enzymes, receptors and wall components are required.

REFERENCES