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Shikimic acid, cyclohexene as a hydroaromatic intermediate, harbors potent *in vitro* antimicrobial activity

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Abstract

Aim: Shikimic acid is a hydroaromatic intermediate in the amino acid biosynthesis pathway in bacteria, fungi, and plants. Shikimic acid is an essential agent in applied sciences, especially in pharmacy and medicine. It can be used as a reactant in organic synthesis to obtain various medicinal drugs. This study aims to evaluate the antibacterial and antifungal properties of shikimic acid comparatively and to determine the minimum inhibitory concentrations (MIC) of shikimic acid against the tested *Candida* and bacterial species.

Materials and Methods: The inhibitory effect of pure (98.85%) shikimic acid was tested on five bacteria (two Gram-positive and three Gram-negative) and five *Candida* species. The broth two-fold microdilution method was used to determine the MIC values against the tested microorganisms, and the viability of microorganisms treated with shikimic acid was determined using resazurin sodium salt.

Results: The MIC values of shikimic acid against *Candida glabrata*, *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* were determined to be between 250 and 31.25 mg/mL. However, the MIC values against bacterial species, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Enterobacter aerogenes*, ranged between 15.625 and 3.906 mg/mL. Therefore, the antibacterial effect of shikimic acid is almost twenty times stronger than its antifungal effect.

Conclusion: This preliminary study shows that shikimic acid is also usable as an antibacterial and antifungal as a potential therapeutic agent in addition to its proven antiviral properties. Further, advanced studies in this sense will be eye-opening.

Introduction

The discovery of shikimic acid dates back to 1885, when it was first isolated from *Illicium religiosum* in Japan [1]. Fifty years later, its entire molecular structure was determined, and in the 1950s, it was determined that this compound played a pivotal role in forming aromatic amino acids. Shikimic acid is a pioneer compound in the biosynthesis of many aromatic compounds and primary metabolites, such as amino acids and folic acid, in microorganisms and plants (not found in mammals). The benzene ring, the essential element of all aromatic compounds, is formed in these organisms via the shikimic acid pathway [2-4].

Shikimic acid, a versatile natural organic compound, is an essential agent in the applied fields of pharmacy and medicine. It is primarily used as a reactant in organic synthesis to obtain various medicinal drugs [5, 6]. Shikimic acid has been reported to have anti-inflammatory, analgesic, antioxidant and antibiofilm properties [7-9]. With its antibiofilm activity, shikimic acid prevents microorganisms that enter the host from forming biofilms, allowing antibiotics to target pathogens more effectively [7]. Shikimic acid is used as a starting material for synthesizing the drug Oseltamivir, which is used as an antiviral to treat the H5N1 influenza virus and all known strains of the influenza virus [10, 11]. At the same time, shikimic acid is used in the synthesis of (−)-zeylenone, which is widely used in the chemotherapy treatment of cancerous diseases. Zeylenon has antiviral, anticancer and antibiotic properties [12]. Shikimic acid is a substrate for synthesizing carbasuguars with various biological activities, such as glycosidase inhibitors. When used in a complex with platinum, shikimic acid acts as a potential antitumor agent [13]. Various antitumor compounds, such as dioxolamycin, pericosin A, and citiaformins B, C, and D, are derivatives of 4-epi- Shikimic acid [14]. Shikimic acid derivatives synthesize substances with anticoagulant and antithrombotic

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activity and can reduce blood clotting when injected intramuscularly [15, 16]. Moreover, shikimic acid derivatives are also used in the food industry and agriculture. Most shikimic acid derivatives can be used as herbicides and antibacterial agents by blocking the shikimate pathway in plants and bacteria [17, 18].

Considering the studies carried out on shikimic acid, more attention has been drawn to its effectiveness on viruses in the literature, and it is currently used as an antiviral drug in the field of pharmacy and medicine. However, very few studies highlight the antibacterial and antifungal properties of shikimic acid. In most of these studies, the antimicrobial properties of any natural source are attributed to the shikimic acid or other components it contains. Therefore, this study we conducted will be one of the rare studies showing the antimicrobial properties of pure shikimic acid. In this study, various opportunistic or pathogenic microorganisms, including Gram-negative and Gram-positive bacterial cells and fungal cells of *Candida* species, were used to test the inhibitory effect of shikimic acid.

Materials and Methods

Shikimic acid and chemical reagents

Shikimic acid, also called shikimate, was obtained from BLDpharm (CAS: 138-59-0) (Shanghai, China). Its molecular formula is $C_7H_{10}O_5$ (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid). The purity level of shikimic acid is 98.85%, and its molecular weight is 174.15 g/mol. Its appearance is crystalline powder, and its chemical structure is displayed in Figure 1. Dimethylsulfoxide (DMSO), used as a solvent to dissolve shikimic acid, was purchased from (Honeywell, Germany). Resazurin sodium salt (an indicator showing whether microorganism growth) was purchased from (Sigma, Darmstadt, Germany).

Microorganisms and media

The antimicrobial activity of shikimic acid was examined by measuring the inhibition effect of shikimic acid on ten different microorganisms. Five bacteria, namely *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (NEB C2987), *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 12600) and *Enterobacter aerogenes* (ATCC 51697); and five different fungal species, including *Candida glabrata* (ATCC 2001), *Candida albicans* (ATCC 14053), *Candida krusei* (ATCC 14243), *Candida tropicalis* (ATCC 13803) and *Candida parapsilosis* (ATCC 22019), were used in the antimicrobial activity test. Muller hinton agar and sabouraud agar were used for microorganism subculture for bacteria and *Candida* species, respectively. In determining antimicrobial activity, muller hinton and sabouraud broth were used for bacteria and *Candida* strains, respectively.

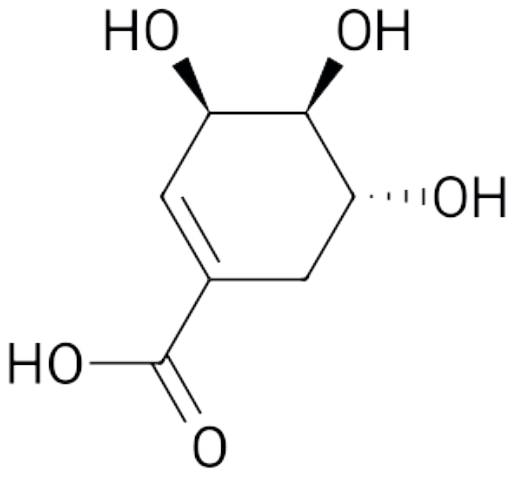

Figure 1. Chemical structure of shikimic acid (shikimate).

Antimicrobial activity

The antimicrobial properties of shikimic acid were determined using the broth microdilution method as applied before [19-22].

As the first step in determining shikimic acid's minimum inhibitory concentration (MIC), 2 g of shikimic acid was dissolved in 250 µL DMSO. For antibacterial activity, 175 µL of muller hinton broth was placed in the first wells of the microplate, and 100 µL of muller hinton broth was placed in the following wells. Sabouraud broth was used in the same proportions for *Candida* species. Afterward, 25 µL of this DMSO suspension (including 200 mg shikimic acid) was added to the first well of the microplates, and a twofold dilution was applied. Thus, the shikimic acid concentration from the first to the tenth well of the microplates varies between 1000 and 1.953 mg/mL.

The density of each microorganism in distilled water was adjusted to 0.5 McFarland, and 1 µL of each strain was inoculated in its own row on the microplate, except for the negative control wells. The eleventh and twelfth wells were designated as negative and positive controls, respectively. The eleventh well contains only broth medium, indicating the absence of contamination.

The twelfth well contains only the medium, and the microorganism culture is inoculated into that row of the microplate, which shows whether or not the microorganism used is alive. Microplates were kept in an incubator at 36.5 °C for 24 hours, and the next day, all wells were treated with resazurin. Afterward, the microplates were left in the incubator at 36.5 °C for approximately 4 hours, and the color change on the microplates was evaluated.

As a result of the antibacterial study, a color change was observed in the last row wells of the microplate, so the antibacterial activity test was repeated at lower concentrations of shikimic acid. Therefore, 31.25 mg of shikimic acid was weighed, dissolved in DMSO, and distributed equally into the first five wells on the microplate. Therefore, the dilution was started with the concentration corresponding to the sixth well of the microplate (31.25 mg/mL) in the first dilution method.

As a result of the two-fold dilution, the shikimic acid concentration from the first well to the tenth well of the microplate varied between 31.25 and 0.061 mg/mL.

Results

Antifungal activity test results

After the treatment of microplates with resazurin, the color change on the microplate indicates whether the tested microorganisms are inhibited by shikimic acid. Well colors changing from dark pink to light salmon color indicate microorganism growth in that well. The antifungal activity of shikimic acid is shown in Figure 2. As the figure shows, *C. glabrata* inoculated in row A started growing from the fifth well. Therefore, the MIC value of shikimic acid is 125 mg/mL, which is the concentration in the fourth well. *C. albicans*, *C. krusei* and *C. tropicalis* grew from the fourth well, so the MIC value of shikimic acid against these three *Candida* species is 250 mg/mL. *C. parapsilosis*, inoculated in the last row, started growing from the seventh well. Therefore, the MIC value against *C. parapsilosis* was found to be 31.25 mg/mL.

Antibacterial activity test results

According to the first dilution results, bacterial microorganisms were observed to grow in the last wells of the microplate containing the lowest concentrations of shikimic acid (Figure 3). As seen in Figure 3, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. aureus* and *E. aerogenes* began to grow in the tenth, ninth, ninth, tenth and eighth wells, respectively. Therefore, lower concentrations of shikimic acid were tested against bacterial strains (Figure 4). As a result, as seen in Figure 4, *P. aeruginosa* and *S. aureus* started to grow from the fifth well. Therefore, the MIC value of shikimic acid against these bacteria was determined as 3.906 mg/mL. *E. coli* and *K. pneumonia*, inoculated in rows B and C, started to grow from the fourth well. Therefore, the MIC value was determined as 7.813 mg/ml. *E. aerogenes*, which was inoculated in the last row, started to grow from the third well, and the MIC value of shikimic acid against this bacteria was found to be 15.625 mg/mL. All results are shown in Figure 5 comparatively.

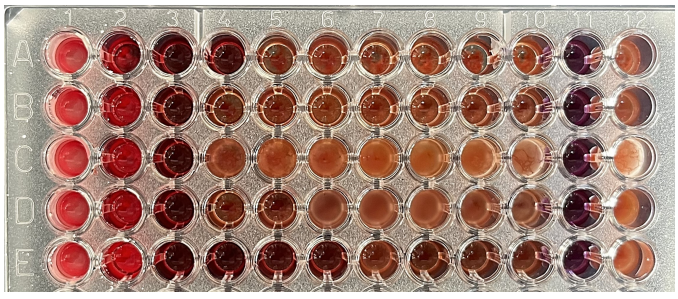

Figure 2. Antimicrobial activity of shikimic acid against *Candida* strains: (A) *C. glabrata*, (B) *C. albicans*, (C) *C. krusei*, (D) *C. tropicalis*, (E) *C. parapsilosis*. The shikimic acid concentration ranges between 1000 and 1.953 mg/mL from the first to the tenth well. The wells showing the MIC values are A4 (MIC:125 mg/mL), B3 (MIC:250 mg/mL), C3 (MIC:250 mg/mL), D3 (MIC:250 mg/mL), and E6 (MIC:31,25 mg/mL). The eleventh and twelfth-row wells are controls.

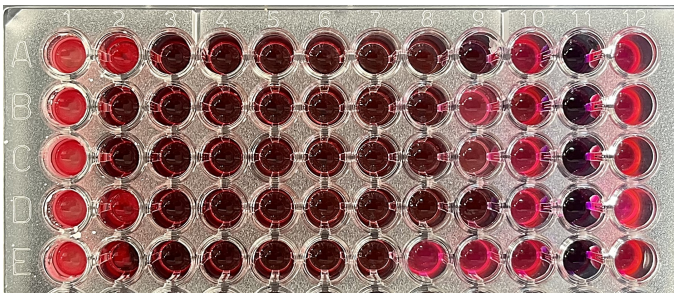

Figure 3. Antimicrobial activity of shikimic acid against bacteria: (A) *P. aeruginosa*, (B) *E. coli*, (C) *K. pneumoniae*, (D) *S. aureus*, (E) *E.aerogenes*. The shikimic acid concentration ranges between 1000 and 1.953 mg/mL from the first to the tenth well. The wells showing the MIC values are A9 (MIC:3,906 mg/mL), B8 (MIC:7,813 mg/mL), C8 (MIC:7,813 mg/mL), D9 (MIC:3,906 mg/mL), and E7 (MIC:15,625 mg/mL). The eleventh and twelfth-row wells are controls.

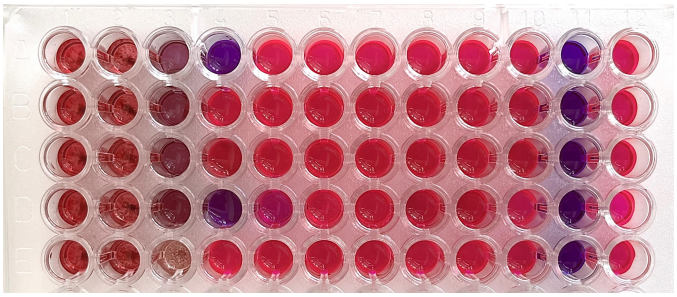

Figure 4. Antimicrobial activity of shikimic acid against bacteria: (A) *P. aeruginosa*, (B) *E. coli*, (C) *K. pneumoniae*, (D) *S. aureus*, (E) *E.aerogenes*. The shikimic acid concentration ranges between 31.25 and 0.061 mg/mL from the first to the tenth well. The wells showing the MIC values are A4 (MIC:3,906 mg/mL), B3 (MIC:7,813 mg/mL), C3 (MIC:7,813 mg/mL), D4 (MIC:3,906 mg/mL), and E2 (MIC:15,625 mg/mL). The eleventh and twelfth-row wells are controls.

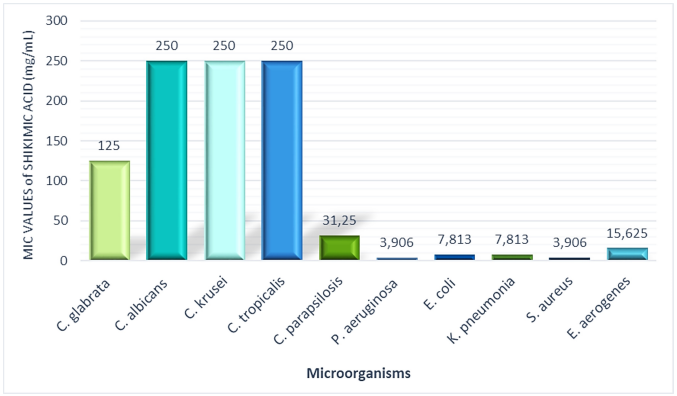

Figure 5. Comparative results of MIC values of shikimic acid against tested *Candida* and bacteria strains.

Discussion

Shikimic acid is an essential intermediate in the biosynthesis of amino acids (tyrosine, tryptophan and phenylalanine), lignin, and alkaloids in plants and microorganisms, which has made it a center of attention in the field of pharmacy [11, 23-25]. The extensive research on shikimic acid has revealed its diverse biological activities. The availability of shikimic acid from microbial and plant sources has opened up new avenues for study. Particularly promising is its potential, when combined with other plant leaf compounds, to inhibit food pathogens. Traditional plant leaf extracts from Thailand, containing shikimic acid, have shown inhibitory effects against food pathogens like *S. aureus*, *E. coli*, *Bacillus cereus*, and *Listeria monocytogenes* [26].

In this study, the inhibitory activity of 98.85% pure shikimic acid against ten different microorganisms, including *C. glabrata*, *C. albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. aureus* and *E. aerogenes* was examined. Promising results were obtained in terms of its usability in infectious diseases. According to the results, bacteria treated with shikimic acid were found to be more sensitive to shikimic acid than fungal cells. The fact that shikimic acid is so effective on bacterial cells and its reduced inhibitory effect on fungal cells may also be related to the fact that *Candida* cells like acidic conditions to grow. However, a study showed that this inhibition of shikimic acid on bacteria was not due to its acidity. The antibacterial activity of shikimic acid is not only due to its acidity but is also closely related to its structural properties [27].

The most detailed study on the antimicrobial activity of shikimic acid was conducted by Bai et al., in which the inhibitory effect of quinic acid and shikimic acid against *S. aureus* was explained together with the mechanism of action [27]. In the study by Bai et al., shikimic acid was able to interact with the cell membrane of *S. aureus* and cause functional impairment in oxidative phosphorylation [27, 28]. Shikimic acid caused membrane fluidity by interfering with the content of glycerophospholipids and fatty acids and was able to alter membrane protein functions. Shikimic acid may alter the normal functions of potassium channels and calcium channels. After the undissociated form of shikimic acid passes through the cell membrane and enters the cell, it can affect ribosome functions and aminoacyl-tRNA synthesis, causing protein synthesis to be impaired. Shikimic acid affects *S. aureus* cell functions by increasing ATP hydrolysis and decreasing succinate dehydrogenase activity, causing a significant decrease in intracellular ATP concentration. It may also reduce DNA synthesis or binding, decreasing DNA content in bacterial cells [29]. At the same time, shikimic acid can disrupt the tricarboxylic acid cycle by interfering with pyruvic acid metabolic pathways. All these results reported that the inhibitory effect of shikimic acid against *S. aureus* is multifaceted [27]. These changes made by shikimic acid in the bacterial cell membrane and functions support the results of our study. In this study, the more potent inhibition of shikimic acid on bacterial cells than on *Candida* cells can be explained by the structural change in microbial cell membranes. Also, as explained above, shikimic acid may have caused inhibition of the tested microorganisms by affecting microbial protein synthesis or reducing the intracellular ATP concentration. This versatile interaction of shikimic acid within the microbial cell explains its potent inhibition on the tested microorganisms.

In the above studies, it was determined that shikimic acid inhibits microorganisms as a result of the structural and physiological damage it causes to microbial cells. At the same time, considering the metabolic activities of microorganisms, enzymes in the shikimic acid pathway have also become the focus for microbial inhibition. The shikimic acid pathway plays a key role in producing structural elements for vitamins, cofactors, protein synthesis and electron carrier compounds such as quinones [30- 32]. Since shikimic acid pathway enzymes are not found in mammals, they are prominent targets in clinical and applied fields to inhibit the growth of microorganisms [33]. Therefore, inhibition of shikimic acid pathway enzymes has gained importance in designing antimicrobial and herbicidal agents that are harmless to humans. As a result, antibacterial drugs have been developed against pathogenic bacteria such as *Mycobacterium tuberculosis*, which causes tuberculosis, and *Helicobacter pylori*, which causes gastrointestinal diseases, by targeting the shikimic acid pathway [34, 35]. The antibacterial activity of shikimic acid on *E. coli* has been proven by inhibiting shikimate dehydrogenase, the fourth enzyme in the shikimic acid pathway [36].

In the last year, studies combining various antibiotics with shikimic acid have revealed its antibacterial mechanism, although no detailed studies have been conducted on its MIC values. It was observed that shikimic acid killed methicillin-resistant *S. aureus* (MRSA) cells treated with the combination of ceftiofur, a broad-spectrum antibiotic of the cephalosporin group, within 2 hours. Although this synergistic effect is supported by *in vivo* and *in vitro* studies, the MIC value is not specified [9]. In another study, the intracellular and extracellular ATP concentrations of MRSA treated with the combination of shikimic acid and oxacillin decreased, and membrane integrity was significantly impaired. The combination of shikimic acid and oxacillin increased the level of radical oxygen scavengers (ROS) and decreased the level of the *mec A* gene. As a result, this combination demonstrated its antibacterial mechanism by disrupting the membrane integrity of MRSA, creating protein leakage, and causing DNA and cellular damage [37].

In light of all the above information, many studies have been conducted on the antimicrobial effect and mechanism of shikimic acid. Realizing this mechanism, especially through inhibiting enzymes in the shikimic acid pathway, has opened up another horizon. However, most studies specified no MIC values of shikimic acid against the tested microorganisms. Although this study did not reveal the inhibitory mechanism of shikimic acid, it numerically expressed the sensitivity of bacterial and fungal cells to shikimic acid. This study proves the usability of shikimic acid as a therapeutic agent against pathogenic or opportunistic microorganisms and is planned to contribute to the literature at this point.

Ethical approval

Ethics committee permission is not required for this study.

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