

ORIGINAL ARTICLE

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A preliminary study of fumaric acid, called allomaleic acid, as a pharmaceutical antimicrobial compound

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Abstract

The emergence of antimicrobial resistance in microorganisms and the adverse effects associated with antibiotic use have necessitated the exploration of new, natural substances for antimicrobial treatments. Fumaric acid, or allomaleic acid, is a natural bioactive compound primarily derived from plant-based products. Fumaric acid has potential analgesic, anti-inflammatory, antioxidant, anti-psoriatic, chemopreventive, immunomodulatory, and neuroprotective properties. So far, studies on the antimicrobial activity of fumaric acid have used a plant ingredient. The antimicrobial activity was attributed to fumaric acid because it was detected in large amounts in herbal products. In this preliminary study, the antibacterial and antifungal properties of pure fumaric acid (99+%) were determined by the microdilution method. As a result of the antimicrobial study, the Minimum Inhibitory Concentration (MIC) values of fumaric acid against *Candida tropicalis*, *Candida krusei*, *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis* were 9.375, 37.5, 4.687, 4.687, and 1.172 mg/mL, respectively. Moreover, bacterial cells were found to be approximately one hundred times more sensitive to fumaric acid than *Candida* cells. The MIC values of fumaric acid against *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes* were determined as 150 µg/mL, and against *Staphylococcus aureus* was 75 µg/mL. These results showed that pure fumaric acid has a potent inhibitory effect against pathogenic or opportunistic microorganisms. Its unique properties and potential as an antimicrobial agent make it a compelling study area. Therefore, the usability of fumaric acid in the pharmacotherapeutic field is promising for future advanced *in vivo* studies.

Keywords: Fumaric acid, allomaleic acid, antimicrobial activity, antibacterials, antifungals, pharmacotherapeutics

Introduction

In recent years, the increasing tendency of microorganisms to develop antimicrobial resistance and the adverse effects due to misuse of antibiotics have made it necessary to search for new, natural substances to inhibit opportunistic and pathogenic microorganisms. Organic acids are one such alternative with proven benefits and have been approved as safe substances by the Federal Drug Administration (GRAS—Generally Recognized as Safe) [1]. Fumaric acid, or allomaleic acid, is a natural, bioactive compound obtained primarily from plant-based products. Fumaric acid is an unsaturated organic acid found ubiquitously in flora and fauna and an essential intermediate of the tricarboxylic acid (TCA) cycle [2]. Fumaric acid, a four-carbon dicarboxylic acid, is widely found in nature and was found for the first time in the fungus *Boletus pseudoignarius* and

isolated from the plant *Fumaria officinalis* in 1832 [3]. Many microorganisms produce fumaric acid, as it is an intermediate in the citric acid cycle [4]. Fumaric acid is a valuable compound called “fumarates” used in the food industry and pharmaceutical products [2,4].

Fumaric acid esters have been used in various skin diseases such as psoriasis, sarcoidosis, malignant melanoma, necrobiosis lipoidica, and granuloma annulare since the 1950s. In the 1990s, fumaric acid esters were launched in Europe as a registered pharmaceutical product for the treatment of psoriasis [5]. Therapeutic use of fumaric acid esters in children with moderate to severe psoriasis has shown promising results [6]. Fumaric acid is an important and promising pharmacological compound as it has a high potential to be converted into active pharmaceuticals. Fumaric acid and fumaric acid esters are

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good antioxidants with their free radical scavenging properties and are also pharmacologically beneficial compounds due to their anti-inflammatory, analgesic, immunomodulatory, and chemopreventive properties [7-11]. Fumaric acid induces brain protection and plays a vital role in expressing neuroprotective factors [7,8]. In tissue engineering, fumarate and fumarate-based oligomers are used to synthesize biomedical and hydrogel implants [12,13]. So far, studies on the usability of fumaric acid in pharmacy and medicine have shed light on future studies, and various studies have been conducted on the use of fumaric acid in infectious diseases. However, these studies were mostly conducted with medicinal plants containing fumaric acid [14-17]. The purpose of the present study was to investigate the antibacterial activity of pure fumaric acid (99+%) against bacterial species frequently isolated from infectious disease units. Furthermore, the antifungal activity of pure fumaric acid against *Candida* species common in fungal infections was investigated, and the broth dilution method was performed for this research.

Material and Methods

Fumaric Acid

Fumaric acid, namely allomaleic acid (Cas No: 110-17-8), was obtained from BLDpharm (Shanghai, China). The purity level of purchased fumaric acid is 99+%, and the molecular formulation of fumaric acid is $C_4H_4O_4$ ($COOH-CH=CHCOOH$) (Molecular Weight: 116.07). The structural formula of fumaric acid is shown in Figure 1.

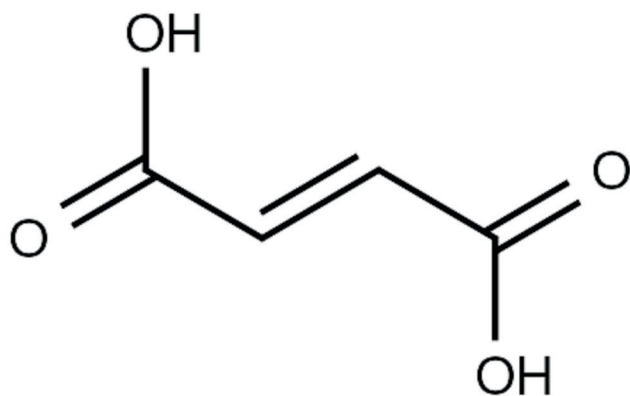


Figure 1. The structural formulation of fumaric acid

Tested Microorganisms and Culture Media

Ten different microorganisms were used in the tests to determine the antimicrobial properties of fumaric acid. These strains are *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (C2987), *Pseudomonas aeruginosa* (ATCC 10145), *Enterobacter aerogenes* (ATCC 51697), *Staphylococcus aureus* (ATCC 12600), *Candida tropicalis* (ATCC 13803), *Candida krusei* (ATCC 14243), *Candida albicans* (ATCC 14053), *Candida glabrata* (ATCC 2001), and *Candida parapsilosis* (ATCC 22019). *E. coli* was purchased from New

England Biolabs (NEB, MA, USA), and other strains were obtained from the American Type Culture Collection (VA, USA). Muller Hinton agar (Merk, Darmstadt, Germany) and Muller Hinton broth (Himedia, Nashik, India) were used for the bacterial strains tested. Sabouraud broth (Bioline, Milan, Italy) and sabouraud 4% glucose agar (Chemsolute, Renningen, Germany) were used for *Candida* species.

Antibacterial and Antifungal Assays

The antibacterial and antifungal effectiveness of fumaric acid was tested by applying the previously used protocol with minor changes, and Minimum Inhibitor Concentrations (MIC) against each microorganism were determined separately [18-20]. The broth microdilution method was used for antimicrobial activity tests. Firstly, 60 mg of fumaric acid was dissolved in 50 μ L dimethylsulfoxide, which was added to the first well of the microplate. Afterward, while 150 μ L of broth medium was added to the first well, 100 μ L of liquid medium was added to the subsequent wells, and then a two-fold dilution was carried out. At the end of the two-fold dilution, fumaric acid concentration from the first well to the 10th well was distributed between 300 mg/mL and 0.586 mg/mL. This protocol was applied for each row of microplates. The density of each microorganism was adjusted to 0.5 McFarland in distilled water, and each strain was inoculated into the specified row of microplates. For control groups, pure muller hinton broth and sabouraud broth without extracts were placed in the eleventh well (negative control), and microorganisms used with muller hinton broth and sabouraud broth were placed in the twelfth well (positive control). Then, microplates were incubated at 35°C overnight. The next day, each well in the microplates was treated with 15 μ L of resazurin (0.15% w/v) (Sigma, Darmstadt, Germany) and incubated at 35°C for 4 hours to determine MIC values.

Although the above protocol for the antibacterial activity test was followed precisely, lower concentrations of fumaric acid were tested for bacterial species due to the results obtained. Therefore, for bacterial species, 60 μ g of fumaric acid was dissolved in 5 μ L of dimethylsulfoxide and added to the first wells of the microplate. After two-fold dilution, fumaric acid concentration ranged from the first well to the 10th well as 300 μ g/mL and 0.586 μ g/mL for the bacterial species.

Results

Antifungal Activity Test Results

The color change on microplates kept in the incubator overnight and then treated with resazurin was observed to determine the MIC values of fumaric acid against *Candida* species. The color change was observed starting from wells A7, B5, C8, D8, and E10, respectively. Therefore, the MIC values of fumaric acid against *C. tropicalis* (row A), *C. krusei* (row B), *C. albicans* (row C), *C. glabrata* (row D), and *C. parapsilosis* (row E)

were determined as 9.375, 37.5, 4.687, 4.687 and 1.172 mg/mL, respectively (Figure 2). The negative control in the 11th row showed no contamination during the experiment. The positive control in the 12th row showed the viability of the microorganism strains used in the same row of the microplate.

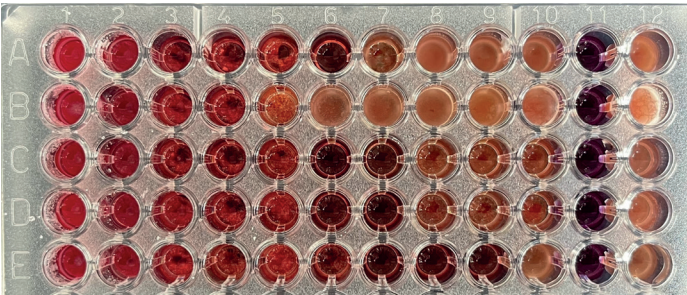


Figure 2. Antifungal test results of fumaric acid against *C. tropicalis* (A), *C. krusei* (B), *C. albicans* (C), *C. glabrata* (D), and *C. parapsilosis* (E). The fumaric acid concentrations in the 1st and 10th wells range between 300 mg/mL and 0.586 mg/mL. The 11th and 12th wells are negative and positive controls, respectively

Antibacterial Activity Test Results

According to the antibacterial test results, no growth was observed in any wells except the control wells (Figure 3). These results showed the dominant inhibitory effect of fumaric acid on bacterial cells at the concentration tested in the antifungal activity test (between 300 mg/mL and 0.586 mg/mL). Therefore, the antibacterial results repeated at lower concentrations of fumaric acid are given in Figure 4. According to the new antibacterial activity test results, MIC values of fumaric acid against *K. pneumoniae* (row A), *E. coli* (row B), *P. aeruginosa* (row C), *E. aerogenes* (row D), *S. aureus* (row E) were determined by observing the color changes in the wells of the microplate. As seen in Figure 4, *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *E. aerogenes* inoculated in rows A, B, C, and D started growing from the third well. Therefore, the MIC value for these four bacterial species was determined as 150 µg/mL. The MIC value for *S. aureus* in the last row, which started to grow from the fourth well, was found to be 75 µg/mL. The MIC values found against all tested microorganisms are shown comparatively in Figure 5.

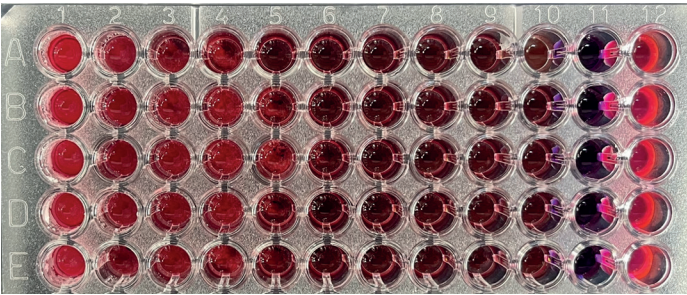


Figure 3. Antibacterial test results of fumaric acid against *K. pneumoniae* (A), *E. coli* (B), *P. aeruginosa* (C), *E. aerogenes* (D), *S. aureus* (E). The fumaric acid concentrations in the 1st and 10th wells range between 300 mg/mL and 0.586 mg/mL. The 11th and 12th wells are negative and positive controls, respectively

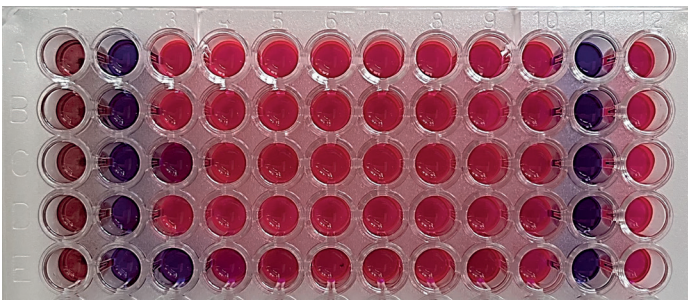


Figure 4. Antibacterial test results of fumaric acid against *K. pneumoniae* (A), *E. coli* (B), *P. aeruginosa* (C), *E. aerogenes* (D), *S. aureus* (E). The fumaric acid concentrations in the 1st and 10th wells range between 300 µg/mL and 0.586 µg/mL. The 11th and 12th wells are negative and positive controls, respectively

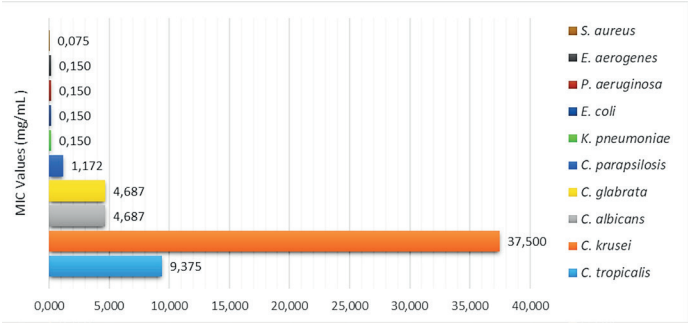


Figure 5. The MIC values against all tested bacteria and *Candida* strains

Discussion

In many studies, compounds obtained from herbal products show profound antimicrobial properties and take their place in the literature as compounds that can be used in the fields of pharmacy and medicine [21-23]. Fumaric acid exhibits stability and disperses in aqueous environments due to its hydrophilic structure. It is a generally safe (GRAS) food-grade acidifier and low molecular weight organic acid [24,5]. It is found in many plants and microbial sources and is used in the food industry, medicine, and pharmacy due to its antimicrobial properties [26,27]. In this study, the antimicrobial activity of fumaric acid was examined using resazurin sodium salt. Resazurin sodium salt is added to the medium as a redox indicator. Living microbial cells reduce resazurin (purple) to resorufin (pink), which can be further reduced to hydroresorufin. Thus, the metabolic activity of microorganisms can be directly measured [28]. Since fumaric acid added to the medium also changes the pH of the medium, the well in which resazurin is added turns from purple to pink-salmon color. However, when the wells in which microorganisms grow and those in which they do not grow are compared, the difference in tone guides us in determining the MIC value. In conclusion, it was found that the MIC value of fumaric acid against *Candida* cells was determined to be between 1.172 and 37.5 mg/mL. It was observed that bacterial cells tested with fumaric acid were approximately a hundred times more sensitive than fungal cells. The MIC values of fumaric acid against bacterial cells were determined to be between 75 and 150 µg/mL. It is thought that this difference between MIC values is due to the different bacterial and fungal cell membranes.

In studies conducted with some plant species that predominantly contain fumaric acid, the antimicrobial activity of plant extracts was attributed to fumaric acid. The disk diffusion method was used in the study conducted with the extract of *Aloe vera* L. As a result, the antibacterial activity of plant extracts against *S. aureus*, *Streptococcus*, *E. coli*, and *Salmonella* was attributed to the fumaric acid contained in it. MIC and MBC values for the tested bacteria were determined as 50 to 780 and 100 to 1560 µg/mL, respectively [29]. In another study, fumaric acid was isolated from petals of *Camellia japonica* L., and the disk diffusion method was used to determine its antibacterial properties against *Salmonella typhimurium*, *E. coli*, *Lactobacillus monocytogenes*, and *S. aureus*. Plant aqueous extracts have been shown to have the potential to inhibit foodborne pathogens. Although this antimicrobial component is associated with the fumaric acid it contains, it has been stated that other yet unidentified compounds in plant extract may also play a role in this inhibition [30]. The antimicrobial activity of some organic acids and fumaric acid was determined using a SYBR Green-based real-time PCR assay. In cells treated with fumaric acid, the potential of the ion-selective electrodes changed, causing an increase in cell cytoplasmic and outer membrane permeability [31]. Research also exists on using fumaric acid as a food preservative due to its antibacterial properties. This potential application of fumaric acid as a food preservative brings hope for a natural and effective solution for food safety. In addition to being used as a food preservative in Europe and America, fumaric acid is also used as an acid regulator and flavor enhancer [32]. Fumaric acid was used to inhibit the *E. coli* population in cider, and it was observed that fumaric acid significantly reduced the *E. coli* population [26].

The inhibitory effects of organic acids on microorganisms, a topic of significant importance in the field of microbiology and food science, are explained by the theory of passive diffusion of undissociated molecules and their intracellular decomposition [33]. This theory suggests that more decomposed organic acids are less antimicrobial, and less decomposed organic acids are more antimicrobial [33]. However, the differences in the action mechanisms of various organic acids and the behavior of microorganisms may cause variability in the antimicrobial mechanism of action as additional factors [34]. The antimicrobial activity of fumaric acid, a weak acid, is attributed to unknown factors, such as intracellular metabolic activities of weak acids, which could involve the disruption of cellular processes or the alteration of pH levels within the microorganism, stress mechanisms, or interactions with other cellular functions [35]. Fumaric acid has been identified as an inhibitor of the glutamate decarboxylase (GAD) enzyme of *E. coli* [36]. In a study, fumaric acid showed high bactericidal properties against three strains of *Listeria monocytogenes*. This situation does not fit well with the intracellular dissociation theory of weak acids, but it shows antibacterial effects that only occur in highly acidic conditions. As a result, it was determined that the antimicrobial activity of fumaric acid was associated with a decreased intracellular GAD system or other systems [35]. These findings underscore the complexity and importance of understanding the antimicrobial

effects of organic acids.

The limitation of this study is that fumaric acid's antimicrobial activity was studied *in vitro*, and its *in vivo* activity needed to be examined. Most studies in the literature to support the use of fumaric acid in medicine and pharmacy are *in vitro* studies, but *in vivo* studies are very limited. Moreover, another limitation is that although it was proven that fumaric acid had a satisfactory inhibitory effect against the microorganisms tested in this study, the inhibitory effect of fumaric acid against microorganisms that cause pathogenic or opportunistic infections other than these microorganisms was not tested. Additionally, the exact mechanism of action needs to be clarified to provide more data and enable fumaric acid to be used clinically in treating diseases. Therefore, further studies are required in more detail. In addition to *in vivo* and *in silico* studies, detailed at the molecular level may be needed.

Conclusion

Fumaric acid is an organic acid found naturally in flora and fauna. Since fumaric acid has various pharmaceutical properties, it is widely used in the pharmaceutical, medicine, and food industries. Fumaric acid is known to be a good antioxidant, analgesic, anti-inflammatory, immunomodulatory, anti-psoriatic, chemo-preventive, and neuroprotective, and there are also various studies on its antimicrobial activity. At the same time, the inhibitory ability of the plant aqueous extract on bacterial cells has been attributed to fumaric acid, which was found in studies conducted with plants. In this study, fumaric acid had a strong inhibitory effect against the bacterial and fungal cells tested and proved to have selective toxicity against bacterial cells. Therefore, this preliminary study obtained information about the usability of fumaric acid as a pharmacotherapeutic agent in infectious diseases. Future advanced studies and *in vivo* and *in silico* techniques should support the findings regarding the usability of fumaric acid in many areas.

Conflict of Interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

The authors declare that they have received no financial support for the study.

Ethical Approval

Ethics committee approval is not required since the study was not an experimental animal study and did not use human-sourced materials.

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