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Evaluation of Antibacterial Effects of Newly Synthesized Derivative Methyl 2'-Methyl-1,3-Dioxo-1,1',2',3,5',6',7',7a'-Octahydrospiro [Indene-2,3'-Pyrrolizidine]-2' Carboxylate against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Objective: Unfortunately, infectious diseases are still one of the most important health problems around the world. The resistance of pathogenic bacteria to several drugs is an important and growing problem in the treatment of infectious diseases and hospital infections. It seems necessary to identify new compounds that have inhibitory or lethal effects on this bacterium. That's why we decided antimicrobial efficacy. The newly synthesized compound (2-methyl-1,3-dioxo-',11',2',3',5',6',7'a,7-octahydrospiro] inden-'2,3-pyrrolizidine 2-carboxylate (5) on *Staphylococcus aureus* and *Escherichia coli* bacteria.

Methods: At first, synthesized of methyl'2-methyl-1,3-dioxo-1',1',2',3',5',6', 7',7'a, -octahydrospiro] inden-'2,3-pyrrolizidine 2- Carboxylate was prepared by green chemistry method and with the help of microwave in one pot. Microbial culture media containing control bacteria of *Staphylococcus aureus* and *Escherichia coli* were prepared and concentrations of 70 to 110 Landa of the above compound were added to the samples. The antimicrobial property of the samples was determined after 3 days using the disk diffusion method and broth micro dilution method was determined, as well as the minimum inhibitory concentration and bacterial lethality of the samples.

Results: The minimum inhibitory concentration and bacterial lethality in the above combination against the same standard strain of *Escherichia coli* bacteria was obtained at the rate of 12.5 µg/mL. The minimum inhibitory and lethal concentration of methyl methacrylate compound against the standard strain of *Staphylococcus aureus* bacteria was 12.5 and 25 µg/mL, respectively.

Conclusion: The use of the above combination was effective in controlling and inhibiting the tested bacteria.

Introduction

Staphylococcus aureus: methicillin-resistant *staphylococcus aureus* (MRSA) is responsible for many antibiotic-resistant infections. This type of golden staphylococcus is resistant to betalactam antibiotics (such as Penicillin, Nafcillin, Oxacillin) and Cephalosporins such that these antibiotics have no effect in treating infections related to this factor. The prevalence of MRSA is especially higher in

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hospitals, patient care centers, patients with open wounds, patients with implanted materials (prosthesis) and patients with weak immune system [1].

S. aureus is a gram positive and facultative anaerobic coccus and is the most important species in the genus staphylococcus in terms of medicine. Staphylococcus aureus is one of the most prosperous pathogenic bacteria. Due to the production of golden pigment staphyloxanthine, this bacterium generates golden colonies. This pigment participates in pathogenicity since it acts as an antioxidant and protects the bacteria against oxygen free radicals. Oxygen free radicals are generated by the immune system of the host (white globules) to kill bacteria [2-5].

Escherichia coli as a strong urinary infection agents, has relatively high resistance to different antibiotics such as third generation fluoroquinolones and cephalosporines. Also known as *E. coli,* this bacterium is a gram-negative bacillus and belongs to enterobacteriaceae which is widely present in the intestine of warm-blooded animals. Most E. coli strains are harmless but some serotypes such as O_{157}:H_7 can cause food poisoning and diarrhea. These harmless strains are a part of normal flora of intestine. They contribute to vitamin K2 production and prevent the placement of pathogenic bacteria in the intestine. These bacteria account for 0.1% of the total flora in the intestine. The most common cause of urinary system infection is this bacterium which accounts for 90% of urinary infections in young women. The clinical symptoms of this infection are frequent urination, dusyria, blood in urine and pus in urine. This bacterium has three enzymes whose genes are placed close to each other and are expressed by one regulating section [4-6].

Asef et al. (2018) investigated the efficiency of 1-(5-((1H-pyrazol-1- yl) methyl)-2-aryl-1,3,4-oxadiazol-3(2H)-yl) ethanone. The results showed that the efficiency of this compound in preventing the growth of three species of *S. aureus*, *E. coli* and pseudomonas aeruginosa at 0.61mg/mL concentration was higher than control antibiotics ciprofloxacin and tetracycline [7].

Hiashi et al. (2021) studied the reciprocal bonding of Pd in methyl iodide with carboxylate (C11) for the synthesis of acetic acid and its active esters, i.e. the acetylation of small, medium and large C11 molecules. The combination of Pd attached to methyl iodide and its esters, due to the presence of methyl iodide, can easily diffract different positrons and therefore, can act as an exchanging agent in the transferring of high-energy particles to microorganisms and in turn, their death [8]. Isidine and indenic alkaloids in urine have important biological and pharmaceutical as well as antibacterial, antifungal and anticancer properties. More than seven thousand such compounds have been verified. Spiro cyclic oxindoles are valuable kinetic reaction intermediates which form main units of many pharmaceuticals and alkaloids [9]. Due to a variety of biological antibacterial, antimicrobial, antifungal, antiviral, and local anesthetic properties, these compounds have attracted the attention of several chemists [10]. Therefore, various synthetic pathways have been devised for their synthesis [11, 12]. Bipolar cycloaddition reaction is an efficient method for the synthesis of 5-memnered heterocycles and Spiro heterocycles such as pirrolidines, pirrazolydines, pyrrolizidines which are commonly present in natural products and biologically active compounds. Despite several methods for their synthesis, production of new Spiro heterocycles is still very popular and great effort has been devoted to their synthesis in several research works [13-15].

The new compound methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2 carboxylate, which is produced via a one pot and spacioselective reaction as a single diastromer with high purity [15]. Heterocycles containing several rings with one spiro center and four chiral centers are produced. Presence of indene and pyrrolizidine cyclic systems intensify the possibility of pharmaceutical properties in these compounds. This compound was synthesized as a single diastromer with high purity and this paves the way for the investigation of their microbiological properties [16].

Application of new synthetic compounds and evaluation of their efficiency are research hot topics and deserve to be extensively investigated. The aim of this research was to investigate and evaluate the new synthetic methyl 2'-Methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2 carboxylate (5) on *S. aureus* and *E. coli* bacteria.

Synthesis of new compounds (5):

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In the beginning of the preparation of methyl 2-methyl-1,3'-dioxo-1,1,2',5',3,6',7',7'a octahydrospiro[indene-2,3-pyrrolozodone] 2- carboxylate was synthesized as follows: in a 50cc round bottom vessel, a mixture of ninhydrin (0.178g, 1mM), proline (0.115g, 1mM) and methyl metacrylate (0.086g, 1mM) in pure ethanol was prepared and put in microwave for 2 minutes. The reaction took place by emitting CO_2 gas. Reaction process and its completion was explored with chromatography technique, after the completion of the reaction, the solvent was discarded under vacuum and yellow crystals were extracted from the mixture (Fig. 1).

Preparation of microbial suspension:

To do so, 24-h pure bacterial cultivation was applied. About 3 or 4 bacterial colonies were taken and dissolved in sterile physiological serum in a tube and for the homogeneity of the suspension, vortex was performed. Then, using a spectrophotometer, initial bacterial concentration in the suspension was set at to be 1.5×10^8 CFU/mL. Then, final bacterial concentration of 5×10^5 CFU/mL was prepared from the initial suspension for broth micro dilution tests. Optical absorption of 0.08-0.13 in the suspension at wavelength 625 nm indicated bacterial concentration of 1.5×10^8 CFU/mL in the suspension.

leaves were crushed in natural conditions in dry shade and then crushed. To prepare the extract, 40 g of dry plant powder was placed in half-liter Erlenmeyer flakes containing 200 mL of 96% ethanol.

The contents of the Erlenmeyer flask were mixed at room temperature for 24 hours with a shaker (130 rpm) and then filtered through Whatman 2 paper. The solvent was separated from the extract by a rotary apparatus using a vacuum pump (distillation in vacuum). The weighted extracts were then dissolved in DMSO solvent.

Preparation of Moller-Hinton broth:

Using the information provided in on the container of the cultivation medium, the amount of required powder was calculated and weighted using a digital scale and dissolved in the required amount of water. Then, the mixture was heated on flame and autoclaved after being divided into test tubes. After autoclave, the tubes were cooled and stored in fridge for later use.

Preparation of Moller-Hinton agar (MHA) cultivation medium:

Using the information provided in on the container of the cultivation medium, the amount of required powder was calculated and weighted using a digital scale and dissolved in the required amount of water in a clean round bottom vessel. Then, the mixture was heated on flame and the mixture vessel was put in autoclave for sterilization. After autoclave, the tubes were cooled (in room temperature to maximum temperature of 50°C) and placed in disposable sterilized plates (this was done beside the flame under hood). After setting, plates were stored in fridge for later use.

Investigation of antimicrobial activities Preparation of minimum inhibitory concentration (MIC)

MIC is defined as the minimum concentration of an antimicrobial material which can hinder microbial growth under laboratory conditions. This test was performed according to broth microdilution approach and based on CLSI protocols (16, 17). To do so, first, in 100 µl Moller-Hinton broth (MHB), serial dilutions of methyl 2'-methyl-1,3-dioxo -1,1',2',3,5',6',7',7a'-octahydrospiro [indene-2,3'-pyrrolizidine]-2 carboxylate in the concentration range of 0.19 to 25 µg and control antibiotics in the concentration range of 0.03 to 200 µg were prepared in a 96-cell microplate. 100 µl prefabricated bacterial suspension with 5×10^5 CFU/ml was added to each of the cells and incubated for 24 h at 37°C. Then, using a microplate spectrophotometry (Epoch- BioTek Co., Winooski, VT, USA), based on the light absorption of the suspension at wavelength 625 nm, MIC of the newly synthesized compound and antibiotics were determined. For this test, sterile and bacteria-free MHB was applied as negative control and MHB containing bacteria was adopted as positive control. In this research, vencomicine and gentamicine antibiotics were applied for staphylococcus aureus and E. coli, respectively, as control to compare with test results.

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Determination of minimum bactericidal concentration (MBC)

MBC is defined as the minimum concentration of an antibacterial compound which can kill 99.99% of th bacteria. MBC of methyl 2'-methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2 carboxylate and antibiotics were determined based on CLSI protocol. To perform these tests, 10 µL of each cells prepared based on MIC approach were cultivated in MHA medium and incubated at 37°C for 24 h. then, the colonies were counted after 18-24 h.

Yellow prism (EtOH), 88–91% yield, m.p. 111–112 ˚C. 1H NMR (CDCl3, 500 MHz) δ 1.67 (3H, s, CH3), 1.68-1.78 (1H, m, 7'-CH), 1.83-1.93 (1H, m, 7'-CH), 1.94-2.03 (2H, m, 6'-CH2), 2.06 (1H, dd, J = 6.2, 12.2 Hz, 1'-CH), 2.43-2.48 (1H, m, 1'-CH), 2.71-2.76 (2H, m, 5'-CH2), 3.29 (3H, s, OCH3), 3.95-4.04 (1H, m, 7a'α-H), 7.83-7.92 (3H, m, ArH), 8.01-8.03 (1H, m, ArH). 13C NMR (CDCl3, 125 MHz) δ 14.26 (CH3), 29.21, 31.21, 33.65, 48.84, 51.91 (OCH3), 55.17, 68.35 (Cspiro), 74.12 (CH-N), 122.12, 123.91, 136.24 (4CH, aromatic), 142.17, 142.22 (2Cipso, aromatic), 171.29, 203.40, 204.71 (3C=O). IR (υmax/cm-1, KBr) 1680, 1584 (2C=O). MS (m/e, %) 313 (M+, 75), 254 (M+-CO2Me, 30), 212 (254-C3H6, 100), Anal. calcd for C18H19NO4 (313.348): C, 68.99; H, 6.11; N, 4.47; O, 20.42%. Found: C, 68.98; H, 6.16; N, 4.48; O, 20.42%.

Antimicrobial activity results

MIC and MBC of methyl metacrylate against standard strain of E. coli bacterium were the same which was found to be 12.5 µg/mL.

Corresponding values against E. coli were 3.2 and 6.4 µg/L, respectively.

MIC and MBC of methyl metacrylate against standard strain of staphylococcus aureus were 12.5 and 25 µg/mL, respectively. MIC

and MBC values for vancomycine antibiotic against staphylococcus aureus were both 0.2 µg/ml (Tables 1, 2 and 3).

Discussion

It could be understood from the antimicrobial activity of methyl metacrylate on the two grams negative and gram positive bacteria *E. coli* and *S. aureus* that this compound at constant concentration of 12.5 µg/mL prevented the growth of both bacteria. Although methyl metacrylate MBC in gram negative bacterium E. coli at the same concentration as MIC, this compound at higher concentrations could kill gram positive bacterium staphylococcus aureus. In other words, to kill gram positive bacteria such as staphylococcus aureus, higher concentrations of methyl metacrylate is required. The reason for this was in the structural differences of cell wall in the two-gram positive and gram-negative bacteria. It seems that penetration of methyl metacrylate into cell wall and its later attachment to target position is easier in a gram-negative bacterium than a gram-positive bacterium with a thicker peptydoglycane wall which could provide physical resistance to the penetration of methyl metacrylate. Anyway, further research is required to understand in more detail the mechanism of transportation through bacterial cell wall, attachment position and bacterial growth inhibition mechanism of methyl metacrylate. Another important issue which could be understood from the action mechanism of methyl metacrylate against *E. coli* is that this compound could completely kill the bacterial at concentration of up to 12.5 µl/mL while at lower concentrations (6.25 µg/mL) bacterial growth, like positive control, occurred completely. For staphylococcus aureus bacterium, unlike *E. coli*, at concentrations of lower than MIC, bacterial growth occurred at mild slope.

Results

In this research, methyl 2'-methyl-1,3-dioxo-1,1',2',3,5',6',7',7a'-octahydrospiro[indene-2,3'-pyrrolizidine]-2' carboxylate was synthesized in a one pot procedure. This compound was obtained through one pot reaction of ninhydrine (1), proline amino acid (2) and estermethyl metacrylic acid (3). The structures of the obtained compounds were verified using spectroscopy (IR, 13CNMR, 1HNMR) and elemental analysis (Figs. 2 and 3). Spectral data of compound (5) are provided below.

Conclusion

This indicated that the antibacterial concentration of methyl metacrylate in gram negative bacteria occurred with a dose-dependent procedure while for gram positive

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staphylococcus aureus bacterium, this effect seemed to be more dependent on attachment position of methyl metacrylate in the bacterium.

Abbreviation

MHB: Müller-Hinton nutrient medium

MIC: minimum inhibitory concentration

MBC: minimum inhibitory concentration

Conflict of interest

None of the authors have any conflict of interest to declare.

Consent for publications

All authors approved the final manuscript for publication.

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