Synthesis and antibacterial activity of new Sulfamethoxazole derivative.
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Abstract:
In the present work, diazonium salt was synthesized by treatment of the primary aromatic amine of sulfamethoxazole with sodium nitrite, which is directly coupled with m-cresol to give a new sulfamethoxazole derivative. Chemical structure of the new derivative was characterized by physical and spectroscopic techniques as UV, FTIR, ¹H NMR and ¹³C NMR spectra. The new derivative [4-(salicylic acid-5”-azo-yl)-N-(5’-methyl-3’-isoxazolyl)benzene-sulfonamide] is characterized by its it lack of the primary aromatic amine in its structure with no possibility to form hydroxylamine metabolite; which is responsible for allergic reactions; In addition, the new derivative is more water soluble due to the presence ofazo-linkage. The new sulfamethoxazole derivative proved to have no antibacterial activity against Pathogenic isolates of G- bacteria (E. coli) used in the present work, and reliable in vitro antibacterial activity against G+ bacteria (Staphylococcus aureus). Mean IC₅₀ against pathogenic Staph aureus isolates used in the study was about 12μg/ml whereas the mean MIC was 20 μg/ml. Presence of azo-bond may change the mode of action of sulfamethoxazole, make it possible to test this a new compound as new antibacterial agent against G+ bacteria.
Introduction
Over the past few decades, bacterial resistance to antibacterial agents has become one of the most important problems in treating infectious diseases. Searching for new compounds, which would combine a non-specific activity against a broad spectrum of bacteria with low toxicity, seems to be a promising way to overcome that problem. Sulfonamides are ones of the least expensive drugs and this factor largely accounts for their greater extent of use in developing countries (1-3). Formerly, sulfamethoxazole was an attractive compound for use in the treatment of urinary tract infections, meningitis, pharyngitis, bacillary dysentery, trachoma, chancreoid, malaria, toxoplasmosis, nocardiasis and conjunctivitis due to susceptible microorganisms (4). But many strains of Meningococci, Pneumococci, Streptococci, Staphylococci, E.coli and Gonococci are now resistant (5). Treatment with sulfamethoxazole is usually associated with a relatively high incidence of immune-mediated hypersensitivity reactions (6). This is believed to be an idiosyncratic consequence of enzymatic generation of the hydroxylamine metabolite and subsequent auto-oxidation to a protein-reactive nitroso species (7, 8). The cellular distribution (9), cytotoxicity (10, 11) and immunogenicity of sulfamethoxazole metabolites have been studied extensively (12). Compound similar to sulfametoaxazole but without problems of resistance and allergic reactions is needed for the treatment of specific bacterial infections, thus the present work aimed to synthesize new sulfamethoxazole derivative and to detect its antibacterial activity against G+ and G− bacteria compared with the parent compound.

Materials and Methods

- **Synthesis of the new sulfamethoxazole derivative:**

Materials:
The parent compound (sulfamethoxazole) was supplied from Nenevah Drug Industry (Iraq) while the chemicals employed in the synthesis were supplied from Fluka Company.

Instruments:
Melting points were determined in open capillaries on electrothermal CIA 9300 melting point apparatus and are uncorrected. The ultraviolet spectrum was obtained via Carrywinn UV. Varian UV. - Visible spectrophotometer while the infrared absorbance was recorded by Buck 500 scientific FTIR spectrophotometer. The 1H-NMR and 13C-NMR spectra of the synthesized compound were recorded by Varian Mercury 400 MHz (France). Thin-layer chromatography (TLC) was carried out on TLC plastic sheets silica gel 60 F5 pre-coated, 20×20 cm, layer thickness of 0.2 mm. The spots on the chromatograms were localized using U.V. light (at 366 nm). The solvent system employed for separation composed of methanol: strong ammonia solution (98.5:1.5).

Experimental (13):
A (12.65 g, 0.05 mol) of sulfamethoxazole was dissolved in a solution composed of equal volume (16 ml) of each conc. HCl and water; the resulting solution was cooled. The cold solution of 20 ml of sodium nitrite (0.057 mol) was then added dropwise to the stirred solution of sulfamethoxazole in an ice bath, temperature of the reaction was kept below (10°C). After the last addition, the resulting solution was stirred for (5 min) in an ice bath and then a drop of
it was diluted with (4 drops) of water, and tested on potassium iodide-starch paper; if no immediate blue color was obtained at the point of contact with paper, a further (1 ml) of sodium nitrite solution was added and tested again after (5 min). In a beaker, m-cresol (5.4 g, 0.05 mol) was dissolved in (45 ml) of (10%) NaOH; the resulting solution was vigorously stirred at temperature below (5°C). The cold diazonium salt solution with 25g of crushed ice were added drop wise to the previously stirred solution in an ice bath; deep red color was readily developed and the crystals soon separated. When the addition was finished, the mixture standing in an ice bath for (30 min), the crystals was filtered off, washed three times with cold water and then recrystallized from ethanol. Purity of the resulted compound established using TLC, by which only a single spot was observed. The melting point was (236-238.5°C) with (78%) yield; the Rf value was (0.42) while the λmax (ethanol) was (611 nm). Synthetic pathway of the new sulfamethoxazole derivative is illustrated in the Scheme (1).
Scheme 1. The synthesis of new sulfamethoxazole derivative.
Biological study
- Detection of the antibacterial activity of the new derivative:

Pathogenic isolates of *Staphylococcus aureus* and *E. coli* were obtained from Bacteriology research lab, Biology Department, College of Science, University of Mosul. Bacteria were identified using macroscopic, microscopic and biochemical tests, depending on Konemann’s (14). Inhibitory effect (Minimal inhibitory and inhibitory concentration of 50% of bacteria) of the new derivative against *G*+ and *G*− bacteria determined using two methods: disc diffusion test (15) and turbidimetric method (16).

**Disc diffusion method:**
Filter paper (Whatman No.1) discs (5mm diameter) were prepared, saturated with different concentrations of the new derivative; 0.1ml of each concentration was added to container, containing 10 sterilized discs (17). Each saturated disc was placed using sterilized forceps on the surface of nutrient agar plates inoculated with the bacteria under study. The plates were incubated at 37ºC for 14-16 hrs, inhibition zone around each disc (concentration) was measured and compared with the pure antibiotic (sulfamethoxazole-supplied from Nenevah Drug Industry/Iraq) discs. Pure antibiotic disc saturated with 20µg sulfamethoxazole in each disc according to WHO protocol (18).

**Turbidimetric method**
Bacterial suspension of about 10⁸ CFU/ml was used in this experiment. Turbidity of bacterial suspension was equivalently to that of McFarland tube No.4 slandered. 0.1 ml of serial dilutions of compound were added to tubes of 9.8 ml nutrient broth, then 0.1 ml of the bacterial suspension was added to each tube. Ingredient concentrations of the new derivative were added to the prepared test tubes (4, 8, 12, 16, 20, 24, 28 and 32µg/ml of the liquid culture), three replicates were applied for each treatment. Negative control test tubes contain 9.8 ml of nutrient broth+0.1ml of bacterial suspension +0.1ml DMF was applied. Turbidity of each prepared test tube was determined at 480nm, after 14-16 hrs of incubation at 37ºC, using SERIS CECIL-CE1021, 2000 spectrophotometer. Minimal inhibitory concentration and inhibitory concentration 50% of the new derivative were estimated. Resistance was considered when the sulfamethoxazole MIC was greater than 12µg/ml.

**Results and discussion**
The biological importance of azo compounds is well known for their use as antineoplastics, antidiabetics, antiseptics and other useful chemotherapeutic agents (19). The hydroxylamine metabolite of sulfamethoxazole is a critical determinant in the pathogenesis of serious hypersensitivity reactions to the sulfonamides; this metabolite produced from oxidation of the primary aromatic amine of sulfamethoxazole by hepatic microsomes (20, 21). In present study, a new sulfamethoxazole derivative was synthesized by reaction of sulfamethoxazole with NaNO₂ under extremely acidic condition at a very cold temperature. Strong acidic medium is needed because a certain amount of amine may be produced by salt hydrolysis unless an excess amount of acid is present (22, 23).The reaction mixture is maintained in an ice bath because the phenyldiazonium salt intermediate is easily decomposed at slightly higher temperature (24, 25).
The FTIR spectrum of new sulfamethoxazole derivative:
The infrared spectrum (KBr) of a new sulfamethoxazole derivative shows that the disappearance of absorbance band at 3436 cm\(^{-1}\) of primary amine of sulfamethoxazole and the appearance of a weak absorbance band at 1473 cm\(^{-1}\) of azo group (unsymmetrical substituted azobenzene) confirmed the formation of azo-compound. The medium bands at (2972 and 2934) cm\(^{-1}\) can be attributed to C-H stretching of methyl groups of aromatic and isoxazole rings respectively. The sharp medium band at 3650 cm\(^{-1}\) indicating the presence of free phenolic OH group while the primary sulfonamide group shows a strong N-H stretching band at 3420 cm\(^{-1}\). The 1H-NMR spectrum of new sulfamethoxazole derivative:
The 1H-NMR (DMSO-d6) spectrum of a new sulfamethoxazole derivative shows that the protons of aromatic rings attached to azo group resonated at \(\delta\) 6.78-6.89 ppm (m, 3H) and at \(\delta\) 7.98-8.09 ppm (dd, 4H) while the proton of the isoxazole ring resonated at \(\delta\) 5.51 ppm (s, 1H). This spectrum clearly indicated the chemical shift of the proton of phenolic-OH at \(\delta\) 7.72 ppm (s, 1H) as well as the proton of sulfonamide group at \(\delta\) 3.16 ppm (s, 1H). The protons of methyl groups attached to aromatic and isoxazole rings resonated at \(\delta\) 2.61 ppm (s, 3H) and at \(\delta\) 2.06 ppm (s, 3H).

The 13C-NMR spectrum of new sulfamethoxazole derivative:
The 13C-NMR spectrum of a new sulfamethoxazole derivative reports that the carbon atom of methyl group attached to aromatic ring resonated at \(\delta\) 16.84 ppm while the carbon atom of methyl group attached to isoxazole ring resonated at \(\delta\) 29.77ppm. The carbon atoms of aromatic rings attached to azo group resonated at \(\delta\) (144.13 and 155.61) ppm. The carbon atoms of aromatic and isoxazole rings attached to sulfonamide group resonated at \(\delta\) (140.13 and 161.83) ppm respectively. The carbon atoms of aromatic ring attached to OH and CH3 resonated at \(\delta\) (170.13 and 129.12) ppm respectively. The FTIR, 1H-NMR and 13C-NMR spectra were confirmed the chemical structure of a new sulfamethoxazole derivative.

Antibacterial activity of the new derivative:
The biological study deals with six pathogenic isolates of G (Escherichia coli), were tested for their sensitivity to the new sulfamethoxazole derivative, using the disc diffusion test. Neither the pure sulphamethoxazole, nor the new derivative demonstrated any inhibitory effects against the tested strains. This result sustains the presence of mutant by which resistant E. coli strains toward antibiotics (including sulfamethoxazole) manifestated. Resistance of E. coli to sulfamethxazole and its new derivatives may result from prolonged use of such antibiotic, especially in developing countries\(^{26}\). Antimicrobial activity of the new sulfamethoxazole derivative against G\(^+\) bacteria (Staphylococcus aureus) detected in vitro using the disc diffusion and turbidimetric methods against six pathogenic isolates of Staphylococcus aureus was used. Whereas the pure sulfomethoxazole had no antibacterial activity against Staphylococcus aureus, the new derivative contribute obvious inhibitory effect. It was found that antibacterial activity of the new derivative was increased with increased concentration (concentration-dependent effect on bacteria growth). See picture. 1. Mean IC\(_{50}\) of the six pathogenic Staph aureus isolates used in the present study was
about 12µg/ml, whereas the mean MIC was 20 µg/ml (Figure. 1). It was found that the new derivative has MIC approximately in the same range of that of pure sulfomethoxazole against the susceptible strains of G- bacteria (25 µg/disc) (27), which were susceptible to sulfamethxazole.

Picture(1):- The extrusive inhibitory effect of the new sulfamethoxazole derivative on Staphylococcus aureus, with the concentration increase of the derivative, compared with the pure sulfamethoxazole (in the middle).
Mechanism of action of the new derivative as antimicrobial agent against G+ bacteria was not known and more investigation was needed. The study proposes that the presence of azo-bond may change the mechanism of sulfamethoxazole action, and the new derivative may be used as a new anti bacterial agent against G+ bacteria. Sulfamethoxazole[4-Amino-N-(5-methyl-isoxazol-3-yl)benzenesulfonamide] inhibits dihydropteroate synthase, the bacterial enzyme that catalyzes the incorporation of para-aminobenzoic acid into dihydropteroic acid, the immediate precursor of folic acid\(^{(28)}\). The new derivative [4-(salicylic acid-5”-azo-yl)-N-(5’-methyl-3’-isoxazoly) benzene-sulfonamide] has advantages over the parent compound; first, it lacks the primary aromatic amine in its structure with no possibility to form hydroxylamine metabolite which is responsible for allergic reactions\(^{(29)}\); second, the new derivative is active against Staph. aureus; third; the new derivative was more water soluble due to the presence of azo-linkage. In conclusion, the new sulfamethoxazole derivative approved to have no antibacterial activity against E. coli, and reliable antibacterial activity against Staphyllococcus aureus in vitro. Thus, there is a need for further investigations about the antimicrobial activity of such compound against other pathogenic genera of G+ bacteria; furthermore, studies about toxicity of the new derivative in vivo and it’s mechanism of action in the targeted bacteria are needed too.
References


