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Sawsan-ahmed-fouaad-2a #274 RT: 4.60 AV: 1 SB: 2 4.45 , 4.45 NL: 1.03E3 T: {0,0} + c El Full ms [40.00-1000.00]























Sawsan-ahmed-fouaad-3b #275 RT: 4.62 AV: 1 SB: 2 4.45 , 4.45 NL: 7.16E2 T: {0,0} + c El Full ms [40.00-1000.00]



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Relative Abundance

0

50

100

150

200

250

Sawsan-ahmed-fouaad-4#275 RT: 4.62 AV: 1 SB: 2 4.45 , 4.45 NL: 1.82E3 T: {0,0} + c El Full ms [40.00-1000.00] 108.19 100-**90** OMe $_{NH_2}$ HN_ 80-0, `S´ ŃΗ 70-O ò NC **60** н NH₂ O **50** 29 ŋ 82.16 94.23 40 (4) 4.38 37 93.37 30-115.36 173.73 261.25 263.54 272.69 Ø 238.72 _____297.53 `307.61 559.80 135.41 2 168.20 333.77 20-4 563,52 200.65 406.95 515.90 354.34 450.38 375.61 229.77 10

300

m/z

400

450

350

500

550













100-

90-





116.20









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Sawsan-anmeo-touaad-10#285 RT: 4.79 AV: 1 Sb: 2 4.45 , 4.45 NL: 0.05E2 T: {0,0} + c El Full ms [40.00-1000.00]













IR spectra of the newly designed compounds

[1]

























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pound name	Company	
]	pound name	pound name Company

Sulfaguanidine	AL Nasr Pharmaceutical Chemicals Company	
Ethyl acetoacetate	Sigma-Aldrich	
4-chloro benzaldehyde	Merck	
4-methoxy benzaldehyde	Merck	
4-hydroxy benzaldehyde	Merck	
3-hydroxy-4-methoxy benzaldehyde	Merck	
Cyanoacetamide	AL Nasr Pharmaceutical Chemicals Company	
DMF	Sigma-Aldrich	
Ethanol (HPLC)	Merck	
Piperidine	Loba Chemical	
Acetyl acetone	AL Nasr Pharmaceutical Chemicals Company	
Amm. Acetate	AL Nasr Pharmaceutical Chemicals Company	
Malononitrile	Loba Chemical	
Salicylaldehyde	AL Nasr Pharmaceutical Chemicals Company	
Sulphur powder	AL Nasr Pharmaceutical Chemicals Company	

Biological Activity

1.1.1. Determination of Inhibition Zones (mm)

The preliminary antimicrobial activity of the newly modified sulfaguanidine derivatives **2a-d**, **3a-d**, **4**, **6**, **7**, **8**, **9a-b**, **10**, **11** and **12** was evaluated against eight microbial pathogen that were divided as three Gram-positive stains, besides three Gram-negative as (*B. subtilis* ATCC 6633, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212), (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *S. typhi* ATCC 6539), as well as two fungal strains *F. oxysporum* (RCMB 008002) and *C. albicans* (ATCC 10231). The antimicrobial activity performed at the bacteriology laboratory, Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. The antimicrobial potential of the newly synthesized organic compounds was investigated towards the test microorganisms and expressed as the diameter of the inhibition zones according to the agar plate diffusion method, where both tetracycline and amphotericin B were used as the positive controls. Briefly, 100 μ L of the test bacteria/fungi were grown in 10 mL of fresh media until they reached a count of approximately 108 cells/mL for bacteria or 105 cells/mL for fungi. One mL of each sample (at 0.5 mg/mL) was added to each well (10 mm diameter holes cut in the agar gel). The plates were incubated for 24 h at 37 °C (for bacteria and yeast) and for 72 h at 27 °C (for filamentous fungi), each test was repeated three times. After incubation, the microorganism's growth was observed. Tetracycline was used as standard antibacterial drugs while amphotericin B was used as a standard antifungal drug. The resulting inhibition zone s accortorls. DMSO was used for dissolving the tested compounds and showed no inhibition zones, confirming that it does not influence the growth of the tested microorganisms.

1.1.2. Determination the Minimal Inhibition Concentration (MIC)

A conventional technique termed paper disk diffusion was used to investigate the MIC of the active compounds through employing a 12.7 mm diameter filter paper (Whatman, Germany). Bacteria were grown in a media of nutrient agar, while fungi and yeasts were grown in a media of Sabouraud's agar. The synthesized compounds were dissolved and loaded on paper disks with different concentrations. Loading the drying disks over the agar plates' surface inoculated with the selected microorganisms was carried out, and then growth inhibition was tested when incubated (at 37 °C for a day) for the bacterial strains and yeasts and fungi (at 27 °C for three days); also, the MIC was confirmed by using the broth micro-dilution procedure described in the Clinical and Laboratory Standards Institute (CLSI) guidelines, where repeating of every experiment for three-times was performed for reproducibility.

1.1.3. Evaluation of the Minimum Bactericidal Concentration (MBC)

An MBC assay was conducted using the broth microdilution assay described in the above section. The MBC was determined by plating 10 μ L of culture volume from the MIC assay onto TSB agar plate and colony formation was examined after 24 h at 37 °C. The MBC is defined as the lowest compound concentration resulting in a \geq 3-log reduction in the number of colony-forming units (CFU).

1.1.4. Multidrug-Resistant Bacteria (MDRB)

To evaluate the antibacterial activity of most potent compounds toward the multidrug-resistant Gram-positive strains (*Staphylococcus aureus* ATCC 43300 and *Staphylococcus aureus* ATCC 33591) and multidrug-resistant Gram-negative strains (*Escherichia coli ATCC BAA-*196 and *Pseudomonas aeruginosa* ATCC BAA-2111). Both of the inhibition zones and MIC were determined by the same methods as described previously. The MBC assay against MDRB was carried out according to the previously reported method. Norfloxacin was chosen to be the control antibiotic.

1.1.5. Immunomodulatory Activity

In vitro intracellular killing activities

Neutrophil isolation: From three healthy volunteers (5 mL blood from each one), a sample of peripheral blood (15 mL) was taken and collected in preservative-free heparin followed by adding 4.5% dextran B in saline (2 mL). The mixed solution was shaken and kept at 37 °C for 1 h to form the erythrocytes sediment. Isolation of the neutrophils through centrifugation was performed. Hypotonic lysis was used to remove the residual erythrocytes followed by washing the neutrophils via Hank's balanced salt solution (HBSS, Sigma-Aldrich (USA), followed by suspending the washed neutrophils in the HBSS with a concentration of 25 × 10⁶ cells/mL to assess the killing efficiency toward the strains. Trypan blue exclusion was used to assess the neutrophils' viability.

Studying the activity of the intracellular killing: The activity of the intracellular killing was studied through a nitro blue tetrazolium (NBT) reduction through the developed Baehner and Nathan technique. In HBSS, the isolated neutrophils and flavonoids were incubated at 37 °C (for half an hour) followed by extracting the blue formazon (reduced dye) through pyridine and the spectrophotometric measurements were carried out at 515 nm. A negative control sample (which includes all the reagents without the neutrophil suspen-

sion) was used for comparative studies. Absorbance variation between the negative control and the cell cultures which actively phagocyte latex particles express the index of the neutrophils' intracellular killing activity. A sample's activity can be calculated by dividing the active compound's percentage ratio in the latex to the positively charged control latex.

In vivo immunomodulatory study: The immunomodulation activity of three synthetic compounds were evaluated in vivo. Male laboratory-bred Swiss albino mice (6–8 weeks old) (n = 84), weighing 20 ± 2 g, were used. They were randomly divided into five groups, three groups receiving compounds **2d**, **8** and **11** besides the positive control, and normal groups receiving vitamin C and normal diet. Immunomodulatory potential was determined by immune organ indices, immune cell population levels using the immune organ weight and index, and flow cytometry.

Relative immune organs weight: After the period of treating, mice were sacrificed, then both the spleen and thymus weight and indices were measured and calculated by divided the weight of spleen or thymus (mg) to body weight (g).

Immune cells population: Male laboratory-bred Swiss albino mice (6–8 weeks old) (n = 84), weighing approximately 20 g, were purchased from Theodor Bilharz Research Institute (TBRI), and were equally randomized into five groups. Each group was treated with the corresponding treatment according to the experimental design for 14 consecutive days. To determine the effect of the compounds on immune cell population, blood was drawn by retro-orbital puncture after a treatment period. T lymphocytes subsets (CD4⁺ and CD8⁺) from peripheral blood were measured and analyzed on FACS Calibur.

Briefly, whole blood was drawn into EDTA pre-coated 1.5 mL tubes by rectal orbital puncture and mixed, and 100 μ L added to the bottom of a well labelled tube and the appropriate primary antibody added to each tube; to a 100 μ L sample was added 0.25 μ g of FITC Hamster Anti-Mouse CD4⁺, and 0.25 μ g of APC Rat Anti-Mouse CD8⁺ (Biolegend Co. Ltd). The tubes were capped, vortexed and incubated in the dark for 25 min at room temperature, followed by the addition of 1.5 mL lysing solution vortexed, incubated at room temperature in the dark for 10–15 min and centrifuged for 5 min at 200x *g*. The supernatant was aspirated; cells fixed by resuspending in 2% paraformaldehyde buffer for 30 min at room temperature and washed. The cells were finally suspended in 500 μ L wash buffer and stored for 24 h at 4 °C in the dark. The samples were then analyzed on FACS Caliber (Merck-Millipore, Bio-Rad Beijing) with InCyte software version 2.2.2.

Ethics statement for both animal models and for using volunteer blood cells: All experimental protocols were approved and performed in compliance with the guide for the care and use of laboratory animals, published by the National Institutes of Health (USA), and performed according to Institutional guidelines. On the other hand, all volunteers gave their consent by signing a consent form before they participated.

Figures of the inhibition zone of the most promising derivatives









2D structure of Ciprofloxacin inside the active site DNA gyrase (2XCT).



3D structure of Ciprofloxacin inside the active site DNA gyrase (2XCT).



2D structure of compound 2a inside the active site DNA gyrase (2XCT).



3D structure of compound 2a inside the active site DNA gyrase (2XCT).



2D structure of compound 2b inside the active site DNA gyrase (2XCT).



3D structure of compound 2b inside the active site DNA gyrase (2XCT).


2D structure of compound 2d inside the active site DNA gyrase (2XCT).



3D structure of compound 2d inside the active site DNA gyrase (2XCT).



2D structure of compound 3a inside the active site DNA gyrase (2XCT).



3D structure of compound 3a inside the active site DNA gyrase (2XCT).



2D structure of compound 8 inside the active site DNA gyrase (2XCT).



3D structure of compound 8 inside the active site DNA gyrase (2XCT).



2D structure of compound 11 inside the active site DNA gyrase (2XCT).



3D structure of compound 11 inside the active site DNA gyrase (2XCT).



2D structure of Methotrexate (MTX) inside the active site DHGR (1DLS).



3D structure of Methotrexate (MTX) inside the active site DHGR (1DLS).



2D structure of compound 2a inside the active site DHGR (1DLS).



3D structure of compound 2a inside the active site DHGR (1DLS).



2D structure of compound 2b inside the active site DHGR (1DLS).



3D structure of compound 2b inside the active site DHGR (1DLS).



2D structure of compound 2d inside the active site DHGR (1DLS).



3D structure of compound 2d inside the active site DHGR (1DLS).



2D structure of compound 3a inside the active site DHGR (1DLS).



3D structure of compound 3a inside the active site DHGR (1DLS).



2D structure of compound 8 inside the active site DHGR (1DLS).



3D structure of compound 8 inside the active site DHGR (1DLS).



2D structure of compound 11 inside the active site DHGR (1DLS).





3D structure of compound 11 inside the active site DHGR (1DLS).