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Preparation and biological evaluation of metronidazole derivatives with monoterpenes and eugenol

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Running title: Preparation of metronidazole derivatives

Abstract

Two series of metronidazole derivatives (ester derivatives and ether derivatives) were prepared reacting metronidazole and its acetic acid oxidized form with menthol, thymol, carvacrol and, eugenol. Both series of compounds were tested in vitro against two strains of *H. pylori* (the ATCC 26695 and P12), and one strain of *Clostridium* (*Clostridium perfringens*). Most of the prepared compounds showed biological activity against the targeted bacteria. Compound **11** was highly active against all tested bacterial strains, especially against P12 with IC₅₀ 0.0011 μM/mL. Compound **6** was highly active against *Clostridium perfringens* with MIC 0.0094 nM/mL. Viability test was conducted for compound **11** to test its selectivity for normal human fetal lung fibroblasts (MRC5), and it was found to be non-toxic with IC₅₀ more than 50 μM/mL.

Keywords: metronidazole, monoterpenes, menthol, thymol, carvacrol, eugenol, *H. pylori*, *clostridium perfringens*

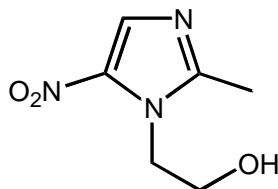
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1. Introduction

Metronidazole (**1**), a 5-nitroimidazole derivative, is a well-known and largely utilized agent in the treatment of anaerobic infections and parasites^[1]. It is considered by far the drug of choice for anaerobic infections^[2]. The use of metronidazole in diverse therapeutic regimens for the treatment of gastric ulcer caused by the facultative anaerobe *Helicobacter pylori* (*H. pylori*), represented one of the most important uses of metronidazole^[3].

Metronidazole was and still is a reliable antibacterial with no evidence of serious bacterial resistance issues^[4]. However, bacterial resistance for some pathogens such as *H. pylori* may occur^[5-7]. Other unwanted side effects and safety issues may also occur, even though it is known to be highly tolerable^{[8][2]}.



Metronidazole (1)

Recently, there were various efforts to develop new nitroimidazole compounds, and specifically metronidazole derivatives possessing improved biological activity, such as the development of metronidazole-triazole hybrids with improved anti-methicillin resistant *Staphylococcus aureus* activity (figure 1 A)^[9], and the preparation of derivatives containing piperazine backbone expressing good anti *Staphylococcus aureus* activity (figure 1 B)^[10]. Metronidazole-triazole conjugates were also developed with noticeable broad-spectrum antimicrobial and antiparasitic activity comprising *Clostridium difficile*, *Entamoeba histolytica*, *Giardia lamblia* and *H. pylori* (figure 1 C)^[11], and the preparation of metronidazole-pyrazole derivatives acting through tyrosyl-tRNA synthetase inhibition and demonstrating growth inhibitory activity of *Pseudomonas aeruginosa* (figure 1 D)^[12]

Monoterpenes such as menthol, thymol, and carvacrol possess multi-biological activities. It is well documented that menthol possesses cooling, local anesthetic, analgesic, antiseptic and antibacterial activities^[13,14]. Thymol, on the other hand, possesses antioxidant, anti-inflammatory, local anesthetic, antinociceptive, cicatrizing, antiseptic, antibacterial, and antifungal properties^{[15][16]}. Carvacrol has much in common with thymol possessing antimicrobial, antimutagenic, antigenotoxic, analgesic, antispasmodic, anti-inflammatory, angiogenic, antiparasitic, antiplatelet, AChE inhibition, anti-elastase, insecticidal, antihepatotoxic and hepatoprotective activities^{[17][18]}.

The phenylpropene derivative eugenol is known for its antioxidant, antibacterial, and antifungal activities^[17,19,20] and in particular, it demonstrated an anti-*H. pylori* activity on *H. pylori* ATCC26695 and other clinical isolates comparable to amoxicillin^[21].

Menthol, thymol, and eugenol, being known for their safe biological profile, were also selected to prepare safe prodrugs or hybrid drugs in combination with other known active entities, such the anti-inflammatory agents ibuprofen, and mefenamic acid in order to reduce their gastrointestinal side effects and potentiate their analgesic activity.^[22,23]

In this paper, we aimed to prepare hybrid drugs consisting of two pharmacologically active entities, the first one being metronidazole with known anaerobic antibacterial activity and selected monoterpenes and phenylpropenes with known antioxidant and antibacterial activities. The aim of this combination is to produce synergistic effects able to combat resistant bacteria or parasites.

2. Materials and Methods

The reference standard of metronidazole, (-)-menthol, thymol, eugenol, carvacrol, N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP), 4-methylbenzenesulfonyl chloride and all other chemicals used for the synthesis were purchased from Sigma Aldrich Chemical Co. (USA). The synthesis was carried out using schemes 1 and 2. Thin layer chromatography was performed to monitor the progress of the reaction and different solvents were used for the developing system. The spots were monitored using iodine chamber and UV lamp at 254 nm. Melting point was recorded for the synthesized compounds using Stuart (SMP3) Melting point apparatus. IR spectra were performed using Tensor 37 FTIR (Bruker Corporation) by

KBr disc method, with OPUS 7.2 software. The ^1H NMR and ^{13}C NMR spectra using CDCl_3 and $\text{DMSO}-d_6$ as solvents were recorded on Bruker 500 MHz spectrometer with TopSpin 3.2 software. Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). The ESI-MS spectra were recorded on a Bruker Q-ToF mass spectrometer.

2.1 General Chemistry

2.1.1 General procedure for the synthesis of metronidazole ester compounds

Synthesis of 2-(2-Methyl-5-nitro-1H-imidazol-1-yl) acetic acid (2)

The synthesis of compound (**2**) was achieved using a reported method with slight modification^[24]. In a stirring suspension of metronidazole (3.42 g, 20 mM) in 10 mL, H_2O at room temperature, (4.36 g 15 mM) of potassium dichromate in 10 mL H_2O was added followed by dropwise addition of 5 mL sulfuric acid 50% in ice water. The mixture was stirred overnight while the progress of the reaction was monitored by using TLC (ethyl acetate: methanol, 9.5: 0.5, v/v). The reaction mixture was carefully neutralized with 4M NaOH and was extracted 3 times with ethyl acetate: tetrahydrofuran (1:1) solvent system. Anhydrous Na_2SO_4 was used to dry the combined organic layer and the solvent was evaporated to get pure compound (66%) (**2**), mp 176-178 $^\circ\text{C}$ ^[25] and data of the IR matched those reported in the literature^[25], IR (KBr, cm^{-1}) ν : 3441 (OH), 1721 (C=O), 1547, 1357 (NO_2); ^1H NMR ($\text{DMSO}-d_6$): δ 8.09 (s, 1H, imidazole), 5.10 (s, 2H, N- CH_2), 2.44 (s, 3H, CH_3 -imidazole). ^{13}C NMR: δ 169 (COOH), 152 (CH_3 -C=N), 139 (O_2N -C=C), 133 (O_2N -C=C), 48 (N- CH_2 -COOH), 14 (CH_3 , imidazole). DEPT-135: 48 (N- CH_2 -COOH) negative signal. MS: m/z (%) 185 (M+, 40), 139 (60), 109 (23), 83 (25), 80 (50), 54 (80), 52 (100)^[25].

Synthesis of metronidazole esters with (-)-menthol, thymol, eugenol, and carvacrol

In a typical reaction^[26], menthol (0.79 g, 5 mM) was dissolved in dichloromethane (20 mL) and 2-(2-methyl-5-nitro-1H-imidazol-1-yl)acetic acid (0.925 g, 5 mM) and 4-dimethylamino pyridine (DMAP) (0.18 g, 1.5 mM) were added at 0 $^\circ\text{C}$. Dicyclohexylcarbodiimide (DCC) (1.24 g, 6 mM) was added after five minutes to the above reaction mixture and then left on stirring for 50 h at room temperature. TLC was used to monitor the progress of the reaction. 20 mL HCl (0.5 M) was initially used to wash the reaction products and then the reaction mixture was basified with saturated NaHCO_3 . The mixture was extracted with dichloromethane, dried with Na_2SO_4 and solvent was evaporated to get crude product. The mixture of the crude product was purified by using silica gel chromatography (ethyl acetate: hexane, 13: 12 to 15: 12) to get pure compound (**3**) (51%) yield (Scheme 1).

For thymol, eugenol, and carvacrol derivatives 2-(2-Methyl-5-nitro-1H-imidazol-1-yl)acetic acid (0.1 mol) was dissolved in a minimum amount of DMF (≈ 2 mL), the solution was stirred in an ice bath (0 $^\circ\text{C}$) for 10 minutes. Thymol (0.12 M) and DMAP (35 mM) were dissolved in dichloromethane (5 mL) then added to the above solution. After 10 minutes DCC (0.12 M) was added to the reaction mixture. The reaction left at room temperature for 50 hours, the reaction was checked by TLC. The reaction was quenched with 20 mL HCl (1.0 M) and then basified with a saturated NaHCO_3 solution. The reaction mixture was extracted with 3 \times 10 mL dichloromethane and the organic layer was combined and washed with water (30 mL) three times. The organic layer was separated and dried with Na_2SO_4 , then evaporated in vacuum to give the crude product. Recrystallization using ethanol gave off-white crystal (48%) with thymol (**4**) and white powder with eugenol (**5**) (59%). For carvacrol, the crude product was purified by using silica gel chromatography (ethyl acetate: hexane, 13: 12 to 15: 12) to get pure compound (**6**) (40%) (Scheme 1).

Metronidazole menthol ester (3)

White solid, Melting point: 135-137 $^\circ\text{C}$ with decomposition; IR (KBr, cm^{-1}) ν : 1747 (C=O), 1575, 1365 (NO_2); 1242, 1273 (C-O, ester); ^1H NMR (CDCl_3): δ 8.05 (s, 1H, imidazole), 5.0 (s, 2H, N- CH_2), 4.7 (m, 1H, HC-OOC), 2.41 (s, 3H, CH_3 -imidazole), 1.89 (m, 1H, HC- $\text{CH}(\text{CH}_3)_2$), 1.61 (m, 6H, cyclohexane), 0.95 (m, 6 H, CH_3 -CH- CH_3). ^{13}C NMR: δ 165.71 (- CH_2 -COO), 157.32 (N-C=N), 150.32 (CH=C- NO_2), 131.84 (N-CH=C), 76.76 (-CH-O-CO), 49.96 (-CH-CH, ring), 47.14 (N- CH_2), 40.60 (CH_2 -CH-O), 33.66 (CH_2 -CH-), 31.40 (CH_3 -CH), 25.36 (CH_3 -CH- CH_3), 23.33 (CH_2 - CH_2 -CH, menthol), 20.66 (3 CH_3 group of menthol), 13.90 (CH_3 , imidazole); DEPT-135; 47.14 (N- CH_2 -COO), 40.6 (CH_2 -CH-O), 23.33, 33.66 (CH_2 - CH_2 -CH, menthol), negative. ESI-MS (m/z): (M+H) 324.1915, calculated: 324.19178

Metronidazole thymol ester (4)

Brown solid, Melting point: 97-99 °C with decomposition, IR (KBr, cm^{-1}) ν : 1769 (C=O), 1530, 1372 (NO_2); 1466 (C=C aromatic), 1272 (C-O, ester); ^1H NMR (CDCl_3): δ 7.97 (s, 1H, imidazole), 7.13 (d, 1H, aromatic), 6.99 (m, 1H, aromatic) 6.77 (d, 1H, aromatic), 5.25 (s, 2H, N- CH_2), 2.86 (m, 1H, CH_3 -CH- CH_3), 2.52 (s, 3H, CH_3 -imidazole), 2.25 (m, 3H, aromatic- CH_3), 1.12 (m, 6 H, $\text{HC}(\text{CH}_3)_2$). ^{13}C NMR: δ 164.92 (- CH_2 -COO), 150.22 (CH=C- NO_2), 147.14 (OOC-C, aromatic), 140.55 (N-C=N), 139.1 (HC-C, aromatic), 136.85 (H_3C -C, aromatic), 131.0 (N-CH=C), 127.47 (CH aromatic), 126.71 (CH aromatic), 122.08 (CH aromatic), 47.38 (N- CH_2), 27.13 (CH_3 -CH- CH_3), 23.01 (2 CH_3 group -CH), 20.85 (CH_3 -aromatic), 13.77 (CH_3 , imidazole); DEPT-135; 47.33 (N- CH_2 -COO) negative. ESI-MS (m/z): (M+H) 318.1449, calculated: 318.1447.

Metronidazole eugenol ester (5)

White powder, Melting point: 78-80°C, IR (KBr, cm^{-1}) ν : 1767 (C=O), 1535, 1366 (NO_2); 1477 (C=C aromatic), 1250, 1206 (C-O, ester); ^1H NMR (CDCl_3): δ 8.03 (s, 1H, imidazole), 6.91-7.15 (m, 3H, aromatic), 5.95 (m, 1H, CH=CH₂), 5.37 (s, 2H, N-CH₂), 5.13 (d, 2H, CH=CH₂), 3.80 (s, 3H, OCH₃), 3.40 (d, 2H, CH₂-aromatic), 2.62 (s, 3H, CH₃-imidazole). ^{13}C NMR: δ 166.25 (-CH-O-CO), 152.06 (aromatic-OCH₃), 150.73 (CH=C- NO_2), 139.93 (N-C=N), 137.81 (-CH=CH₂), 137.34 (aromatic-CH₂), 132.91 (aromatic-OCO), 122.34 (CH aromatic), 120.80 (CH aromatic), 116.16 (CH aromatic), 113.56 (-CH=CH₂), 56.26 (N-CH₂), 47.58 (CH₃-O), 40.04 (aromatic-CH₂-CH=), 14.07 (CH₃, imidazole); DEPT-135; 136.78 (aromatic-CH₂), 112.75 (-CH=CH₂), 55.93 (N-CH₂) negative. ESI-MS (m/z): (M+H) 332.1241, calculated: 332.1241

Metronidazole carvacrol ester (6)

Yellow solid, Melting point: 102-104°C, IR (KBr, cm^{-1}) ν : 1758 (C=O), 1531, 1366 (NO_2); 1466 (C=C aromatic), 1269, 1221 (C-O, ester); ^1H NMR (CDCl_3): δ 7.91 (s, 1H, imidazole), 7.2 (s, 1H, aromatic), 6.9-6.6 (m, 2H, aromatic), 5.07 (s, 2H, N-CH₂), 2.74 (m, 1H, CH_3 -CH- CH_3), 2.48 (s, 3H, CH_3 -imidazole), 2.14 (dd, 3H, aromatic- CH_3), 1.24 (m, 6 H, CH_3 -CH- CH_3). ^{13}C NMR: δ 166.17 (- CH_2 -COO), 150.41 (CH=C- NO_2), 148.30 (OOC-C, aromatic), 138.41 (N-C=N), 131.89 (HC-C, aromatic), 130.78 (H_3C -C, aromatic), 121.0 (N-CH=C), 118.47 (CH aromatic), 113.00 (CH aromatic), 47.26 (N-CH₂), 31.01 (CH_3 -CH- CH_3), 25.94 (2 CH_3 group -CH), 23.95 (CH_3 -aromatic), 13.93 (CH_3 , imidazole); DEPT-135: 47.26 (N-CH₂) negative. ESI-MS (m/z): (M+CAN+H) 359.1004, calculated: 359.1713

2.1.2 General procedure for the synthesis of metronidazole ether compounds (8-11)

2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methyl benzenesulfonate (7)

The reported procedure followed for the synthesis of mentioned compound [27]. Metronidazole (3.4 g, 20 mM) and triethylamine (3.0 mL, 22 mM) were dissolved in CH_2Cl_2 (20 mL), and 4-methylbenzenesulfonyl chloride (4.0 g, 21 mM) in CH_2Cl_2 (10 mL) was added. The reaction took place at 0 °C and continued for 5 h. Ice water (30 mL) was then added. Ethyl acetate was used to extract the product (4x30 mL). The organic layer was extracted a final time with a saturated solution of NaHCO_3 . The combined organic layer was then dried over anhydrous Sodium Sulfate. Removal of the solvent and crystallization in ethanol gave slight yellow crystals of required compound (7) (85% yields) (Scheme 2).

For menthol derivative (8), 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methyl benzenesulfonate (7) (6.2 mM, 2.01 g) and K_2CO_3 (15 mM, 2.1 g) were dissolved in DMF (20 mL). Then menthol was added (6.5 mM, 1.02 g) in DMF (10 mL), the reaction mixture was stirred at 70–80 °C for 14–16 h. After the reaction, the yellow solid was precipitated by addition of crushed ice. The precipitate was washed thoroughly with water to remove DMF and then filtered, dried and target product was separated by column chromatography (ethyl acetate: hexane, 1: 9, 6: 4) to get pure compound (8) (73%). For thymol derivative, 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methylbenzenesulfonate (6.2 mM, 2.01 g) and K_2CO_3 (15 mM, 2.1 g) were dissolved in DMF (20 mL). Add thymol (6.5 mM, 1.0 g) in DMF (10 mL) and the reaction mixture was stirred at 70–80 °C for 14–16 h. After the reaction, crushed ice was added and oily liquid appeared which extracted by addition of ethyl acetate. The target product was separated by column chromatography (ethyl acetate: hexane, 1: 9 to 6: 4) to get pure compound (9) (59%). For eugenol derivative, 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methylbenzenesulfonate (6.2 mM, 2.01 g) and K_2CO_3 (15 mM, 2.1 g) were dissolved in DMF (20 mL). Add eugenol (6.5 mM, 1.06 g) in DMF (10 mL) and the reaction mixture was stirred at 70–80 °C for 14–16 h to complete the reaction. The off-white color precipitate was appeared after addition of crushed ice, was extracted by addition of ethyl acetate. The target product (10) was separated by column chromatography to get pure compound (ethyl acetate: hexane, 3: 7 to 4:

6) (40%). For carvacrol compound, 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methyl benzenesulfonate (6.2 mM, 2.01 g) and K_2CO_3 (15 mM, 2.1 g) were dissolved in DMF (20 mL). Add carvacrol (6.5 mM, 1.0 g) in DMF (10 mL) and the reaction mixture was stirred at 70–80 °C for 14–16 h. The crushed ice was added and the compound (**11**) obtained was extracted by addition of ethyl acetate. The target product was separated by column chromatography (ethyl acetate: hexane, 3: 7 to 4: 6) to get pure compound (51%).

Synthesis of metronidazole menthol ether (**8**)

Yellow Solid, Melting point: 135-136 °C; IR (KBr, cm^{-1}) ν : 1449, 1368 (NO_2); 1226, 1173 (C-O, ether); 1H NMR ($CDCl_3$): δ 8.05 (s, 1H, imidazole), 5.0 (t, 2H, N- CH_2), 4.7 (t, 2H, CH_2 -O), 2.7 (t, 1H, O-CH, ring), 2.42 (s, 3H, CH_3 -imidazole), 2.0 (m, 1H, HC- $CH(CH_3)_2$), 1.01 (m, 6H, CH_3 -CH- CH_3). ^{13}C NMR: δ 155.0 (N-C=N), 130.41 ($CH=C-NO_2$), 127.70 (N-CH=C), 71.59 (-CH-O-C), 50.16 (-CH-CH, ring), 46.86 (CH_2 .ring), 45.05 (CH_2 .ring), 40.16 (CH-N), 34.55 (CH_2 -CH-), 31.65 (CH_3 -CH), 25.88 (CH_3 -CH- CH_3), 23.16 (CH_2 - CH_2 -CH, menthol), 22.22 (CH_2 .ring), 21.74 (3 CH_3 group of menthol), 16.12 (CH_3 , imidazole); DEPT-135; 46.76 (N- CH_2), 45.03 (CH_2 .ring), 34.55 (CH_2 -CH-), 23.16 (CH_2 - CH_2 -CH, menthol) (negative). ESI-MS (m/z): (M+H) 310.2337 Calculated: 310.2125.

Synthesis of metronidazole thymol ether (**9**)

Brown solid, Melting point: 116-118 °C with decomposition; IR (KBr, cm^{-1}) ν : 1536, 1367 (NO_2); 1235, 1188 (C-O, ether); 1H NMR ($CDCl_3$): δ 7.98 (s, 1H, imidazole), 7.02 (d, 1H, aromatic), 6.72 (d, 1H, aromatic), 6.52 (s, 1H, aromatic), 4.72 (t, 2H, O- CH_2), 4.23 (t, 2H, N- CH_2), 2.87 (m, 1H, CH_3 -CH- CH_3), 2.69 (s, 3H, CH_3 -imidazole), 2.23 (s, 3H, H_3C -O-aromatic), 1.03 (m, 6H, HC(CH_3) $_2$). ^{13}C NMR: δ , 154.58 (C=C-O), 150.76 ($CH=C-NO_2$), 138.09 ($O_2N-C=C$), 136 (CH, aromatic), 133.79 (CH, aromatic), 129.40 (N-CH=C), 126 (CH Ar), 126.79 (CH aromatic), 122.65 (=CH-CH=, aromatic), 112.43 (CH=CH, aromatic), 66.89 (O- CH_2), 46.76 (N- CH_2), 26.43 (CH_3 -CH- CH_3), 22.77 (2 CH_3 group -CH), 21.24 (CH_3 -aromatic), 13.78 (CH_3 , imidazole). DEPT-135; 66.89 (O- CH_2), 46.76 (N- CH_2) negative. ESI-MS (m/z): (M+H) 304.1660, calculated: 304.1655.

Synthesis of metronidazole eugenol ether (**10**)

Yellow solid, Melting point: 96-98°C; IR (KBr, cm^{-1}) ν : 1593, 1366 (NO_2); 1260, 1191 (C-O, ether); 1H NMR ($CDCl_3$): δ 8.06 (s, 1H, imidazole), 6.75 (dd, 3H aromatic), 5.9 (m, 1H, $CH=CH_2$), 5.08 (m, 2H, $CH=CH_2$), 4.77 (m, 2H, O- CH_2), 4.32 (m, 2H, N- CH_2), 3.78 (s, 3H, OCH_3), 3.39 (d, 2H, CH_2 -aromatic), 2.85 (s, 3H, CH_3 -imidazole). ^{13}C NMR: δ 151.91 (N-C=N), 149.45 (C=C- OCH_3 , aromatic), 146.45 ($CH=C-NO_2$), 145 (O-C=C, aromatic), 137 ($CH_2=CH$, aliphatic), 136 (CH, aromatic), 134.44 ($O_2N-C=C$), 120.40 (=CH-CH=, aromatic), 115.90 ($CH_2=CH$, aliphatic), 113.88 (CH=CH, aromatic), 112.0 (CH=CH, aromatic), 67.85 (CH_2 - CH_2 -O), 55.58 (O- CH_3), 47.10 (aromatic- CH_2), 39.80 (N- CH_2), 13.45 (CH_3 , imidazole), DEPT-135; 115.90 ($CH_2=CH$, aliphatic), 67.65 (CH_2 - CH_2 -O), 47.10 (aromatic- CH_2), 39.80 (N- CH_2) all are negative. ESI-MS (m/z): (M+H) 318.1444 calculated: 318.1449.

Synthesis of metronidazole carvacrol ether (**11**)

Yellow solid, Melting point: 87-89°C with decomposition; IR (KBr, cm^{-1}) ν : 1578, 1360 (NO_2); 1254, 1187 (C-O, ether); 1H NMR ($CDCl_3$): δ 7.98 (s, 1H, imidazole), 6.95 (d, 1H, aromatic), 6.72 (d, 1H, aromatic), 6.50 (s, 1H, aromatic), 4.6 (t, 2H, O- CH_2), 4.23 (t, 2H, N- CH_2), 2.85 (m, 1H, CH_3 -CH- CH_3), 2.60 (s, 3H, CH_3 -imidazole), 1.98 (s, 3H, H_3C -O-aromatic), 1.10 (m, 6H, HC(CH_3) $_2$). ^{13}C NMR: δ 155.32 (N-C=N), 147.5 ($CH=C-NO_2$), 131.94 (aromatic, CH_2), 130 (N-CH=C), 129.80 (CH, aromatic), 122.35 (CH, aromatic), 117.0 (CH, aromatic), 116.39 (CH, aromatic), 110.76 (CH, aromatic), 109.0 (CH, aromatic), 67.1 (CH_2 - CH_2 -O), 34.5 (N- CH_2), 27.13 (CH_3 -CH- CH_3), 23.50 (2 CH_3 , carvacrol), 15.0 (CH_3 , carvacrol), 13.90 (CH_3 , imidazole). DEPT-135; 39.80 (N- CH_2), 47.10 (aromatic- CH_2), 67.65 (CH_2 - CH_2 -O), 115.90 ($CH_2=CH$, aliphatic) all are negative. ESI-MS (m/z): (M+H) 304.1660, calculated: 304.1655.

2.2 Biology

2.2.1 MTT based Anti *H. pylori* activity

Two strains of *H. pylori* the ATCC 26695 and *H. pylori* P12 were screened. Antibacterial activity of the eight compounds were evaluated by MTT assay as previously described in literature with some modifications^{[28][29]} Each of the two strains was cultured in heart infusion broth (BHI). Final working solution (optical density 0.2)

was taken by diluting the bacterial stock suspension in BHI. This bacterial dilution (200 μL) was added into the wells of a 96-microtiter plate and incubated at 37 $^{\circ}\text{C}$ overnight. Final compound concentrations were 0.001-100 μM (DMSO 0.1%). Each concentration was tested in triplicates, and experiments were repeated three times. Plates were incubated for 48 h, then freshly prepared and filtered MTT (20 μL) was added to each well. Plates were incubated for 4 h, then centrifuged at 4000 rpm for 2 minutes. The supernatant was aspirated, and 50 μL of DMSO was added to each well for 20 mins. Absorbance was read on the multi-plate reader. The optical density of the purple formazan A_{570} is proportional to the number of viable cells. When the amount of formazan produced by treated cells is compared with the amount of formazan produced by untreated control cells, the strength of the drug in causing growth inhibition can be determined, thus compound concentration causing 50% inhibition (IC_{50}) compared to control cell growth (100%) was determined. GraphPad Prism version 5.00 for Windows, GraphPad software, San Diego California USA was used for analysis.

2.2.2 Anti *Clostridium perfringens* activity

Antimicrobial activity was assayed by diffusion method, according to diffusion assay on agar plates as described in National Committee for Clinical Laboratory Standards^[30].

Tested bacterial strain, *Clostridium perfringens* (ATCC 13124) was cultivated on blood agar medium in anaerobic conditions for 48 h. The bacterial strain was suspended in Mueller Hinton broth and adjusted to 0.5 McFarland turbidity. The surface of blood agar plates was swabbed in three directions by Standard Inoculum, using sterile cotton swabs. The plates were allowed to dry within 10 minutes before cutting the wells 7mm into blood agar using sterile sharp glass rods. After that, the wells were filled with 150 μL from different dilutions: 1.25, 2.5, 5, 10 mg/mL of the synthesized compounds plus metronidazole as a reference.

All plates were incubated at 37 $^{\circ}\text{C}$ for 48 hours in anaerobic conditions. After the incubation period, the plates were examined and the diameter of each zone was measured for inhibition zone formed around the well for each compound, and the results were recorded. MIC (minimum inhibitory concentration) was then calculated in $\mu\text{g}/\text{mL}$ and recalculated as nM/mL using regression analysis as reported in the literature^[31].

2.2.3 Cell culture

MRC5 (Normal human fetal lung fibroblast), which was obtained from the ATCC, was sub-cultured in Eagles minimum essential medium (EMEM, 10% FBS) at 37 $^{\circ}\text{C}$, 5% CO_2 , and 100 % relative humidity.

2.2.4 MTT assay for MRC5 viability

The viability of normal fibroblast cells (MRC5) was tested by MTT assay, following its treatment with the reference drug (metronidazole) and compound **11** separately, MRC5 cells were cultured in 96-well ($3 \times 10^3/\text{well}$) and incubated at 37 $^{\circ}\text{C}$ overnight. Final compounds concentrations: 0, 0.1, 1, 10, 25, 50 $\mu\text{M}/\text{mL}$ (DMSO 0.1%; n= 3). Plates were incubated for 72 h, followed by addition of MTT to each well. Plates were incubated for 3 h, then the supernatant was aspirated, and DMSO was added to each well. Absorbance was read on multi-plate reader at A_{570} ^[32].

3. Results

3.1 Chemistry

Metronidazole conjugates were prepared in two different series. The first series key reagent metronidazole carboxylic acid was obtained from the oxidation of the commercially available metronidazole employing potassium dichromate, as shown in scheme 1^[24]. Metronidazole carboxylic acid was then reacted with selected monoterpenes (menthol, thymol, and carvacrol) and the phenylpropanoid eugenol, the reaction was completed using DCC/DMAP^[22].

The second series preparation was conducted in two steps, the first step consisted in the preparation of the precursor, metronidazole tosylate (**7**), The second step involved the conjugation of the tosylate with the same monoterpenes of the first series and with eugenol^[27]. The reactions proceeded easily and with good yield. All the synthesized compounds were characterized by ^1H NMR, ^{13}C NMR, IR and HRMS.

3.2 Biology

3.2.1 MTT based anti-*H. pylori* test

The MTT based anti *H. pylori* test which was implemented using the two different strains, ATCC 26695 and *H. pylori* P12, revealed very good IC₅₀ values in the micromolar range for most of the derivatives, especially the carvacrol ether (**11**), which showed activity in nanogram for the *H. pylori* P12 strain (as shown in table 1). It is worth noting that ether derivatives were, in general, more active than the esters.

3.2.2 Anti *Clostridium perfringens* assay

The activity of the prepared compounds against *Clostridium perfringens* (ATCC 13124) was tested using the diffusion method. Appreciable activity for most of the prepared derivatives was observed, showing values in the nanomolar concentrations in most cases. The most positive results are observed in the carvacrol hybrids (compounds **6** and **11**), especially compound **6** which had a comparable activity to metronidazole which was used as a reference (table 1).

3.2.3 MRC5 viability assay

To investigate the selectivity and safety of the synthesized products towards normal human cells, compound **11** was selected to investigate its effect on MRC5 using MTT assay and compared with the safety of metronidazole. Compound **11** showed no cytotoxicity at IC₅₀ 50 µM/mL, while metronidazole showed toxicity at 49.4 µM/mL. Compound **11** was selected for this test because it was the derivative with the most pronounced anti-bacterial activity in all tested strains.

4. Discussion

Two new series of metronidazole conjugates with selected monoterpenes and eugenol were synthesized. The first series consisted of ester conjugates and was prepared by reacting the metronidazole oxidized to acetic acid and monoterpenes and eugenol using DCC according to procedures reported in the literature (scheme 1)^[24,26]. The synthesized compounds were confirmed by FTIR, NMR and MS. Infrared spectra showed the characteristics band of C=O stretching in range of 1750-1800 cm⁻¹, C-O stretching in range of 1200-1280 cm⁻¹, and disappearance of OH stretching, which confirms the formation of the ester. The ¹H NMR spectra showed characteristic chemical shift, and mass spectra showed the parent peak which anticipated the structure of compounds. The second series consisted of ether derivatives resulting from the reaction between metronidazole tosylate and monoterpenes and eugenol (scheme 2)^[33]. Infrared spectra showed the characteristics band of C-O stretching in range of 1200-1280 cm⁻¹, and disappear of OH stretching, which confirm the formation of ether. The ¹H NMR spectra showed characteristic chemical shift which anticipated the structure of compounds, and mass spectra showed the parent peak confirming the molecular weight of target derivatives. Both reactions proceeded smoothly and with good yields.

The primary purpose of this strategy is to combine the well-known anti-bacterial activity of metronidazole, with the established anti-bacterial and anti-oxidant activity of monoterpenes and eugenol. And to obtain a synergistic effect, especially against gut pathogens. Similar approach was previously reported with tetracycline and benzalkonium chloride in the same culture^[34].

The investigation of these new derivatives against *H. pylori* ATCC 26695 and P12; and against *Clostridium perfringens* demonstrated good activity against *H. pylori* strains, especially in favour of the ether series revealing IC₅₀ values in the micromolar range.

Compound **11**, as seen in table 1, has demonstrated high potency in all three strains, being active in micromolar concentration (0.0011 µM/mL) against *H. pylori* P12 strain. And in the nanomolar range (IC₅₀: 4.92 µM/mL in ATCC 26695 strain; and MIC 2.7nM/mL against *clostridium perfringens*, table 1).

Six out of the eight synthesized compounds exhibited activity against *Clostridium perfringens*; however, in this case there are no net differences between the ether series and the ester one. In fact, it can be observed that both carvacrol ester and ether derivatives (comp. **6** and comp. **11**) have high potencies (MIC=0.0094 nM/mL and MIC =2.7nM/mL respectively), while the absence of activity in the ester and ether derivatives of menthol can be observed (comp. 3 and comp. 8, table 1).

Taking in consideration these results, it is assumed that the main factor in the anti-*H. pylori* activity is primarily related to metronidazole itself; while the anti-*Clostridium* activity seems to be related to the monoterpene, focusing the anti-*clostridium* activity within the carvacrol derivatives.

Compound **11**, which was the derivative with the highest potency against bacterial strains, was found to be selective and non-cytotoxic to the normal human fetal lung fibroblasts, which is a clear indication of the safety profile of these products to host cells.

5. Conclusion

Metronidazole ester and ether derivatives with selected monoterpenes and eugenol were successfully and easily prepared through two different synthetic schemes.

Biological testing against two strains of *H. pylori* (the ATCC 26695 and P12), and one strain of *Clostridium (clostridium perfringens)*, showed appreciable activity for most of the derivatives and in special way for compound **11**. The latter is the metronidazole carvacrol ether derivative, which has shown excellent activity against P12 *H. pylori* strain achieving 0.0011 $\mu\text{M}/\text{mL}$ activity against the *H. pylori* P12 strain, 4.9 $\mu\text{M}/\text{mL}$ in the ATCC 26695 strain, and MIC 2.7 nM/mL in *Clostridium perfringens*. Compound **11**, being the most promising derivative, was tested for its selectivity against normal human fetal lung fibroblasts and was found safe at IC₅₀ 50 $\mu\text{M}/\text{mL}$, even safer than the reference compound (metronidazole) which showed toxicity at 49.4 $\mu\text{M}/\text{mL}$.

These results clearly show that the prepared derivatives represent serious candidates, which could be qualified for future development and for further testing against other potential susceptible microorganisms.

Supplementary Materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

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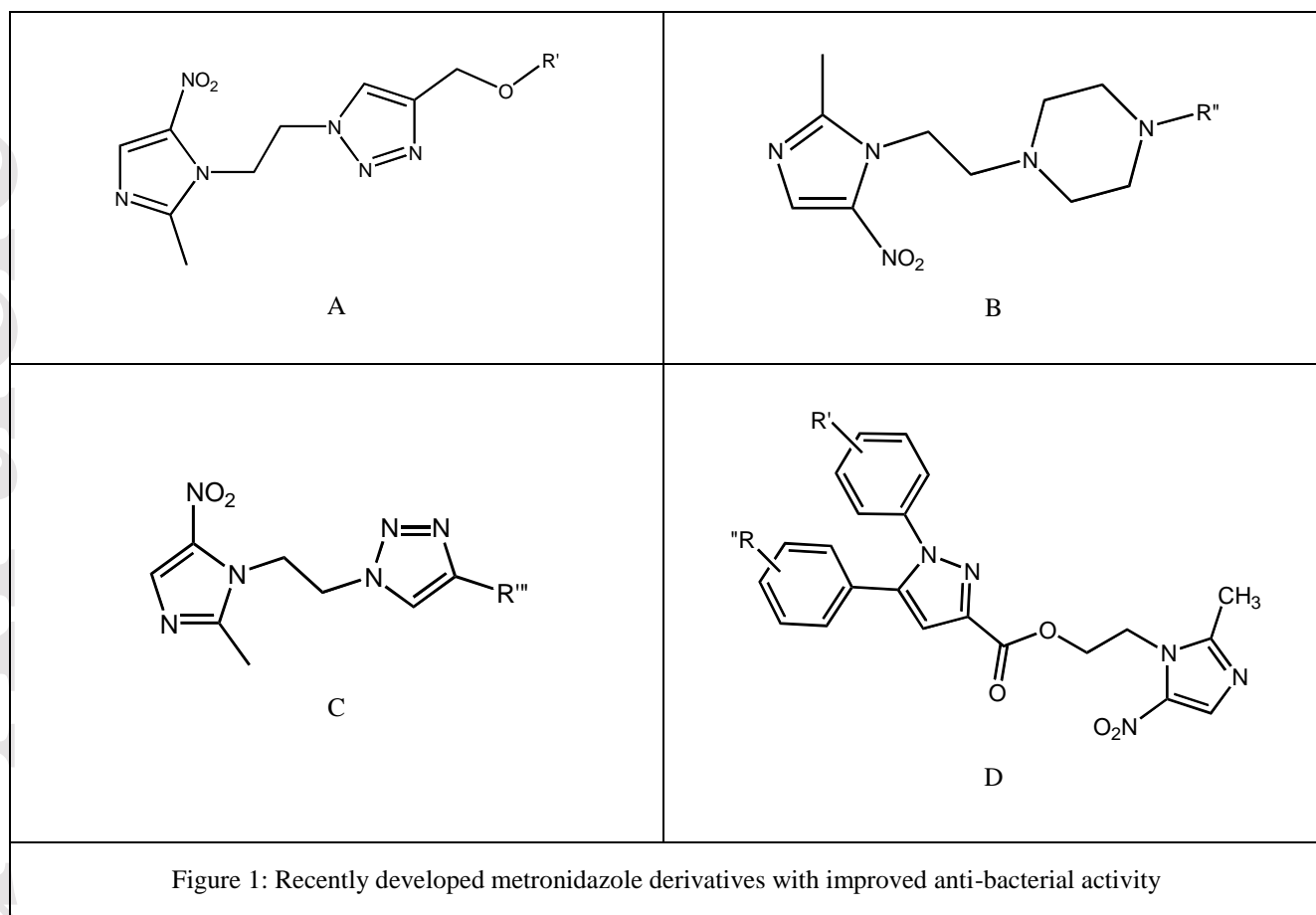
Competing interests

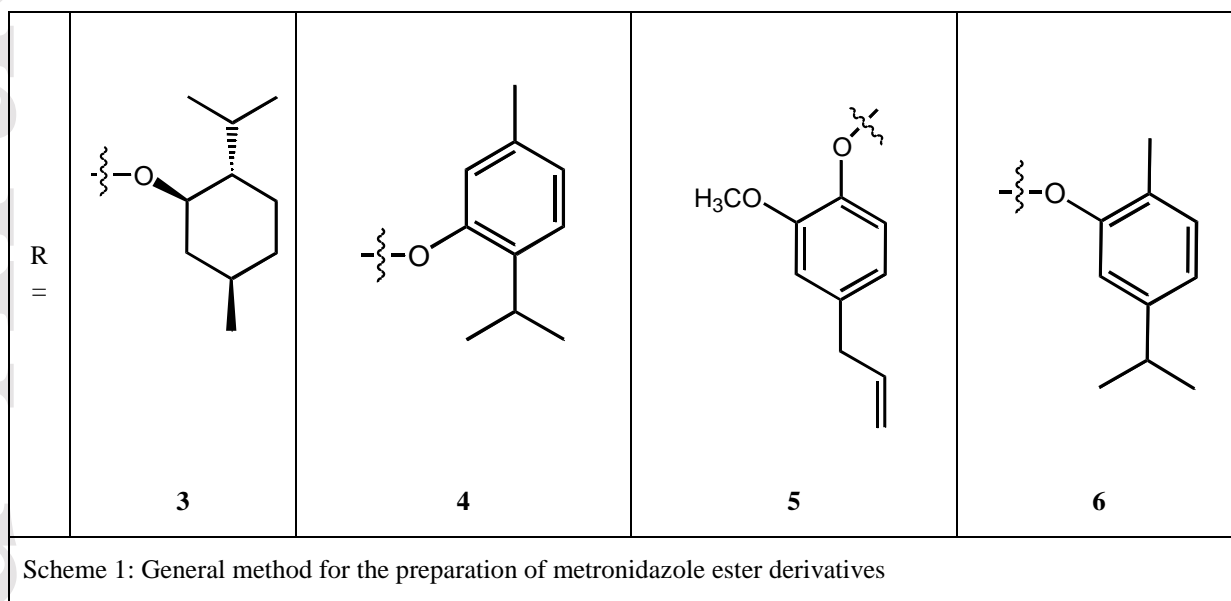
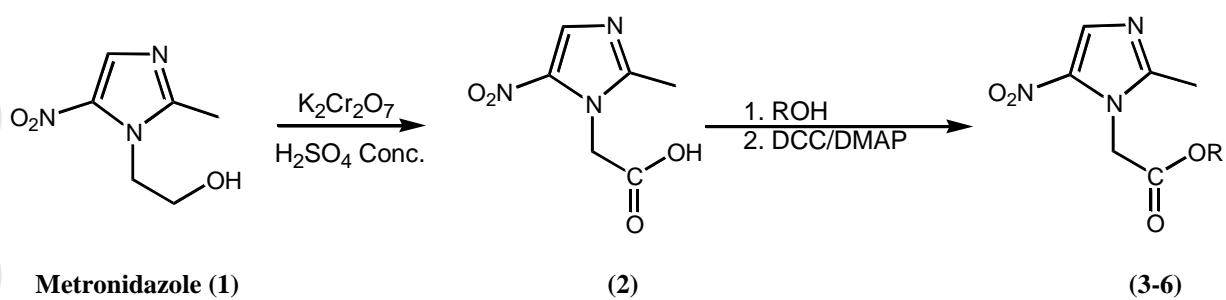
The authors declare that they have no competing interests.

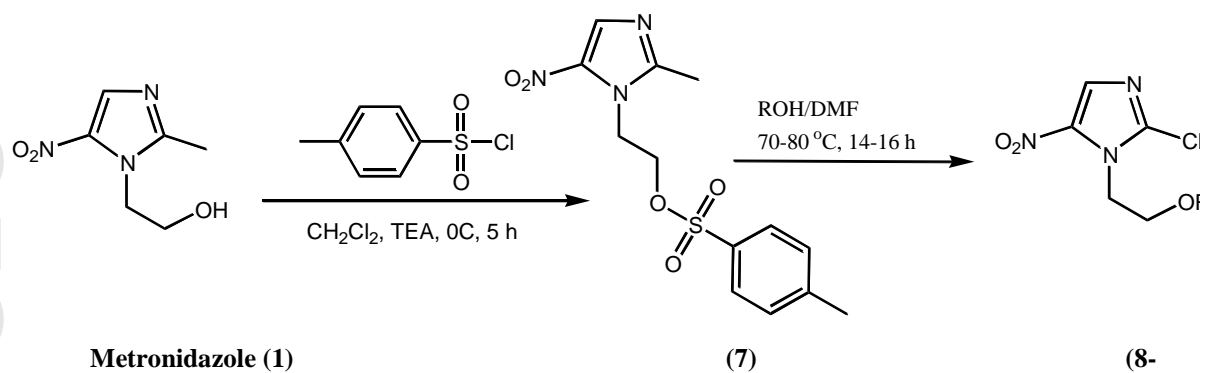
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11)

R=	<p>8</p>	<p>9</p>	<p>10</p>	<p>11</p>
Scheme 2: General method for the preparation of metronidazole ether derivatives				

Table 1. Activity of the eight compounds against two strains of *H. pylori*, MRC5 (MTT), and *clostridium perfringens* (MIC)

Compound/strain	IC ₅₀ (μM/mL)			MIC (nM/mL)
	<i>H. pylori</i> 26695	<i>H. pylori</i> P12	MRC5	<i>Clostridium perfringens</i>
3	30.2166±0.8542	15.3833±3.9233	-	R
4	27.0750±6.9791	41.8100±12.3319	-	1.9
5	35.2333±8.1926	21.6350±8.5772	-	3.8
6	52.3500±18.3847	35.1700±1.1030	-	0.0094
8	11.8200±3.3762	36.7200±4.8766	-	R
9	10.8750±1.2232	14.1733±1.5212	-	1.9
10	31.2700±2.9865	100<	-	1.9
11	4.9210±1.6829	0.0011±0.0003	50<	2.7
Metronidazole	-	-	49.453±3.0934	0.0058

R= resistant