

## Synthesis of new metronidazole derivatives with suspected antimicrobial activity

Yasser F. Mustafa\*, Karam A. Al - dabbagh\*\* Manal F. Mohammed\*\*

\* Department of Pharmaceutical Sciences, \*\* Department of Pharmacology, College of Pharmacy, University of Mosul.

Received: Accepted

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### ABSTRACT

Metronidazole has a bactericidal effect against many anaerobic gram positive and gram negative bacteria, but has no activity against aerobic bacteria.

Metronidazole activity is mostly contributed to its reduced form that produced from anaerobic reduction inside the micro-organisms.

In this research, the reduced form of metronidazole was synthesized and termed compound ( I ) which diazonated to form a diazonium interme-diate, this intermediate coupled with two phenolic compounds to give compounds ( II ) and ( III ) which are azo derivatives.

The structures of these compounds were identified by physical and spectroscopic ( U.V. and FTIR ) techniques.

The antibacterial activity of the synthesized compounds were examined against two aerobic gram positive bacteria ( Staphylococcus aureus, Streptococcus pneumoniae ); two aerobic gram negative bacteria (Pseudomonas aeruginosa, Escherichia coli ) and one anaerobic gram positive bacteria ( Clostridium Perfringens ) by using two techniques: dilution and disc diffusion methods.

Opposite to our expectance, these products showed no antibacterial activity against the tested bacteria at the concentrations used.

### الخلاصة

ميترونيديازول هو عامل قاتل للعديد من الجراثيم اللاهوائية سالبة وموجبة الغرام، لكنه غير فعال تجاه الجراثيم الهوائية. فعالية ميترونيديازول في اغلب الأحيان تعزى إلى شكله الكيميائي المختزل، الذي ينتج عن الاختزال اللاهوائي للميترونيديازول داخل الجراثيم.

في هذا البحث، تم تحضير الشكل الكيميائي المختزل للميترونيديازول وسمي المركب ( I ). المركب ( I ) تأيذن ليكون المركب الدايزوني الواسطي الذي باقترانه مع مركبين من الفينولات كون المركبين ( II ) و ( III ) وهما من مشتقات الأزو.

تم تشخيص المركبات المحضرة بالوسائل الفيزيائية والطيفية المتوفرة وتم دراسة الفعالية المضادة للجراثيم للمركبات المحضرة ضد الجراثيم التالية: الهوائية موجبة الغرام ( Staphylococcus aureus, Streptococcus pneumoniae )، الهوائية سالبة الغرام ( Pseudomonas aeruginosa, Escherichia coli ) واللاهوائية موجبة الغرام ( Clostridium Perfringens ) باستعمال تقنيتي التخفيف والانتشار. لقد أظهرت النتائج وبالعكس المتوقع بان المركبات المحضرة لم تبدي أي نشاط كمضاد جرثومي ضد الجراثيم المستخدمة للبحث ضمن التراكيز المستخدمة.

Metronidazole is the prototype of aomycin (nitro imidazole) anti-infective agents. Nakamura was discovered the aomycin group; Horie was demonstrated the anti-infective property of these compounds leading to their chemical synthesis in various laboratories throughout the world.<sup>1</sup>

Metronidazole is a bactericidal against anaerobic bacteria, its spectrum of activity includes the anaerobic gram negative bacilli including most Bacteroides, Fusobacterium and Veillonella, anaerobic gram positive cocci including Peptococcus and Peptidostreptococcus species and anaerobic gram positive bacilli including Clostridium and Eubacterium species.<sup>2</sup>

Metronidazole is also active against E.histolytica and G.lambliia. It acts primarily against the trophozoite forms and has limited activity against the encysted forms<sup>(3)</sup>.

The mechanism of metronidazole activity against anaerobic micro-organisms occurs through four steps. First of all is the entry into the micro-organism; metronidazole is a low molecular weight compound which diffuses across cell membranes of anaerobic micro-organisms<sup>4</sup>.

The second step is a reductive activation by intracellular transport proteins; metronidazole is reduced by the pyruvate- ferredoxin oxide-reductase (PFOR) system in the mitochondria of obligate anaerobes, which alters its chemical structure.

Pyruvate-ferredoxin oxidoreductase normally generates ATP via oxidative decarboxylation of pyruvate. With metronidazole in the cellular environment, its nitro group acts as an electron sink, capturing electrons that would usually be transferred to hydrogen ions in this cycle.

Reduction of metronidazole creates a concentration gradient which drives uptake of more drug, and promotes formation of intermediate compounds and free radicals that are toxic to cell.<sup>5,6</sup>

Then the reduced intermediate particle interact with host cell DNA, resulting in DNA strand breakage and fatal destabilization of the DNA helix<sup>7,8</sup>, and the last step is the breakdown of cytotoxic intermediate products into inactive end products.<sup>9</sup>

The resistance to metronidazole have been increased<sup>10,11</sup>. Two distinct types of resistance have been recognized for metronidazole and named aerobic and anaerobic according to conditions required for demonstration of resistance by in vitro susceptibility assays.<sup>12</sup>

The aerobic resistance is manifested only if some oxygen is present. Under anaerobic conditions, the resistant organisms succumb to the drug, as they possess a functional drug-activating pathway.

The resistance apparently results from defective oxygen scavenging and subsequent interference of intracellular oxygen with the drug activation.<sup>13</sup>

Alternatively, defective redox properties of ferredoxin or decreased ferredoxin level due to alter transcription of the ferredoxin gene have been proposed as potential mechanisms, However, a causal relationship of these alterations with the aerobically resistant phenotype has not been convincingly demonstrated.<sup>14</sup>

The resistant anaerobic micro-organisms are typically deficient in drug activation system because of the modification of proteins involved in pathways responsible for the reductive activation of the drug<sup>15</sup>.

The loss of ( PFOR ) activity holds for a key attribute of the anaerobic resistance but in vitro obtained lines deficient in ( PFOR ) activity showed only low levels of anaerobic resistance to metronidazole, thus indicating involvement of an additional pathway(s) which can provide electrons for the drug reduction.<sup>16</sup>

Therefore, there is an urgent need to create new metronidazole deriva-tives to overcome the problem of bacterial resistance to metronidazole.

The laboratory reduction of a nitro-containing compound proceeds either by catalytic hydrogenation via molecular hydrogen or by chemical reduction via acid and metal.

Hydrogenation of a nitro-containing compound to a primary amino derivative takes place smoothly when a solution of the nitro compound in alcohol was shaken with finely divided nickel or platinum under a supply of hydrogen gas.

This method can not be used because metronidazole contains some easily

hydrogenated groups (١٩\*), Therefore, the was carried out by adding a mixture of conc. hydrochloric acid and a metal.

When a primary aromatic amine (dissolved or suspended in a cold aqueous mineral acid ) was treated with sodium nitrite, diazonium salt will be formed. Since the diazonium salts are slowly decomposed even at ice-bath temperatures, the solutions of diazonium salts were used immediately after their preparations(١٩\*\*).

The azo compounds were prepared by coupling reaction which involves the interaction of a diazonium salt and phenol in slightly alkaline medium ١١.

The biological importance of azo compounds was well known for their use as antineoplastics, ١١ antidiabetics, ١٢ and antiseptic. ١٢

It has been found that the incorporation of azo linkage with pyrimidine and pyrazole as coupling compounds may decrease the antibacterial activity ١٤ while the incorporation with phenyl ring contains an electron-withdrawing group may increase the antibacterial activity of azo linkage. ١٥

In this research, the antibacterial activity of azo linkage incorporates with phenyl ring containing an electron-donating group(s) and with imidazole ring containing electron-donating groups was examined.

The determination of micro-organism susceptibility to the new synthesized compounds were carried out by two principal methods; dilution and diffusion methods.

These methods can be employed to estimate either the potency of the new synthesized compounds as antibacterial agents or the susceptibility of the tested bacteria to these compounds(٢٦).

## Materials and instruments

### Materials

The parent compound ( metronidazole ) was supplied from a Ninevah Drug Industry ( NDI ). All other chemicals used in preparations were supplied from Fluka-company.

### Instruments

preferred chemical reduction of metronidazole

- Melting points were determined on electrothermal CIA ٩٣٠٠ melting point apparatus and they are uncorrected.
- The ultraviolet spectra were obtained via carrywinn UV varian U.V. – visible spectrophotometer ( Australia ).
- The infrared absorptions were recorded by Buck ٥٠٠ scientific I.R. spectrophotometer ( U.S.A. ).
- Thin-layer chromatography ( TLC ) was carried out on TLC plastic sheets silica gel ٦٠ F٥ precoated , ٢٠ x٢٠ cm, layer thickness ٠.٢ mm. The spots on the chromatograms were localized using U.V. light (٢٦٦ nm)( Whatmann ). The solvent system employed for separation was (Tc) which composed from chloroform: methanol (١:١).
- Structures were drawn by chemdraw office ٢٠٠١ software.

## Experimental methods

### • Synthesis of compound I

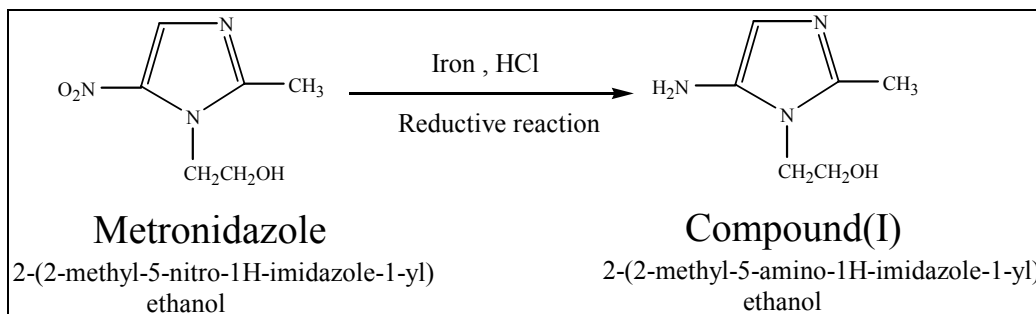
A (١٥٥ ml) of water and ٥٢ g of grease-free iron were placed in a (٥٠٠ ml) round bottom flask. The mixture was warmed on a water bath to about (٦٠°C). A (٤٢.٢٥g, ٠.٢٤٧ mole) of metronidazole was divided into parts, each part weights about (٢g).

Two grams of metronidazole and (١٠ ml) of conc. HCl were added to the previous warmed mixture, the temperature should remain between (٨٠-٩٠°C) throughout the reaction.

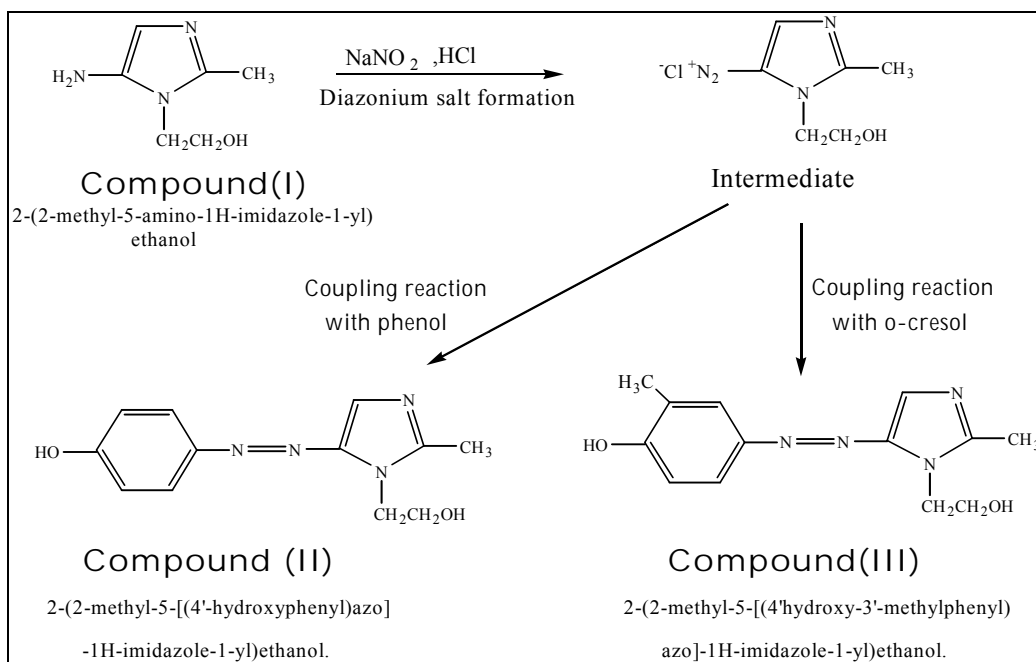
Every ٢٠ minutes; two grams of metronidazole was added with stirring. After the last addition, the mixture was stirred for ٢٠ minutes

The mixture was cooled; filtered ; and the product washed with cold water three times; then recrystallized from a mixture of chloroform : ethanol (١:١).

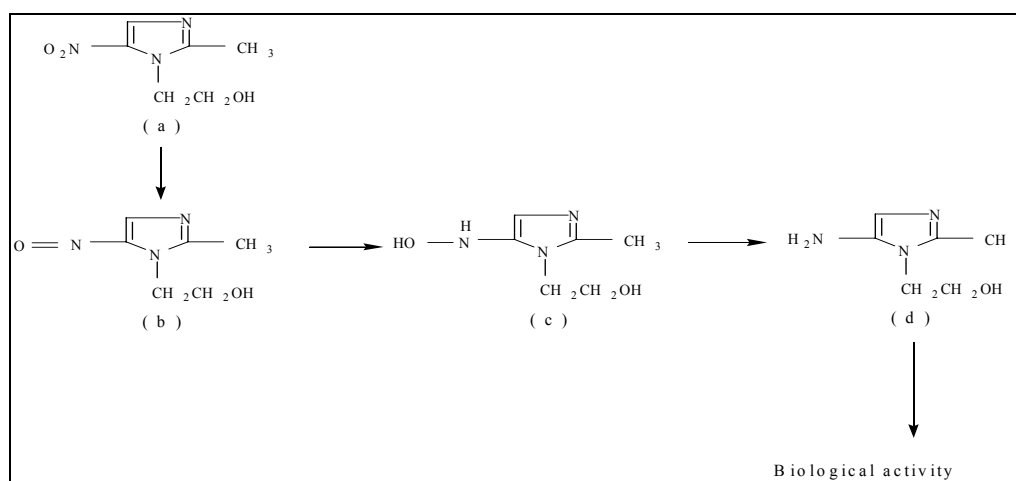
The compound purity was established by thin-layer chromatography using Tc solvent and the TLC result showed that only a single spot was observed.



Scheme 1. Synthesis of compound ( I ).



Scheme 2. Synthesis of compounds ( II ) and ( III ).



Scheme 3. Reduction of metronidazole (a) to form nitroso(b), hydroxylamine(c) and amino(d) derivatives in an anaerobic bacteria.

Compound I has the following data: the m.p. was 168-170°C, the percentage of yield

was ٧٦%, the molecular formula was established as  $C_7H_{11}ON_7$  and the Rf value was ٠.٢٤.

While for Metronidazole, the m.p. was (١٥٨-١٦٠°C), the molecular formula was  $C_7H_8O_7N_7$  and the Rf value was ٠.٣٦<sup>٢٧</sup>.

- **Synthesis of compounds II and III :**

A (٧.٥٨g, ٠.٠٥٤ mole) of compound I was dissolved in a mixture of equal quantity (١٦ ml) of each conc. HCl and water in a large beaker; the resulted solution was cooled by immersing in a bath of crush ice; throughout the reaction, the reaction temperature should be remain below ٥°C.

The cold solution of ٤g, ٠.٠٥٨ mole sodium nitrite in ٢٠ ml water was placed in a separating funnel and then added drop by drop to the stirred solution in an ice bath; the reaction temperature should be remain below ١٠°C by adding few grams of crushed ice when necessary.

The resulted solution was stirred very slowly for ٥ minutes; on the other hand, the phenolic solution was prepared by mixing ٥.٠٥g, ٠.٠٥٤ mole of phenol with ٤٥ ml of ١٠% NaOH in a beaker immersed in the ice bath.

The solution was vigorously stirred and the temperature kept below ٥°C; this can be assisted by a direct addition of crushed ice.

The cold diazonium salt solution (with few grams of crushed ice) was placed in a separating funnel and was added drop by drop to the stirred phenolic solution in an ice bath; yellow color will developed and yellow crystals soon separated giving compound II .

After the diazonium salt solution was added; allow the mixture to stand in an ice bath for ٢٠ minutes; filtered off and washed the crystals three times with cold water, then recrystallized from ethanol.

The compound purity was established by thin-layer chromatography using Tc solvent and the TLC result showed that only a single spot was observed.

The previous procedure can be carried out by using o-cresol instead of phenol to give compound( III ).

Compound II has the following data: the m.p. was ١٣٠-١٣٢°C, the percentage of yield was (٦٤%), the molecular formula was established as  $C_{17}H_{15}O_7N_7$  and the Rf value was ٠.٣٢.

While for compound III , the m.p. was (١٤٦-١٤٨°C), the percentage of yield was ٧١% the molecular formula was established as  $C_{17}H_{17}O_7N_7$  and the Rf value was ٠.٣٨.

### Antimicrobial study

- Sensitivity test method for aerobic bacteria:

This test proceeds by transferring ٤-٥ pure colonies from the tested bacteria (three isolates for each bacterial strain) into nutrient broth media which incubated at ٣٧°C for ١٤-١٦ hours; then the bacterial suspension was diluted with normal saline and compared with controlled tube which contains  $١٠^8$  cells/cm<sup>٣</sup>. From the tested bacterial suspension, (٠.١ cm<sup>٣</sup>) was inoculated into a nutrient agar media and spread on the disc surface by using a sterile cotton swab, the discs were incubated at ٣٧°C for ٢٠ minutes, during this period, diffusion was occur.

A sterile filter paper disc with (٦ mm) in radius saturated with different concentrations (١٢.٥, ٢٥, ٥٠, ١٠٠, ٢٠٠ mg/ml) was placed by using a sterile forceps on the bacterial discs, the resulted discs were incubated at ٣٧°C for (١٤-١٦ hours); then the inhibition zones were identified.

Triplet runs were carried out for two gram positive bacteria (Staphylococcus aureus , Streptococcus pneumoniae); and two gram negative bacteria( Pseudomonas aeruginosa , Escherichia coli).

- Sensitivity test method for anaerobic bacteri

Successful culture of anaerobes requires fresh media and reliable system for maintaining an anaerobic atmosphere, which should included ١٠ % carbon dioxide. This system is unavailable in our college laboratory, therefore, anaerobic bacteria discs which contain Clostridium Perfringens were supplied from general health laboratory in Mosul.

As in a sensitivity test for aerobic bacteria, the same concentrations were applied and triplet runs were carried out .

### Results and discussion

- **Metronidazole and compound I:**

Metronidazole is a potent anaerobic bactericidal agent, its activity is mostly contributed to its reduced form that produced from anaerobic reduction inside the micro-organisms, therefore, metronidazole is ineffective against aerobic bacteria due to lack the ability to form the active metabolite (reduced form).

In anaerobic micro-organisms, metronidazole was metabolized as shown in scheme (٣). Beaulieu B. B. indicated that there is a direct correlation between bactericidal activity of the

metronidazole and the speed of reduced form production<sup>(٢٨)</sup>.

The research proposed that the reduced form of metronidazole (compound I) has enhanced bactericidal activity against anaerobic bacteria, if proven; the reduced form may be more potent in terms of concentration than parent drug.

In addition, metronidazole is ineffective against aerobic bacteria due to the inability of these bacteria to reduce the nitro group of metronidazole to form the active metabolite.

The research proposed that the application of an active metabolite of metronidazole to the aerobic bacteria may cause antibacterial effect; if proven; the compound I may have broader spectrum of activity than the parent drug.

The chemical reduction of metronidazole in laboratory was carried out by adding conc. HCl to a mixture of metronidazole and iron.

Compound (I) was generally formed in a high yield (about ٧٦%) as light brown crystals.

I.R. spectrum was in quite agreement with the structure of compound I, the I.R. spectrum shows the principal following peaks:

٣٢٢٤ cm<sup>-1</sup>(br)for strong intra molecular hydrogen bounding for

hydroxyl group of the ethanol side chain; ٣١٠١ cm<sup>-1</sup>(m)for (C-H) of

CH<sub>2</sub> group; ٢٩٥٧ cm<sup>-1</sup>(m)for (C-H) of CH<sub>2</sub> group at position ٢ of

imidazole ring; ١٨٠٥ cm<sup>-1</sup>(m)for tri substituted imidazole ring;

٢٤١٧cm<sup>-1</sup>(m)for (N-H) of primary amine; ٢٢٩١ cm<sup>-1</sup>(br)for primary amino salt (<sup>+</sup>NH<sub>2</sub>Cl<sup>-</sup>).

The appearance of absorption band at ٢٤١٧cm<sup>-1</sup> confirmed the reduction of a nitro group of metronidazole into a primary amino group.

The U.V. spectrum of compound I in an aqueous acidic solution shows λ<sub>max</sub> at ٢٧٩ nm, while the λ<sub>max</sub> of metronidazole in an aqueous acidic solution was at ٢٧٧ nm<sup>(٢٩)</sup>.

• *Synthesis of compounds II and III :*

A primary aromatic amine ( compound I ) was dissolved in a cold aqueous mineral acid solution then treated with sodium nitrite to form a diazonium salt, this process called diazotization of primary amine.

Two important points must be taken into consideration in the preparation of diazonium

salts; which are: the amine is comparatively a weak base, so that, a certain amount of amine will be produced by salt hydrolysis unless an excess of acid present; and the reaction mixture must be kept very cold during the process (the reaction is exothermic); otherwise, the diazonium salt may be partially converted into the corresponding hydroxyl compound.

The aromatic ring undergoes attack by the diazonium ion must be in general contains a powerful electron-donating group( —OH, —NH<sub>2</sub>, —NHR ).

Substitution usually occurs at para position to the activating group. According to this fact, phenol and o-cresol were chosen.

The most important point in the coupling reaction using phenol is the degree of acidity.

Phenol is appreciably acidic in aqueous solutions and exists in equilibrium with phenoxide ion; thus; in the preparation of compounds II and III , a low acidity level which give high amounts of ionized phenols to be coupled, and then a relatively high percentage of yields were obtained.

For compound II , the I.R. shows the following principal peaks:

٣٣٥٦ cm<sup>-1</sup>(m)for weak inter molecular hydrogen bounding of the

phenolic hydroxyl group; ١٨٩٩ cm<sup>-1</sup>(w)for disubstituted aromatic ring; ١٧٥٤ cm<sup>-1</sup>(w)for tri

substituted imidazole ring; ١٦١٢ cm<sup>-1</sup>(m)for (C=C) of imidazole ring; ١٤٩٤ cm<sup>-1</sup>(w)for (-

N=N-) group (unsymmetrical p-substituted azobenzene); ١٠٨٥ cm<sup>-1</sup>(m)for (C-N).

The disappearance of absorption band at ٢٤١٧cm<sup>-1</sup> of primary amine and the

appearance of a weak absorption band at ١٤٩٤ cm<sup>-1</sup> of azo group confirmed the formation of

azo compound, these data were quite in agreement with the structure of compound II .

The U.V. spectrum of compound II in an aqueous acidic solution shows the λ<sub>max</sub> at ٣١٢ nm, the increasing in the magnitude of

λ<sub>max</sub> may indicates the presence of a new conjugation.

For compound III , the I.R. shows the following principal peaks: ٣٢٢٠ cm<sup>-1</sup> (br)for

strong inter molecular hydrogen bounding of the phenolic hydroxyl group; ٣١٠١ cm<sup>-1</sup>(w)for

(C-H) of aromatic ring; ٢٩٥٧ cm<sup>-1</sup>(w)for (C-H) of methylene group; ١٦١٤ cm<sup>-1</sup>(m)for (C=C) of

imidazole ring;  $1487 \text{ cm}^{-1}$  (w) for (-N=N-) group (unsymmetrical p-substituted azobenzene);  $1384 \text{ cm}^{-1}$  (s) for strong interaction between (O-H) bending and (C-O) stretching of phenol;  $1074 \text{ cm}^{-1}$  (m) for (C-N).

The disappearance of absorption band at  $3417 \text{ cm}^{-1}$  of primary amine and the appearance of a weak absorption band at  $1487 \text{ cm}^{-1}$  of azo group confirmed the formation of azo compound, these data were quite in agreement with the structure of compound ( III ).

The U.V. spectrum of compound ( III ) in an aqueous acidic solution shows the  $\lambda_{\text{max}}$  at  $313 \text{ nm}$ , the increasing in the magnitude of  $\lambda_{\text{max}}$  may indicates the presence of a new conjugation.

#### • In vitro antibacterial assay

The new metronidazole compounds ( I , II and III ) were tested in vitro for their antibacterial activity against five strains of bacteria which are: Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli and Clostridium Perfringens by using different concentrations of these compounds (  $12.5$ ,  $25$ ,  $50$ ,  $100$  and  $200 \text{ mg/ml}$ ). Opposite to our expectance, no antibacterial activity for these compounds were detected.

#### Conclusion

In this research, three new metronidazole derivatives were synthesized. The compound ( I ) { $\gamma$ -( $\gamma$ -methyl-o-amino- $\gamma$ -H-imidazole- $\gamma$ -yl)ethanol} was prepared by reducing metronidazole { $\gamma$ -( $\gamma$ -methyl-o-nitro- $\gamma$ -H-imidazole- $\gamma$ -yl)ethanol} via conc. HCl and iron. Compound ( I ) represents the reactive intermediate of metronidazole.

Compound ( II ) { $\gamma$ -( $\gamma$ -methyl-o-[( $\epsilon$ '-hydroxyphenyl)azo]- $\gamma$ -H-imidazole- $\gamma$ -yl)ethanol} and compound ( III ) { $\gamma$ -( $\gamma$ -methyl-o-[( $\epsilon$ '-hydroxy- $\gamma$ '-methyl phenyl)azo]- $\gamma$ -H-imidazole- $\gamma$ -yl)ethanol} were prepared by diazotization of compound I and then coupling with phenol and o-cresol respectively.

The antibacterial activity of these compounds (I, II and III) was tested against: Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli and Clostridium Perfringens by using different concentrations for each compound (  $12.5$ ,  $25$ ,  $50$ ,  $100$  and  $200 \text{ mg/ml}$ ).

Triplet runs were carried out for each bacterial strain.

The results of this research showed that these compounds have no anti-bacterial activity against the tested bacteria at the concentrations used; and the reactive intermediate of metronidazole (compound I ) was only active when formed inside the anaerobic bacteria and inactive when applied directly to these micro-organisms.

In addition, the incorporation of azo linkage with phenyl ring containing electron-donating group(s) and with imidazole ring containing electron-donating groups may diminish the antibacterial effect of azo bond, as in compounds II )and III .

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