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Extracting and Analyzing Antibiotics from Natural Human Urine.

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ABSTRACT

Antibiotics that are widely used in human and veterinary medicine, over the past few years, antibiotics have been considered emerging pollutants due to their continuous input and persistence in the aquatic ecosystem even at low concentrations. Antibiotics as pollutants are detected nowadays in wastewater and surface water. They are carried with animal excreta and liquid manure into streams. Two extraction methods have been studied for detection of antibiotics in human urine using several solvents. The antibacterial and antifungal potentialities of the urine samples extract were determined against yeasts, bacteria and molds studied. The extracted residue was analyzed for antibiotic present using ultraviolet UV, Infrared IR, Nuclear Magnetic Resonance NMR, Mass spectra and C^{13}. This work provided a preliminary investigation of Erythromycin aglycone which is not known in nature, but its presence by hydrolysis were detected in human urine samples.

Keywords: antibiotic, human urine, pharmaceutical contaminates, yeast, bacteria.

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INTRDUCTION

Measurements conducted during the last decade showed that the concentrations of antibiotics in municipal sewage, hospital effluents, surface water and ground water are mostly in the same range, respectively [1]. The term antibiotic originally referred to any agent with biological activity against living organisms, however, "antibiotic" now refer to substances with antibacterial, antifungal, or anti-parasitical activity [2]. Currently, antibiotics are obtained by chemical synthesis, such as the sulfa drug (e.g. sulfametho xazole), or by chemical modification of compounds of natural origin. The classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another microorganism.

Antibiotics can be grouped by either their chemical structure or mechanism of action. Therefore, under different pH conditions antibiotics can be neutral, cationic, anionic or zwitterion. Their physic-chemical and biological properties [3], sorption behavior, photo reactivity and antibiotic activity toxicity may change with pH. Several hundred different antibiotic and antimitotic substances are used in human and veterinary medicine, e.g. more than 250 in Germany. International comparable data on antibiotic consumption is scarce, and whatever information is available is heterogeneous. Usage patterns may be different in different countries [4]. In the USA for instance the use of streptomycin in fruit growing is widespread, whereas its use for this purpose is banned in other countries such as Germany. Wise [5] estimated antibiotic consumption world wide to lie between 100,000and 200,000 ton annum. According to data supplied by European Federation of Animal Health [6], in 1999 there were a total of 13,216 ton of antibiotics used in European Union and Switzerland, 65% of which was applied in human medicine. A more recent report estimated that US livestock producers use approximately 11,200 metric tons of antimicrobials for non-therapeutic purposes primarily to promote the growth of cattle, hogs, and poultry. Clinical uses are estimated at about 10% of total antimicrobial use [7].

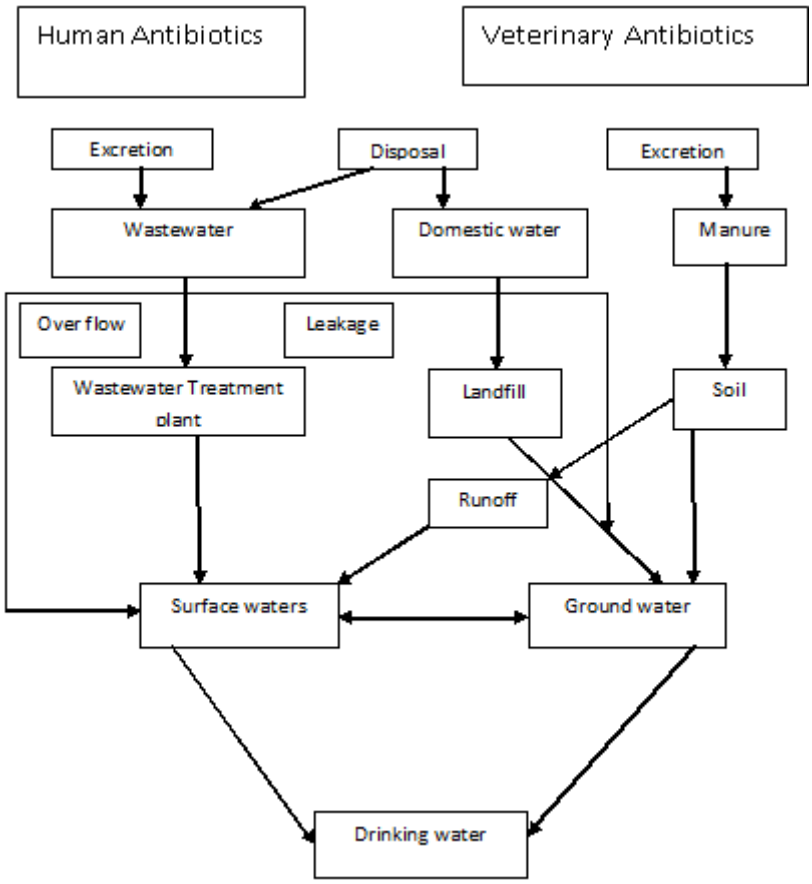

Figure 1: Human Antibiotics reaches natural waters via several pathways

It was found that β-lactam antibiotics, including the sub-groups of penicillin, cephalosporin and, as a marginal fraction carbopenems and others, make up the largest share of human use antibiotics in most

countries. Extraction rate for the unchanged active compounds cover a broad range (10 – 90%, Ceftazidime for example less than 10 %). On the average, if the volume for all antibiotics used is totaled the metabolic rate is estimated to be 30% [2], i.e. 70% of the used antibiotics is excreted unchanged into wastewater. As for the metabolism of active compounds in humans there is a wide range in the degree to which these compounds are metabolized [2] and [8]. Some compounds are metabolized by (90% or more, while others are metabolized by only 10% or even less. Often the metabolites are more water soluble than the parent compounds, leading to their excretion with urine. However, sometimes the formation of metabolites can result in compounds which are more toxic to humans than the parent compounds [9].

Antibiotics are only partially eliminated in sewage treatment plants. If they are not eliminated during the purification process, they pass through the sewage system and may end up in the environment. In general, concentrations were in the higher µ -per – liter range in hospital effluent, in the lower µ -per – liter range in municipal wastewater [10]. Antibiotics have also rarely been found in drinking water [11].

Antibiotics at sub-inhibiting concentrations can have an impact on cell functions and change the genetic expression of virulence factors on the transfer of antibiotic resistance [12]. The capacity of bacteria to adapt to changes in their environment and true survive is called resistance. For antibiotics resistance is usually quantified as the minimum concentration required asserting a definable effect [13] on a population of cells. However, there is evidence that antibiotic resistance is already present in natural environments and that it can be exchanged between bacteria for at least decade [13]. An important source for the resistance material found in hospital effluents, municipal sewage and sewage treatment plants STPs is the impute of bacteria that have already become resistant through the use of antibiotics in medical treatment. There are reports that the widespread use of biocides such as triclosan and quaternary ammonium compounds used in hospitals and homes could select for antibiotic – resistant bacteria [14]. The concentration of antibiotics may be much higher if the active compounds are persistent and accumulate e.g. by sorption to solid surface in certain environmental compartments such as sewage sludge, sediments or soil. In these cases, the role of antimicrobial concentration could differ to that in water. It is not known how strong the antibiotics are sorbet and under what circumstances they are still biologically available and active after sorption. The concentration of antibiotics in municipal sewage and in sewage treatment plants STPs are typically lower by factor of 100 compared to hospital effluent [15]. Antibiotics are rarely found in ground water and, when they are found, they usually only occur at concentrations far below the μL^{-1} range.

Leaching from fields fertilized with animal slurry or passing through sediments into the groundwater might be a source of antibiotics in ground water [16]. However, antibiotic – resistant bacteria were detected in drinking water as early as the 1980`s and later in the 1990`s. The treatment of raw water and its subsequent distribution selects for antibiotic-resistant bacteria. In agreement with these data, increased phenotypic resistance rates were also detected at drinking water sampling points [17]. Resistant bacteria may be present in sediments because of the application of antibiotics in fish farming or because of selection through the antibiotics present in sediments. High antibiotic load in sediments and at concentrations potent enough to inhibit the growth of bacteria have been reported for aquaculture. Antimicrobials can have qualitative and quantitative effects upon the resident microbial community in sediments. Therefore, the product use of antibiotics and the restriction of their input into the aquatic environment are necessary.

The introduction of these compounds into the environment can constitute a potential risk for aquatic and terrestrial organisms. The aim of this work is to detect and evaluate antibiotics in human urine execrate.

EXPERIMENTA AND METHODS

Materials

All materials used, amyl acetate, ethyl acetate, petroleum ether, benzene, chloroform, methanol, acetone, butanol and diethyl ether are pure grade. The natural urines were collected using clean plastic jars from six healthy males and females aged between 22 and 40 years in 24 hr. The content of the natural urine was analyzed as shown in Table (1).

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Table1: Properties of human Urine Under Test

Materials	Concentration in Authentic urine
pH	5.38
conductivity	19.62 mS/cm
COD	37216 mg/L
Total P	3.769 g/L
NH_4	784 mg/L
TDS	9.8 mg/L
Mg	9.963 mg/L
Fe	70 mg/L
Mn	0.038 mg/L
B	0.1 mg/l
Ca	Hiegh
Cu	14 mg/L
Zn	42 mg/L
MO	Not Detected
CO	Not Detected
Cr	Not Detected
Cd	20 mg/L
SO_4	0.0475 g/L
Pb	3.4 mg/L
K	2.48 mg/L

Antimicrobial Quantification

Antibacterial and antifungal potentialities of the studied human urine samples were determined against the following molds, yeast and bacteria; *Aspergillus niger, Botrytis allii, Macrophomina phaseoli, Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Candida albicans, Saccharomyces chevalii, Rhodotorula minuta ,Staphylococcus aureus, Pseudomonas auroginosa, Bacillus cereus, Bacillus subtilus, and Sarcina* sp.

The tested fungi, yeast and bacteria were obtained from the culture collection of the Microbial Chemistry Laboratory, National research center, Cairo, Egypt.

Molds, yeasts and bacteria were cultivated on Czapek's Dox agar, malt-extract agar and nutrient broth agar respectively.

The antimicrobial potentialities were expressed as the diameter of the inhibition zones following the agar plate diffusion method [18].

Extraction of the bioactive substance

Two methods were used to purify the studied bioactive substance: solvent-solvent extraction and precipitation.

Solvent-solvent extraction

Solvent-solvent extraction involves the use of different solvents of different polarities for the extraction of the bioactive substance. Different solvents immiscible with water were tested for their ability to extract the substance: Ethyl ether, petroleum ether, benzene, chloroform, butyl acetate, butyl alcohol and ethyl acetate.

Precipitation

600 ml of urine sample was divided into three portions, the first portion was made alkaline with NH_4OH solution to pH10, the second kept nautral, while the third was acidified with acetic acid to pH2. each portion was divided into five equal parts (40ml), to each part equal volume of methyl alcohol, acetone, saturated solutions of ammonium sulphate, calcium chloride or ammonium chloride were added. Formed

precipitates were separated, dried then dissolved in 0.5ml sterile distilled water and tested for its antimicrobial potentiality.

Partial purification of the bioactive material

About 5 liters of urine sample were collected and bioassayed by the agar diffusion method, the active substance was precipitated with equal volume of methanol at neutral pH value. The obtained precipitate was bio assayed using the test organism *Bacillus cereus.* The methanol precipitate was dried and the solid residue was dissolved in diethyl ether. The diethyl ether layer was collected, bio assayed against the test organism *Bacillus cereus*, the diethyl ether layer was concentrated under vacuum till dryness.

Some characteristics of the partially purified substance

Chromatographic spectrum

The concentrated methanol precipitation was spotted at the start of a 1 cm wide and 30 cm long strips of Whatman paper No.1. Dried strips were developed in the following solvent systems: diethyl ether, chloroform, carbon tetrachloride, petroleum ether, acetone, benzene, methanol, ethyl acetate, n-butanol, H_2O saturated with n-butanol, 3% NH_4Cl in water, n-butanol-acetic acid-water (2:1:1) or amyl acetate. These strips were placed on the surface of agar plates seeded with *Bacillus cereus*. The R_f value of the extracted antibiotic was calculated according to the method of [19].

The ultraviolet and infrared spectra

The ultraviolet and infrared spectra of the partially purified substance were estimated using bio active extracted substance for the first test , and Fourier Transform 300 E Infrared spectrometer, using KBr discs for the second test at the National Research Center, Cairo, Egypt.

Nuclear magnetic resonance (NMR)

The proton (^{1}H) and (^{13}C) NMR spectra were estimated using DEMSO by bio active extracted substance NMR spectrometer, at National Research Center, Cairo, Egypt.

Antimicrobial potentialities

The antimicrobial characteristics of urine sample Table (2) show that this sample inhibits the growth of *Bacillus cereus, Bacillus subtillus, Pseudomonas auroginosa, Rhodotorula minuta, Staphylococcus aureus, Sarcina* sp., *Saccharomyces chevalii, Saccharomyces carlsbergensis, Saccharomyces cerevisia* and *Candida albicans* but not *Aspergillus niger, Botrytis allii* or *Macrophomina phaseoli.*

Table2: Antimicrobial potentialities of urine sample

Test organism	Zone of inhibition (mm)
Bacillus cereus	31
Bacillus subtillus	29
Pseudomonas auroginosa	29
Rhodotorula minuta	29
Sarcina sp.	38
Staphylococcus aureus	27
Saccharomyces chevalii	32
Saccharomyces carlsbergensis	28
Saccharomyces cerevisia	30
Candida albicans	35
Aspergillus niger	0
Botrytis allii	0
Macrophomina phaseoli	0

Extraction of the bioactive substance

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Solvent-solvent extraction

The results presented clearly reveal that ethyl ether was the best solvent for extraction of the bioactive substance at neutral pH value, which was followed by chloroform, ethyl acetate or butanol- ethyl acetate (1:1) while the other tested solvents failed to extract the bioactive material Table (3).

Precipitation

Results given in table (4) indicate that, acetone and methanol was the best precipitating agents that able to precipitate the bioactive substance from urine sample at different pH values.

Table 3: Extraction of the bioactive substance of urine sample

Solvent system	Activity
Chloroform	+
Diethyl ether	+++
Ethyl acetate	+
Butyl alcohol	-
Petroleum ether	-
Butanol -ethyl acetate (1:1)	+
Benzene	-

Test organism: *Bacillus cereus*

Table 4: Suitability of different precipitating agents at various pH values for the precipitation of the bioactive substance

Precipitating agents	pH value		
	2	7	10
Calcium chloride	19	20	20
Ammonium chloride	15	13	12
Ammonium sulphate	31	23	18
Methyl alcohol	33	34	37
Acetone	30	38	37

Test organism: *Bacillus cereus*

RESULTS AND DISCUSSION

Characterization of the bioactive substance isolated from urine sample

Solubility in different solvents

The bioactive substance was soluble in ethyl ether, ethyl acetate and chloroform but insoluble in petroleum ether, benzene, methanol, acetone, butanol or amyl acetate.

Chromatographic spectrum

The bioactive substance showed high R_f values in diethyl ether (0.9), ethyl acetate (0.95), butanol-acetic-water (0.72) and 3%NH4Cl in water (0.64) But give 0 R_f values with other tested solvents Table (5).

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Table 5: R_f values of the bioactive substance isolated from urine sample in different solvent systems

Solvent system	Rf value
Petroleum ether	0
Benzene	0
Methyl alcohol	0
Acetone	0
Diethyl ether	0.9
Ethyl acetate	0.95
Amyl acetate	0
Butyl alcohol	0
3% NH_4Cl in H_2O	0.64
N-butanol-acetic acid-water (2:1:1)	0.72

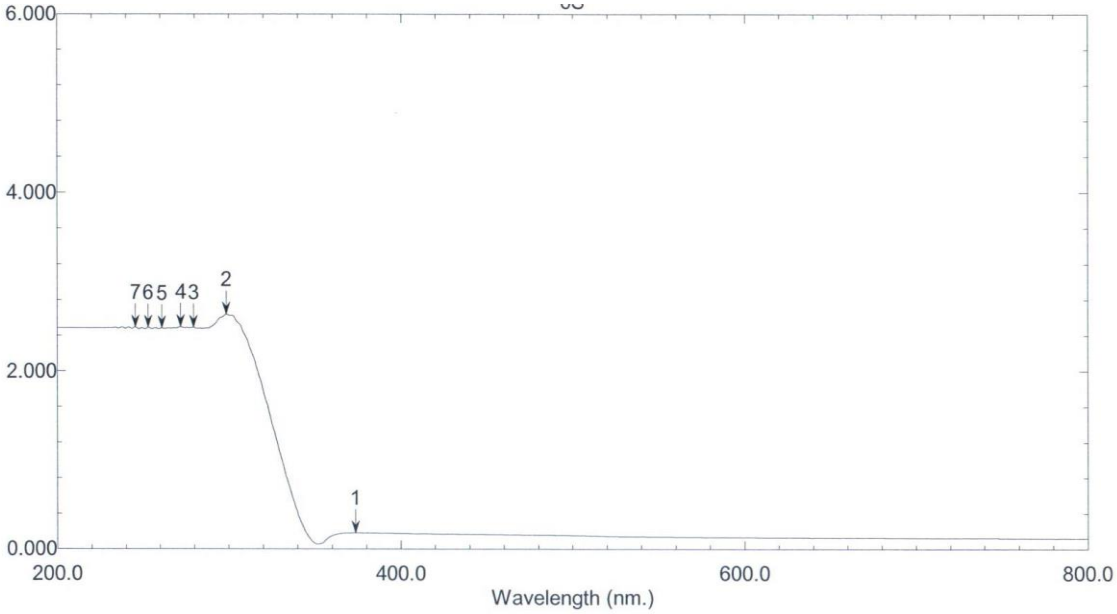

Figure 2: UV Chart Showing the extracted antibiotic within the range of erythromycin

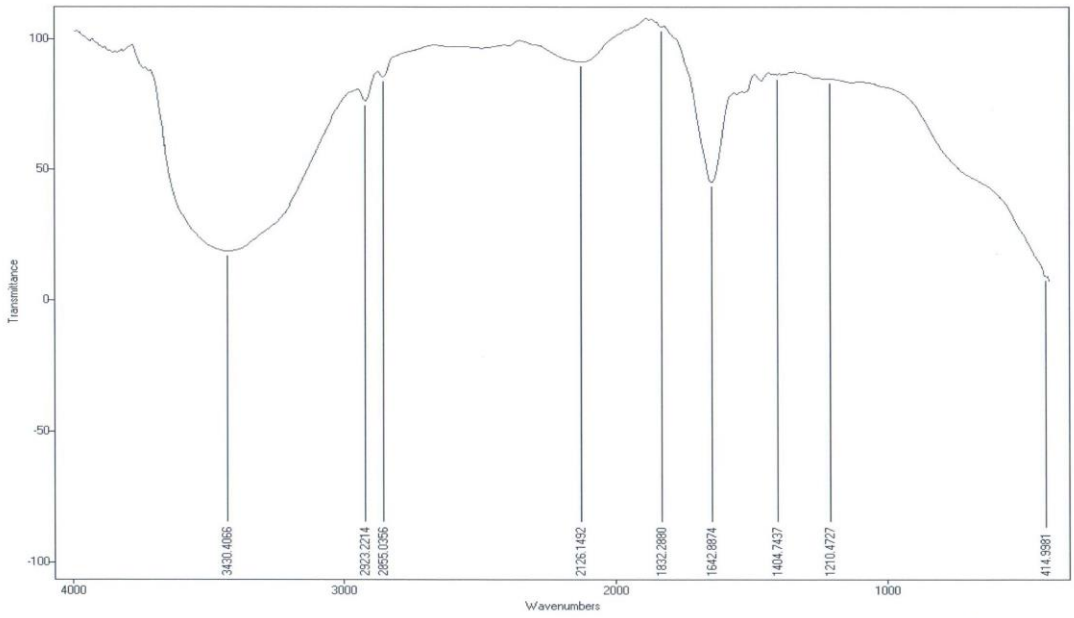

Figure 3: Infrared Spectra for partially purified bioactive Substance

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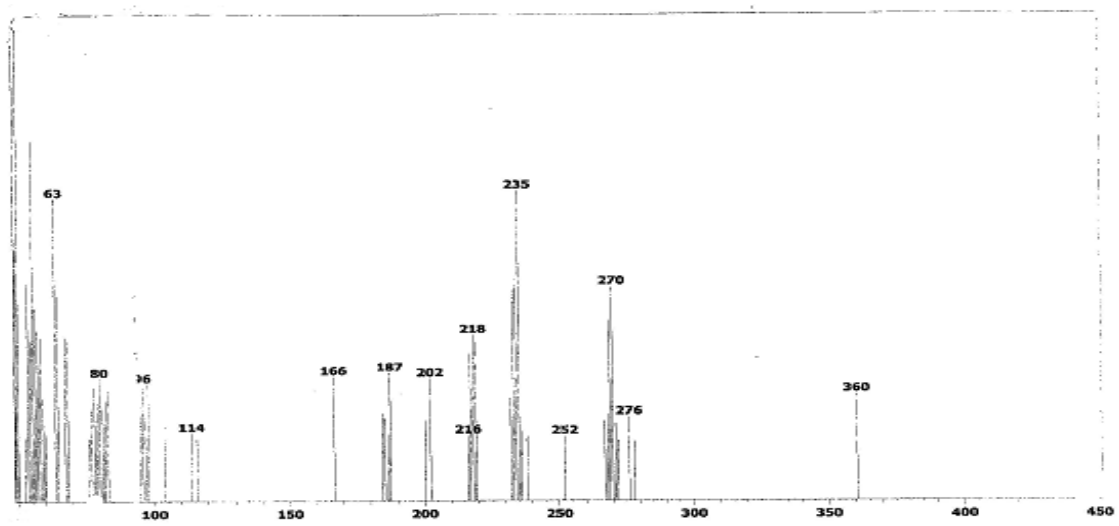

Figure 4: Mass spectra of the extracted antibiotic at range of 55

Various kinds of pharmaceuticals and hormones are used around the world and many of them are excreted via urine. These pharmaceuticals commonly include amoxicilline, carbamazepine, atenolol, erythromycin, ibubrufen, trimethoprine, tetracycline, acetyl salicylic and others. The antibacterial substance (X) extracted, and detected in this research was very close to erythromycin. Erythromycin contains in its structure a nitrogen containing sugar, however substance (X) Erythromycin gives negative molishes test for sugar and its spectral data (IR and NMR) did not show nitrogen. Also this substance gives positive Libermann for isoprenoid skeleton. The former finding may suggest erythromycin in aglycone from (i.e. without sugar moiety). The spectral data (MS, UV, IR,

HNMR and C^{13} NMR) supported deduced structur Fig (5).UV maximal at 298 nm Fig (2) and IR bands at cm^{-1} 3430 (OH), 2923, 2855 (CH, CH_2, CH_3), 1642 (C=O), 1404 (CH_3) and 1210 (OH), are close to erythromycin Fig (3). MS showed molecular weight at 418 for C_{21} H_{38} O_8 and base peak of ion M/Z = 55 and another fragment M/Z= 360 which gives fragments, at M/Z= 235 (loss of C_7H_9 O_2) and M/Z = 218 (loss of OH), also different losses of CH, CH_2, CH_3 Fig(4). A typical isoprenoid skeleton MS (360 (21), 270(43), 285(63), 218 (33), 202(24), 187 (25), 114(12), 63(61), 55(100%) are illustrated too. NMR data are shown in Table (6) which are very similar to erythromycin.

Table 6: HNMR and C^{13} NMR of substance (X) erythromycin extracted

C	∂ H ppm	∂ C^{13} ppm	C	∂ H ppm	∂ C^{13} ppm
1	-	205	11	-	73
2	2.013	160	12	1.5	39.5
3	3.4	76	13	2.01	43
4	-	39.5	14	1.15	10
5	3.46	83	15	1.12	14.4
6	-	167	16	1.26	22.8
7	2.64	45	17	1.1	18
8	3.44	79	18	1.25	11
9	2.8	40	19	1.01	9
10	2.8	85	20	1.29	22.6
			21	1.17	14.5

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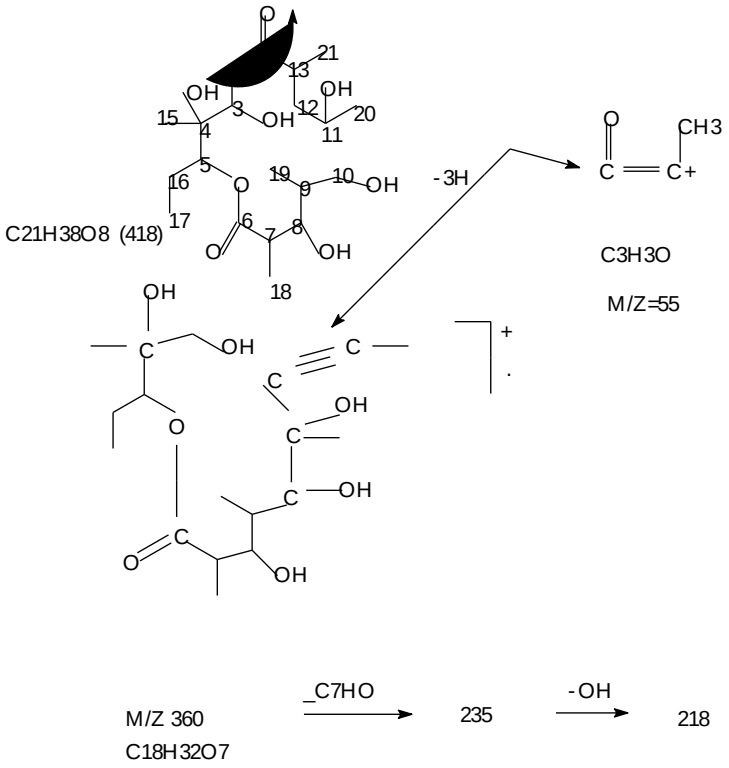

Figure 5: The suggested structure of substance under study Erythromicine aglycone.

CONCLUSION

From the above data we can conclude that substance (X) is erythromycin in a glycone form (without sugar moity). The erythromycin aglycone is not known in nature, and its presence in this research may be explained by a propable hydrolysis occurs to erythromicine in urine and also during extraction and isolation procedures.

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