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Sterility and Radiostability of Amoxicillin and Cefaclor Antibiotics Sterilized by Gamma Irradiation

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Authors' contributions

This work was carried out in collaboration between all authors. Author HNE designed the study, author SSF performed the statistical analysis, author HNE wrote the protocol, wrote the first draft of the manuscript, author AMH managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present investigations aimed at studying the effect of sterilization by gamma irradiation on amoxicillin and cefaclor antibiotics. They have been irradiated in solid dry state and the probable changes in physicochemical and microbiological properties were studied
Place and Duration of Study: The study was carried out from 2011 to 2013 in the Drug Radiation Research Department, Egyptian Atomic Energy Authority.
Methodology: Amoxicillin and cefaclor compounds in solid states were exposed to γ

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irradiation in air atmosphere at room temperature, with a dose of 25kGy and afterwards they had been subjected to microbiological and analytical tests checking their sterility and antibacterial activitiy it was tested against different pathogenic bacterial species by measuring MIC using Microdilution technique and microplate reader.
Then their chemical stability were evaluated by different techniques. EPR, FTIR, UV analysis, mass spectroscopy, and melting point.
Results and conclusion: The results showed that the majority of initial unirradiated compounds had a slight degree contamination with *Bacillus*, *Micrococcus* genera, and fungi. By applying **γ** irradiation at 25kGy it showed sterilization of the tested antibiotics and keeping their antibacterial activity. The EPR analysis results showed formation of free radicals. The other analytical tests (FTIR), (UV) analysis ,mass spectroscopy, and melting point results proved that the antibiotics analyzed are radioresistant and can be sterilized by irradiation with a dose of 25kGy, without any detrimental effect on their properities and antibacterial activity.

Keywords: Gamma-irradiation; sterilization; amoxicillin; cefaclor.

ABBREVIATIONS

NCRRT=National Center for Radiation Research and Technology; FT-IR =Fourier Transform Infrared spectrometer EPR=electron paramagnetic resonance; MIC=minimum inhibitory concentration UV=ultraviolet γ= gamma irradiation

1. INTRODUCTION

In modern medicine, a number of sterilization methods are applied, including tempering, cauterization, hot air sterilization, steam sterilization, sterile filtration, radiation sterilization(e.g., by ionizing radiation or UV light), gas sterilization (e.g., by ethylene oxide or formaldehyde), and chemical sterilization [1-3].

Sterilization is intended to kill or remove all vegetative and sporing microbes from the environment or material [4]. In the case of medical substances, choice of the sterilization method depends on the type, properties, and production method of the substance in question. Irradiation of drugs, or other medical products, by a suitable dose of ionizing radiation, conducted in an appropriate environment, ensures sterile conditions [5-8]. Basic terms and sterilization protocols can be found in ISO11137-1 [9]. Radiation sterilization is especially useful in the case of thermolabile products, because irradiation causes only a small rise in the temperature of sterilized substances [8] In the case of gamma radiation, a substance to be sterilized does not directly interact with the reagents and, as a result, lacks any traces of chemical pollution [2] Moreover, packaged products may also be irradiated as gamma radiation possesses excellent penetrative properties; this constitutes one of its economic advantages [1-2]. Medical products subjected to gamma radiation sterilization do not become radioactive [2]. It was found that the sterility assurance level (SAL) of 10^{-6} is normally achieved at 25kGy according to pharmacopoiea, which is a dose generally applicable to products manufactured under good manufacturing practice [10,11].

2. MATERIALS AND METHODS

2.1 Materials

In the present study Chemotherapeutic agents as raw materials of antibiotics were supplied from companies agents in Egypt ,i.e. amoxicillin samples were mainly supplied by Glaxo Smith kline, and cefaclor from Ranbexy.

2.2 Irradiation

The process of irradiation was carried out at NCRRT. It was performed by using Cobalt 60 source (Gamma cell 4000-A-India) at the dose rate 2.78kGy/h at activity was 2914 curie (ci) All samples were stored at room temperature in air, in the dark. Then 0.1g of each sample was placed in colourless jars and closed with a plastic stopper, then every tested antibiotic was subjected to a radiation dose equal to 25kGy according to [10].

2.3 Micrbiological Tests

2.3.1 The microbial load isolation

The isolated strains were recovered using Nutrient agar (oxoid) and Tryptone glucose yeast extract agar (oxoid), and Sabraud's dextrose agar then identified by, API20Strep for *streptococci/enterococci* and API50CHB for *Bacillus* species and other endospore-forming genera (bioMérieux, Marcy 'Etoile, France) except one strain was identified according to the method described by [12]) biolog ^{TM} ID assay biochemical features were analyzed using 96 well BIO LOG TM ID technology (Biolog Inc Haywar, CA, USA) the GN 3 (Gram positive bacteria). The data were analyzed with Microlog 3, 5 software (Biolog inc.)

2.3.2 Microdilution technique (mlcrobiological assay and MIC)

The microbial inoculum of the test organisms under Study (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633), and *Pseudomonas aeruginosa* ATCC 27853, were prepared according to the method described by [13]).Isolates were removed from storage, streaked onto a Trypticase soy agar plate (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and incubated for 18 to 24h at 37ºC. A working bacterial suspension was prepared by suspending 3–5 isolated colonies in 3 ml of Mueller–Hinton media. The turbidity of this suspension was carefully adjusted photometrically to equal that of a 0.5 McFarland standard. For the test, the final inoculum was further diluted in Mueller–Hinton media to achieve a final concentration of 0.5×10^5CFU/ml. Microdilution plate was prepared according to the method described by [14]) as the following: Sterile 96-well U bottomed, microtitre plates (Tarsons, India) were used. The stock suspensions of the drugs were prepared in Mueller–Hinton medium and 0.2ml of the highest concentration of each drug was added to the respective wells of the first row of the plates except the first and the last well.

2.3.3 The plates were read by two methods

a- Visually by comparison with the drug free controls.
b- With micro plate reader at a wavelength of 450nm

2.4 Sterility Test

Sterility tests were carried out according to USP 34 [15] by filtration method.

2.5 Statistic Analysis

The microbiological stability tests were carried out in triplet. For comparison and statistical analysis, observations were obtained for each combination of isolate and antibiotic agent. then was analyzed by statistics program SPSS-version 15 (independent sample .T. test).

2.6 UV Spectrophotometry

UV spectrophotometric determinations were carried out on aqueous solutions of the unirradiated and irradiated samples of antibiotic using JASCO UV 560 spectrophotometer (JASCO International Co. LTD ,Japan).

2.7 IR Spectroscopy

The dry powder of both unirradiated and irradiated samples of antibiotics were mixed and compressed with KBR then analysed by (FT/IR-6300 FT-IR Spectrometer).

2.8 Mass Spectroscopy

The dry powder of antibiotics was dissolved in 95%methanol. Then analayzed by mass spectrophotometer by direct inlet unit (DI-50) of Shimamadzu GC/MS-QP5050A.

2.9 Electron Paramagnetic Resonance (EPR)

It has matured into a powerful, versatile, non-destructive, and non intrusive analytical method using Bruker EMX spectrometer (X-band) product of Bruker, Germany.

2.10 Melting Point

The samples were analyzed in solid state by using stuart melting point apparatus (Stuart Analogue Melting Point Model SMP11).

3. RESULTS

3.1 The Microbial Load Isolation

The results obtained from isolation and identification of isolated strains from antibiotics were shown in (Table 1).

Table 1. Microbiological contamination of tested antibiotics before and after irradiation at 25kGy

Compound	Microorganisms observed growth	
	0kGy	**25kGy**
Amoxicillin	1- *Bacillus sphearicus* 2-*Bacillus pumilus* and fungi	No growth observed
Cefaclor	1-*Bacillus.subtilis* 2-*Micrococcus luteus* and fungi	No growth observed

3.2 Evaluation of the Stability of Biological Activity of Antibiotic Amoxicilin & Cefaclor

Evaluation the stability of biological activity of the both antibiotics amoxicillin and cefaclor was tested against the previously mentioned four standard bacterial organisms (2.3.2); the effect of the antibiotic was measured by micro plate reader. It was found that the antibacterial activity of un-irradiated amoxicillin and cefaclor are the same as the irradiated samples at 25 kGy. The results of evaluation of biological activity of antibiotic amoxicillin and cefaclor against the bacterial test organisms are summarized in (Figs. 1-8). In order as follows, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, and *Pseudomonas aeruginosa* ATCC 27853, respectively when unirradiated sample (blue) and irradiated sample (red). Pseudomonas showed the same resistance to both unirradiated and irradiated antibiotics samples it showed growth for all used concentrations. *Pseudomonas auregienosa.* recorded a great resistance to many antimicrobials [16], and specially resistance to amoxicillin and cefaclor [17] MIC values are shown in (Table 2).

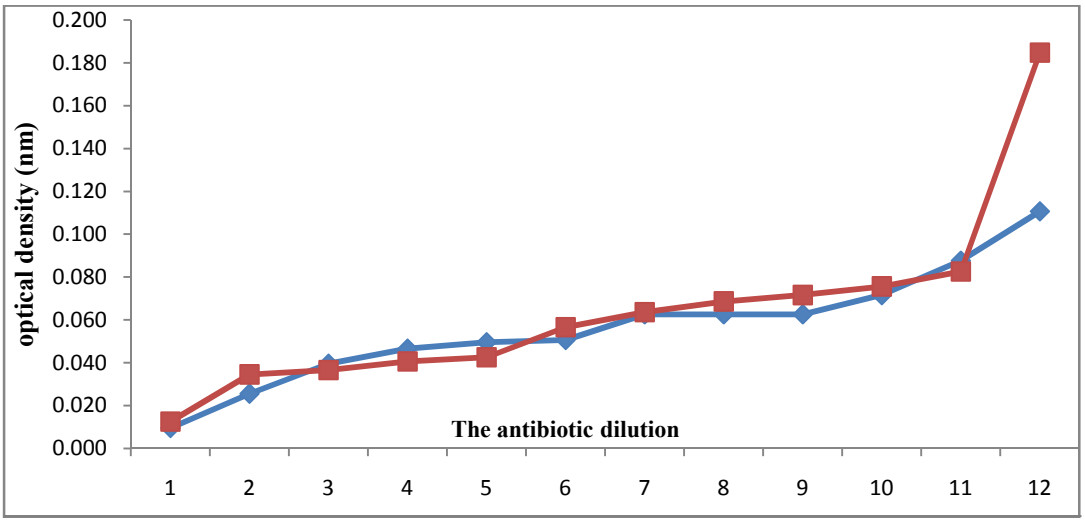

Fig. 1. MIC of both unirradiated (blue) and irradiated (red) amoxicillin for *Staphylococcus aureus*

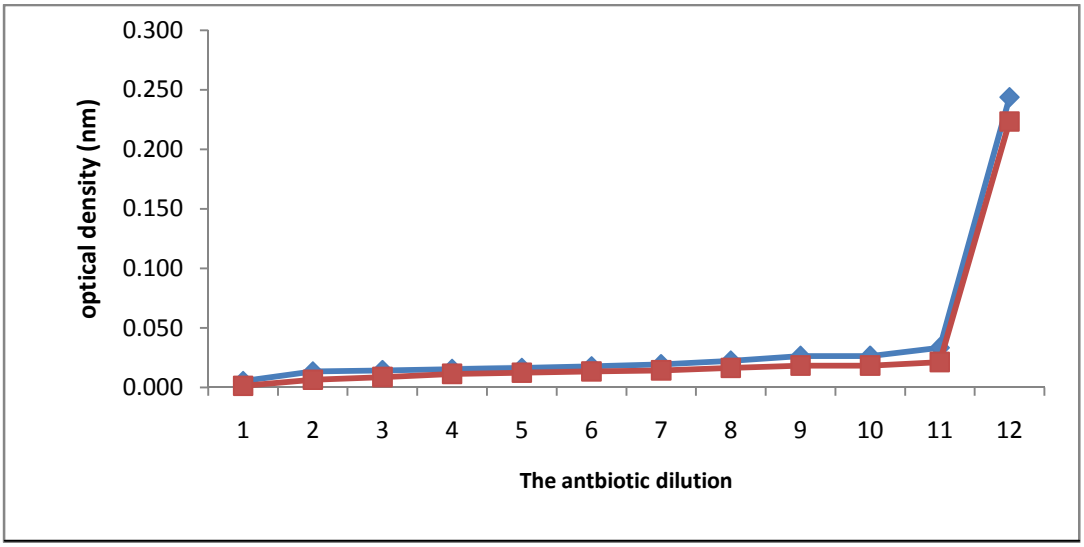

Fig. 2. MIC of both unirradiated (blue) and irradiated (red) amoxicillin for *Escherichia coli*

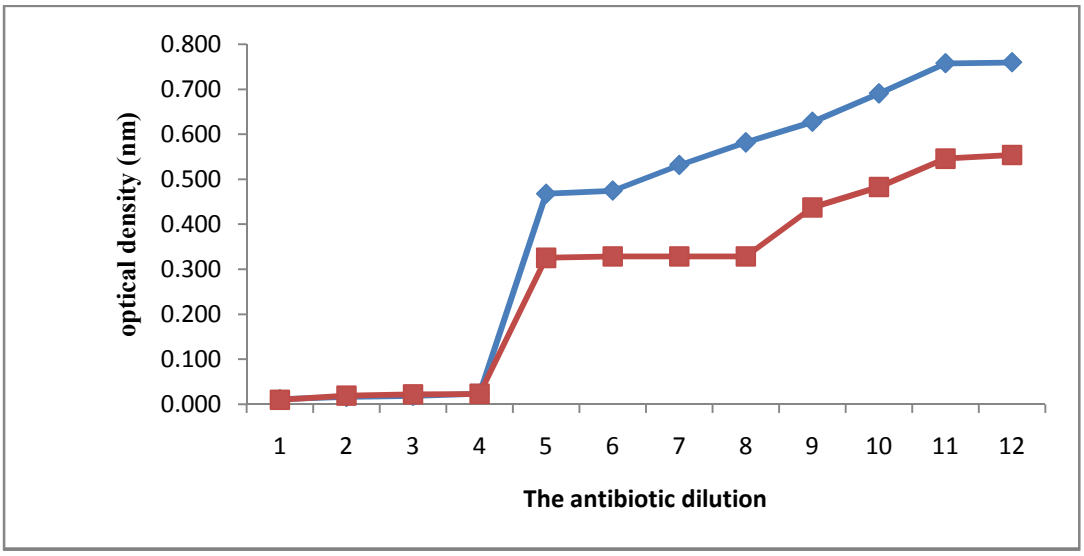

Fig. 3. MIC of both unirradiated (blue) and irradiated (red) amoxicillin for *Bacillus subtilis*

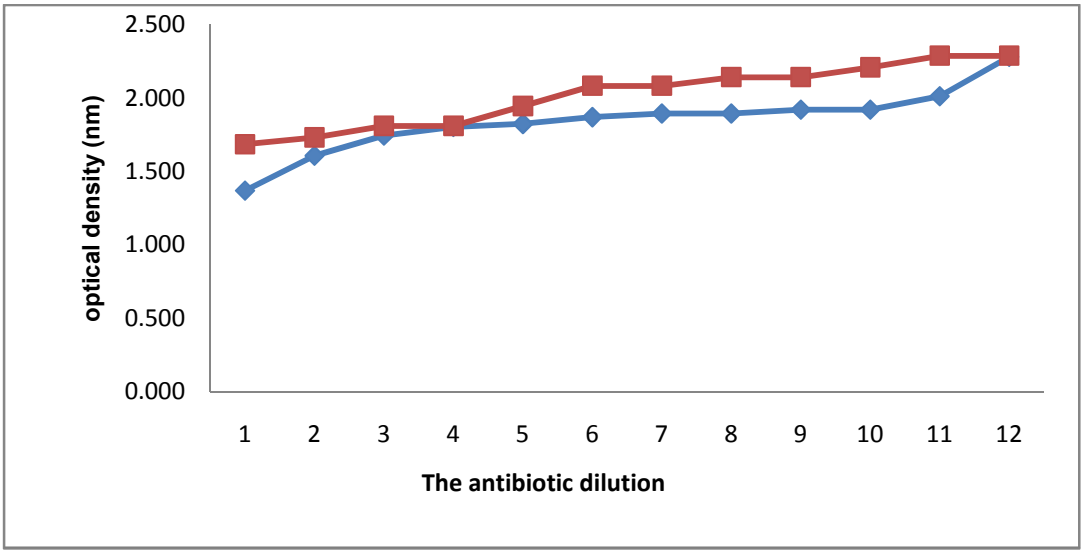

Fig. 4. The response (resistance) of *Pseudomonas aeruginosa* for both unirradiated (blue) and irradiated (red) amoxicillin

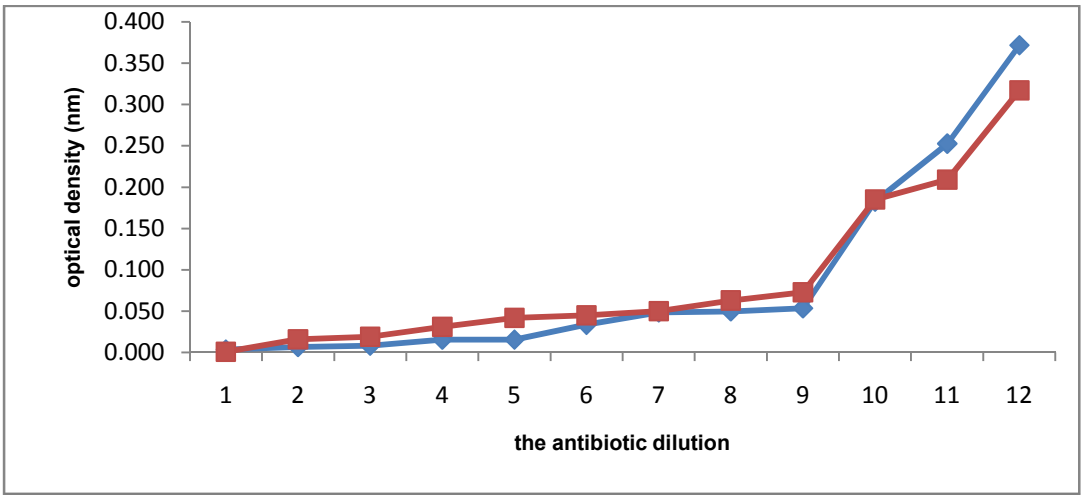

Fig. 5. MIC of both unirradiated (blue) and irradiated (red) cefaclor for *Staphylococcus aureus*

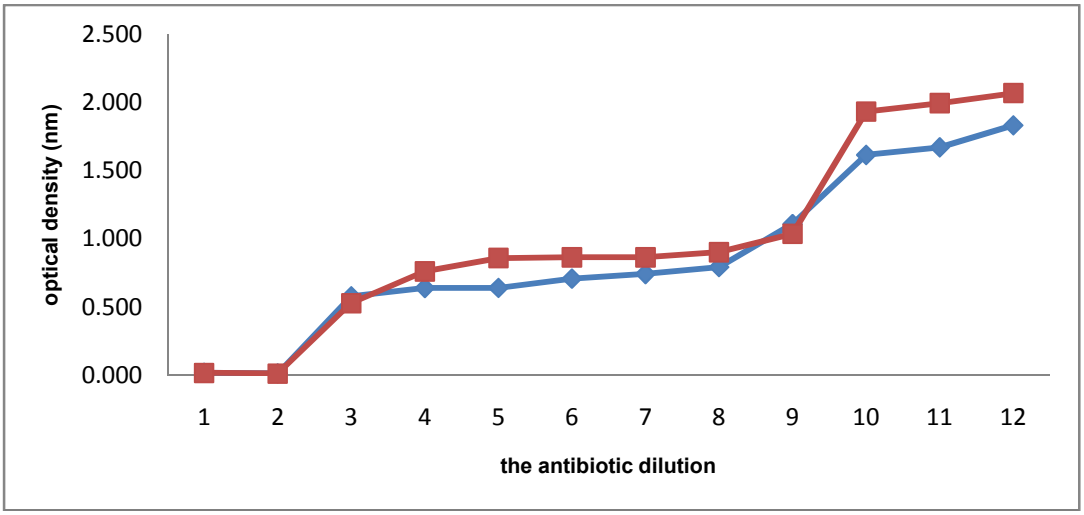

Fig. 6. MIC of both unirradiated (blue) and irradiated (red) cefaclor for *Escherichia coli*

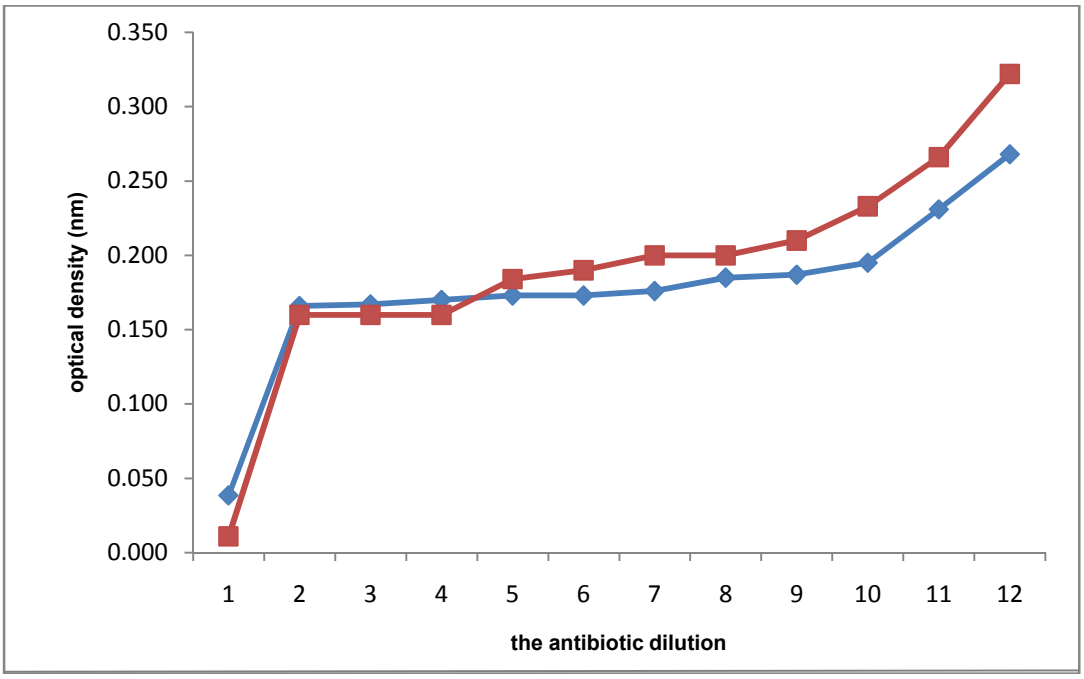

Fig. 7. MIC of both unirradiated (blue) and irradiated (red) cefaclor for *Bacillus subtilis*

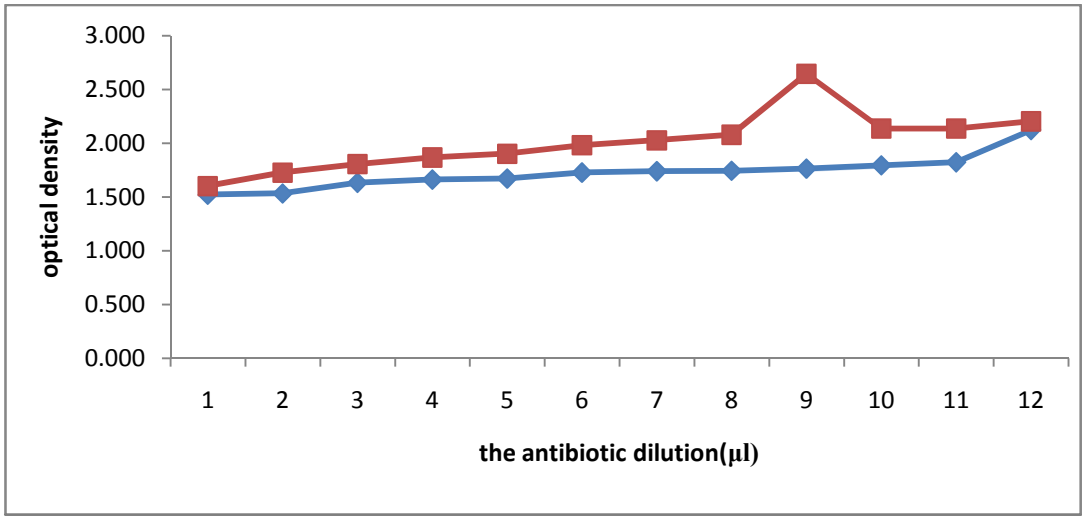

Fig. 8. The response of *Pseudomonas aeruginosa* for both unirradiated (blue) and irradiated (red) cefaclor

Table 2. The MIC values of both unirradaited and irradiated antibiotics

The antibiotic		The microorganism growth observed (in nm) and its equivalent MIC value of antibiotic (µg/ml)			
		***Staphylococcus aureus* ATCC 25923**	***Escherichia coli* ATCC 25922**	***Bacillus subtilis* ATCC 6633**	***Pseudomonas *aeruginosa* ATCC 27853**
Amoxicillin	unirradiated	0.088 (1µg/ml)	0.029 (1µg/ml)	0.023 (128µg/ml)	Recorded resistance*
	irradiated	0.083 (1µg/ml)	0.026 (1µg/ml)	0.023 (128µg/ml)	Recorded resistance*
Cefaclor	unirradiated	0.183 (4µg/ml)	0.013 (512µg/ml)	0.166 (512µg/ml)	Recorded resistance*
	irradiated	0.185 (4µg/ml)	0.012 (512µg/ml)	0.160 (512µg/ml)	Recorded resistance*

** P. aeruginosa showed resistance to all used concentrations, (the MIC of P. aeruginosa growth inhibition was found to be 200-400µg/ml, according to (18) (Where,,dilution1=1024 µg/ml, dilution 2=512µg/ml, dilution 3=256µg/ml, dilution dilution 4=128µg/ml, dilution 5=64µg/ml, dilution 6=32µg/ml , dilution 7=16µg/ml, dilution 8=8µg/ml, dilution 9=4µg/ml, dilution 10=2µg/ml, dilution 11=1µg/ml, and dilution 12=0.5µg/ml)*

3.3 Sterility Test

(Figs. 9-10) shows the sterility test of both unirradiated and irradiated antibiotic samples. Irradiated amoxicillin and cefaclor on right side shows no growth. On other hand in the unirradiated antibiotic sample on left side shows turbidity which indicating the presence of microbial load.

Fig. 9. Sterility test of unirradiated and irradiated amoxicilin

Fig. 10. Sterility test of unirradiated and irradiated cefaclor

3.4 UV and Discoloration

Irradiation discolored the amoxicillin powders from off-white to yellow, as shown in (Table 3) and (Figs. 11,12) but kept the same λ max absorption peak. Irradiation did not change the color of cefaclor as shown in (Table 4) and (Figs. 13,14) and kept also the same λ max absorption peak.

Table 3. UV analysis results of amoxicillin before and after irradiation

Test	Unirradiated sample	25kGy
Color	Off White	yellow
Dispersibility	Uniform suspension	Uniform suspension
Clean of suspension	Free from visible impurities	Free from visible impurities
λ max absorption peak	280nm	280nm

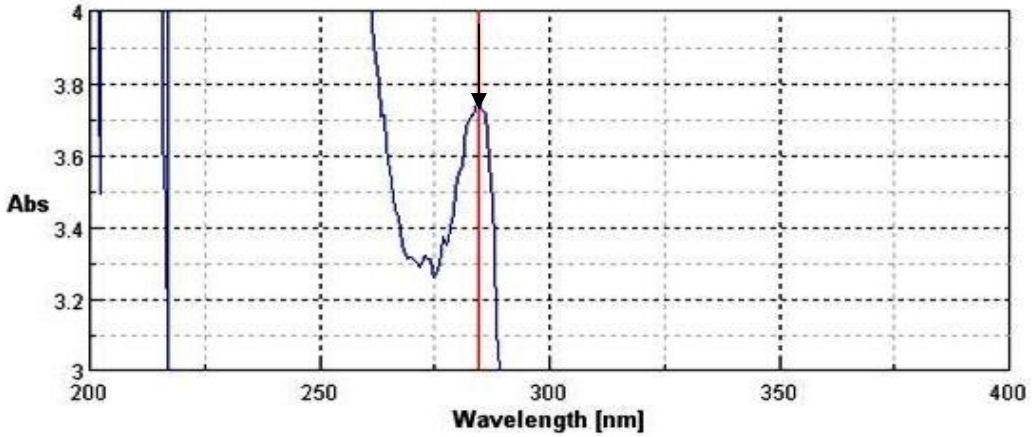

Fig. 11. UV-spectrum of unrradiated amoxicillin powder

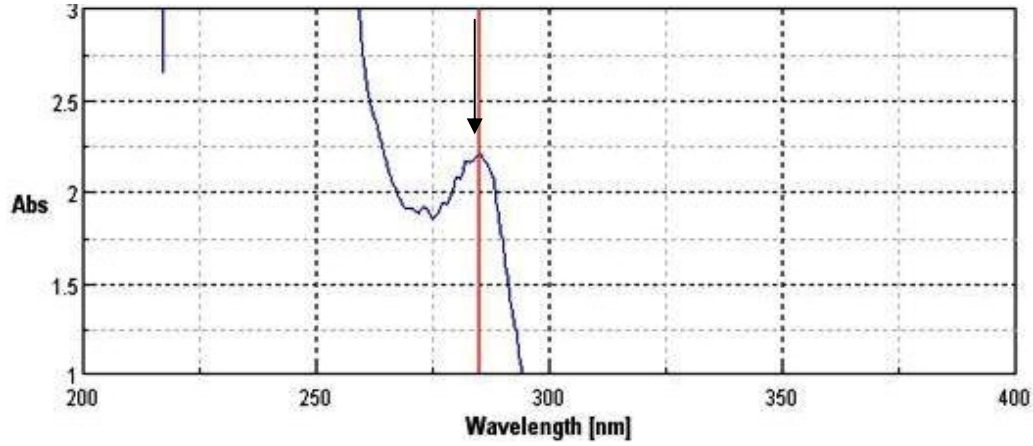

Fig. 12. UV-spectrum of irradiated amoxicillin powder at 25kG

Table 4. UV analysis results of cefaclor before and after irradiation

Test	Unirradited sample	25kGy
Color	Off white	Off white
Dispersibility	Uniform suspension	Uniform suspension
Clean of suspension	Free from visible impurities	Free from visible impurities
λ max absorption peak	580nm	580nm

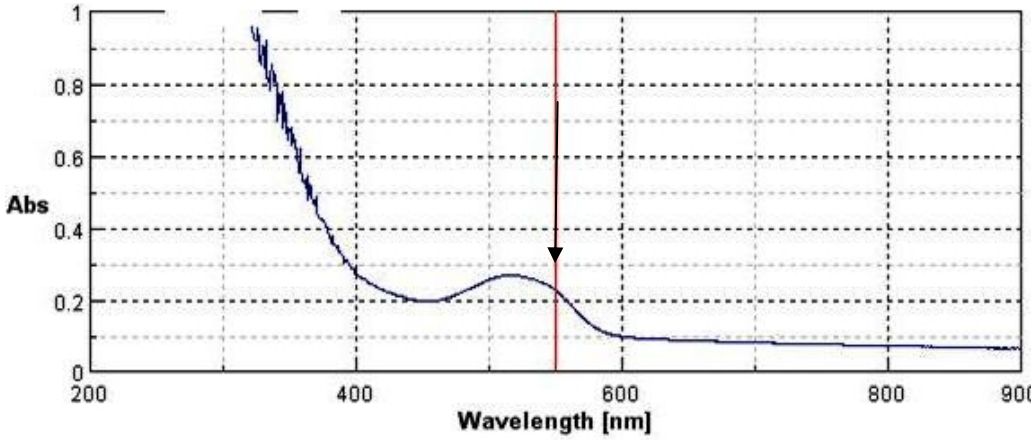

Fig. 13. UV-spectrum of un-irradiated powder of Cefaclor

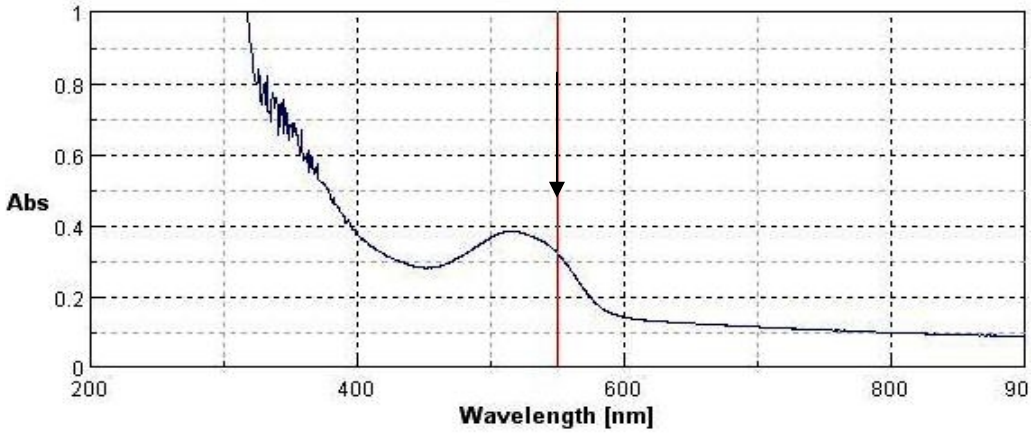

Fig. 14. UV-spectrum of irradiated powder of Cefaclor at 25kGy

3.5 IR Spectroscopy

The infrared spectrum of unirradiated amoxicillin and cefaclor showed the same strong absorption peaks of irradiated at dose 25kGy as shown in (Tables 5-6) and (Figs. 15-18) for amoxicillin and cefaclor respectively.

Table 5. IR most absorbant function groups of amoxicillin before and after irradiation

Wavelength of Peak in cm^{-1}	Unirradiated	Irradiated	Comments
3200-3459	present	present	characteristic to OH,NH and NH2 groups
3043.12	present	present	characteristic to C-H aromatic
1774.19	present	present	characteristic for C=O group of the β-lactam ring and C=O of amide group, respectively
2356.59	present	present	characteristic for C-N bond

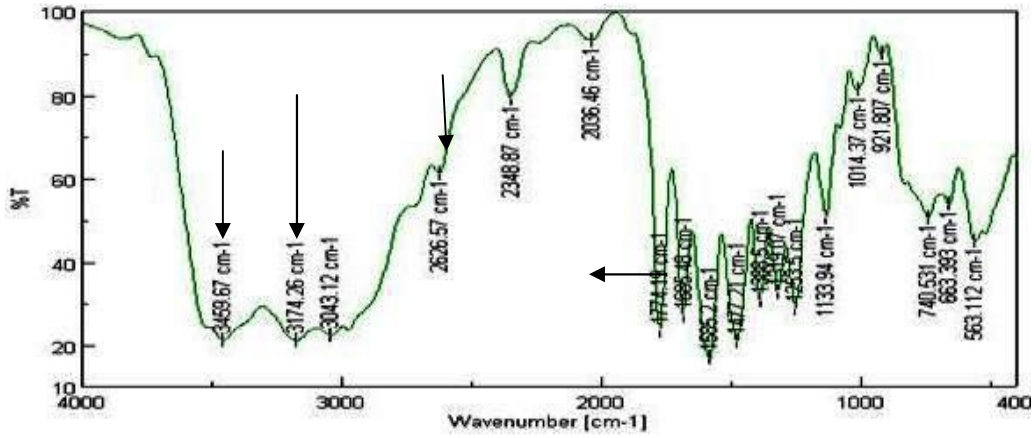

Fig. 15. FT-IR analysis of unirradiated amoxicillin

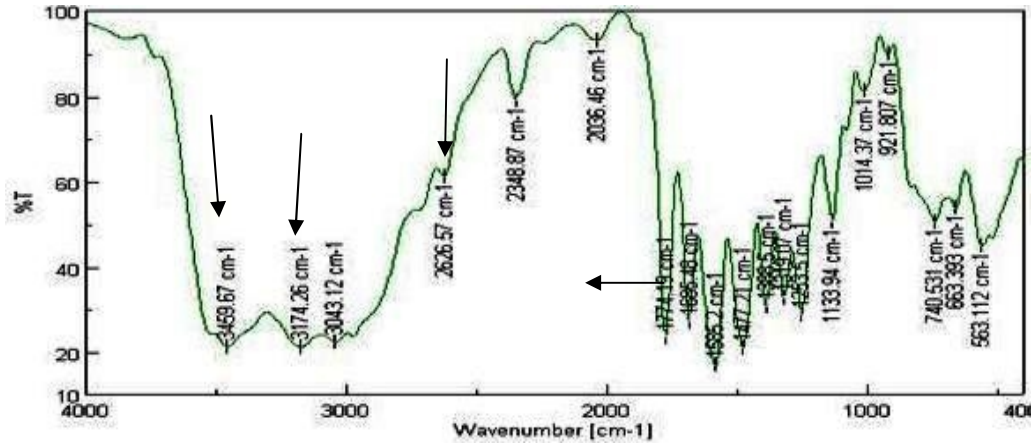

Fig. 16. FT-IR analysis of irradiated amoxicillin

Table 6. IR most absorbant function groups of cefaclor before and after irradiation

Wavelength of peak cm^{-1}	Unirradiated	Irradiated	Comments
3332.39	present	present	characteristic to OH and NH2 groups
3031.56	present	present	characteristic to C-H aromatic
2356.59	present	present	characteristic for C-N bond
1720.33	present	present	characteristic for C=O group of the β-lactam ring and C=O of amide group, respectively

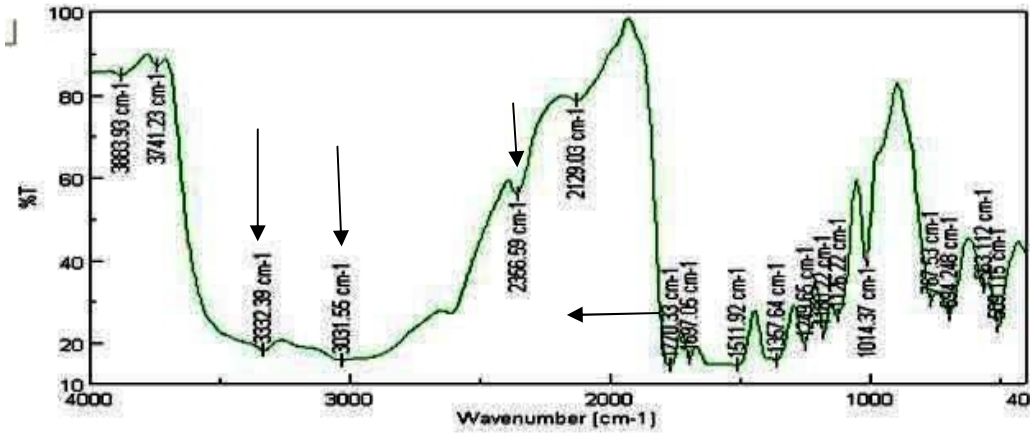

Fig. 17. FT -IR analysis of unirradiated cefaclor

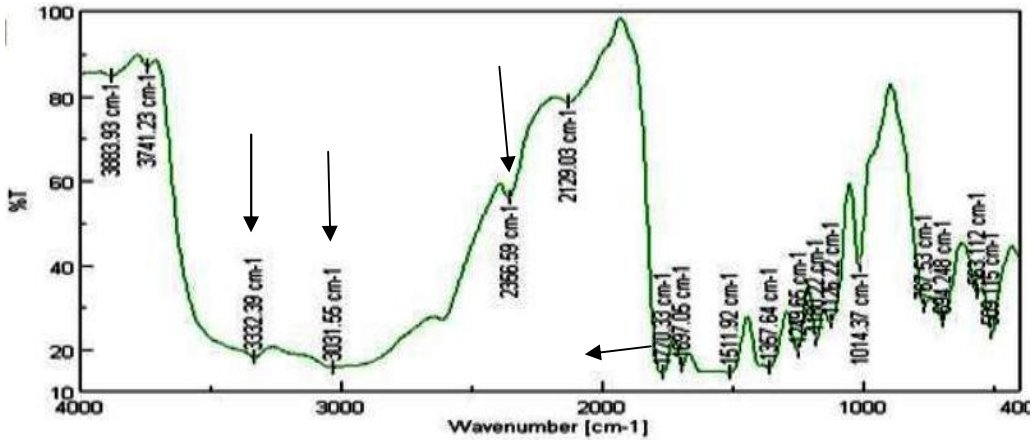

Fig. 18. FT-IR analysis of irradiated cefaclor

3.6 Mass Spectroscopy

The mass spectra of the unirradiated and irradiated samples of both antibiotics showed almost the same fragmentation pattern. Summarized in (Tables 7 and 8) and (Figs. 19-22) for amoxicillin and cefaclor respectively.

Table 7. The mass spectra of amoxicillin before and after irradiation

Fragmentation peaks	Fragmentation abundance of antibiotics	
	Unirradiated amoxicillin	Irradiated amoxicillin
479	Present	Present
402	Present	Present
361	Present	Present
357	Present	Present
337	Present	Present
313	Present	Present
282	Present	Present
216.7	Present	Present
210	Present	Present
193	Present	Present

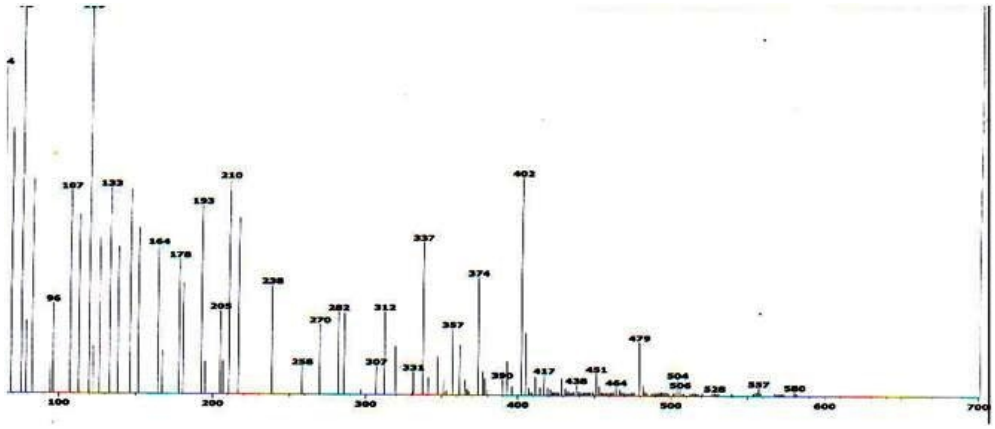

Fig. 19. Mass spectra of unirradiated amoxicillin

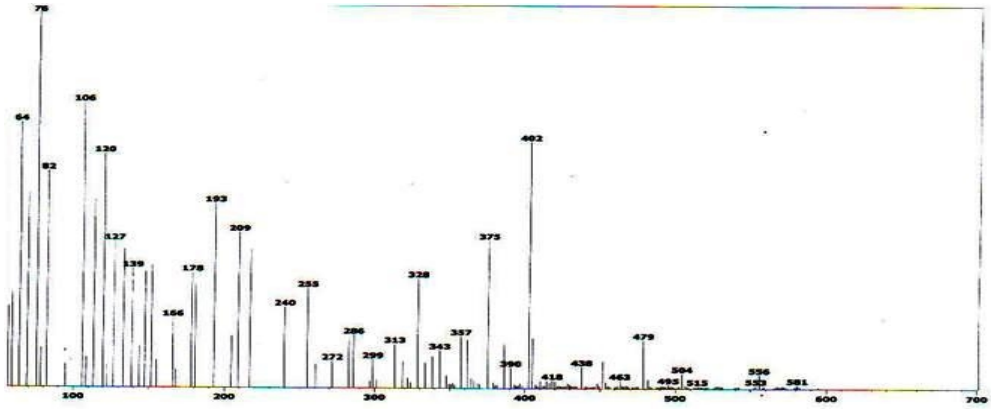

Fig. 20. Mass spectra of irradiated amoxicillin

Table 8. The mass spectra of cefaclor before and after irradiation

Fragmentation peaks	Fragmentation abundance of antibiotics	
	Unirradiated cefaclor	Irradiated cefaclor
402	present	present
375	present	present
358	present	present
329.2	present	present
204	present	present
193	present	present
164	present	present
151	present	present
147	present	present
132	present	present
119	present	present

Fig. 21. Mass spectra of unirradiated cefaclor

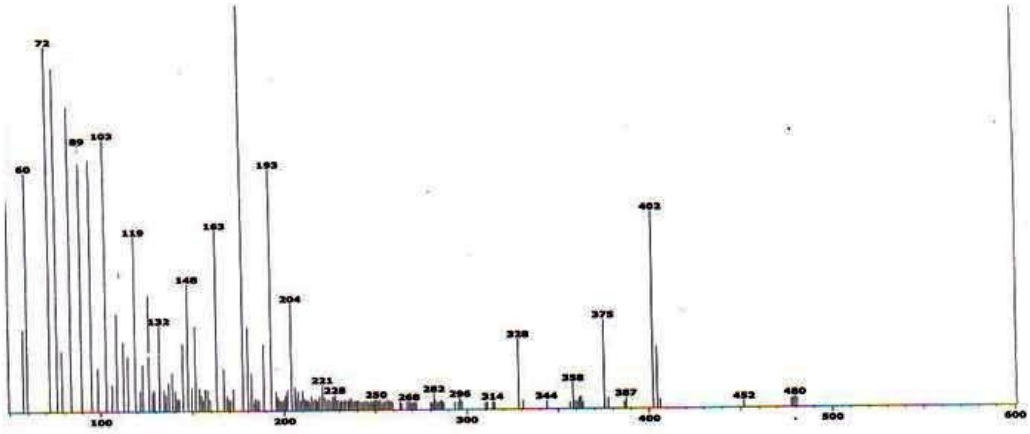

Fig. 22. Mass spectra of irradiated cefaclor

3.7 Electron Paramagnetic Resonance (EPR)

Unirradiated antibiotics samples showed no signal for amoxicillin but for cefaclor there was a small singlet. The irradiated samples showed a slight increase in peak intensity indicating formation of free radical after irradiation. (Figs. 23 and 24) for amoxicillin and (Figs. 25-26) for cefaclor.

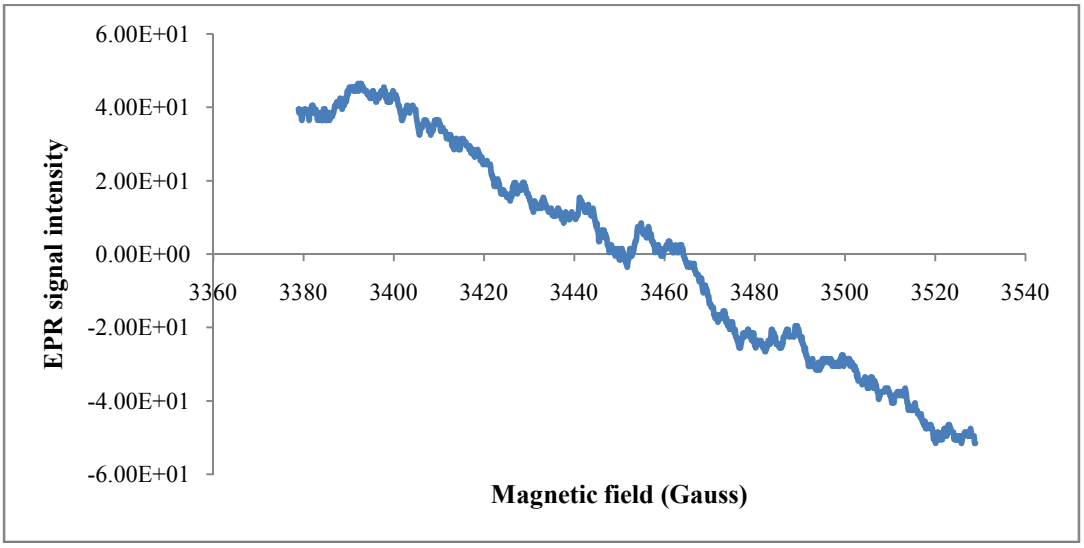

Fig. 23. EPR analysis of unirradiated amoxicillin

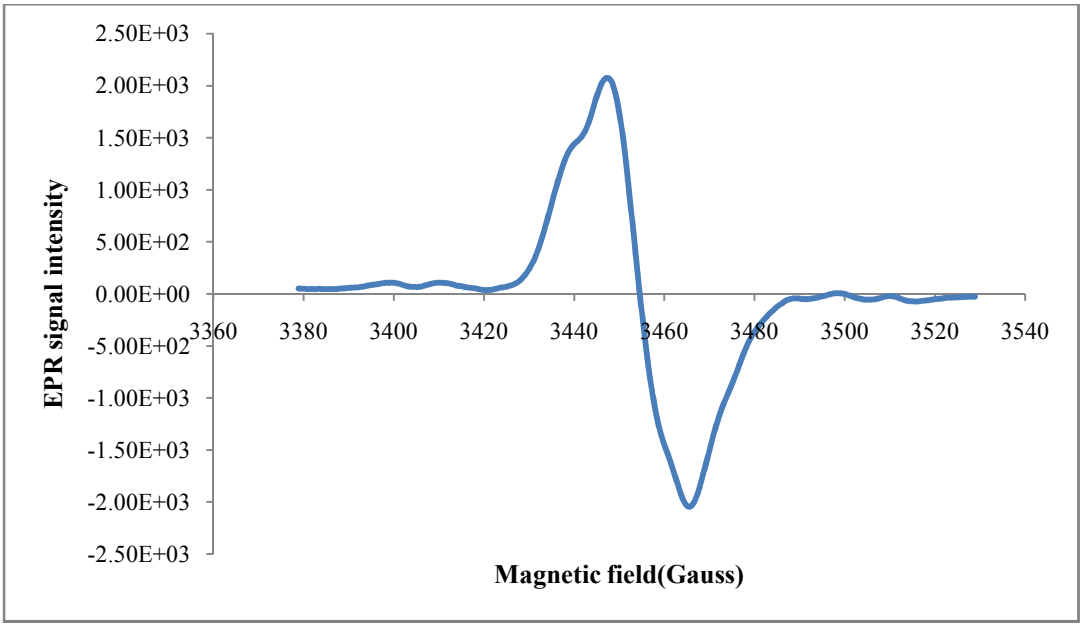

Fig. 24. EPR analysis of irradiated amoxicillin

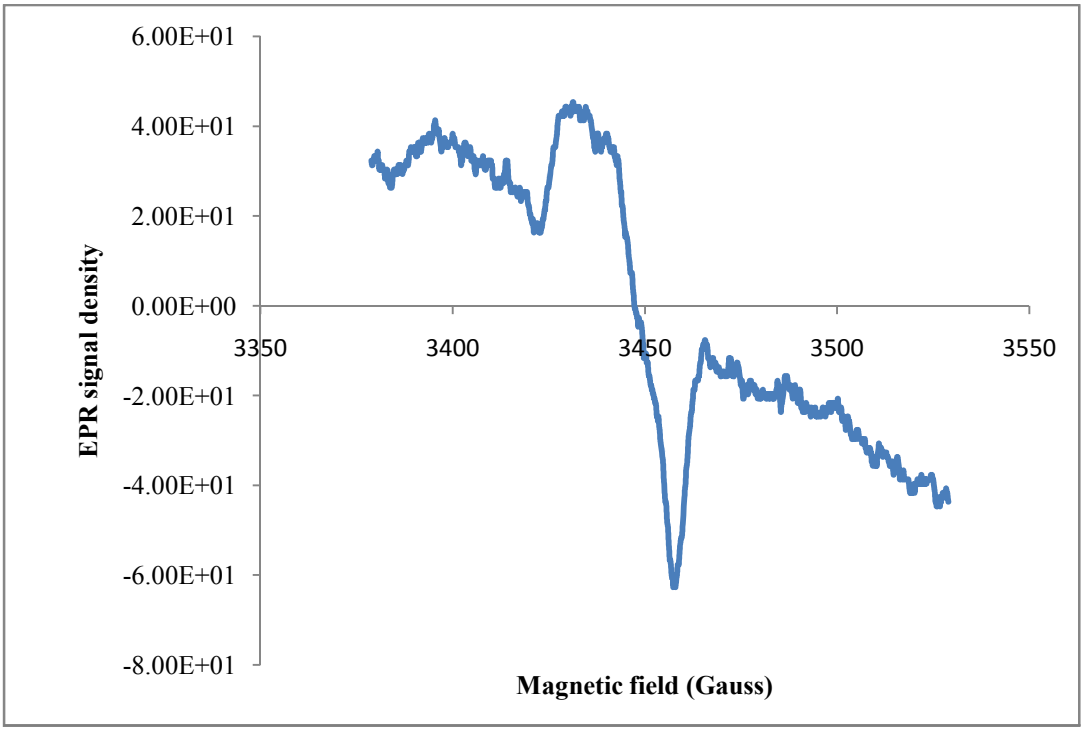

Fig. 25. EPR analysis of unirradiated cefaclor

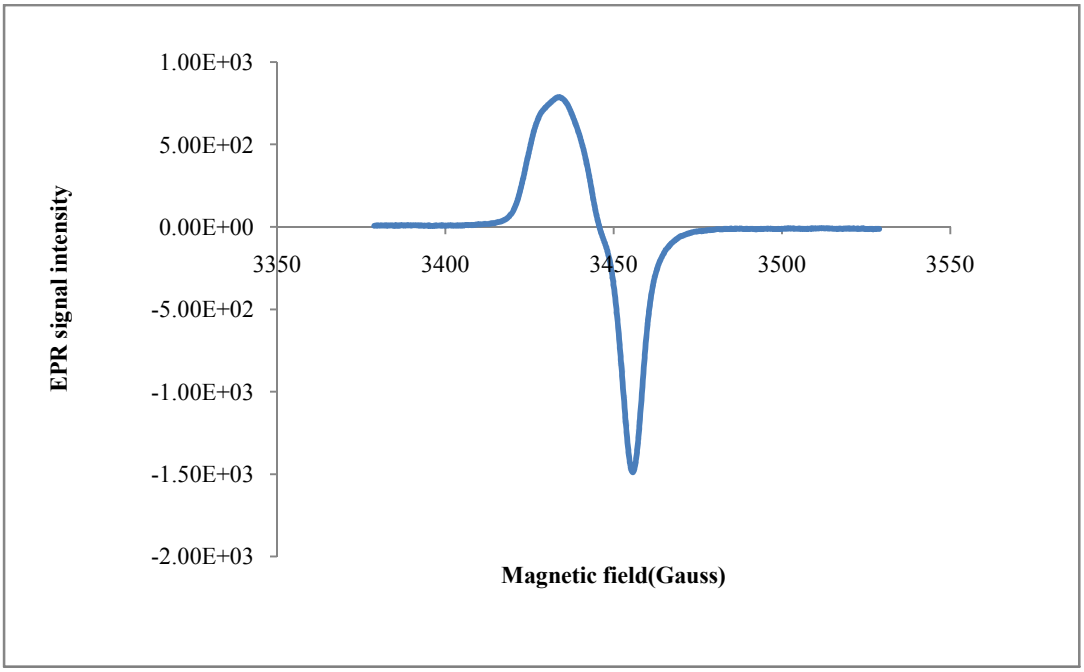

Fig. 26. EPR analysis of irradiated cefaclor

3.8 Melting Point Result

The results showed there is no difference in the melting point of the antibiotics before and after irradiation (Table 9).

Table 9. Melting points before and after irradiation

The antibiotic name	Melting point range		
	Before radiation	After radiation	The USP standard
Amoxicillin	192ºC-194ºC	192ºC-194ºC	192ºC-194ºC
Cefaclor	327ºC	327ºC	327ºC

4. DISCUSSION

The results of the of microbiological purity tests of unirradiated antibiotics revealed that there was a different range of contamination with microorganisms. The microorganisms isolated were Gram positive *Bacillus sphearicus* and *Bacillus pumilus* for amoxicillin and Gram–positive *Microccocus luteus* and *Bacillus subtilis* for cefaclor and a slight contamination with fungi for both drugs. These results are in accordance with [19] who found that some penicillins and their salts, gentamycin and neomycin had been contaiminated to a slight degree by bacteria from genera *Bacillus* and *Micrococcus* and fungi.

After gamma irradiation of the antibiotics under study at 25kGy their sterility was tested and it was found that there was no observed microbial growth.This result is inaccordance with [20] who recorded that some penicillins (piperacillin, cabenicillin and benzyl penicillin) did not reveal any microbial growth after gamma irradiation at 25kGy.

Biological & antibacterial activity of the antibiotics that evaluated by microdilution technique suscepility tests ,was not affected significantly *(P*>.05). These results were in accordance with [21] who reported that the biological activity of penicillin and ampicillin was not affected significally after irradiation at doses 10 and 25kGy.

UV absorption of unirradiated sample of amoxicillin was the same as irradiated sample at the λ max 280, except darkening from off-white to yellow color was observed in the irradiated sample, same results were obtained by [22] that recorded that gamma radiation has effected on the color of the ampicillin and crystalline penicillins, at 25kGy caused discoloration. While for cefaclor no change in color was observed and absorption was at λ max 580 nm. These results are in agreement with [23] who found that no changes of the UV spectra were noticed up to 50kGy for cephradine.

The melting point of unirradiated and irradiated amoxicillin was the same the mass spectra of unirradiated and irradiated amoxicillin samples showed nearly the same fragmentation pattern. Also similar results were recorded by [24] that there were no differences of melting point of both unirradiated and irradiated cephradine.

The EPR results for unirradiated and irradiated amoxicillin and cefaclor showed that there is a slight increase in peak intensity after treating with gamma indicating formation of free radicals, [25] mentioned that in the commercial market of drugs, radicals should be detected up to two years after irradiation. Also our results are ingreement with [22,26] who observed that decrease in free radicals concentration for irradiated samples of ampicillin is as a

function of storage time can be explained by the interaction of the free radicals with oxygen molecules O_2.

It was found that the FT-IR of unirradiated and irradiated samples of amoxicillin and cefaclor have the same characteristic absorption bands indicating that there is no change in the structure of both antibiotics, also [23] observed that infrared spectra of both unirradiated and irradiated cephradine were identical.

The mass spectra of unirradiated and irradiated amoxicillin and cefaclor samples showed nearly the same fragmentation pattern, the same result was obtained by [27] who found that the fragmentation pattern of MS-MS spectrum of cefatoxime and those of radiolytic compounds were very similar suggesting that they were structural analogues to the main drug.

These collective results disagree with those obtained by [28] who reported that the sterilized penicillin-derived antibiotics: piperacillin, ampicillin, and crystalline penicillin antibiotics drugs following gamma sterilization showed very different properties therefore may reduce the therapeutic properties of the pharmaceuticals. Free radicals formed in sterilized drugs may have an effect not only on the pharmacological activity of the drug [29], but also on its pharmacokinetic properties [30], which is often neglected in the scientific literature.

5. CONCLUSION

This study showed that amoxicillin and cefaclor antibiotics are radioresistant from a chemical point of view and for the antimicrobial activity our tests also showed no change in the antibiotics activity i.e no qualitative or quantitative differences were observed. Therefore they could be suitable candidates for radiosterilization studies by gamma rays in solid state. Briefly, this research considered the feasibility of radiation sterilization for cefaclor and amoxicillin antibiotics in dry state.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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