Azhar Int J Pharm Med Sci 2023; Vol 3 (2):85-98

Open Access

Print ISSN: 2735-4598
Online ISSN: 2735-4601

(Research Article)

Microbiological evaluation of antimicrobial agents potency in some Egyptian pharmaceutical products

Ahmed M. Saber[1*], Fathy M. Elkady[2], Mohamed S. Ashour[2]

[1] *Microbiology Laboratory, Quality Control, Egyptian International Pharmaceutical Industries Company (EIPICO).*
[2] *Department of Microbiology and Immunology, Faculty of Pharmacy (Boys), Al–Azhar University, Cairo, Egypt.*

*** Correspondence**: amsasd@ymail.com**.**

Article history: **Received** 26-10- 2022 **Revised** 25-01-2023 **Accepted** 16-03-2023.

Abstract: Microbiological bioassay, utilizing sensitive microbial indicator, commonly applied in quality control to determine the potency of antimicrobial agents. This method depends on the biological inhibitory activity of the tested antimicrobial agent on the growth of suitable microbial indicator in comparison to reference standard. The current study aimed to follow up the potency of three of the most prescribed antimicrobial agents namely, amikacin, ceftriaxone, and ciprofloxacin, in various commercial pharmaceutical preparations. The 5 X 1 microbiological bioassay in presence of *Staphylococcus aureus* ATCC 6538P strain, as a sensitive indicator, was used to evaluate the potency of the tested antimicrobial agents. It was observed that the products decomposition rates varied throughout test intervals; however, the potency of all products remained within the range established by the US Pharmacopeia (USP) by the end of the study. In conclusion, microbiological bioassay using *Staphylococcus aureus* ATCC 6538P standard strain is appropriate for the assessment of amikacin, ceftriaxone, and ciprofloxacin, in different commercial antimicrobial products, and can be routinely applied in their quality control.

Keywords: Microbiological bioassay, amikacin, ceftriaxone, ciprofloxacin, quality control.

1. INTRODUCTION

Ciprofloxacin is the most clinically and economically successful fluoroquinolones derivative that represents one of the most widely used antimicrobial agents in treating the infection of urinary, respiratory, and gastrointestinal tracts [1].

Similarly, amikacin is one of the popularly prescribed antimicrobial agents. It is effective against wide range of severe microbial diseases especially Gram–negative bacterial infections [2].

As well, ceftriaxone is a 3rd generation cephalosporin effective in treatment of wide range of Gram–positive and Gram–negative bacterial infections. Compared with 1st or 2nd generations, it has been widely used due to its only once–daily administration and better stability against traditional penicillinase enzyme [3].

The minor variation of these antimicrobial agents concentration in their pharmaceutical preparations may affect the genuine efficacy. Accordingly, accurate potency evaluation of the active constituent in these preparations is critical for their activity and particularly essential for quality assurance and quality control [4].

Quantification of active pharmaceutical ingredients is carried out using either physicochemical or microbiological assay [5]. Unfortunately, the attractive and convenient modern physicochemical automated methods are unable to truly estimate the antimicrobial activity in case of antimicrobial combination [6]. Not only that, but when assessing the stability of antimicrobial drugs, these techniques are incapable of picking up the subtle changes that are linked to a decline in efficacy [7].

Cite this article: Saber, A., Elkady, F., Ashour, M.. Microbiological evaluation of antimicrobial agents potency in some Egyptian pharmaceutical products. Azhar International Journal of Pharmaceutical and Medical Sciences, 2023;3(2):85-98 doi: 10.21608/AIJPMS.2023.171015.1176

DOI : **10.21608/AIJPMS.2023.171015.1176**

On the other hand, no highly toxic solvents or particular apparatus are required in microbiological assays [8].

Accordingly, microbiological bioassay is commonly used in quality control, industrial, clinical, and research activities [9]. In this method, the potency of the antimicrobial agents is determined depending on their biological inhibitory effect on the growth of a sensitive microbial indicator in comparison with a corresponding reference standard substance [10].

Similar meaning of the previous in this method, this study aimed to assess the potency of commercially available ciprofloxacin, amikacin, and ceftriaxone in various antimicrobial pharmaceutical preparations following the microbiological bioassay method.

2.2. METHODS

2.1. Tested antimicrobial agents.

A total sample number of 300 antimicrobial pharmaceutical dosage units from different batches of amikacin, ceftriaxone, and ciprofloxacin produced by nine Egyptian pharmaceutical manufacturers (coded as A, B, D, E, O, P, R, S, and U) were assayed **(Table 1)**.

2.2. Standard strains and antimicrobial agents.

The amikacin, ceftriaxone, and ciprofloxacin secondary standard powders as well as, *Staphylococcus aureus* ATCC 6538P standard strain **(Microbiologics, USA)** were kindly supplied by the Egyptian International Pharmaceutical Industries Company (**EIPICO)**.

Table 1. The tested antimicrobial agents samples.

Active constituents	Dosage Units (Total; 300)	Company*	Manufacturing Date	Expiry Date
Amikacin (500 mg/2ml vial)	5 vials	A	May/2021	May/2024
	5 vials	E (batch E1)	Jan**/2021	Jan/2024
	5 vials	E (batch E2)	Jan/2021	Jan/2024
	5 vials	E (batch E3)	Feb**/2021	Feb/2024
	5 vials	E (batch E4)	June/2021	June/2024
Ceftriaxone (500 mg/vial)	5 vials	B	Jan/2021	Jan/2024
	5 vials	S	April/2021	Feb/2024
	5 vials	E	Feb/2021	Feb/2024
	5 vials	P	Jan/2021	Dec**/2023
	5 vials	D	April/2021	Feb/2024
Ciprofloxacin (500 mg/tablet)	50 tablets	U	Feb/2021	Feb/2024
	50 tablets	P	March/2021	March/2024
	50 tablets	R	Feb/2021	Jan/2024
	50 tablets	O	Jan/2021	Jan/2024
	50 tablets	E	March/2021	March/2024

**** Companies' names are coded to protect companies' privacy.** Jan; January, Feb; February, and Dec; December.***

2.3. Bioassay of antimicrobial agents.

The 5 X 1 cylinder plate agar diffusion method, according to the USP [11], was used to evaluate the potency of the selected commercial antimicrobial agents, against their corresponding standards, over a period of one year at three months intervals as the following:

2.3.1. Preparation of the standard antimicrobial solutions.

The secondary standard powders of amikacin sulfate, ciprofloxacin, and ceftriaxone sodium were used for the preparation of working standard solutions S_1, S_2, S_3, S_4, or S_5 with final concentrations of 4.1, 5.12, 6.4, 8, or 10 µg/ml, respectively in case of amikacin sulfate and ciprofloxacin. For ceftriaxone, the final concentrations were 10, 15, 20, 25, or 30 µg/ml, respectively.

2.3.2. Preparation of the tested antimicrobial solutions.

The final tested concentration of 6.4 µg/ml from each tested amikacin (T_A) and ciprofloxacin (T_F) samples were prepared. As well, from each tested ceftriaxone sample, a final tested concentration (T_C) of 20 µg/ml was prepared.

2.3.3. Bacterial inoculum standardization [12].

The *Staphylococcus aureus* ATCC 6538P fresh stock suspension was prepared after incubation at 30–35°C for 18–24 hours (hrs) on tryptic soya agar (TSA) plates. The harvested cells were then suspended in sterile phosphate buffered saline (PBS) and homogenized to provide a 25 % light transmission at 530 nm.

2.3.4. Agar diffusion bioassay [13].

Every 100 ml of the melted M11 agar medium **(Difco, Detroit, MI, USA)** was inoculated with 1 ml of the standardized bacterial suspension after cooling to 45–50°C. To each sterile Petri dish (9 cm), 25 ml of the inoculated M11 agar medium was dispensed and left to congeal. Using a sterile cork–borer, and considering the even spacing, 6 wells each of 6 ± 0.1 mm diameter were then made in each plate.

In a set of 3 plates (containing 18 wells), 50 µl from the 3rd reference standard dilution (S_3) was used to fill each of the alternative wells, number 1, 3, and 5, in each plate. For standard curve derivation, 50 µl of the 1st standard dilution (S_1) was then used to fill each of the remaining wells, number 2, 4, and 6, in each plate. This procedure was repeated 3 times using either the 2nd (S_2), 4th (S_4), or 5th (S_5) standard dilution instead of the 1st standard dilution (S_1).

For subsequent estimation of the tested sample potency, 50 µl of the corresponding tested sample dilution (TA, TC, or TF) was used to replace the standard dilution to fill each of the remaining wells, number 2, 4, and 6, in the 3 plates. Following plates incubation for 18–24 hrs at 35–37°C, each growth inhibition zone diameter, in the 18 wells of each tested 3 plates sets, was measured to the nearest 0.01 mm by the aid of protocol 3 plus software **(Synbiosis, UK)**.

2.3.5. Determination of the potency of the tested sample [11].

In each set of 3 plates, the average, standard deviation, and percentage relative standard deviation for the 9reference standard zones of inhibition values as well as, the other 9 standard zones of inhibition values were initially calculated. To confirm the variability suitability of the results, the relative standard deviations for the reference and standards were determined.

Additionally, within each set, a plate–to–plate variation correction was carried out via corrected standard mean calculation:

$$X_C = X_S - (X_R - P)$$

Where;

X_C =Corrected standard mean.

X_S =Original standard mean.

X_R =Reference (S_3) mean.

P =Correction point; the overall reference mean

The standard curve line was constructed by plotting the measurements of the corrected zone readings against the log of the standards concentrations. Additionally, on these mentioned values, the standard curve regression equation was determined through the creation of a standard unweighted linear regression. The standard curve line is considered valid when the value of the coefficient of determination (% R^2) is not less than 95%.

For determination of the tested sample potency, the correction point was used to correct the average zone measurement and the logarithmic concentration of the tested sample was calculated as the following.

$$L_U = (U - a)/b$$

Where;

L_U=Logarithmic concentration of the tested sample (T).
U =Corrected average for the tested sample.
a =Intercept of the regression line.
b =Slope of the regression line

The tested antimicrobial agent sample (T) potency was then calculated as the antilog of L_U multiplied by any further dilution factor. The potency of each tested sample was expressed as a percentage in relation to the reference standard.

2.3. 6. Calculation of decomposition rate [14, 15].

The rate of decomposition of antimicrobial agents, after each assay interval, was calculated according to the first order kinetic equation:

$$K = (2.0303/t) * \text{Log} (C_0/C)$$

Where

K = Decomposition rate constant.
C_0 = Starting concentration.
C = Concentration after a period of (t) months.

3. RESULTS

In all assays, clearly distinguished zones of growth inhibition were obtained after assay conditions optimization. The triplicate wells of each tested antimicrobial agent concentration gave zone diameters with not more than 1 mm variation.

3.1 Assay of amikacin.

The values of the correction point, which is equal to the overall reference S_3 mean, were 17.6 mm in the 1st, 2nd, and 3rd assays, 17.7 mm in the 4th assay, and 17.4 mm in the 5th assays. The corrected standard means for S_1, S_2, S_4, and S_5 ranged from 15.3 to 15.8, 16.5 to 16.8, 18.3 to 18.6, and 19.2 to 19.6 mm, respectively. While the corrected average for all the tested amikacin samples ranged from 17.4 to 17.6 mm **(Table 2)** indicating the high precision of the assay method.

Additionally, suitable assay results were confirmed by the low relative standard deviation (RSD) values in all assays (not exceeding 2.6%) for the reference, standards, and the tested amikacin samples. Optimum linearity of the standard curve lines was obtained indicated by the high values of the

Table 2. Relative standard deviation and corrected mean of growth inhibition zone replicates for standards and tested amikacin products.

Date \ Tested sample*		S_1	S_2	S_3	S_4	S_5	A_1	A_2	A_3	A_4	A_5
June/2021	**RSD** (%)**	1.4	1.9	1.5	1.5	0.8	0.8	1.6	0.7	0.9	1.2
	Corrected mean (mm)	15.3	16.5	17.6	18.4	19.6	17.5	17.5	17.4	17.5	17.5
September/2021	**RSD (%)**	1.8	1.6	1.2	1	1.1	1.1	1.2	0.9	1.3	1.5
	Corrected mean (mm)	15.7	16.6	17.6	18.4	19.5	17.6	17.6	17.5	17.6	17.6
December/2021	**RSD (%)**	1.1	1.3	1.2	0.8	1.0	1.1	1.0	0.8	1.2	1.1
	Corrected mean (mm)	15.8	16.6	17.6	18.6	19.5	17.6	17.6	17.5	17.6	17.6
March/2022	**RSD (%)**	1.1	1.4	1.0	1.5	0.7	1.0	0.9	1.4	1.4	1.0
	Corrected mean (mm)	15.7	16.5	17.7	18.4	19.2	17.4	17.4	17.4	17.4	17.4
June/2022	**RSD (%)**	2.6	2.1	2.1	1.8	2.0	1.8	1.5	1.3	1.6	1.6
	Corrected mean (mm)	15.8	16.8	17.4	18.3	19.5	17.4	17.4	17.4	17.4	17.5

*S_1, S_2, S_4, and S_5; working standard amikacin solutions, S_3; reference standard amikacin solution, A_1; tested amikacin 500 mg/2ml vial (A), A_2; A_3; A_4; and A_5; tested amikacin 500 mg/2ml vial (E); batches E1, E2, E3, and E4, respectively).
**RSD: relative standard deviation.

percentage coefficient of determination (%R^2) starting from the first assay through the fifth assay (99.7%, 99.83%, 99.79%, 99.51%, and 98.94%, respectively).

Table 3. Potency ratios of different amikacin products at different assay periods.

Product	June/2021	September/2021	December/2021	March/2022	June/2022
Amikacin 500mg/2ml vial (A)	**100.94%**	**99.95%**	**99.17%**	**98.11%**	**97.29%**
Amikacin 500mg/2ml vial (E1)	**100.56%**	**99.64%**	**99.01%**	**98.05%**	**97.32%**
Amikacin 500mg/2ml vial (E2)	**99.54%**	**99.04%**	**98.47%**	**97.92%**	**97.34%**
Amikacin 500mg/2ml vial (E3)	**100.82%**	**99.90%**	**99.25%**	**98.38%**	**97.66%**
Amikacin 500mg/2ml vial (E4)	**101.51%**	**100.53%**	**99.74%**	**98.82%**	**97.93%**

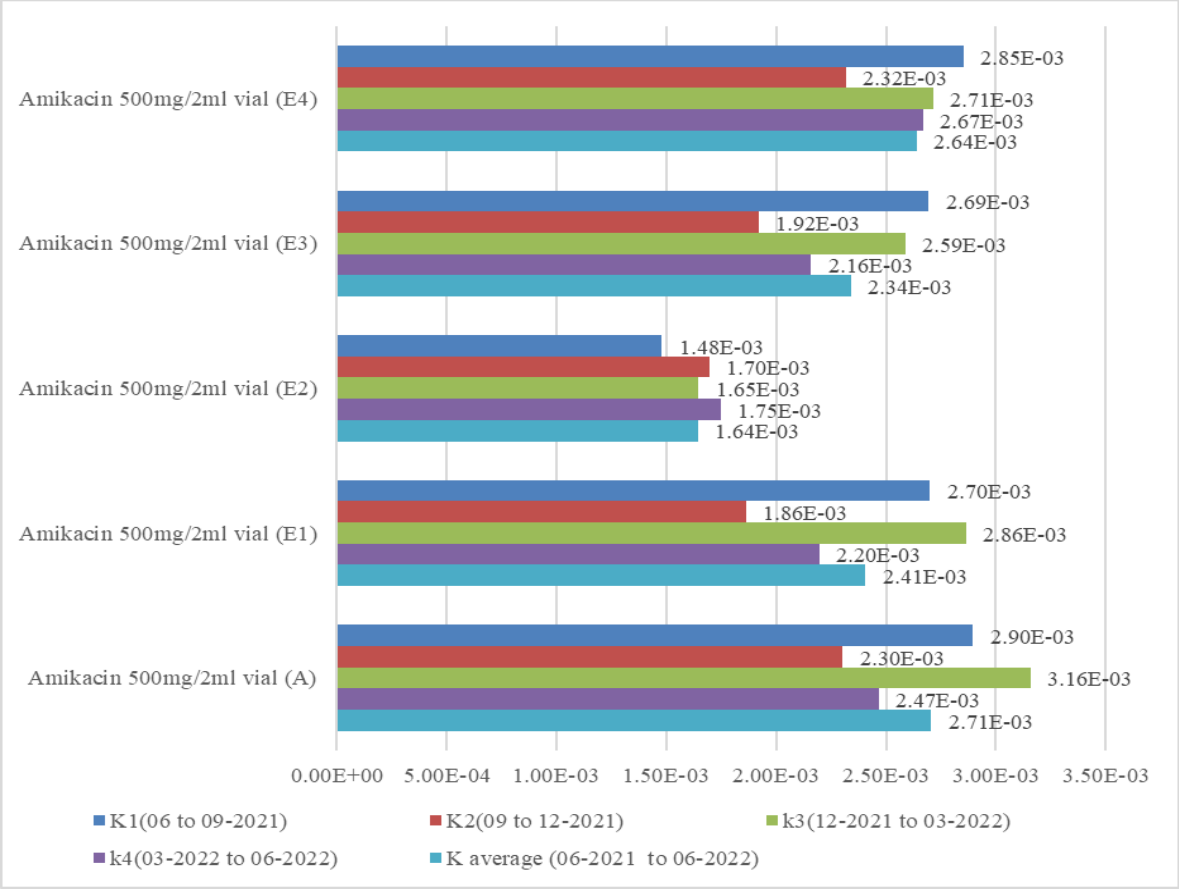

Figure 1. Decomposition of amikacin products at different time intervals.
K_1, **K_2**, **K_3**, and **K_4**; rate of decomposition after the 1st, 2nd, 3rd, and 4th three months, respectively from the beginning of the study, **K** average; mean rate of decomposition during the whole study.

The calculated potency ratios illustrate moderate variability in potency ratios amongst the tested amikacin products at the beginning of the study and during the 3, 6, 9, and 12 months intervals **(Table 3)**. The results showed that amikacin 500 mg/2 ml vials (E4) had the highest potency ratio all over the assay period, while amikacin 500 mg/ 2 ml vial (A) showed the lowest potency at the end of the assay period.

The variation in the decomposition rate of each amikacin product was noticed amongst the assay intervals **(Figure 1)**. Comparison between the mean value of decomposition rates of the products revealed the highest rate of decomposition (2.71E–03 month^{-1}) of amikacin 500 mg/ 2 ml vial (A) followed by (2.64E–03 month^{-1}) for amikacin 500 mg/ 2 ml vial (E4), then (2.41E–03 month^{-1}) for amikacin 500 mg/ 2 ml vial (E1), then (2.34E–03 month^{-1}) for amikacin 500 mg/ 2 ml vial (E3), and finally (1.64E–03 month^{-1}) for amikacin 500 mg/ 2 ml vial (E2) which has the lowest decomposition rate.

3.2. Assay of ceftriaxone:

The repeatable values of the correction point of the reference (S_3) and the corrected means of each standard (S_1, S_2, S_4, and S_5) and tested ceftriaxone samples denote the high precision of the assay method. Over the whole assay period, the corrected standard means for S_1, S_2, S_4, and S_5 ranged from 13.4 to 15.7, 15.8 to 16.4, 17.2 to 18.7, and 17.5 to 20 mm, respectively. While the corrected mean for all the tested ceftriaxone samples had typically the same range, 16.7 to 17.7 mm, of the correction point values of the reference standard **(Table 4)**.

Moreover, the variability suitability of the results was proved because the RSD values not exceeding 2.9% for the reference and standards in all assays in comparison to 2% for the tested ceftriaxone samples. As well, acceptable linear regression of the assay results was demonstrated from the high values of the percentage coefficient of determination (%R^2) from the 1st assay to the 5th assay (99.73%, 99.90%, 99.88%, 99.86%, and 98.97%, respectively).

Assay of different commercial ceftriaxone products illustrates the variable maximum and minimum potency ratios at the beginning of the study and at different time intervals (3, 6, 9, and 12 months) throughout all the products **(Figure 2)**. At the beginning of the study, ceftriaxone 500 mg/vial (D) started with the maximum potency ratio (102.38%) followed by ceftriaxone 500 mg/vial (E), while ceftriaxone 500 mg/vial (B) showed the minimum ratio (99.63%). At the end of the study, ceftriaxone 500 mg/vial (E) recorded the highest potency ratio (97.53%), while ceftriaxone 500 mg/vial (B) remained with the lowest ratio (94.24%).

Variable rates of decomposition at each assay interval, as well as, the overall mean rate of decomposition, of each ceftriaxone product, were observed **(Figure 3)**. Amongst the tested ceftriaxone samples, ceftriaxone 500 mg/vial (B) was the least stable ceftriaxone product with a K average of 4.09E–03 (month^{-1}), whereas ceftriaxone 500 mg/vial (E) was the most stable product with K average of 2.77E–03 (month^{-1}).

3.3. Assay of ciprofloxacin:

The closely related values of the correction point of the reference ciprofloxacin standard (S_3) and corrected means of each of the ciprofloxacin standards (S_1, S_2, S_4, and S_5) and the tested ciprofloxacin samples in the different assays demonstrate the high repeatability of the method **(Table 5)**. The results over the whole assay period showed that the corrected standard means for S_1, S_2, S_4, and S_5 ranged from 14 to 14.7, 15.2 to 16.1, 17.7 to 18.2, and 18.7 to 19.1 mm, respectively. While the corrected mean for all the tested ciprofloxacin samples ranged from 16.4 to 16.9 mm compared to the correction point values range of the reference standard (16.5 to 17.3 mm).

Suitable assay findings were demonstrated by the low RSD values in all assays (not more than 2.8%) for the reference, standards, and the tested ciprofloxacin samples. Optimum linearity of the obtained standard curve lines was indicated by the high values of the percentage coefficient of determination (%R^2) starting from the first assay through the fifth assay (99.41%, 99.74%, 99.77%, 99.5%, and 98.83%, respectively).

The five assays for the potency of each tested ciprofloxacin products are illustrated in **Figure (4)**. At the beginning of the study, ciprofloxacin 500 mg/tablet (P) demonstrated a maximum potency ratio of 102.76% followed by ciprofloxacin 500 mg/tablet (U) with a potency ratio of 102.66% while ciprofloxacin 500 mg/tablet (R) showed the minimum ratio (100.76%). Conversely, at the end of the study, almost all the tested products maintained the same potency ratio of 96% with a slight fractional variation.

Table 4. Relative standard deviation and corrected mean of growth inhibition zone replicates for ceftriaxone standard and tested products.

Date \ Tested sample*		S_1	S_2	S_3	S_4	S_5	CX_1	CX_2	CX_3	CX_4	CX_5
June/2021	**RSD** (%)	0.3	1.1	1.4	2.9	0.6	0.6	2.0	1.1	1.0	1.7
	Corrected mean (mm)	13.4	15.8	17.7	18.7	20.0	17.5	17.5	17.6	17.5	17.7
September/2021	**RSD (%)**	0.5	0.5	0.5	0.4	0.4	0.4	0.4	1.1	1.1	0.5
	Corrected mean (mm)	15.6	16.3	16.8	17.2	17.6	16.8	16.8	16.8	16.8	16.9
December/2021	**RSD (%)**	0.7	0.9	1.0	0.4	0.3	0.6	0.9	0.7	0.5	0.6
	Corrected mean (mm)	15.5	16.3	16.7	17.2	17.5	16.7	16.7	16.8	16.7	16.8
March/2022	**RSD (%)**	1.6	1.2	1.4	1.1	1.5	1.4	0.9	1.2	1.0	1.5
	Corrected mean (mm)	15.7	16.4	16.9	17.2	17.6	16.8	16.8	16.8	16.8	16.9
June/2022	**RSD (%)**	2.2	1.4	1.6	1.7	1.3	1.6	1.2	1.1	1.3	1.4
	Corrected mean (mm)	15.7	16.4	17.0	17.3	17.9	16.9	16.9	17.0	16.9	17.0

* **S_1**, **S_2**, **S_4**, and **S_5**; working standard ceftriaxone solutions, **S_3**; reference standard ceftriaxone solution, **CX_1**; tested ceftriaxone 500 mg/vial (B), **CX_2**; tested ceftriaxone 500 mg/vial (S), **CX_3**; tested ceftriaxone 500 mg/vial (E), **CX_4**; tested ceftriaxone 500 mg/vial (P), and **CX_5**; tested ceftriaxone 500 mg/vial (D).
**RSD: relative standard deviation.

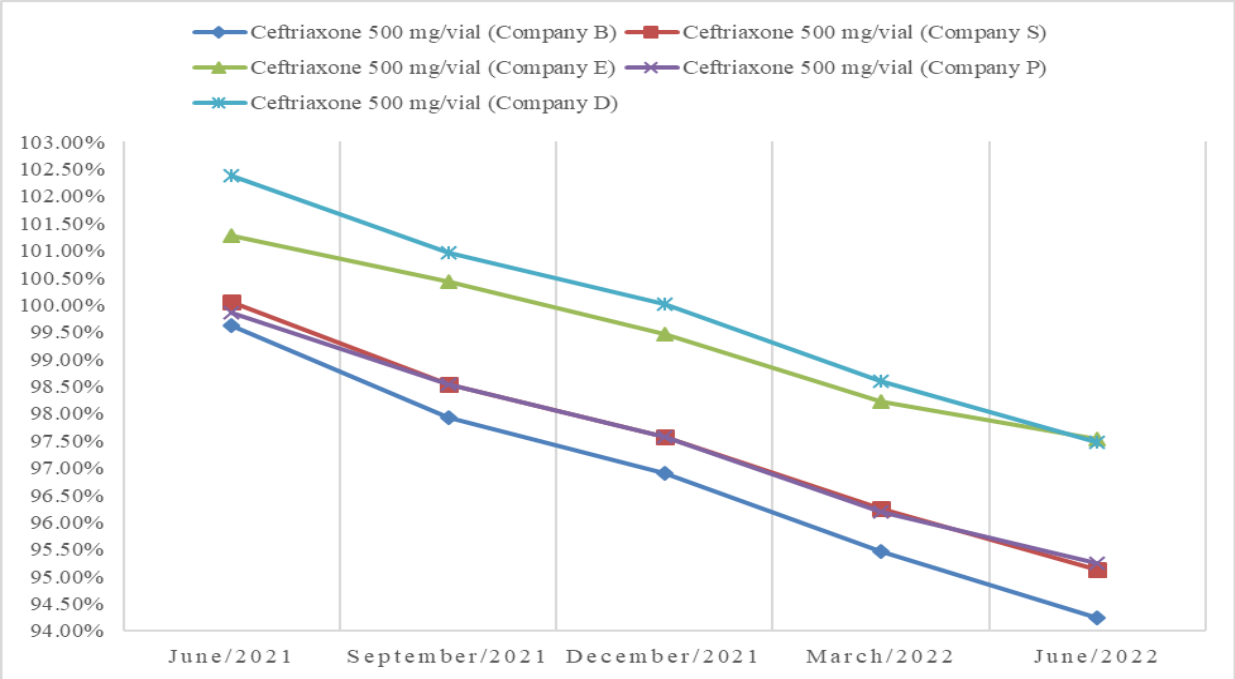

Figure 2. Potency ratios of different ceftriaxone products at different time intervals.

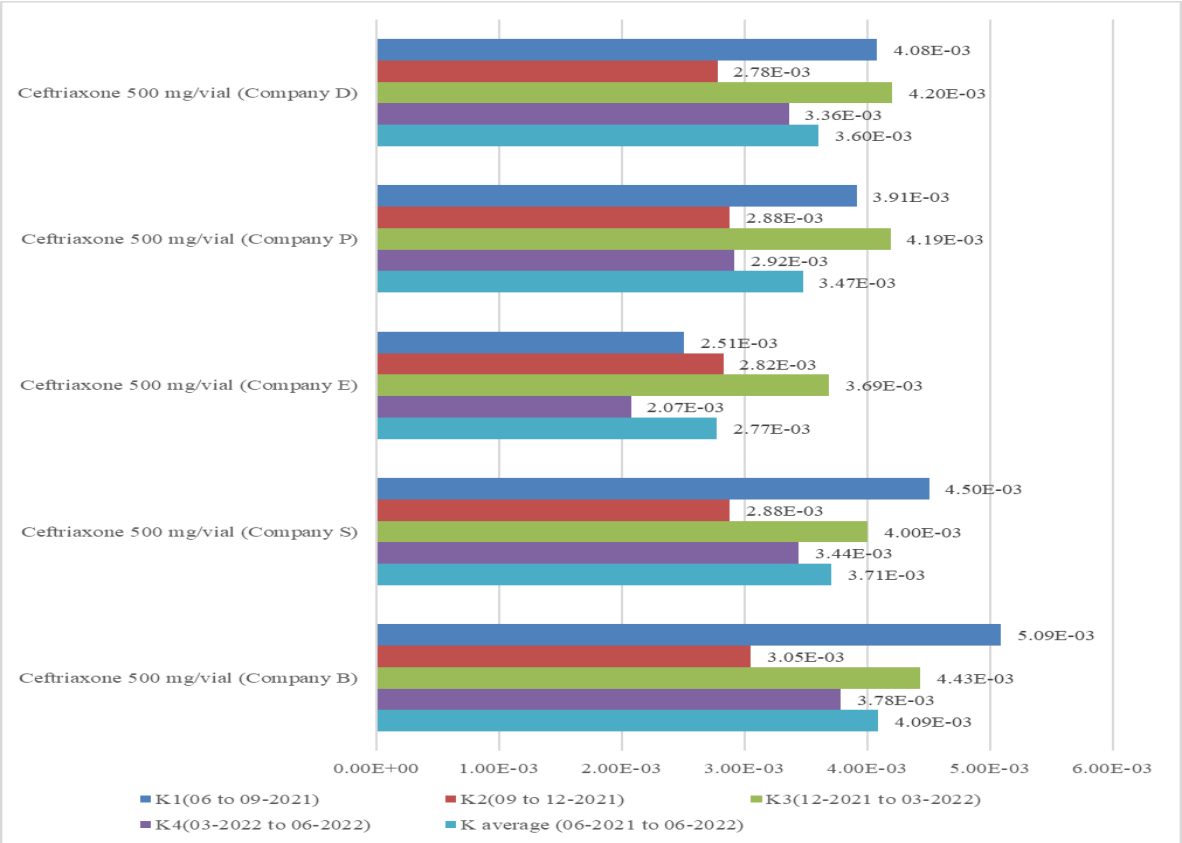

Figure 3. Rate of decomposition of different ceftriaxone products at different time intervals. **K_1**, **K_2**, **K_3**, and **K_4**; rate of decomposition after the 1st, 2nd, 3rd, and 4th three months, respectively from the beginning of the study, **K** average; mean rate of decomposition during the whole study.

Table 5. Relative standard deviation and corrected mean of growth inhibition zone replicates for ciprofloxacin standard and tested products.

Date \ Tested sample*		S_1	S_2	S_3	S_4	S_5	C_1	C_2	C_3	C_4	C_5
June/2021	**RSD** (%)**	1.1	2.8	1.7	2.0	1.8	1.2	0.9	0.6	1.9	1.2
	Corrected mean (mm)	14.6	15.4	16.8	17.7	18.7	16.7	16.8	16.7	16.7	16.7
September/2021	**RSD (%)**	2.0	2.8	2.6	1.3	2.2	2.4	2.8	1.8	2.5	2.5
	Corrected mean (mm)	14.0	15.2	16.5	17.8	18.7	16.5	16.5	16.4	16.4	16.5
December/2021	**RSD (%)**	0.8	1.5	1.4	1.0	0.9	0.8	0.8	0.7	0.9	0.7
	Corrected mean (mm)	14.1	15.2	16.6	17.7	18.7	16.5	16.5	16.4	16.4	16.4
March/2022	**RSD (%)**	0.8	1.0	1.0	0.6	1.1	0.9	1.2	1.3	1.6	1.5
	Corrected mean (mm)	14.6	15.7	17.1	18.1	19.0	16.8	16.8	16.8	16.8	16.8
June/2022	**RSD (%)**	2.1	1.9	1.9	1.1	1.4	1.4	1.5	1.1	1.2	1.6
	Corrected mean (mm)	14.7	16.1	17.3	18.2	19.1	16.9	16.9	16.9	16.9	16.9

*S_1, S_2, S_4, and S_5; working standard ciprofloxacin solutions, S_3; reference standard ciprofloxacin solution, C_1; tested ciprofloxacin 500 mg/tablet (U), C_2; tested ciprofloxacin 500 mg/tablet (P), C_3; tested ciprofloxacin 500 mg/tablet (R), C_4; tested ciprofloxacin 500 mg/tablet (O), and C_5; tested ciprofloxacin 500 mg/tablet (E). **RSD: relative standard deviation.

According to the first order kinetic, the decomposition rate of each ciprofloxacin product, after each assay interval and their overall mean were inconsistent **(Figure 5)**. The results illustrate the slowest decomposition rate (3.46E–03 month^{-1}) of ciprofloxacin 500 mg/tablet (R), while ciprofloxacin 500 mg/tablet (P) was found to have the fastest rate of decomposition (4.30E–03 month^{-1}).

4. DISCUSSION

Careful storage conditions are required to maintain antimicrobial product activity and integrity with subsequent ensuring that patients receive optimum therapeutic benefits [16]. Microbiological bioassay of antimicrobial agents potency is an easy, cost–effective, and accurate method [17]. It is more sensitive than chemical methods that might fail to adequately demonstrate the decreased biological activity [11].

In this study five potencies of Egyptian pharmaceutical products containing amikacin, ceftriaxone, or ciprofloxacin were determined using the agar diffusion assay at 0, 3, 6, 9, and 12 months following sample collection. Additionally, after each interval, the rate of antimicrobial agent decomposition was determined using the first order kinetic equation.

Dafale *et al.*[5], described several variables that can affect the zone diameters in a traditional agar diffusion bioassay including type and concentration of the used microorganism, type and pH of dilution buffer, concentration range and inoculated volume of each antimicrobial agent working solution, the type, pH, and thickness of media, the temperature and period of incubation. In our experiment, many trials were performed to adjust the optimum assay conditions and parameters with subsequent achievement of distinct zones of growth inhibition in all assay experiments. The replicate growth inhibition zones, for each concentration in each assay, gave diameter values with less than 1 mm variation.

Concerning amikacin, all products were selected with the same labeled potency and with manufacturing dates ranging from 1/2021 to 6/2021 and expiration dates ranging from 1/2024 to 6/2024 to avoid the variation in potency due to differences in production dates. The 5 X 1 assay design was employed with cylinder plates containing antibiotic M11 agar medium and S. aureus ATCC 6538P (1ml containing around 10^8 CFU/ml per 100 ml of media). Aboubakr *et al.*[18], utilized the same assay design (5 X 1) in cylinder plates but containing Mueller–Hinton agar medium seeded with *B. subtilis* ATCC 6633 (160 µl of 10^8 CFU/ml per 100ml of media). These differences in the assay medium and sensitive microorganism indicates the need for the performance of several assay trials, with different microorganisms and microbiological media before starting the analysis, to apply the optimum conditions with suitable inhibition zone diameter.

During the amikacin assay, the standards S_1, S_2, S_4, and S_5 exhibited corrected means ranging from 15.3 to 15.8, 16.5 to 16.5, 18.3 to 18.6, and 19.2 to 19.6 mm, respectively. The correction points equal to the overall reference S_3 mean ranged in all assays between 17.4 and 17.7 mm compared to a range of 17.4 to 17.6 mm for all tested amikacin samples, indicating high assay precision. The low RSD values in all assays for the reference, standards, and the tested amikacin samples further supported the suitability of the assay results. The high percentage coefficient of determination (%R^2) values from the first assay through the fifth assay (99.7%, 99.83%, 99.79%, 99.51%, and 98.94%, respectively) show that the standard curve lines are linearly optimal. These findings were comparable to those of Christ *et al.* [19], who validated a linear (%R^2 = 99.95) and precise (%RSD = 2.58) turbidimetric microbiological assay.

At the baseline and after 3, 6, 9, and 12 months of testing, the estimated potency ratios showed moderate variability in potency ratios across the evaluated amikacin products. The results showed that among the amikacin 500 mg/2 ml vials tested, amikacin product (E2) had the highest potency ratio throughout the entire testing time, while amikacin (A) had the lowest potency (97.29%) by the end of the study. Until the end of the assay period, all tested amikacin products retained a potency ratio of around 97% which remains within the limit set by the USP [20], which stipulates that the injectable amikacin products should contain not less than 90% and not more than 120% of the indicated potency along the shelf life. Similarly in a previous study, Zuluaga *et al.*[21], used the agar diffusion technique in large rectangular plates to compare the potency of generic versus brand pharmaceutical products containing amikacin and the potency range was 99.8 to 100.5%.

Across the various assay periods, there was a noticeable difference in the rate of decomposition of each amikacin product. The highest rate was found to be 2.71E–03 (month^{-1}) for amikacin 500 mg/2 ml vial (A), followed by 2.64E–03 (month^{-1}) for amikacin 500 mg/2 ml vial (E4), 2.41E–03 (month^{-}

^{1}) for amikacin 500 mg/2 ml vial (E1), 2.34E–03 (month^{-1}) for amikacin 500mg/2ml vial (E3), and finally 1.64E–03 (month^{-1}) for amikacin 500mg/2 ml vial (E2) which had the lowest rate of decomposition.
\

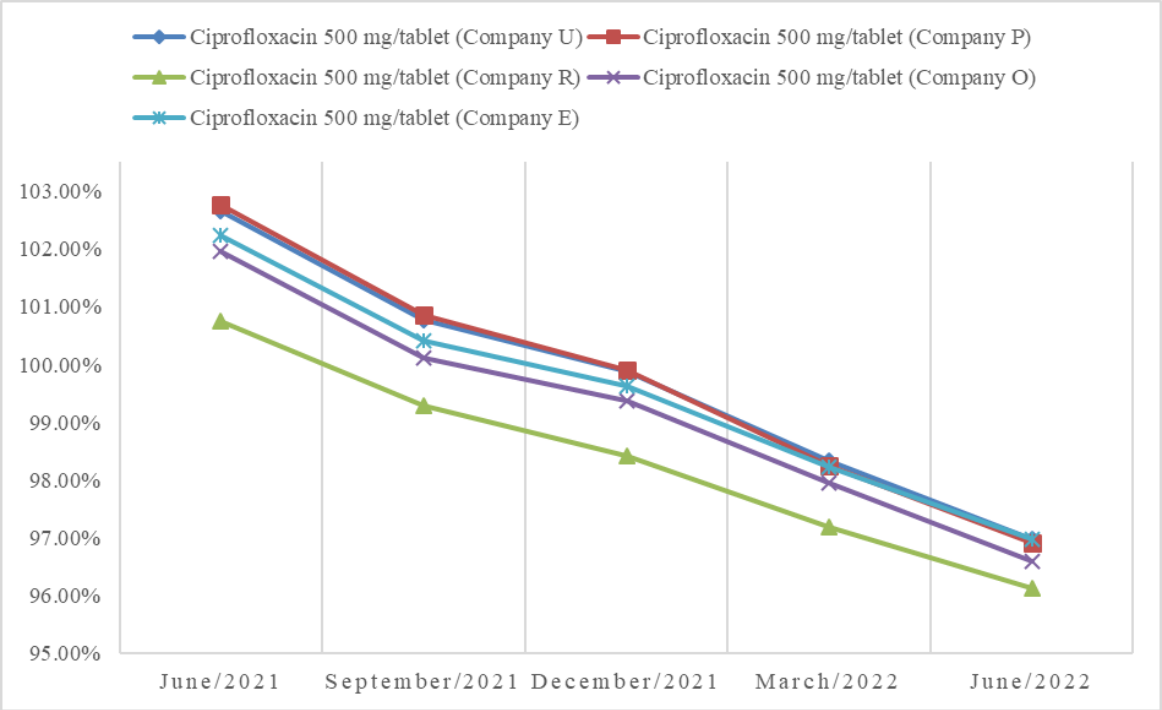

Figure 4. Potency ratios of different ciprofloxacin products at different time intervals. B.N. batch number.

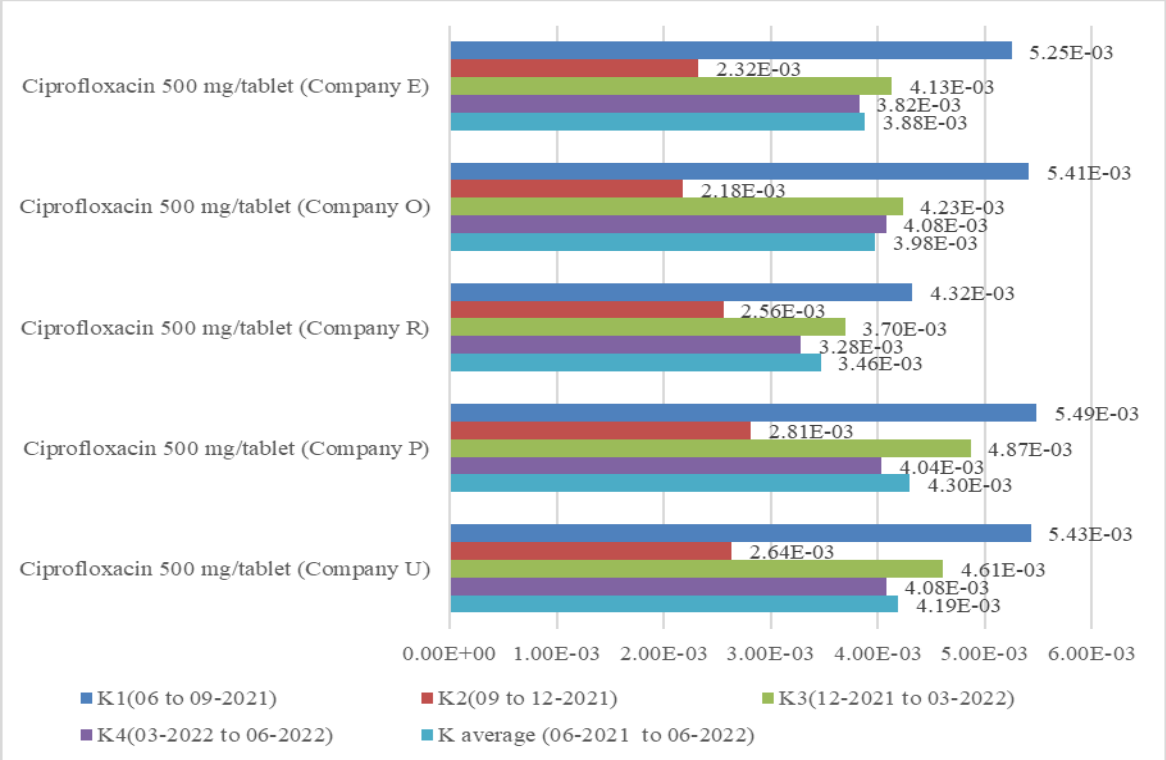

Figure 5. Rate of decomposition of different ciprofloxacin products at different time intervals. **K$_1$**, **K$_2$**, **K$_3$**, and **K$_4$**; rate of decomposition after the 1st, 2nd, 3rd, and 4th three months, respectively from the beginning of the study, **K** average; mean rate of decomposition during the whole study.

Regarding ceftriaxone, to eliminate variations in potency due to variances in manufacture dates, we chose only ceftriaxone products having the same listed potency (500 mg/vial) and a manufacturing date range of 1/2021 to 4/2021 and an expiration date range of 12/2023 to 4/2024. Using a 5 X 1 assay design, *S. aureus* ATCC 6538P (1 ml containing around 10^8 CFU/ml per 100 ml of media) was plated out on cylinder plates containing antibiotic M11 agar medium in the current study. In a former study, Dafale *et al.*[12], employed the same 5 X 1 assay design using antibiotic M11 agar medium, but they considered *Kocuria rhizophila* ATCC 9341 as a sensitive test organism (3 ml containing about 10^7 CFU/ml per 100 ml of media).

During the ceftriaxone assay, the repeatability of the values of the reference correction point (S_3) and the corrected means of each standard (S_1, S_2, S_4, and S_5) and tested ceftriaxone samples indicated the assay method's excellent precision. The corrected standard means for S_1, S_2, S_4, and S_5 ranged from 13.4 to 15.7, 15.8 to 16.4, 17.2 to 18.7, and 17.5 to 20 mm, respectively, across the entire assay period. While the corrected mean for all of the tested ceftriaxone samples showed the same range of correction point values as the reference standard (16.7 to 17.7 mm). The relative standard deviation values not reaching 2.9% for the reference and standards in all assays, as well as 2% for the tested ceftriaxone samples, further demonstrated acceptable variability suitability of the results. The high percentage coefficient of determination (%R^2) values from the first assay to the fifth assay (99.73%, 99.90%, 99.88%, 99.86%, and 98.97%, respectively) showed that the assay findings could be linearly regressed acceptably. Manfio *et al.*[22], found comparable outcomes, proposing a microbiological technique using agar diffusion that was both reproducible (RSD = 2.5%) and linear (%R^2 = 99.98%) in assessing ceftriaxone concentration.

Additionally, measurements of maximum and minimum potency made at the beginning of the trial and again at 3, 6, 9, and 12 months show that maximum and minimum ratios vary among all tested items. The first results of the study showed that the potency ratio for ceftriaxone 500 mg/vial (D) was the highest (102.38%), followed by the ratio for ceftriaxone 500 mg/vial from (E) (101.28%), where the ratio for ceftriaxone 500 mg/vial (B) was the lowest (99.63%). These results were in agreement with those of Raceme *et al.*[23], whose potency ranges for generic cephalosporins (cefixime) was 90 to 110%. Additionally, the final results in this study, showed that the potency ratio for ceftriaxone 500 mg/vial (E) was the greatest (97.53%), whereas the ratio for that of (B) was only 94.2% which is the least value, however, all products were still within the pharmacopeial limit of ceftriaxone for injection (90 to 115%), according to the USP [24].

Furthermore, the degradation rates of ceftriaxone products were found to vary with time, both at individual assay intervals and on average. The average decomposition rate constant (K) for the tested ceftriaxone samples ranged from 2.77E–03 ($month^{-1}$) for the product (E), the most stable tested product, to 4.09E–03 ($month^{-1}$) for the least stable (product B). These variable decomposition rates of ceftriaxone products were also demonstrated in a relatively similar study, but under different conditions where Diego *et al.*[25], demonstrated that ceftriaxone decomposes according to first order decomposition kinetics, where the decomposition rate of reconstituted ceftriaxone from two separate pharmaceutical laboratories was assessed over a period of 26 days at ambient temperature with and without light protection, and the rate constants were 2.14E–02 and 1.98E–02 (day^{-1}), respectively with light protection, and 2E–02 (day^{-1}) for both samples when exposed to light.

With regard to ciprofloxacin, we only selected a manufacturing date range of 2/2021 to 3/2021 and an expiration date ranging from 2/2024 to 3/2024 of ciprofloxacin products with the same indicated potency (500 mg/tablet), in order to remove potency differences caused by variations in manufacture dates.

In a previous study, Cazedey and Salgado [26], proposed a turbidimetric microbiological assay method for ciprofloxacin hydrochloride concentration in ophthalmic solutions using *S. epidermidis* ATCC 12228, as the test microorganism. The test method was linear (%R^2 of about 99.8%) and highly precise (RSD of 2.3%). In the current experiment, with a relatively similar linearity (%R^2 not less than 98.8%) and excellent precision (RSD not exceeding 2.8%), we presented the agar diffusion microbiological assay technique for the determination of ciprofloxacin hydrochloride concentration utilizing *S. aureus* ATCC 6538P as the test microorganism.

Additionally, the great repeatability of the approach is demonstrated by the closely comparable values of the correction point of the reference ciprofloxacin standard (S_3) and corrected means of each of the ciprofloxacin standards (S_1, S_2, S_4, and S_5) and the tested ciprofloxacin samples in the various assays. The results throughout the course of the entire assay period demonstrated that the corrected standard means for S_1, S_2, S_4, and S_5 varied between 14 and 14.7, 15.2 and 16.1, 17.7 and 18.2,

and 18.7 and 19.1 mm, respectively. While the corrected mean for all of the ciprofloxacin samples tested ranged from 16.4 to 16.9 mm compared to the reference standard's correction point values (16.5 to 17.3 mm).

The low relative standard deviation values (less than 2.8%) across all assays for the reference, standards, and the tested ciprofloxacin samples verified relevant assay results. Additionally, from the first assay to the fifth assay, excellent linearity of the standard curve lines was achieved over the used concentration range of 4.1 to 10 µg/ml, as shown by the high values of the percentage coefficient of determination (%R^2) (99.41%, 99.74%, 99.77%, 99.5%, and 98.83%). In a related study by Abdelaziz *et al.*[27], a cylinder–plate agar diffusion microbiological assay method was used to quantify moxifloxacin using *Escherichia coli* ATCC 25922 as the test organism. The method was precise (%RSD = 6.39) with good linearity (%R^2> 98).

Initial results of the assay of the ciprofloxacin products showed that the maximum potency ratio of 102.76% was demonstrated by ciprofloxacin 500 mg/tablet (P), followed by product of (U) with a ratio of 102.66%, and finally by that of (R) with the lowest ratio of 100.76%. On the other hand, by the end of the trial, nearly all the products examined maintained a 96% potency ratio, with only a few decimal places of variation. According to the USP [28], all tested products remained within the pharmacopeial range for ciprofloxacin tablets (90% to 110%).

Regarding stability considerations of the tested ciprofloxacin products, the first order kinetic analysis revealed variations between the rates of the breakdown of individual ciprofloxacin products after different time intervals and between these rates and their overall mean. The findings show that ciprofloxacin tablets (P) decomposed at the fastest rate (4.30E–03 $month^{-1}$) whereas (R) decomposed at the slowest rate (3.46E–03 $month^{-1}$). The variation of ciprofloxacin decomposition rates was also noticed in a comparatively related study applying wide range of temperature, where Onyechi and Igwegbe [29], proved that ciprofloxacin decomposes according to first order decomposition kinetics, where the decomposition rate of ciprofloxacin was determined over a period of one month at 27, 45, 60, and 70°C and the rate constants were 1.95E–04, 2.21E–02, 3.61E–02, and 4.32E–01 ($week^{-1}$), respectively.

The variation in the decomposition rate between amikacin, ceftriaxone, and ciprofloxacin products from different manufacturing sources may be due to differences in the formulation of each product [30] or differences in the active materials source and their method of preparation [15].

5. CONCLUSION

The proposed agar diffusion 5 X 1 microbial assay methods represent cheap, easy–to–use, repeatable, and accurate analytical methods for determining the potency of amikacin, ceftriaxone, and ciprofloxacin in pharmaceutical products. Although different rates of decomposition were observed between the tested antimicrobial agents, all the tested pharmaceutical products were within the pharmacopeial potency limit through all the assay intervals and by the end of the study period.

Acknowledgments: The authors express their sincere thanks to the Faculty of Pharmacy (Boys), Al-Azhar University, Cairo, Egypt for providing the necessary research facilities. Also, thanks to EIPICO for supplying the research materials.

Funding Information: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest: The authors declare that they have no conflict of interest.

Ethical Statement: Not applicable.

Authors' Contributions: Conceptualization, Mohamed S. Ashour (M.S.A.); methodology, Ahmed M. Saber (A.M.S.); software, Fathy M. Elkady (F.M.E.) and A.M.S.; formal analysis, M.S.A., F.M.E., and A.M.S.; investigation, M.S.A., F.M.E., and A.M.S.; resources, A.M.S.; writing original draft preparation, A.M.S. and F.M.E.; writing review and editing, M.S.A. and A.M.S. All authors have read and agreed to the published version of the manuscript.

List of Abbreviations: ATCC: American Type Culture Collection; USP: United States Pharmacopeia; EIPICO: Egyptian International Pharmaceutical Industries Company; Jan: January; Feb: February; Dec: December; T_A: tested amikacin sample; T_F: tested ciprofloxacin sample; T_C: tested ceftriaxone sample; TSA: Tryptic Soya Agar; Hrs: hours; PBS: phosphate buffered saline; K: Decomposition rate constant; % R^2: percentage coefficient of determination; RSD: relative standard deviation; *S. aureus*: *Staphylococcus aureus*.

REFERENCES

1. Suaifan, GARY, Mohammed, AAM, Alkhawaja, BA. Fluoroquinolones' biological activities against laboratory microbes and cancer cell lines. Molecules. 2022; 27:1–28.

2. Xu, L, Cheng, X, Zhu, G, Hu, J, Li, Q, Fan, G. Therapeutic drug monitoring of amikacin: Quantification in plasma by liquid chromatography–tandem mass spectrometry and work experience of clinical pharmacists. Eur J Hosp Pharm. 2021; 0:1–6. https://doi.org/10.1136/ejhpharm–2021–003049.

3. Hirai, Y. Treatment options for Gram–positive and Gram–negative bacteria: Focus on Ceftriaxone. Clinical Medicine Reviews in Therapeutics. 2011; 3:215–234.

4. Nwosu, CN, Udobi, CE, Onele, DU, Asuquo, GE. Use of alternative indicator organisms for the microbiological assay of four antibiotics commonly sold in Uyo, Nigeria. European Journal of Medical and Health Sciences. 2022; 4:112–118.

5. Dafale, NA, Semwal, UP, Rajput, RK, Singh, GN. Selection of appropriate analytical tools to determine the potency and bioactivity of antibiotics and antibiotic resistance. Journal of Pharmaceutical Analysis. 2016; 6:207–213. https://doi.org/10.1016/j.jpha.2016.05.006.

6. Eissa, ME, Rashed, ER, Eissa, DE. Microbiological antibiotic assay validation of gentamicin sulfate using two–dose parallel line model (PLM). High Tech and Innovation Journal. 2021; 2:306–319.

7. Lourenço, FR, Traple, MAL, Okamoto, RT, Pinto, TJA. Development and validation of microbiological assay for ceftriaxone and its application in photo–stability study. Current Pharmaceutical Analysis. 2013; 9:77–81.

8. Saviano, AM, Francisco, FL, Lourenço, FR. Rational development and validation of a new microbiological assay for linezolid and its measurement uncertainty. Talanta. 2014; 127:225–229.

9. Eissa, DE, Rashed, ER, Eissa, ME. Validation of microbiological assay design of neomycin sulfate in 30 x 30 cm rectangular antibiotica plate. Journal of Advanced Biomedical and Pharmaceutical Sciences. 2022; 5:54–63.

10. Pati, P. Microbial assay. Journal of Microbiology and Pathology. 2021; 5:1–2.

11. United States pharmacopeia. National formulary. Rockville (MD): United States Pharmacopeial Convention, 2021a. <81> Antibiotics–Microbial Assays. https://doi.org/10.31003/USPNF_M98820_04_01.

12. Dafale, NA, Semwal, UP, Agarwal, PK, Sharma, P, Singh, GN. Quantification of ceftriaxone sodium in pharmaceutical preparations by a new validated microbiological bioassay. Analytical Methods. 2012; 4(8):2490–2498. https://doi.org/10.1039/c2ay25145k.

13. Suke, SG, Chahande, AD, Kasliwal, RH, Asnani, AJ. Roxithromycin potency quantification in pharmaceutical preparation by applying a validated bioassay method and comparison with HPLC analysis. Annales Pharmaceutiques Francaises. 2015; 73(5):340–350. https://doi.org/10.1016/j.pharma.2015.04.007.

14. Ghimire, P, Shrestha, AC, Pandey, S. Guidelines on Stability Studies of Pharmaceutical Products and Shelf Life Estimation. Int. J. Adv. Pharm. Biotech. 2020; 6(1):15–23.

15. Omara, WAM. 1997, 'Microbiological quality control of some pharmaceutical preparation commonly used in the local market', Master thesis. Department of Microbiology, Faculty of Pharmacy, Al–Azhar University, Cairo, Egypt.

16. Waterman, KC, Adami, RC Accelerated aging: Prediction of chemical stability of pharmaceuticals International Journal of Pharmaceutics. 2005; 293(1–2):101–125. https://doi.org/10.1016/j.ijpharm.2004.12.013.

17. Schmidt, CA, Agarrayua, DA, Laporta, LV, Machado, JC, Manfio, ML, Bittencourt, CF. Development and validation of a microbiological agar assay for determination of cefuroxime sodium in pharmaceutical preparations. Journal of Microbiological Methods. 2009; 77(3):308–315. https://doi.org/10.1016/j.mimet.2009.03.012.

18. Aboubakr, M, Elbadawy, M, Medhat, A. Pharmacokinetics, bioavailability and tissue residues of amikacin in broiler chickens. World Journal of Pharmacy and Pharmaceutical Sciences. 2016; 6(1):96–105

19. Christ, AP, Machado, MS, Ribas, KG, Schwarzbold, AV, da Silva, C de B, Adams,

AIH. A fully validated microbiological assay for daptomycin injection and comparison to HPLC method. Brazilian Journal of Pharmaceutical Sciences. 2015; 51(4):775–783.

20. United States pharmacopeia. National formulary. Rockville (MD): United States Pharmacopeial Convention, 2021b. Amikacin Sulfate Injection. https://doi.org/10.31003/USPNF_M2640_04_01.

21. Zuluaga, AF, gudelo, M, Rodriguez, CA, Vesga, O. Application of microbiological assay to determine pharmaceutical equivalence of generic intravenous antibiotics. BMC Clinical Pharmacology. 2009; 9:1–11. https://doi.org/10.1186/1472–6904–9–1.

22. Manfio, ML, Agarrayua, DA, Machado, JC, Schmidt, CA. A fully validated microbiological assay to evaluate the potency of ceftriaxone sodium. Brazilian Journal of Pharmaceutical Sciences. 2013; 49(4):753–762. https://doi.org/10.1590/S1984–82502013000400015.

23. Raceme, ZM, Siddique, AB, Babar, SE. Chemical and Microbial Analysis of Post Marketed Antibiotics. Greener Journal of Medical Sciences. 2013; 3(3):98–110.

24. United States pharmacopeia. National formulary. Rockville (MD): United States Pharmacopeial Convention, 2021c. Ceftriaxone for Injection. https://doi.org/10.31003/USPNF_M14149_04_01.

25. Diego, MD, Godoy, G, Mennickent, S. Chemical stability of ceftriaxone by a validated stability–indicating liquid chromatographic method. J. Chil. Chem. Soc. 2010; 55:335–337.

26. Cazedey, ECL, Salgado, HRNA. Novel and rapid microbiological assay for ciprofloxacin hydrochloride. Journal of Pharmaceutical Analysis. 2013; 3(5):382–386.

27. Abdelaziz, AA, Elbanna, TE, Gamaleldeen, NM. Validated microbiological and HPLC methods for the determination of moxifloxacin in pharmaceutical preparations and human plasma. Brazilian Journal of Microbiology. 2012; 43(4):1291–1301.

28. United States pharmacopeia. National formulary. Rockville (MD): United States Pharmacopeial Convention, 2021d. Ciprofloxacin Tablets. https://doi.org/10.31003/USPNF_M17880_01_01.

29. Onyechi, KK, Igwegbe, CA, & Pharma, D. Shelf Life Determination of Picralima Nitida, Glibenclamide, Ciprofloxacin and Pefloxacin using UV Spectrometry Physicochemical Technique. Der Pharma Chemica. 2018; 10 (6):67–74.

30. Al Hanbali, OA, Hamed, R, Arafat, M, Bakkour, Y, Al-Matubsi, H, Mansour, R, Al-Bataineh, Y, Aldhoun, M, Sarfraz, M, Dardas, AK. Formulation and evaluation of diclofenac controlled release matrix tablets made of HPMC and Poloxamer 188 polymer: An assessment on mechanism of drug release. Pakistan Journal of Pharmaceutical Sciences. 2018; 2(31): 345–351.