

STUDY OF TOXICITY OF VETERINARY DRUG- ENROFLAXACIN ON ORGANISMS ISOLATED FROM DRUG AFFECTED SOIL AND ITS DEGRADATION

¹Neha J. Shaikh, ²Dr. Reena G. Desai

¹Student, ²Guide (Head of department)

¹Microbiology

¹Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management and Commerce, Valsad, Gujarat, India.

Abstract: Enrofloxacin (EFX) is one of the most frequently used broad-spectrum synthetic quinolones veterinary drugs and its concentration ranges from 0.3 to 142mg/kg in manure. The misuse/ overuse of antibiotics release residues in various environment, such as soil, agricultural farms, waste water treatment plants, lakes, rivers and ground water. Samples were collected from the veterinary hospital of Valsad district and Pardi (drug affected area). Isolation was performed using Thiosulfate citrate bile salts sucrose medium and Sabourauds medium, 12 isolates of bacteria (YS-1, GM-1, BS, YM, YNL, YS-2, GM-2, GNS, G-1, G-2, GNL and OS) and 3 isolates of fungi (FY, FB, FG) were obtained. These isolates were exposed to three different concentration (5,25,70mmol) of 50 and 150 mg of EFX each to check their tolerance. From which 11 isolates of bacteria were susceptible and 1 isolated bacteria (YM-1) and 3 fungi (FG, FB, FY) were resistant. Field test was performed to check the effect of higher concentration of EFX on isolate YM-1 and all three fungi in soil collected from drug affected area which are able to tolerate the known amount of EFX in bioassay. After 4 days at 200 mmol concentration of EFX no growth of bacteria was observed by SPC but all the three fungi are able to tolerate this concentration of EFX. As no growth was observed in field test at 200mmole concentration, this concentration was exposed to Physical and Biological degradation. Physical degradation was performed using Ultraviolet rays at different interval of time from 1min,...,21min and the smallest zone size of 38mm with strain GM-1 was observed at 21minutes, which shows degradation of EFX after 21 minutes of exposure. Biological degradation of EFX was performed using all 3 isolated fungi in Sabourauds medium and consideration amount of breakdown of EFX was obtain using chemical oxygen demand (COD) colorimetric method. The fungi FB was able to degrade more amount of EFX after exposure for 15 days. OD was measured at 420nm at an interval of 5 days. TLC was performed to check the degraded EFX and the R_f value of degraded EFX was 0.05 and that of standard EFX was 0.7. The fungi FB which can degrade higher amount of EFX in biological method, was identified to be related to *Aspergillus niger* DTO 370-17 by molecular identification.

IndexTerms - Enrofloxacin, Quinolones, antibiotic, Thiosulfate citrate bile salt, Degradation.

INTRODUCTION

Since, their first discovery, antibiotics have played incomparable roles in treatment of diseases and promotion of animal feed efficiency. There were more than 70 billion clinical doses of this antibiotics employed globally in 2010, and 162,00 tons of antibiotics were used in china in 2013 making upto 52% of veterinary drugs. Enrofloxacin (EFX) is one of the most frequently used broad-spectrum synthetic quinolones veterinary drugs and its concentration could range from 0.3 to 1,421mg/kg in manure. Fluoroquinolones constitute one of the largest groups of antibacterial pharmaceuticals used worldwide. They are relatively new group of synthetic antimicrobial agents derived from 3-quinolone carboxylic acid. The predecessor of the class was non-fluorinated nalidixic acid with a narrow spectrum of activity that was mainly limited to treating urinary tract infections. The spectrum of antibacterial activity was considerably enhanced by introducing a fluorine atom on the number 6-carbon atom. The new 6-fluoroquinolones chemotherapeutics have become very useful in a variety of infections, especially caused by pathogens resistant to older antibiotics. Excretion of these antibiotics and improper waste disposal has led to substantial concentrations of these compounds. Even the processing of communal wastewater in sewage-treatment plants cannot prevent entry of antibiotics into surface water because of their stability. Possible effects of antibiotics include both toxicity and the emergence of bacterial resistance to the antibiotics. Although the transformation of enrofloxacin by fungi has not been reported, cultures of wood-decaying fungi have been shown to convert Enrofloxacin to CO₂ and several other metabolites. The fungi transforms enrofloxacin to enrofloxacin N-oxide, desethylene-enrofloxacin, and N-acetylprofloxacin. The misuse or overuse of antibiotics results in their residues in various environment, such as soils, agricultural farms, waste water treatment plants, lakes, rivers and ground water. This calls the attention to the problematic of the environmental release of these compounds and to the effects that they may exert in the environment, highlighting the need of developing efficient treatment strategies for their removal and for restoring eco-systems impacted by this type of pollution.

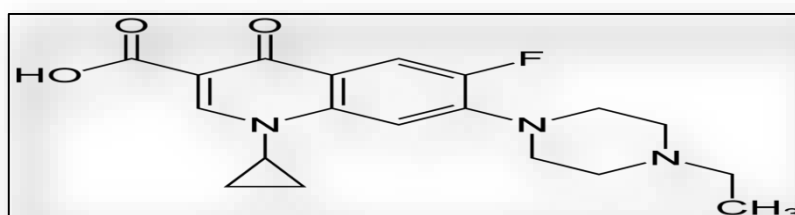


figure 1: Structure of Enrofloxacin (Source-en.wikipedia.org)

II. MEDIA

1. Enrofloxacin drug (150mg and 50mg).
2. Thiosulfate citrate bile salt sucrose medium(TCBS) and Sabouraud's medium.
3. Biochemical media and reagents (Brenner *et al.*, 1988)
4. Reagents for COD(Chemical oxygen demand)
 - Potassium ferricyanide
 - Sulphuric acid
6. Reagents for Extraction
 - Ethyl acetate
 - Sodium sulphate crystals

III. RESEARCH METHODOLOGY

3.1 Sampling

Soil samples were collected from of different regions of Drug affected area of Valsad district and Pardi (Veterinary hospital) with temperature 24°C and pH 9 during monsoon. Samples were collected in a clean plastic bag by a clean spatula. Sample were collected from a depth of 1-10 inches from the top of the soil (Wei *et al.*, 2018).

3.2 Enrichment

0.2 gm of freshly collected soil samples were enriched in 20ml of Thiosulfate citrate bile salt sucrose (TCBS) and Sabouraud's broth and incubated at 37°C and 28°C respectively for 48 hrs for isolation (Wei *et al.*, 2018).

3.3 Screening and Isolation

The enriched samples were then serially diluted from 10^{-2} , 10^{-4} and 10^{-6} for each sample and plated on both TCBS and Sabourauds plates for primary screening. Plates were incubated at 37°C and 28°C for 24hrs. After incubation different types of colonies were observed and their characteristics were noted down. Then from the isolates obtained from the primary screening, secondary screening was performed on TCBS medium. Then Gram's staining and fungal mounting were performed. Various biochemical test were also performed.

3.4 Bioassay

Suspension of the isolates were prepared in 1ml of d/w and 0.1ml of suspension were spreaded on 2 different plates of each medium. With the help of cup borer three wells were prepared in each plate of TCBS and Sabourauds. In one plate of both medium three different concentration (5,20,75mmole) of 50 mg of EFX were added in each well. Similarly, in another plates three concentration (5,20,75mmole) of 150mg of EFX were added in each well. All the plates were incubated at 37°C and 28°C for 24hrs. Zone of inhibition were observed (Ninama *et al.*, 2012).

3.5 Field Test

100gm of soil from drug affected area (veterinary hospital) was autoclave. Isolated bacteria and fungi which can tolerate the EFX in bioassay were enriched in TCBS and Sabourauds broth respectively. SPC from each broth was performed. 10 ml of the culture from each enriched broth were added to 100 gm of soil along with EFX(100mmole/10ml-first day). SPC of 1st day was performed and growth was observed on the next day. Then 125mmole, 150mmole, 175mmole and 200mmole of EFX were added on 2nd, 3rd, 4th and 5th day respectively and growth was observed by performing SPC. Results were observed (Brill *et al.*, 1985)(modified).

3.6 Degradation of EFX

200 mmol EFX was subjected to degradation by both Physical and Biological method.

Physical degradation

Solution of EFX was prepared in 20 ml of d/w. This solution was then exposed to Ultraviolet light at different intervals of time i.e., 1minutes, ..., 21minutes and 0.1 ml of exposed solution were poured in well, which were prepared in TCBS plate with organisms(GM-1) spreaded on it. Zone of inhibition was observed on vary next day (Wang *et al.*, 2019)(modified).

Biological Degradation

Degradation of EFX by 3 isolated fungi was performed by chemical oxygen demand(COD) colorimetric method. All fungi were enriched in 100ml of Sabourauds broth for each (Parshikov *et al.*, 2000). Then 1ml of EFX was added in each broth and Enrofloxacin before degradation and after degradation was measured by COD colorimetric technique At the regular interval of 5 days COD from each broth was performed. OD was measured regularly to determine the concentration of EFX which was degraded by the fungi. Results were obtained (APHA guidelines).

table 1: COD testing

Tubes	Sample/d/w (ml)	Digestion Solution(ml)	Sulphuric acid Reagent(ml)	Total final Volume(ml)
Sample	2.5	1.5	3.5	7.5
Sample	5	3	7	15
Sample	10	6	14	30
Blank	2.5	1.5	3.5	7.5
Blank	5	3	7	15
Blank	10	6	14	30

After which COD vials were kept on cod digester for 24 hours and then cooled. Absorbance was measured at 420 nm.

The difference between absorbance of a given digested sample and the digested blank is measure of the sample COD.

COD was measured by the following formula:

$$\text{COD as mg O}_2\text{/L} = \frac{\text{mgO}_2 \text{ in final volume} \times 100}{\text{ml sample}}$$

3.7 Extraction of EFX

The mycelia from Sabouraud's broths used in biological degradation were harvested by using Whatman filter paper in a Buchner funnel and then extracted with three equal volumes of ethyl acetate. Then it was dried over anhydrous sodium sulphate crystals and evaporated in vacuo. The residues were dissolved in methanol: acetonitrile: acetic acid (10:10:2) (Parshikov and Khasaeva., 2018).

3.8 Characterization of Degraded EFX

Thin layer Chromatography

TLC of enrofloxacin was performed in the plates prepared with silica gel taken from Si60F₂₅₄. The solvent system used was dichloromethane/methanol/2-propanol/25% aqueous ammonia (3:3:5:2) volume. With the help of capillary tube one drop of standard EFX and one drop of dissolved EFX were placed on TLC sheet at equidistant. After development, plate was dried in air. Spots were detected in UV irradiation as purple spots. R_f values of the spots were calculated (Asperger *et al.*, 2006).

Formula for calculation of R_f value:
$$\frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

3.9 Molecular identification of Isolated fungi

The suspected fungi isolate, which can degrade high amount of EFX was further identified by 18s rRNA sequencing. DNA was isolated from the culture provided by the client. Quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 18s rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500x1 Genetic Analyzer. The 18s rRNA sequence was used to carry out BLAST against the NCBI GenBank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple sequence alignment software programs (Gene explore, Ahmedabad).

IV. RESULT AND DISCUSSION

4.1 Enrichment of Sample

For the enrichment, TCBS broth and Sabouraud's broth were used. As there were 3 samples, 4 flask of TCBS broth were prepared for bacteria, 1 for control and 3 flask for samples S1, S2 and S3. Similarly, 4 flask of Sabouraud's broth were also prepared for fungi, 1 for control and 3 flask for samples as F1, F2 and F3. After inoculation TCBS and Sabouraud's broth were incubated at 37°C and 28°C respectively. Turbidity was observed after 48 hrs in all flask except the flask label as control.

4.2 Screening and Isolation

4.2.1 Screening of bacteria

In primary screening after enrichment, samples were diluted and were plated on TCBS medium. Because of the fermentation of sugar, the pH of the medium shift which leads to the production of yellow brown colonies. Colonies showing yellow brown color were selected and were further used for secondary screening. Total 12 isolates were collected for secondary screening. Total 12 isolates were obtained from primary screening were selected and were used for secondary screening on TBS medium for further isolation. Isolates were coded as YS-1, GM-1, BS, YM, YNL, YS-2, GM-2, GNS, G-1, G-2, GNL and OS. Then colony characteristics of all the isolates were noted down and gram's staining were performed.

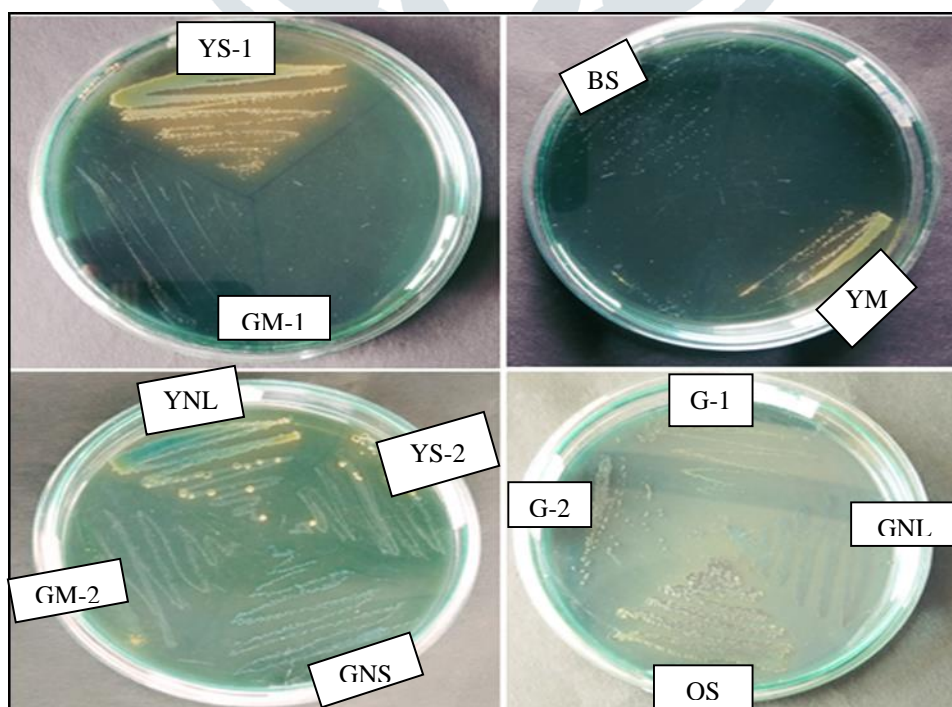


figure 2: Secondary screening of bacteria on TCBS medium

4.2.2 Screening of fungi

All the 3 fungi after enrichment were isolated on Sabouraud’s agar medium. After incubation of 4 days. Fungal mounting of all three isolates were performed. By fungal mounting, some fungal isolates belong to the genera *Aspergillus*, *Rhizopus* and *Penicillium*. The isolate FY belongs to the genera of *Aspergillus*, isolate FB belongs to the genera *Rhizopus* and isolate FG belongs to the genera *Penicillium*.

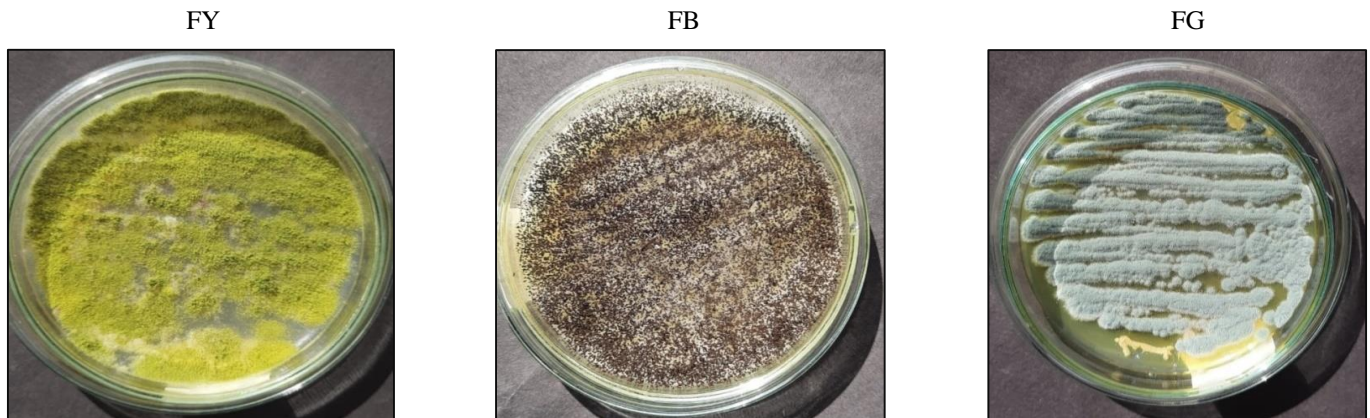


figure 3: Screening of fungi on Sabouraud’s agar medium

4.3 Bioassay

Bioassay is use to measure the concentration or potency of a substance by its effect on living cells i.e. microorganisms, here it is used to measure the potency of a enrofloxacin against all 12 isolates of bacteria and 3 isolates of fungi. Here, the potency of 3 different concentration 5, 20, 75 mmol of 50 and 150 mg tablets was tested. Among all the 12 isolates of bacteria 11 isolates (YS-1, YS-2, G-1, G-2, GM-1, GM-2, YNL, GNL, BS, OS, GNS) were found to be susceptible and one (YM) was found to be resistance with growth in the medium. While, all the three isolates of fungi (FY, FB and FG) were found to be resistance with all the concentration of enrofloxacin used.

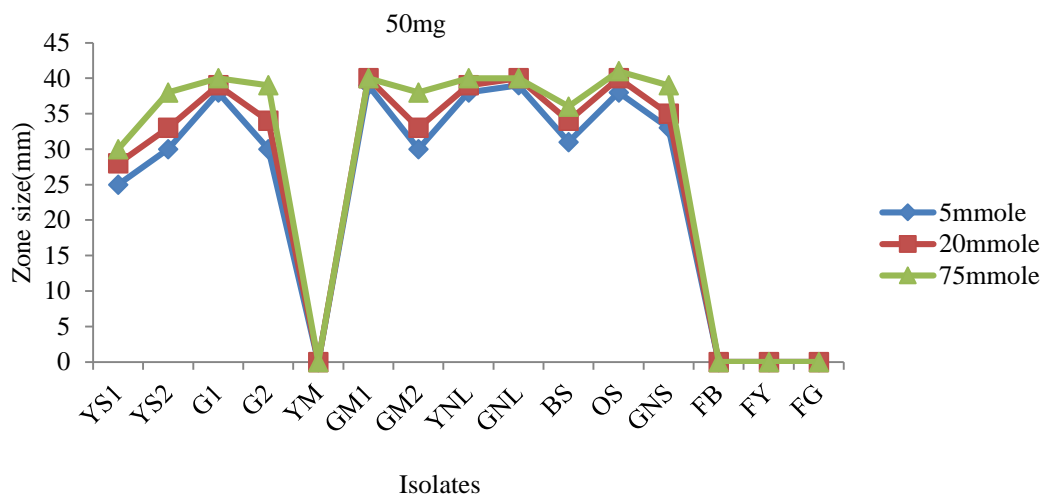


figure 4[A]: Inhibitio zone of all isolates with 50 mg of EFX

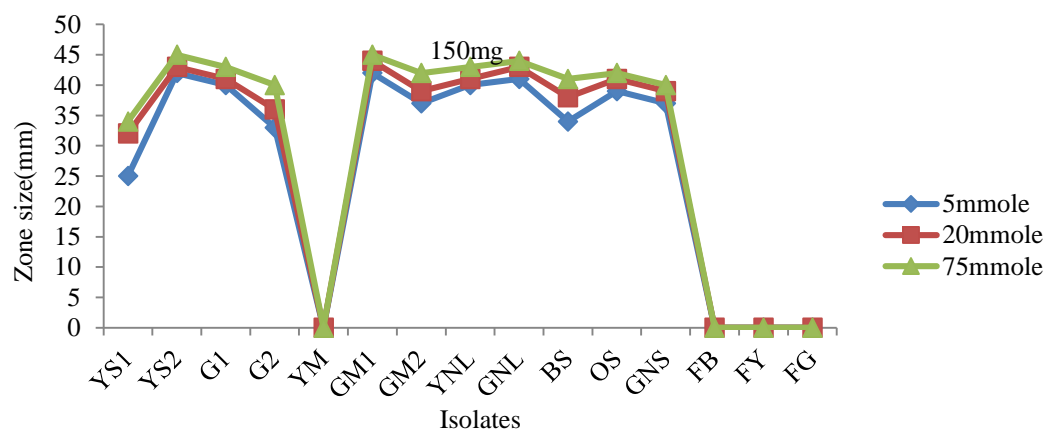


figure 4[B]: Inhibition zone of all the isolates with 150 mg of EFX

4.4 Field Test

The field test was performed in the field of application to study that how much concentration of EFX the isolates can tolerate. Here, the isolates, which showed resistance towards EFX during bioassay were used.. 1 isolate (YM) and 3 isolates of fungi (FY, FB, FG) were used in the field. These isolates were enriched and were added in the soil simultaneously. Different concentration of EFX (100 mmol, 125 mmol, 150 mmol, 175 mmol and 200 mmol) were added upto 5 days respectively. There SPC was performed and it was found that the bacterial isolate cannot tolerate upto 200 mmol of EFX in the soil, while all the fungal isolate can tolerate 200 mmol of soil in the area of application.

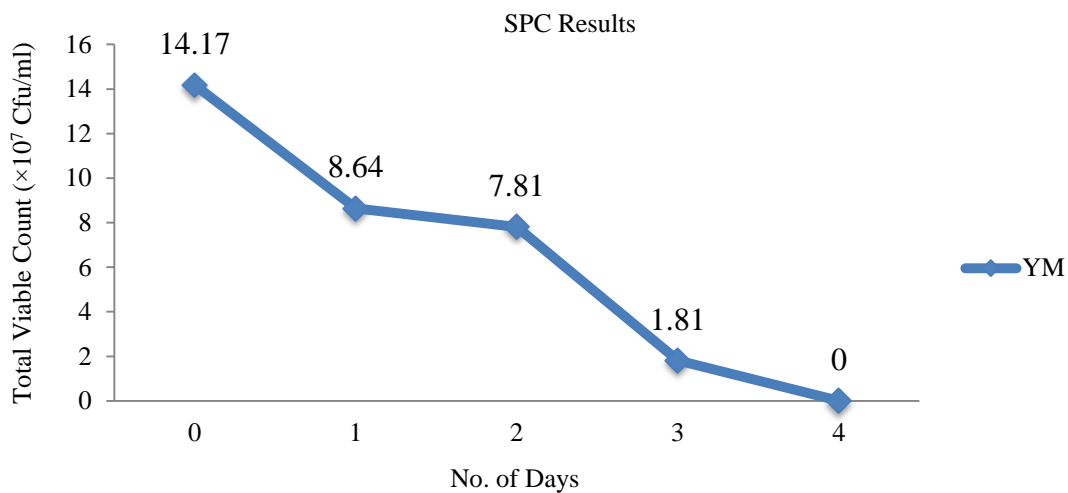


figure 5: Results of field test from 0 to 4th day

4.5 Degradation

4.5.1 Physical degradation

Physical degradation was used to degrade the EFX using Ultra-violet rays. 200mmole concentration of EFX was exposed to the UV light from 1 min to 21 min. 0.1 ml of the exposed solution was then poured in well made in TCBS agar plate with isolate (GM-1) spreaded on it. It was observed that the smallest zone size of 38mm was obtained at 21 minutes, which shows the maximum degradation of EFX.

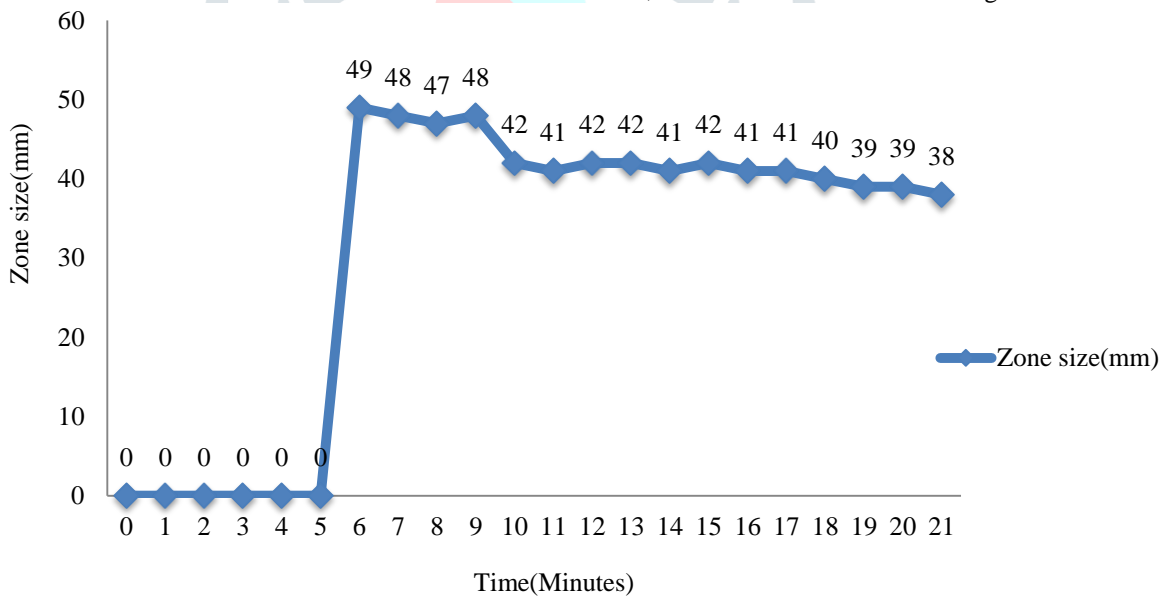


figure 6: Graph of Physical degradation of EFX

4.5.2 Biological degradation

Biodegradation of EFX was measured using COD colorimetric method. Here, all the three fungi (FB, FY and FG) tolerating 200 mmole of EFX as observed in field test were exposed to 200 mmole of EFX in Sabouraud’s broth. Here cod method measured the remaining amount of EFX after interval of 5 days during these 5 days fungi were supposed to intake the carbon present in the broth due to degradation of EFX. Here in this method EFX is an organic matter and it releases carbon and oxygen during degradation and cod colorimetric method measures these carbon released from structure of EFX. On 1 day cod of standard EFX was performed and measured and i.e. OD is 348 nm. At an interval of 5 days cod was performed until 15 days. On day 15 FB fungi was found to have the lowest OD and FY fungi was found to have the highest OD. As the dilution increases the OD decreases, this indicates that fungi FB can degrade and intake a considerable amount of EFX.

table 2: Result of COD colorimetric method

Isolates		FB	FY	FG
Day	Sample(ml)			
1	2.5	348	352	340
	5	180	200	240
	10	100	182	196
5	2.5	342	350	333
	5	179	196	213
	10	88	167	187
10	2.5	332	326	316
	5	176	177	204
	10	76	156	134
15	2.5	320	314	302
	5	100	158	189
	10	26	126	120

4.6 Extraction of degraded EFX

Thin layer chromatography

The R_f values of both standard and degrade EFX. In TLC sheet one drop of Standard EFX and one drop of degraded EFX were placed. After development drops were visualized in UV light. Different R_f values of both the samples were calculated. The R_f value of standard EFX was 0.7 and degraded EFX was 0.05.

table 3: Results of TLC

Samples	R_f value
Standard EFX	0.7
Degraded EFX	0.05

4.7 Molecular identification of fungi

Isolate FB was identified by 18s rRNA sequencing at Gen explore. The isolate was found to be closely related to *Aspergillus niger* based on nucleotide homology and phylogenetic analysis and comparison with the available sequence at NCBI GenBank. With *Aspergillus niger* strain DTO 390-I7, the isolate FB showed 100% query cover and 100% identity. This isolate FB was obtained from soil sample of Valsad veterinary hospital.

V. CONCLUSION

In this study total 12 isolates of bacteria were obtained from the drug affected area. Along with that, 3 isolates of fungi were also obtained. Also the 11 isolates of bacteria were gram negative and 1 isolate was gram positive cocci. Further characterization of isolates was done by performing biochemical characterization. Fungi were also further isolated on Sabourauds agar medium. And fungal mounting were also performed. Bioassay of all the isolates i.e. 12 of bacteria and 3 of fungi were performed, in which it was observed that among the 12 isolate 11 were found to be susceptible to EFX and 1 isolate(YM) was resistant, where all the fungi(FY, FB, FG) were resistance to all the EFX concentration i.e. 5mmol, 20mmol and 75mmol. Further this one isolate (YM) and all three fungi isolates were exposed to field test. After 4 days it was found from SPC that bacterial isolate was not able to tolerate 200mmol concentration of EFX in soil, while all the fungi isolate can tolerate 200mmole of EFX. This fungi is further used for biological degradation of EFX. Physical degradation of EFX was performed by using UV rays. It was observed that at around 21 minutes of exposure the zone size decreases to 38mm with isolate GM-1, the 200mmol concentration of EFX can be degraded. Biological degradation of EFX was also performed using all 3 fungi. Here the degradation is measured by COD colorimetric method. The COD was performed at an interval of 5 days till 15 days of exposure. OD was measured at 420nm and on 15th day FB fungi was found to have the lowest OD, which shows more degradation of EFX and FY fungi was having highest OD. Therefore FB fungi can degrade considerable amount of EFX. Degraded EFX was characterized by Thin layer chromatography. The pattern of bands were observed and the R_f value of standard EFX was 0.7 and degraded EFX was 0.05. The isolated fungi FB which can degrade more amount of EFX, was identified to be related to *Aspergillus niger* DTO 370-I7 by molecular identification. It can be concluded that the EFX used as veterinary drug can be degraded physically with the help of UV rays and it can

also be degraded by the fungi. However the bacteria are susceptible to the EFX. Thus fungi can be used for the biodegradation of higher amount of EFX in future..

VI. ACKNOWLEDGMENT

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