Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2021; 9(2): 151-156

Received: 15-12-2020
Accepted: 20-01-2021

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Determination of strepto-penicillins residues in cow milk after its intramuscular administration

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Abstract
The present study aimed to evaluate the persistence of strepto-penicillins in lactating Holstein Frisian cow milk followed by an intramuscular injection of strepto-penicillins consecutively for three days at 24 hrs interval. The collection of milk samples was performed twice daily up to the 10th milking followed by strepto-penicillins injection and milk samples were analysed by MS/MS. The detection limit of the method was determined as 0.1 µg/kg. The residues of the penicillin were not observed in the any of the milking. The highest concentrations of streptomycin were determined in the milking at 24Hrs after injection last injection and mean concentration of this milking was found to be as 1008.17µg/L, however at 72 hrs it was 151.5µg/L. streptomycin residue in all milk samples after 72 hrs of last administration was lower than the maximum residue limit (200 µg/kg). In conclusion, this study determined the persistence of streptomycin in cow milks based on an MS/MS method. In addition, results of the study showed that cow milk after intramuscular administration of strepto-penicillins showed withdrawal period of 72 hrs.

Keywords: streptomycin, strepto-penicillins, milk residues, MS/MS

Introduction
As Milk contains important nutrients such as fat, proteins, and carbohydrates, essential vitamins and minerals such as calcium, selenium, magnesium, riboflavin, pantothenic acid and vitamin B12, it is an important component of human diet [1, 2]. Cow milk also possesses rich nutrient content and it is the most produced milk source worldwide. Many dairy products including cheese, cream, butter, and yoghurt are prepared from the milk [3, 4].
Antibiotics are considered as the magic bullets which are helpful in curing the many infectious diseases of the human as well as animals. Streptomycin is a parenteral aminoglycoside antibiotic derived from cultures of *Streptomyces griseus*. Dihydrostreptomycin and streptomycin are aminoglycoside antibiotics used for the treatment of bacterial infections in food-producing animals. It was the first of the aminoglycosides to be isolated and used therapeutically. Clinical use of streptomycin includes treatment of *Mycobacterium tuberculosis* infections (second- or third-line agent), tularemia (Francisella tularensis), plague (Yersinia pestis), and, until recently, in combination with a penicillin for the treatment of endocarditis. Streptomycin was approved by the FDA in 1945. Dicrysticin-S injection is commonly used in large ruminants to treat mixed infections (Mixed infections caused by Penicillin and Streptomycin sensitive organisms and infections in which the organisms cannot be readily identified). It is routinely used medicine, containing combination of Streptomycin Sulphate, Procaine Penicillin G and Penicillin G Sodium. However, the literature citing amount of all or any of the ingredient excreted in milk of lactating animal is scanty and hence it became essential to study antimicrobial residues for such combinations regarding public health view point. Several detection methods including immunoassays, capillary electrophoresis, high-performance liquid chromatography, gas chromatography, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were developed for aminoglycoside and penicillin residue analyses with different sensitivities. However, LC-MS/MS is accepted as the most reliable confirmatory method based on its high sensitivity and accuracy [5, 6]. Several studies were reported on pharmacokinetic features of aminoglycosides for farm animals [7-9]. However, the information about the persistence of these antibiotics in the milk of cattle in Indian conditions are lacking. Therefore, the present study was planned to investigate the residues of streptomycin and penicillin in milk after intramuscular administration of strepto-penicillin injection consecutively for three days at 24 Hrs interval in lactating cows.

Materials and Methods

Streptomycin sulphate, Procaine penicillin G and Penicillin G Sodium obtained from Zydus AHL, Ahmadabad. Ammonium formate and formic acid (LCMS grade), Acetonitrile required for LC-MS/MS analysis from commercial sources. The LC/MS/MS analysis of milk samples was carried out via Agilent Technologies 1260 series, attached with a binary high-pressure gradient pump. Agilent Eclipse plus C18 column (100x 4.6mm, 5µm) was employed for LC separation at 40 °C. The mobile phases consisted of solvent A (0.1% formic acid solution and 5mM Ammonium formate) and solvent B (Methanol containing 0.1% formic acid and 5mM Ammonium formate).

Six clinically healthy female HF cross lactating cows, weighing 400-500 kg were selected for this study. The experimental animals were kept at Institutional Livestock farm Complex of Krantisinh Nana Patil College of Veterinary Science Shirwal District Satara. This study was approved by the Institutional Animal Ethics Committee of the KNPCVS, Shirwal, Dist- Satara. All animals were kept under similar conditions having standard ration and free access to water. Streptopenicillin injections (Dicrysticin-S 2.5g) in powdered form was provided by Zydus AHL, Ahmadabad. The powdered drug was reconstituted by adding 7.5ml water for injection. The prepared solution was administered at the rate of 2ml/50kg live body weight to the lactating cows at 24 Hrs interval, consecutively for three days. The milk samples from each animal was collected in sterile 15ml vials at 12 Hrs interval upto five days after last administration. Collected milk samples were directly stored at -20 °C for further analysis. All samples were transported to the laboratory immediately after sampling under cold conditions and stored at -20 °C in a deep freezer for further analysis.

The stock solutions of streptomycin, Procaine penicillin G and Penicillin G Sodium was prepared in distilled water (1 mg/mL) and stored at -20 °C prior to use. Working solutions of streptomycin, Procaine penicillin G and Penicillin G Sodium were also prepared in distilled water by serial dilution. To generate eight-point concentrations (0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL) of the calibration curve, calibration standard samples were prepared in milk by spiking with an appropriate volume of serially diluted stock solution.

Methods: The extraction of milk samples was performed as previously described by Jank *et al*. [10] with some modifications.

For streptomycin briefly, each milk sample (5 mL) was transferred into a polypropylene centrifuge tube and then mixed with 4.9 ml water and 100 µL of 0.5 % HCL and vortexed it for one minute. After this 10ml acetonitrile was added and vortexed for three minutes and then Centrifuged at 11000 rpm for 10 Minutes. Two ml supernatant was pipetted out and filtered through 0.22 µm Nylon Syringe filters. Volume of injection was 5 µL for LC-MS/MS. The recovery was 90-100%.

Similarly, for Penicillin briefly 5 ML Homogenized Milk Sample was taken into centrifuge tube and 5ml water was added to it and vortexed for one minute. Then 10 ml Acetonitrile was added and vortexed for 3 minutes and centrifuged for 10 min at 11000 RPM. Lastly 2ml supernatant was pipetted out and filtered through 0.22 µm syringe filters recovery was 95-100%.

The gradient of LC separation was as follows: 0.0 min, A/B (80/20); 2.0 min, A/B (80/20); 4.0 min, A/B (20/80); 7 min, A/B (20/80); 9 min, A/B (80/20); 14 min, A/B (20/80). The flow rate of the mobile phase was set at 0.6 ml/min and the injection volume of the sample was 5 µL. Agilent 6460 LC/MS Triple Quadrupole instrument was used for mass spectrometry analysis. A nitrogen generator was employed to produce nebulizer and drying gas (250 °C). All MS parameters including sheath gas flow, nebulizer gas, capillary voltage and sheath gas temperature were as 8 L/min, 55 PSI., 3500 V, 250 °C, and 15 eV, respectively.

The methods were validated by spiking raw milk samples. The quality parameters established were linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery, and intra- and inter-day precisions. The limit of detection (LOD) was defined as the lowest concentrations of streptomycin and penicillin that the analytical process can reliably differentiate from background levels (signal-to-noise ratio≥3), while the limit of quantification (LOQ) was defined as the lowest concentration of streptomycin and penicillin that can be quantified (a signal-to-noise ratio ≥10). The retention times for streptomycin and penicillin G were 1.119 and 6.397 minutes respectively.

Results

Milk samples when spiked with standard pharmaceutical ingredients such as streptomycin and penicillin showed more than 90% recovery on LC-MSMS. The typical chromatogram of streptomycin is shown in Fig. 1. The method was validated by determining linearity, recovery, precision and accuracy, LOD, and LOQ.

The quantification of penicillin in cow milk samples was performed by LC-MSMS. However none of the sample was positive for the residues of penicillin's.

The quantification of streptomycin in cow milk samples was performed by LC-MS/MS. Chromatographic separation was also performed using an LC technique in line with Kim *et al*. [17]. The linearity of the calibration curve showed an appropriate correlation (r2 =0.999) in the range from 0.5 to 100 µg/kg (Fig. 2 and 4) for streptomycin and penicillin both. Any of the milk sample was not showing the residues of the penicillin. Streptomycin concentrations at different time points are depicted in the table 1. During the study, the highest concentrations of streptomycin was detected was 1008.17± 151.35 microgram/liter at 24hrs after last administration. Also, streptomycin milk concentration decreased consequently during milking period and was observed under the maximum residue limit (200 µg/kg) at the sixth milking (at 72hrs) onwards.

Table 1: Mean ± SE concentrations of streptomycin in milk at different time points.

Milking No.	Time point in Hrs After last Injection of Dicrysticin-S	Streptomycin levels (Mean) in microgram/liter
1	After 12 Hrs	863.83± 250.85
2	24 Hrs	1008.17± 151.35
3	36 Hrs	380±104.63
4	48 Hrs	473±147.91
5	60 Hrs	229.33±11.70
6	72 Hrs	151.5±17.92
7	84 Hrs	102.67±21.62
8	96 Hrs	82.17± 21.02
9	108 Hrs	20.83± 11.04
10	120 Hrs	66± 10.12
11	132 Hrs	18.67± 11.10

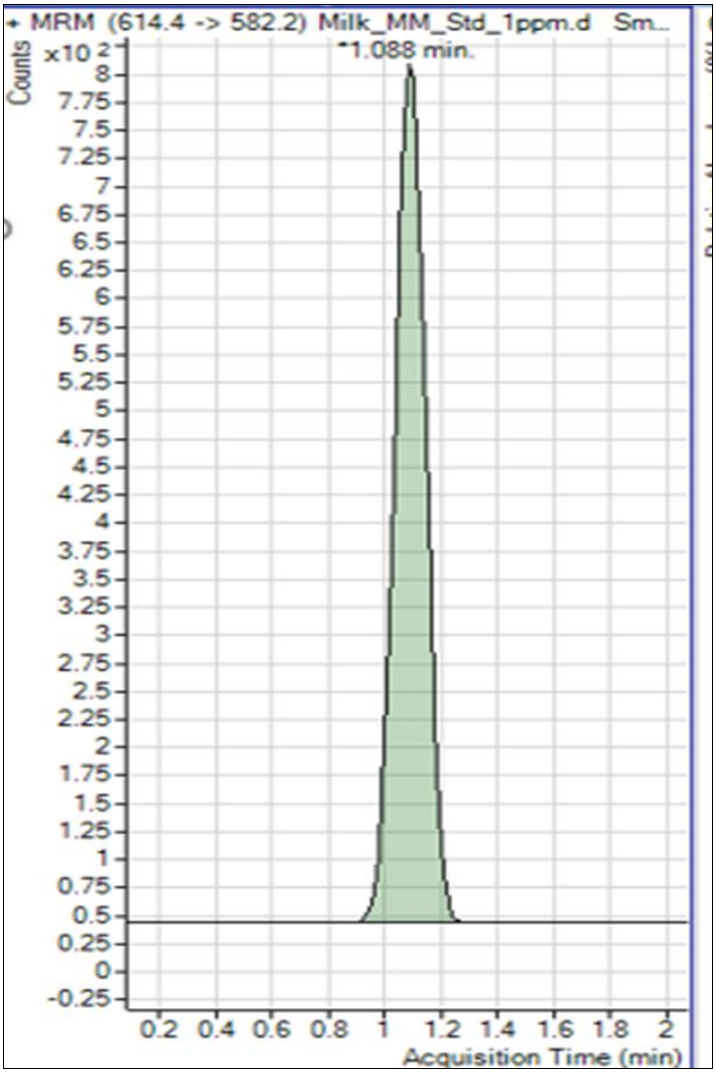

Fig 1: Chromatogram of Streptomycin

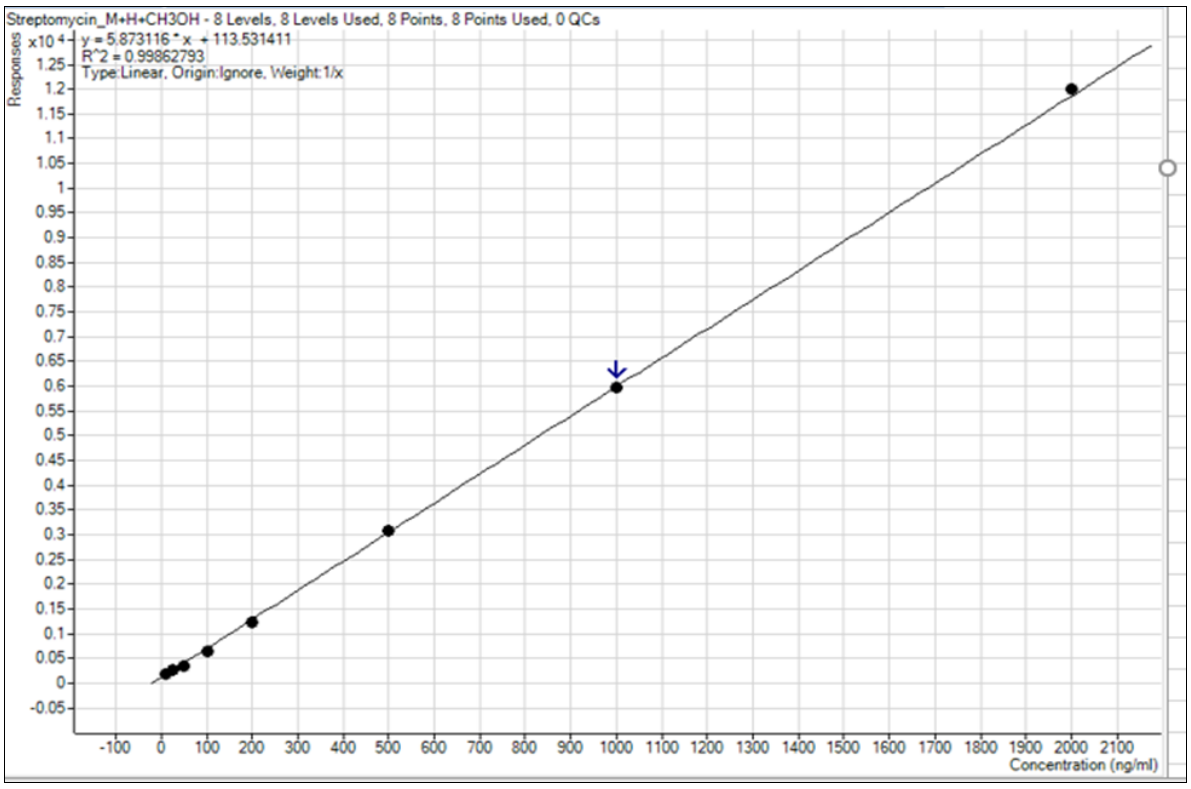

Fig 2: Calliberation Curve for Streptomycin

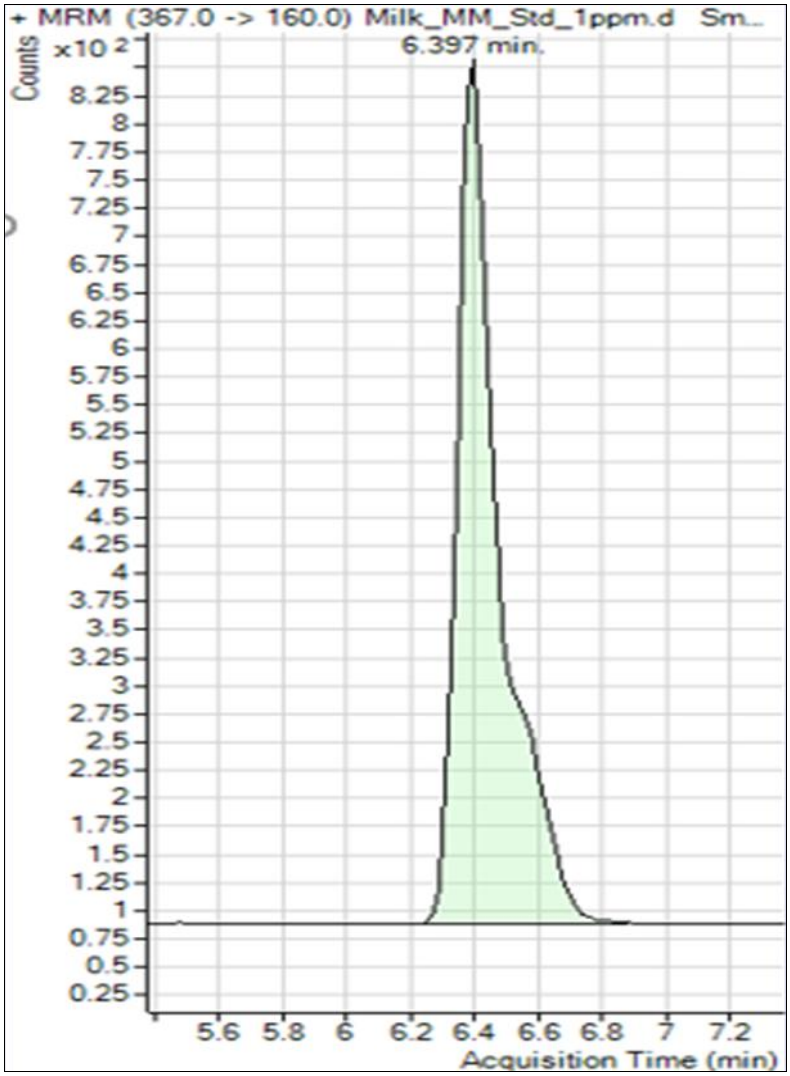

Fig 3: Chromatogram for Penicillin

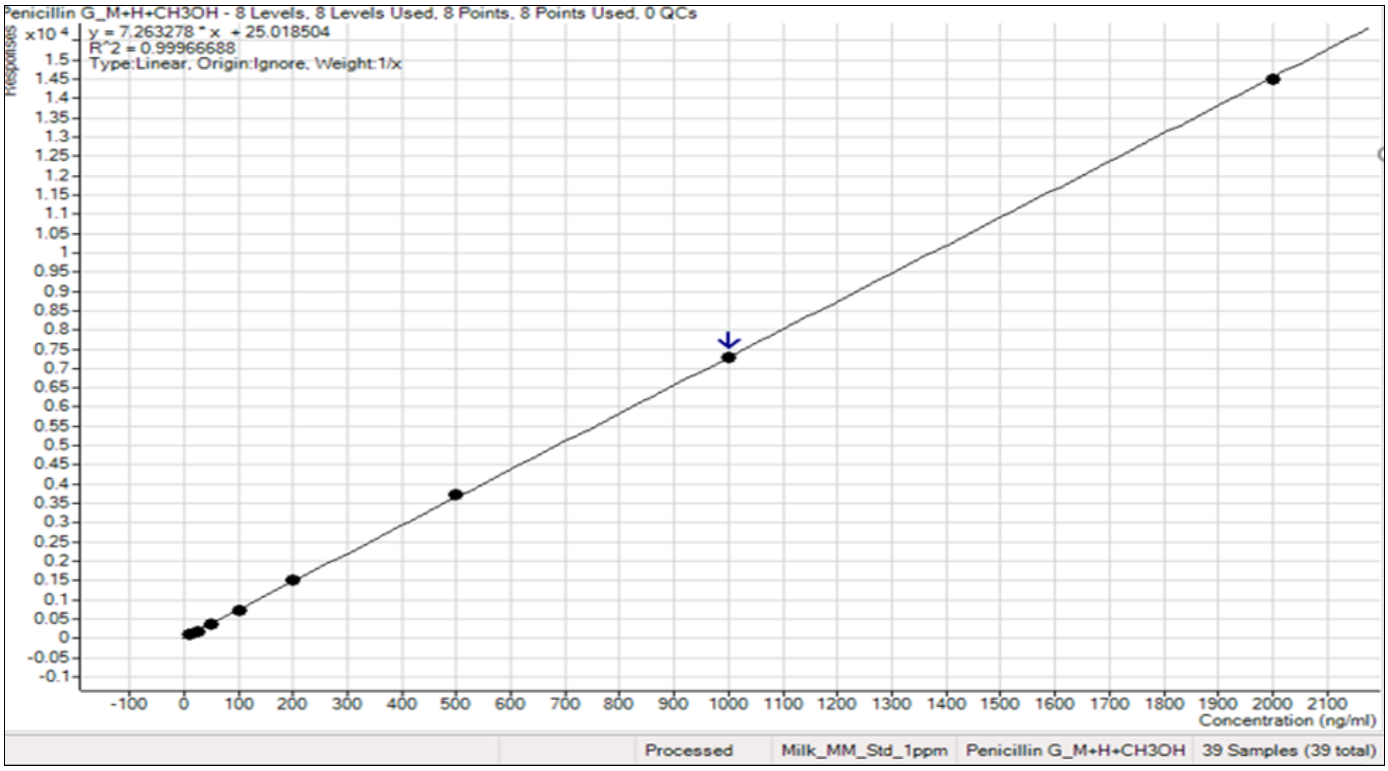

Fig 4: Calibration curve for Penicillin

Discussion

Several previous studies have described the detection methods for antibiotics in bovine milk, including high-performance liquid chromatography (HPLC) [11], HPLC-MS/MS [12], UPLC-MS/MS [13], screening methods and immunoassays [14]. Acetonitrile was chosen for the extraction of streptomycin from milk matrix due to its protein precipitation capacity. Aminoglycosides have good stability under cold circumstances. Therefore, Streptomycin was prevented from degradation by centrifugations at low temperatures (4 °C). The LOD of streptomycin was determined as 0.1 µg/kg. [15-19].

In the present study, the highest concentrations of streptomycin was detected was 1008.17± 151.35 microgram/liter at 24hrs after last administration. Several authors reported residual analysis of streptomycin through milk. However in most of the reports the intramammary infusion studies are reported. In the present study penicillin was not detectable in any of the milk sample. However streptomycin was observed upto 11th milking. Jaychandran *et al*. (1987) studied pharmacokinetics of streptomycin (10mg/kg) after single intramuscular administration in she buffaloes and reported that streptomycin was detectable upto 8 Hrs after administration [20]. In one of the study by Siddique *et al*. (1965) reported concentrations observed in milk of cattle after intramuscular and intramammary use of dihydrostreptomycin that the after intramuscular administration were found upto 12 hrs and 96 hrs respectively [21]. Park *et al*. (2016), reported that after intramammary administration in cow, Penicillin G residue in milk was not detected two days after administration of the drug, whereas streptomycin residues in milk were detected until 4 days after treatment of the drug following the manufacturer's recommended dose of 5 g per quarter per day [22]. In another study both NEO and dihydrostreptomycin residue detected 5 days post-treatment was under the MRL in milk from dairy cows administered with a drug (6 g) containing 200 mg of NEO once daily for 2 days and a drug (4 g) containing 100 mg of dihydrostreptomycin once [23].

The withdrawal time for milk after administration of streptomycin is 72Hrs with MRLs below 200 microgram/kg [24]. In the present study the streptomycin concentrations at 72 Hrs as mean ±SE was 151.5±17.92 µg/kg. Jaychandran *et al*. (1987) reported withdrawal time of 96 Hrs [20]. The present findings regarding withdrawal time are as per the regulations set by the regulatory authorities.

The above-mentioned reports support the present findings regarding the strepto-penicillins used in cows by intramuscular route.

Conclusion

The residues of Streptomycin in milk samples was found to be below MRL (200 mcg/L) after 72 hours of administration of Strepto-penicillin injection (Streptomycin @ 10 mg & Penicillin @ 8000 IU per Kg body weight). Levels of Penicillin G Sodium and Procaine Penicillin G were not detected in the given milk samples or below levels of quantification. However, residues of Streptomycin were detected in milk below MRL up to 5 days after administration of the drug (Dicrysticin S). It is recommended to observe 3 days withdrawal periods after administration the formulation in lactating animals. During the study, susceptibility of commonly occurring pathogens was also assessed where pathogens like *Pseudomonas, E. coli, Salmonella, Staphylococcus* species found to susceptible to Strepto-penicillins.

Conflict of Interest: Authors declares that there is no any conflict of interest.

Acknowledgement: Authors acknowledges the support given by Associate Dean, KNPCVS, Shirwal as well Zydus AHL, Ahemadabad.

For Journals Format: Author(s) of article (surname initials). Title of the manuscript. Journal title. Year of publication; volume number (issue number): page numbers.

Standard journal article (If more than six authors, the first six shall be listed followed by *et al*.) Gupta MP, Sharma K, Khan BU, Kapoor BC, Bajpai PK, Kumar A *et al*. Tissue specific esterase isozyme variation in Clarias batrachus and C. gariepinus. Global Journal of Pharmacology. 2009; 3(1):1-5.

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