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DETERMINATION OF AMOXICILLIN RESIDUES USED BY VETERINARIANS TO TREAT CAMEL DISEASE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) IN TRIPOLI REGION, LIBYA

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ABSTRACT

Background: Residues of antibacterial are a serious issue in the world in both veterinary and human fields. Based on the toxicity of antibiotics to animals and consumer health. Our research build on a method for the simultaneous analysis of amoxicillin residues in edible camel muscle, liver, kidney and fat samples via liquid chromatography-Mass spectroscopy (LC-MS) technique was used. **Methods:** Forty samples of slaughtered camel's tissues (10 of muscle, 10 of liver, 10 of kidney, and 10 of fat) were collected from different carcasses at different slaughter houses in Tripoli districts. The LC-MS method was validated according to specificity, sensitivity, linearity, matrix effects, precision, accuracy, decision limit, detection capability, and stability, as defined by the European Union and Food and Drug Administration. **Results:** Amoxicillin residues have been detected in 30% in muscle, 20% in liver, 20% in kidney and 40% in fat samples. The samples, although contaminated, yet decided acceptable as the detected levels were less than that were regulated by Codex Alimentarius Commission (CAC) for amoxicillin maximal residual levels (50, 50, 50 and 50 µg/Kg muscle, liver, kidney or fat, respectively). **Conclusions:** The authors recommended avoiding irrational use of amoxicillin in veterinary practice and camel in particular; and sticking to the withdrawal time regulated and labelled for drugs used in therapy among veterinary personnel, organizations, and governmental agencies in Libya.

KEYWORDS: Drug residues, Amoxicillin, Camel, Tripoli.

INTRODUCTION

Veterinarians in Tripoli region have a broad spectrum of applications exist in the Libya drugs market to treat camel disease. Among Libyan veterinarian, antibiotics are extensively prescribed medications. One of these antibiotics is amoxicillin aminopenicillin derivative, is a prophylactic drug belonging to b-lactams drug groups that are habitually prescribed medication against microbial infections. β-lactam ring which is responsible for its anti-bacterial property but differs from other members of the group by the side-chain which account for the major differences in their chemical and pharmacological properties.[1] Amoxicillin known as drug of choice and most commonly used antibiotics to treat bacterial infections in both human and animals.[2]

Antimicrobial resistance (AMR) and failure of antimicrobial therapies in humans is a mounting public health problem in whole world and several countries struggling against treating bacterial infections.[3] Antimicrobial resistance is driven by many factors including the use of antimicrobial drugs without planned strategy in both humans and animals. World Health Organization report announced urgent need for action in countering antibiotic resistance and all approach that should focus on all efforts to reduce unnecessary use of antibiotics, one of these approach that antibiotics be administered to animals only when prescribed by a veterinarian.[4] In Ibadan, south-western Nigeria engage in indiscriminate administration of antibiotics without any consideration of the fate of these antibiotics in the slaughtered beef cattle.[5]

In livestock breeding farms a low level of hygiene, inadequacy of husbandry zone planning and the lack of state management result several new problems such as uncontrolled epidemic diseases.[6] In Libya farmers consider antibiotics as one of the solutions to fight against camel diseases and to improve the animal productivity. Rather than allergy and bacterial resistance, there are more serious health hazards are attributed abuse and unobserving the withdrawal times of different

antimicrobials might lead to anaphylaxis and other toxicological implications such as mutagenesis and carcinogenesis.[7] Antibiotics are usually prescribed to treat bacterial infections in human and animal health. Once the antibiotics are prescribed and given by the veterinarian, the antibiotic residues promote the contamination of the animal's products. Most antibiotics are not metabolized after ingestion through reactions exist in the liver; instead, a significant amount of antibiotics residues are still found in waste as urine and faeces.[8]

Camels in Libya is considered as an important source of meat protein supply.[9] Several drugs are approved for use in camels which may carry risk of presence of residues in blood and tissues of administered animals slaughtered carelessly without considering withdrawal times of the given drugs Even a low level of antibiotics in the camel body's tissue could enhance microbial resistance, which results in adverse health problems to for society who consume camel products. Amoxicillin, It is used as a single drug or in combined forms with clavulanic acid. Its log Kow is 0.87, pKa: 3.23 (acidic) and 7.43 (basic), and is not lipophilic, thus easily absorbed in the intestine. FAO/WHO categorized amoxicillin as "highly important antimicrobial agent' based on their usage both in human and veterinary medicine. Amoxicillin was considered as the drug of choice in 71 countries from 2000 to 2010.[10] Amoxicillin residues are found in different dairy products.[11] Presence of amoxicillin residues in animal products holds the risk of undesirable health hazards for the consumers, ranging from allergic reactions, development of mechanisms of antibiotic resistance and other related diseases.[12] To avoid these consequences, animal product exposed to antibiotic treatment need to be analyzed for the presence of these residues before consumption. Kim research group reported that amoxicillin affects the liver and could activate the enzyme. Elevated transaminases are an indication of hepatomegaly, which is caused due to amoxicillin toxicity.[13] Different analytical methods exist for the detection and separation of Amoxicillin including high performance liquid chromatography (HPLC),[14,15] and capillary electrophoresis,[16,17] HPLC required large amount of high purity organic solvents, long equilibration and derivatization treatment.

Antibiotic resistance developed by eating product contaminated by antibiotics residues is probably the most threatening difficulty for treatment in the world. The resistance phenomenon due to the antibiotics residues has contributed to scientists' finding newer antibiotics. Therefore, our study to preserve the level of amoxicillin residues in camel meat in Tripoli region to develop specific data that can be use in future by veterinarian in order to improve antibiotics uses in Libya.

MATERIALS AND METHODS

Study Area and Time

This study was carried out in Tripoli area, Libya. Samples were collected from different slaughter houses and meat shops located in Al-Hadba, Souq-Al-Jomaa, Hay Al-Andalus, Gorjy, El-Dereby, El-Fallah, Gergaresh, Al-Mansoura, Al-Dahra and Mizran.

Feedback information

A questionnaire survey was conducted by personal interviews with the meat shops' owners, veterinarians and farmers in different districts in Tripoli. It was carried out to determine associations between the occurrence of antibiotic residues in camel edible tissues and various risk factors like management practices, treatment factors and residues prevention methods. In management practices of the meat shops' owners, the information collected were the sources of purchasing camels be slaughtered, use of any drugs for keeping animal live status. Information on treatment factors from field veterinarians included sources of antibiotics, type person who administered antibiotics to camels, route of antibiotic administration, record keeping. Regarding residue prevention methods, the information gathered were marking of treated camels and use of antibiotic test kit and knowledge of withdrawal periods of antibiotics.

Experimental design

Samples were collected randomly from the above mentioned districts in Tripoli area for the detection of residues of amoxicillin. Samples were prepared for detection of residues of amoxicillin by liquid chromatography-Mass spectrometry (LC-MS). Data were statistically analysed and incidence of residues was determined as a percentage. Acceptability or not of the positive samples was decided according to Codex Alimentarius Codex (CAC).[18]

Collection of samples

Forty random samples of slaughtered camel's tissues (10 of liver, 10 of muscle, 10 of kidney, and 10 of fat) were collected from different carcasses at different slaughter houses in Tripoli districts mentioned above. Each sample was put in a separate clean plastic bag, labelled and transferred to the laboratory and frozen at -50 °C till analysis.

Equipment

The following devices have been used in the present study: Homogenizer (PRO Homogenizer, Pro scientific, Oxford, USA), Vortex (Maxi mixII, Thermo scientific, Watham, MA, USA), Balance (Vibra HT, Intelligent Weighing Technology, CA, USA), Centrifuges (Pro-SepE, Centurion, West Sussex, UK), Elmasonic X-tra ultrasonic cleaner (Laval Lab Inc., Quebec, Canada), Filtration device (Puradisc TM 25NYL, Whatman, Kansas, USA), Rotary evaporator (Büchi, Switzerland) and SPE sorbent (Strata® SPE, Phenamenex, CA, USA).

Reagents and Chemicals

All reagents and chemicals were of analytical grade. Reference standards of the tested antibacterial drugs have been purchased from Sigma-Aldrich, Saint Louis, USA. The standard was of 95% purity or higher. It was used for calibration curve preparation (see below). Acetonitrile, methanol and other chemicals were supplied by Sigma, Saint Louis, USA. Ultrapure water was generated by a Milli-Q system (Millipore, Billerica, Massachusetts, USA).

Sample preparation

One gram of homogenized camel meat, fat, kidney or liver was extracted using 9 mL of a 2:8 (v/v) mixture of water and acetonitrile (forming a total volume of 10 mL) and vortexed for 5 minutes in 50 mL polypropylene centrifuge tube. The sample was then centrifuged at 5000 rpm for 5 minutes and the supernatant was decanted into a 15 mL tube containing 500 mg Strata® C18E SPE sorbent. The sample was shaken and again briefly vortexed for 30 seconds and centrifuged again for 1 minute. A 5 mL aliquot of the resulting supernatant was transferred to a graduated tube and the contents evaporated down to less than 1 mL. The sample was then brought up to 1 mL total volume with water, and filtered (0.45 µm PVDF; polyvinyl difluoride) prior to performing LC-MS analysis on the same day as the extracts were prepared.[19] Standard curve and calibration Individual stock solution (1000 ng/mL viz 1000 ppb) was prepared in the solvent mixture (acetonitrile: water, 80: 20, v/v). Working diluted concentrations have been prepared for each antibiotic under investigation ranging between 0.5 and 150 ppb according to the expected residue levels. LC-MS Analysis The chromatographic system consisted of an Agilent 1100 series (Agilent Technologies Inc., CA, USA) including binary pump (G1312B), equipped with on-line solvent degasser (G1379B), Auto-sampler (G1367E), thermo-stated column (G1316A; kept at 40°C) and temperature module (Palo Alto, CA, USA). This chromatographic system was interfaced with an Applied Biosystems 4000 QTRAP® LC-MS system with Turbo V™ ion source. The whole system was controlled using Analyst® software version 1.4.1.[20] The chromatographic conditions were as follows:

- Column: Kinetex 2.6 µm C18
- Dimensions: 50 x 2.1 mm
- Part No: 00B-4462-AN
- Mobile phase: A: 0.1 % Formic Acid in Water and B: 0.1 % Formic Acid in Methanol
- Injection volume: 10 µL - Flow rate: 0.5 mL/min
- Temperature: 40 ºC
- Detector: Mass spectrometer (MS)

The MS determination was performed in electrospray (ESI) positive or negative mode combined with monitoring of the most abundant MS/MS (precursor → product) ion transitions (dwell time of 75 ms for each transition). The following MS conditions were used:

- Entrance potential: 10 V
- Ion spray voltage: 4500V
- Ion source temperature: 525 °C
- The curtain gas regulator was set at 40 psi with optimum setting (in the Analyst software).
- The nebulizer and collision gas regulators were set at 90 psi with optimum settings.

At the above mentioned settings, amoxicillin was detected after retention time of 2.08 min.

Data Management and Statistical analysis

The obtained results were statistically analysed using SPSS software. The number of positive samples against the total number of examined samples was calculated as %. Among the positive samples, those which were found exceeding the MRL (maximum residual level) were considered significant. The overall mean ± STDEV of the detected residue values have been calculated for each type of samples (muscle, liver, kidney and fat).

RESULTS

Camels are imported from Sudan, and drugs are given by herd managers including, benzylpenicillin, oxytetracycline, tylosin, neomycin, sulphonamides, chloramophenicol, enrofloxacin, amoxicillin and ivermectin. Usually, because of unavailability of doses for camels, those of ruminants are applied without differentiation. Moreover, dosage “dose regimen” including frequency of dosing and period of medication are inaccurate and mostly performed in a haphazard manner. The aforementioned drugs are used for treatment of infections that occur accidentally to animals and/or for prophylaxis. Most interviewees said that there is no veterinary inspection procedures are being undertaken at the Libyan ports. Additionally, animals are being slaughtered directly without residual check, where there were no routine tests are carried out at abattoirs before slaughter. Almost all farmers, breeders and some of Veterinarians are unaware and are not updated about the maximum residue levels (MRLs) and withdrawal times of the used drugs. Taking history of medication and calculating withdrawal times are almost neglected at slaughter houses.

Standard calibration curve for Amoxicillin is depicted in Figure 1; the curve was linear throughout the prepared concentrations.

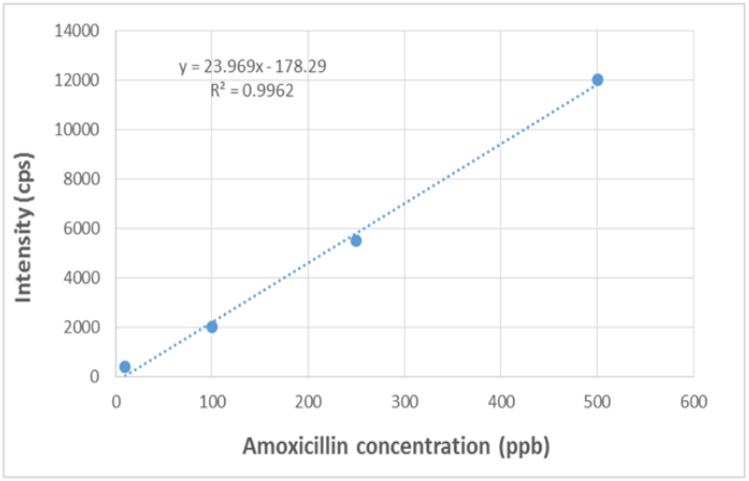

Figure 1: Standard calibration curve for amoxicillin.

Results of the residual analysis of amoxicillin (ppb) in the examined camel samples (n= 40), including muscles (M), livers (L), Kidneys (K) and fat (F) were shown in table 1. And figure 2 shows Residual concentrations of Amoxicillin in the examined camel tissue samples (n = 40).

Table 1: Results of residual analysis of Amoxicillin (ppb) in the examined camel tissue samples (n= 40).

Tissue	*Negative samples*		*Positive samples*		*Min.*	*Max.*	*Mean ± SD*
	No	*%*	*No*	*%*			
Muscle	7	70	3	30	15.3	591	227.67 ± 316.15
Liver	8	80	2	20	28.4	34.7	31.55 ± 4.454
Kidney	8	80	2	20	86.4	123	104.7 ± 25.88
Fat	6	60	4	40	45.8	1220	675.7 ± 428.359

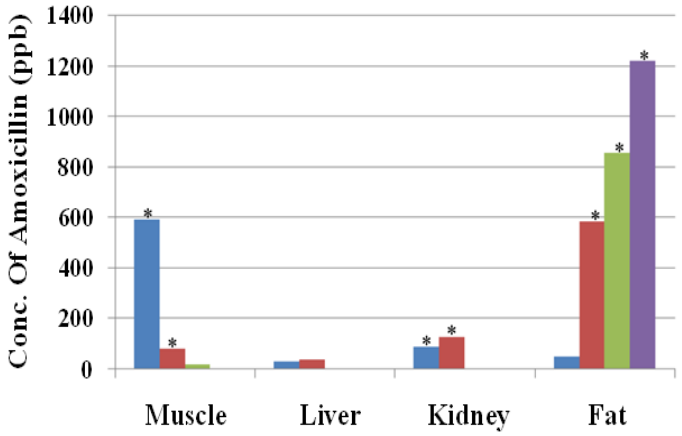

Figure 2: Residual concentrations of Amoxicillin in the examined camel tissue samples (n = 40).

*****Unaccepted according to Codex Alimentarius Commission 2012.[18]

Table 2 shows acceptability of the examined camel tissue positive samples based on their maximum residue levels (MRLs) of amoxicillin according to Commission of the European Communities; Veterinary drug residues, 2nd Ed. 1994.[19]

Table 2: Acceptability of the examined camel tissue positive samples based on their Maximum residue level (MRL) of Amoxicillin (n= 40).

Tissue	*MRL (ppb)**	*Accepted samples*		*Unaccepted samples*	
		No	*%*	*No*	*%*
Muscle	50	1	33.33	2	66.67
Liver	50	2	100	0	0
Kidney	50	0	0	2	100
Fat	50	1	25	3	75

*ppb: part per billion according to the 35th Session of the Codex Alimentarius; (Codex Alimentarius Commission 2012.[18]

Figure (3) represents LC-MS chromatograms of maximum residual amounts of amoxicillin detected in camel muscle (A), liver (B), kidney (C) and fat (D) samples (n = 40).

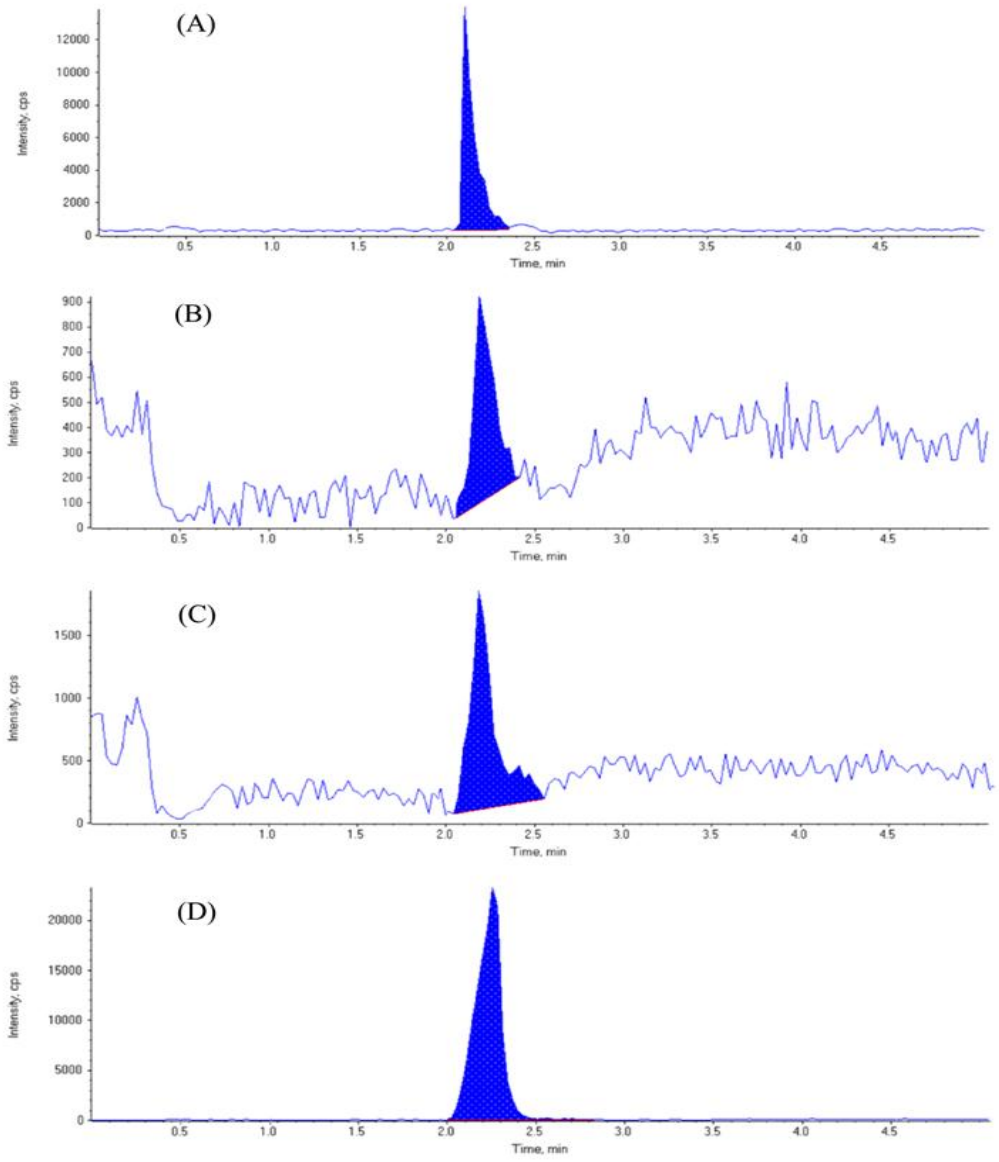

Figure 3: represents LC-MS chromatograms of maximum residual amounts of Amoxicillin detected in camel muscle (A), liver (B), kidney (C) and fat (D) samples (n = 40).

In the present study, amoxicillin residues have been detected in 30% (muscle), 20% (liver), 20% (kidney) and 40% (fat) of examined samples. The samples, although positive, yet decided acceptable as the detected levels were less than those regulated by CAC for amoxicillin maximal residual levels (50, 50, 50 and 50 µg/Kg muscle, liver, kidney or fat, respectively). The present study revealed the findings stated and discussed below.

DISCUSSION

Camel meat is not universally eaten. However, camel is a good source of meat and milk in areas where the climate adversely affects other animals.[21] In the pastoral communities, camel meat is only eaten on special occasions. These include festive gatherings following the return of the herd from grazing.[22] However, in Libya, meat from camel, especially Youngers (GAOOD) is considered as an important source for protein supply to Libyan population. A variety of antimicrobial agents have been administered to camelids (Camel, Llama and Alpaca). However, most have not been studied scientifically, and the attending veterinarian must assume the responsibility for extra-label drug use and potential adverse effects on the animal. As a general rule, antibiotics appear to have longer elimination half-lives in camelids than in domestic ruminants, potentially prolonging their therapeutic effect but also increasing their risk of toxicity. This may be due to a lower rate of urine production in camelids [23], which may increase half-life of antibiotics excreted primarily through the kidneys (e.g., penicillins, aminoglycosides). As another general rule, volume of distribution varies tremendously among individual camelids. Higher dosages are generally recommended to avoid subtherapeutic drug concentrations in some camelids. The unsupervised extra-label use of these drugs may carry the risk of presence of residues in blood and tissues of administered animals slaughtered carelessly without considering withdrawal times of the given drugs.

Camels are imported from Sudan, and drugs are given by herd managers including, benzylpenicillin, oxytetracycline, tylosin, neomycin, sulphonamides, chloramophenicol, enrofloxacin, amoxicillin and ivermectin. Usually, because of unavailability of doses for camels, those of ruminants are applied without differentiation. Moreover, dosage "dose regimen" including frequency of dosing and period of medication are inaccurate and mostly performed in a haphazard manner.

In the present study, amoxicillin residues have been detected in 30% (muscle), 20% (liver), 20% (kidney) and 40% (fat) of examined samples. The samples, although positive, yet decided acceptable as the detected levels were less than those regulated by CAC for amoxicillin maximal residual levels (50, 50, 50 and 50 µg/Kg muscle, liver, kidney or fat, respectively). The percentage of amoxicillin incidence as residue in different tissues of slaughtered camels are much higher when compared with data reported by Chowdhury research group in 2015; they found that amoxicillin residues among 200 milk samples analyzed, Amoxicillin residue was detected in raw local milk (14%), raw commercial milk (38%), boiled local milk (12%), boiled commercial milk (37%). In TLC testing, Amoxicillin was also detected in raw local milk (13%), in raw commercial milk (35%), in boiled local milk (12%), and in boiled commercial milk (35%).[24] A study conducted by Hassan team revealed presence of amoxicillin residues declared that the incidence of amoxicillin in the examined samples of chicken heart, gizzard and liver was 6.67%, 16.67% and 20%, respectively. Besides, the concentrations (µg/kg) of amoxicillin in the examined samples of chicken giblets varied from 24 to 71 for heart, 25 to 180 for gizzard and 35 to 210 for liver.[25]

A study conducted by Neeley and Connolly, 2004; to evaluate amoxicillin in edible tissues of bovine revealed the presence of amoxicillin is (kidney: 25 µg/kg; liver: 25 µg/kg; muscle: 10 µg/kg; fat: 10 µg/kg) using LC-MS/MS, [26]; however, the detected levels were higher than the other report issued by Luo and Ang, 2000; using HPLC fluorescence.[27] In other research on amoxicillin multi-residue analytical methods on bovine tissue (muscle, liver, and kidney) showed presence lover than previous mention reports.[28] However, because the metabolites of amoxicillin also fluoresce under the acidic conditions used to generate the fluorescent derivative for amoxicillin, from here we understand the exist of the analytical methods that use fluorescence for detection cannot be used to make regulatory decisions because those methods tend to overestimate the concentration of residual amoxicillin in treated samples.

The results of the present study indicate that amoxicillin residues were detected at higher incidence rates that reached 40% in the fat tissues in Tripoli, Libya. The remainder of sample, although positive, yet decided acceptable as the detected levels were less than that was regulated by CAC for amoxicillin MRLs (50 µg/Kg muscle, liver, kidney or fat; equivalent to 50 ppb). Although the detected levels were lower than the legislated MRLs, yet we imply that they are of unsafe for the consumer in Tripoli as they may pose a potential hazard to public health as they might induce drug resistance of pathogenic micro-organisms or teratogenic effects if consumed by pregnants. We strongly recommend making Libyan individuals and organizations aware of the problem. Even if the camel products containing residues amoxicillin levels are lower than MRLs.

Competing of interest

The authors declare that there is no competing of interests regarding the publication of this paper.

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Ethical approval:
The study was approved by the Institutional Animal Ethics Committee.

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