Research Article

Toxicopathological Studies on Effects of Ectoparasites and Ivermectin Residue in Cow Hide Industerial Value

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Abstract | **Background:** Cowhide quality is crucial in the manufacturing of leather products. Ectoparasites are one of the major problems that hinder the quality of the skin and urge the use of insecticides to control them. One of the commonly used anti-parasitic drugs is ivermectin in many food-producing animals. This study investigates the harmful effect of ectoparasites, and the side effects of commercial ivermectin drugs on the quality of skin collected from slaughterhouses in Egypt. **Methodology:** Overall, Ivermectin, pesticide, and veterinary drug residues were detected in 50 random bovine skin samples. Each sample was kept in a separate sterile plastic bag and transferred to the lab in an insulated icebox for detection of the presence of ivermectin residues using the high-performance liquid chromatography (HPLC) technique. Skin histopathology and immunohistochemistry of collagen were performed. **Results:** Ivermectin was detected in 36 samples, out of them 15 contained high ivermectin levels (100 ppb). Chlorpyrifos, piperonyl butoxide, and acetamiprid were below the limit of quantification in 3 samples only. Histopathology of tick-infested skin revealed severe multifocal eosinophilic dermatitis and inflammation of subcutaneous hypodermis (panniculitis) whereas, in the skin with high ivermectin residue, there was mild multifocal epithelial hyperplasia with mild perivascular mononuclear cells infiltration. **Conclusion:** Different levels of ivermectin residue were detected in bovine skin samples collected from slaughterhouses in Egypt however the degree of damage caused by ectoparasites exceeds the damage caused by ivermectin.

Keywords | Ectoparasites, Ivermectin, Histopathology, Immunohistochemistry, Oxidative stress, Pesticides, Skin damage, Slaughterhouses.

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INTRODUCTION

Cowhide quality is crucial in the manufacturing of leather products. Hides are raw materials that are used in the leather industry (Bai et al., 2022). Several factors affect their production like the management, rearing, and disposal of the livestock. Major constraints affecting the leather industry are correlated to skin diseases, flay cuts, branding, scratches, poor pattern, scabs, putrefactions, and poor substances (Abadi, 2000). Skin damage due to major skin diseases like Lumpy skin disease, and bovine papillomatosis transmitted by blood-sucking insects represent a challenge to overcome (Sanz-Bernardo et al., 2020; Gallina et al., 2020). One of the major diseases that damage

skin is tick bites (tick marks) which may further contribute to disease spread as they act as a vector for tick-borne diseases like east coast fever, anaplasmosis, babesiosis, and dermatophilosis. They feed on host animal blood causing blood loss and anemia besides causing inflamed skin and sometimes permitting entry of other microbes in areas where they attach themselves. Furthermore, pinpoint scars are formed at the attachment sites hindering the use of rawhide for full-grain leather. Consequently, rawhide materials are downgraded and of less value especially to the tanning industry (Jabbar et al., 2002).

Based on the aforementioned reasons a lot of farmers provide extensive veterinary services for the protection of their animals against the effect of ticks. Sarli et al. (2021) demonstrated the efficacy of long-acting ivermectin formulation but at a high dose resulting in its accumulation in the plasma and possibly increasing its resistance in the tick population.

In cattle, gastrointestinal roundworms, lungworms, cattle grubs, sucking lice, and mites are mostly treated by ivermectin. Ivermectin belongs to the macrocyclic lactones recognized as avermectins, which exhibit broad-spectrum anti-parasitic activity and consist of two homologs mixture 22, 23- dihy-droavermectin B1a and 22, 23- dihydroavermectin B1b (Fent, 2014). It boosts the release of gamma amino butyric acid (GABA) at presynaptic neurons. GABA inhibits neurotransmission blocking the post-synaptic stimulation of nearby neurons in nematodes or arthropods. Thus, the parasite is paralyzed and dies (Plumb, 2011).

To detect drug residues in different tissues, highly sensitive analytical assays are required. In recent years, several chromatographic methods were established for the measurement of veterinary drug residues in different tissues like muscle, liver, milk, plasma, serum, and salmon (Degroodt et al., 1994; Abjean, 1995; Samsonova et al., 2002).

The residues of ivermectin can cause many health hazards in humans and animals (Baudou et al., 2020), it can result in mild Mazzotti reaction manifested as pruritus, fever, arthralgia, postural hypotension, myalgia, edema, lymphadenopathy, headache, gastrointestinal symptoms, sore throat, and cough. therefore, it's necessary to regulate the presence of residues to preserve food safety (James and Reynolds, 1993; Koesukwiwat et al., 2007).

Ivermectin residues can be found in animal products such as milk and meat (Bassissi et al., 2004). The distribution of ivermectin is mainly in fat and liver tissues and was less concentrated in muscle tissue as demonstrated in sheep, cattle, and rats (Chiu et al., 1990). Tissue redistribution is not affected by the route of administration (Adam, 2001). The ivermectin maximum residue limit (MRL) has been stated to be 100 ppb (European Medicines Agency, 2014).

This study investigates the damage caused by the tick parasitic infection and also investigates the ivermectin residue in bovine skin and its adverse effect on the quality of the hide needed for the leather industry.

MATERIALS AND METHODS

Animals

In this study, 50 skin samples from cattle (weighing 100g each) were collected from slaughterhouses by stratified purposeful sampling. Part of the samples was collected on formalin for histopathology and another part was translocated to the laboratory in an ice box for preparation and analysis of residues.

Chemicals

All chemicals were of HPLC grade. Ivermectin standard, Methanol, Ethyl acetate, acetonitrile, 1-methylimidazole, trifluoroacetic anhydride (99%), Sodium sulfate anhydrous, sodium chloride, glacial acetic acid were obtained from Sigma-Aldrich (Chemical Co., St. Louis, Mo, USA).

Analysis of pesticides

Pesticides including organochlorine, nitrogen, organophosphorus, carbamate, pyrothirdate, benzimidazole, methyl bromide, and dithiocarbamate compounds were analyzed in the skin samples by GC-MS/MS in the Central Laboratory for analysis of pesticide residues and heavy metals in food (Dokki, Giza, Egypt) according to the method described by Usui et al. (2012) and Nassar et al. (2016).

Analysis of drug residues

The residues of veterinary drugs including Nitrofurran compounds, Hormones (progesterone, testosterone, zeranol, and trenbolone acetate), Chloramphenicol, antibiotics (sulfonamides, tetracycline, macrolides, Fluoroquinolones, and Trimethoprim), and Ractopamine were analyzed in the skin using LC MSMS and methodologies in compliance with European Union requirements (Central Laboratory for analysis of pesticide residues and heavy metals in food, Dokki, Giza, Egypt) according to a previous method (Kennedy et al., 1993).

Analysis of ivermectin residues

Preparation of samples for analysis: At the time of the assay, frozen skin tissue samples were partially thawed at room temperature (23°C) for 30 min and were crushed in a food processor four times for 20–30 sec at high speed. The material after each time was subjected to stirring to obtain a uniform paste-like consistency, and the samples

were then stored at -70°C until analyzed within 30 days.

Extraction and determination of drug residues: Extraction of the drug residues from the samples was carried out according to Stoilova (2008). Frozen samples were thawed in centrifuge tubes at room temperature (23˚C) and then 1 g was accurately weighed into a polypropylene centrifuge tube. 10 ml of acetonitrile was added and shaken for 1min; the sample was then shaken for 10 min and centrifuged for 10 min at 9500 rpm. The supernatant was evaporated under a nitrogen stream at 50˚C, and the extraction was repeated using acetonitrile with the sample residues. The additional supernatant was added to the initial one and evaporated under a nitrogen stream at 50˚C. The residue was then dissolved in 5 mL of 0.02 M ammonium acetate pH=9 and then vortexed for 1 min. The extract was then applied to the SPE C18 cartridge using the following steps:

• SPE cartridge was previously activated with 3 ml acetonitrile and 3 ml 0.02 M ammonium acetate pH=3.0.
• After sample loading, the cartridge was washed with 2 ml water, and then dried for 3 min.
• The analyte was eluted with 10 ml 0.2% formic acid in acetonitrile. The sample was evaporated to dryness and dissolved into a 1 mL mobile phase. Finally, filtration was performed using a 0.45 µm nylon syringe filter.

HPLC operating conditions: Ivermectin residues were assessed by HPLC ultraviolet (UV) in cattle skin samples. The HPLC apparatus (Agilent1100) equipped with a diode array detector was used in which the injection volume of 20 µL, flow rate: 1 ml/min, column: Zorbax SBC 18 (150 mm×4.6 mm×0.5 um film thickness); column temperature: 50°C, UV- detector: 280 nm and the mobile phase: 50 mL/L acetic acid: acetonitrile: methanol (900:50:50) according to Kamberie et al. (1998).

The ivermectin residues in skin samples were related to those given from analogous injections of the standard solutions. These residues were quantified by using software according to the peak areas in the chromatogram. The validation of the method was performed.

Histopathology

Skin tissue specimens were fixed in 10 % neutral buffered formalin. Ascending grades of alcohol for dehydration and xylene (2 changes) for clearance were used in the processing of samples which were then embedded in paraffin. Tissues were sectioned by rotary microtome (Leica RM2125, Germany) into 4 µm thick and stained by hematoxylin and eosin stain (Suvarna et al., 2012).

Immunohistochemistry

On paraffin-embedded tissue sections, immunohistochemistry of collagen 1 (COLI) was performed. Antigen retrieval using citrate buffer was performed. Primary antibodies against COLI (1:100, ab34710, Abcam, Cambridge, UK) were applied to sections followed by the Horseradish peroxidase-conjugated antibodies (Abcam, Cambridge, UK). Diaminobenzidine was used as a substrate and Mayer's hematoxylin was used as a counterstain (Ramadan et al., 2022; El Miniawy et al., 2022).

RESULTS

Pesticides and veterinary drug residues

Only three samples had chlorpyrifos, piperonyl butoxide, and acetamiprid below the limit of quantification (LOQ). Otherwise, all examined samples were free from pesticides and veterinary drugs.

HLPC analysis of ivermectin

High-performance liquid chromatography analysis recorded that the corresponding peak responses (area under the peak) of 22, 23 Dihydroavermectin B1a (H_2B_{1a}) standard concentrations of 10, 20, 50, 100, 200, 500, and 1000 µg/gm as illustrated in Table (1) and Figure (1). The analytical method linearity, range, LOD, LOQ, recovery, and intraday & interday precision: the obtained results were summarized in Table (2). H2B1a distribution in cattle skin tissues was represented in Table (3). The typical chromatogram of H2B1a is shown in Figure (2). There was a widespread distribution of the H2B1a in the tested tissues.

Specificity: The equilibrated chromatograms of H_2B_{1a} in skin samples were demonstrated specific at a retention time of 1.3 min showing no interference between the extracted different spiked matrixes and pure standard (Table 1, Fig 1).

Table 1: The concentrations of ivermectin spiked tissues (µg/gm) and their corresponding peak response automatically using HPLC

Retention time	Conc.	Area
1.3	10	65.2
	20	125.3
	50	320.95
	100	648.2
	200	1278.7
	500	3220
	1000	6466

Histopathology and immunohistochemistry of skin

Microscopy of the skin infested by the tick showed chronic severe multifocal dermatitis with abundant eosinophils and MNC infiltration (Fig. 2a, b). The epidermis showed necrosis of the epidermis with neutrophil cell infiltration

Table 2: Validation sheet of the HPLC method

Parameter	Skin+ fat	Acceptance criteria
Range (ppb)	10- 1000	
Retention time (min.)	1.3± 0.002	
Slope	7.0011	
Intercept	-54.111	
Correlation coefficient (R)	0.9996	≥0.99
LOD (ppb)	0.3	
LOQ (ppb)	0.8	
Recovery %	92-102	85-115
Intra-day precision (RSD %)	0.2	≤1
Inter-day precision (RSD %)	0.6	≤2
Robustness (pooled RSD %)	1.1	≤6
Tailing factor	1.2	≤2
Theoretical plates	13000	≥2000

LOD = Level of Detection LOQ = Level of Quantitation

Table 3: The concentrations of 22, 23 Dihydroavermectin B1a in tissues of slaughtered cattle automatically using HPLC

Ivermectin residue level (µg/kg)	Number of samples	Concentration
ND	14	-
0-100	21	45±0.02
More than 100	15	110±0.6

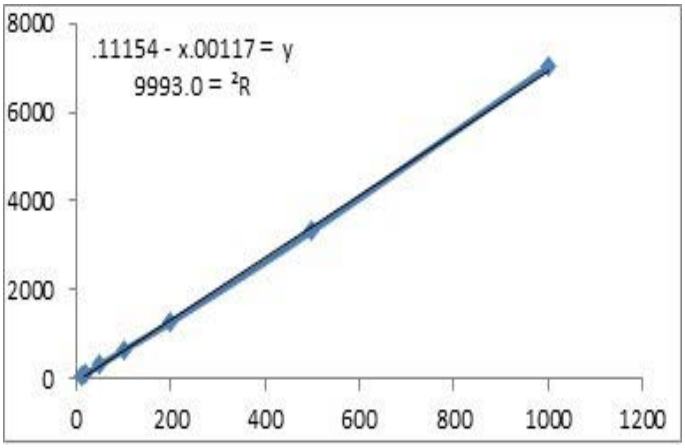

Figure 1: Calibration curve of H_2B_{1a} in blank skin

and fibrin exudation at the site of tick attachment. The epidermis had multifocal erosions, acantholysis with the widening of intracellular spaces, and loss of cellular contacts. The dermis and hypodermis were infiltrated by numerous eosinophils, especially around the blood vessels (Fig. 2c). The skin also showed edema, pustule formation in the dermis and hypodermis, eosinophilic folliculitis and furuncles, and collagen degeneration. Pustule formation was also sometimes observed in the epidermis (Fig. 2d).

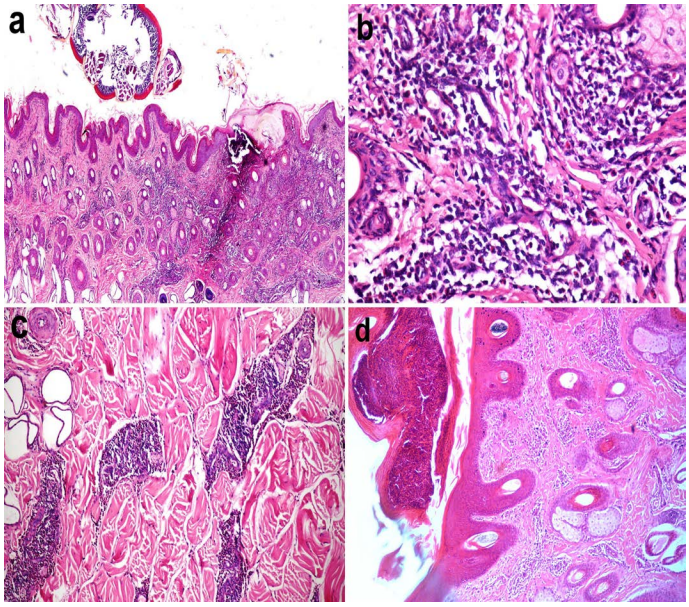

Figure `2: Histopathology of Bovine skin showing (a) tick attached to the surface of the skin with diffuse dermatitis (X40), (b) mononuclear and few eosinophils infiltration in the dermis (X200), (c) panniculitis in the hypodermis, (d) pustule formation in the epidermis (X100). (H and E stain)

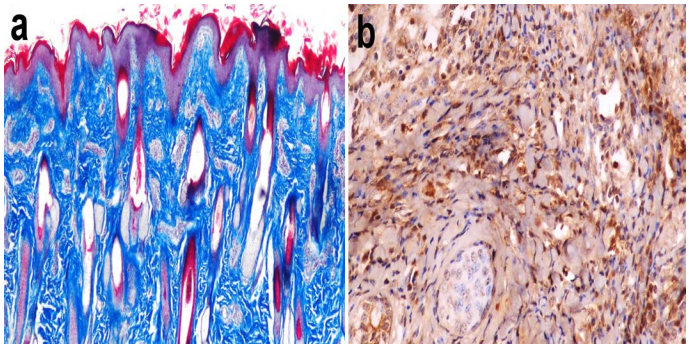

Figure 3: Histopathology of Bovine skin showing (a) Edema, separation, and moderate disorganization of collagen bundles in the dermis in addition to perivasculitis (Masson's Trichrome stain X 40), (b) loss of spatial orientation of grains and dislodgement of poorly stained collagen bundles (Immunoperoxidase and Mayer's Hematoxylin counterstain X 400).

The collagen fibrils were destructed with loss of spatial orientation of grains and dislodgement of collagen bundles (Fig. 3a). The skin infested with tick showed a severe decrease in dermal type I collagen when compared to noninfected skin (Fig. 3b).

Histopathology of skin with high ivermectin residue revealed mild focal epidermal hyperplasia (Fig. 4a) and perivascular leukocyte infiltration (Fig. 4b).

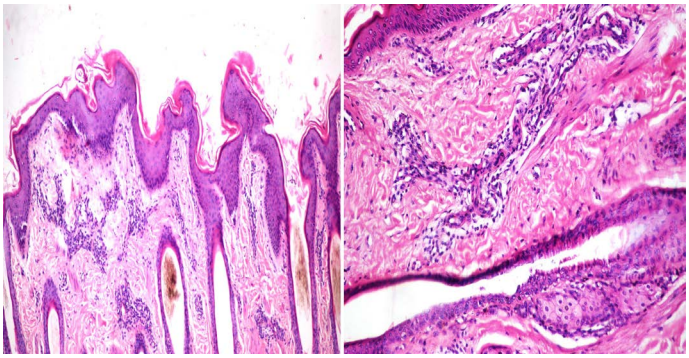

Figure 4: Histopathology of skin with high ivermectin residue (a) mild focal epidermal hyperplasia (X40), (b) perivascular mononuclear cells infiltration (H and E stain X 200).

DISCUSSION

This study describes the skin lesions caused by ticks and evaluates for the first time in Egypt the residues of veterinary drugs, pesticides, and ivermectin in the skin. One of the main cattle products is leather (Durrani et al., 2006). The presence of ticks attached to the skin of the cattle is confirmative for infestation as it is a macroscopic external parasite. In the present study, the only external parasite encountered during the external examination of 50 cattle slaughtered was the ticks. Ticks are the most common external parasites causing reduced milk production, blood loss, low hide quality, and weight loss, and help in the spread of diseases (Shemshad et al., 2012). Unfortunately, ticks play an important role as a vector to several diseases besides the occurrence of non-specific symptoms such as toxicosis and anemia (Solomon et al., 2001). Furthermore, low productivity causes economic loss in some ruminants (Whatford et al., 2022). Bites from some tick species may result in fatal paralysis in their hosts (Ejima and Ayegba, 2011).

The skin damage caused by ticks is mainly at the attachment sites. Tick mouthparts injure the skin as it penetrates its different layers but at the same time trigger wound healing through growth factors present in the tick saliva (Bartíková et al., 2020). Similar to our findings, the lesions observed are mainly inflammatory with eosinophilic and sometimes neutrophilic cell infiltration. Delayed hypersensitive reactions with intra-epidermal pustulation were also observed in high-resistance cattle (Latif et al., 1991). At the site of the tick bite, it was reported that the thickness of collagen bundles was increased causing narrowing or the absence of interspaces (Mihara, 2017). Likewise in our study in which an alteration in the organization of collagen was also observed.

For the prevention and control of ticks many acaricides have been used but among them is ivermectin. Ivermectin is a water-insoluble lipophilic compound that mainly persists in the adipose tissue of humans, cattle, goats, etc. (Bloom and Matheson III, 1993; Baraka et al., 1996; Lanusse et al., 1997; Lespine et al., 2005; Canga et al., 2008). However, adipogenesis is hindered by ivermectin due to hyperpolarization and an increase in intracellular calcium levels resulting in activated calcineurin (Qi, 2019). Treatment of skin with ivermectin resulted in histopathological alteration in the skin such as perivascular cuffing with eosinophils, neutrophils, and monocytes in addition to excessive fibrous connective tissue formation as reported in previous research (Jameel et al., 2014). Similarly in the current study, the skin with high ivermectin residue had almost similar lesions in which the perivascular leukocyte infiltration was the most dominant.

The withdrawal time of ivermectin varies between species and ranges from 35-days for cattle to 18-days for swine. Nevertheless, derived food may still have traces of ivermectin (Crooks et al., 1998; Crooks et al., 2000).

CONCLUSION AND RECOMMENDATIONS

Our study revealed the presence of ivermectin residue in bovine skin in some samples indicating that it's the drug of choice of most breeders in Egypt. The skin having high residues exhibited several lesions which however were less than those observed with tick infestation. Results showed that hide production facing a serious challenge in Egypt. It was downgraded and rejected because of various disorders which are represented here in tick infestation and accumulation of parasiticides (drug residues). Many recommendations could be summarized in frequent awareness for all responsible persons beginning from farmers to tanneries owners about the importance of hide and leather production at local and international levels. Decrease the prevalence of dermatologic infections that affect hide's quality. Besides the necessity of finding alternative solutions to eliminate external parasites that infect the skin to eliminate the problem of drug residues that affect hide production.

ETHICS COMMITTEE STATEMENT

This study was granted ethical approval permission from the Institutional Animal Care and Use Committee, Cairo University (Vet CU12/10/2021/375).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

LIST OF ABBREVIATION

PPb: part per billion
HPLC: high-performance liquid chromatography
GABA: gamma amino butyric acid
MRL: maximum residue limit
GC-MS/MS: Gas chromatography with a triple quadrupole mass spectrometry system
LC MSMS: Liquid Chromatography with tandem mass spectrometry
LOD: limit of detection
LOQ: limit of quantification

NOVELTY STATEMENT

The leather industry is one of the important industries that contribute to the national income of the country and it is necessary to pay attention to it. This research highlights the importance of this industry and some of the risks it faces.

AUTHORS CONTRIBUTION

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Marwa Khattab, Ahmed Osman, Heba Farag, Rehab Azouz, Huda Omar, and Asmaa Azouz. The first draft of the manuscript was written by Marwa Khattab and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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