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ON THE COVER

The Vespa orientalis, a tough and aggressive predator, poses a significant threat to honey bees and public health (Photo by R. Dehghani-see page 13).

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Venomous and poisonous arthropods in Iran, West Asia, and the Middle East: an overview of their identification, bites, stings, behavior, biology and geographical distribution

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ABSTRACT

Arthropods belong to the invertebrate Phylum Arthropoda, which contains the most species on Earth. Venomous arthropods are among the most important animals that live in abundance in the human environment. The current study gives an overview of the importance of their identification, bites, stings, behavior, biology, and distribution in the geographical region of Iran, West Asia and the Middle East. The databases have been searched comprehensively and the most relevant Published articles and books from 1978 to 2023 were carefully selected based on the most appropriate keywords. Biting and stinging venomous creatures of arthropods, class, order, and family were presented in the tables and their importance and role of each order in the bite, sting and the occurrence of hazards were determined separately. Finally, the methods of preventing their bites and stings were recommended. The phylum of arthropods in Iran has two subphylums including Chelicerata with one Class, five Orders, and 25 families, and Mandibulata with three Classes, nine Orders, and 29 families. They are scattered all over Iran. Their venom apparatus includes venom gland, modified pedipalps, chelicers, stinger in the tail (telson), mouthparts such as hypostome, fangs or forcipules, appendages (mandibles), proboscis, ovipositor (stinger), and hair (urticating bristles). The importance of venomous animal stings and bites in the training programs of physicians, the medical and nursing staff is very weak or does not exist at all. To achieve and enhance the management efficacy of bites and envenomation, more accurate information about venomous creatures and their venom composition is required.

Keywords

Venomous arthropods, poisonous, Insect, Bites and stings, Iran, Middle East

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Abbreviations

No abbreviations

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Introduction

Venomous animals have undergone evolutionary adaptations, leading to the development of venoms with a distinctive chemical composition that can interfere with the physiological systems of vertebrates. Consequently, this interference can result in biological malfunction or even death. Throughout evolution, numerous animal groups have independently evolved venom-producing organs and specialized injection apparatuses. Over the course of this extensive process, venoms have acquired unique properties and high potency, enabling them to effectively target their molecular counterparts [1]. Approximately 15% of the estimated 1.7 million animal species are venomous, with a significant portion of these creatures belonging to the phylum Arthropoda [2].

Arthropods employ their venom for various purposes, including immobilizing and capturing prey, deterring predators, and facilitating food digestion. Additionally, some arthropods utilize their venom for intraspecific competition. Despite humans harboring a deep-seated fear of venomous animals, there has always been a fascination with them [3]. This fear can be attributed to historical myths, narratives, and the negative portrayal of these creatures in the media. It is important, however, to acknowledge that certain venomous animals, such as snakes, are responsible for tens of thousands of reported deaths and injuries worldwide. The World Health Organization (WHO) has even reclassified snake envenomation as a neglected tropical disease. Paradoxically, over the past few decades, there has been an increase in global popularity and interest in keeping poisonous snakes, spiders, scorpions, and centipedes as pets in captivity.

Recent scientific research has been focused on exploring the potential benefits of animal venoms and toxins in various applications. These include their use as molecular research tools to study physiological processes, as templates for developing novel drugs with diagnostic and therapeutic purposes, and even as agents for pesticides and anti-parasitic treatments [4-8]. Researchers have been actively involved in continuous studies aimed at producing effective and safe drugs, and they have made promising discoveries regarding the beneficial effects of animal toxins. Despite their toxicity, venoms contain components that exhibit a wide range of therapeutic potential. These scientific advancements have altered the perception of venoms from being solely deadly substances to having therapeutic value. Currently, numerous studies are underway to identify their molecular targets, such as ion channels and receptors, as well as to explore their pharmacological properties for the development of new drugs [9-11]. More than one million species of arthropods have been identified. These invertebrates, belonging to one of the most successful and prevalent phyla in the animal kingdom, inhabit various environments including land, air, and water [12-17]. Venom has evolved in different groups of arthropods, enabling them to adapt to their respective habitats. Over millions of years of evolution, arthropods have developed sophisticated mechanisms for delivering their venoms to prey, effectively fulfilling their defensive and predatory goals. The morphological diversity of arthropods' venom apparatuses is astonishing, encompassing various structures such as modified pedipalps, tails (telson), mouthparts like hypostomes, chelicerae, proboscises, ovipositors (stingers), and specialized hairs (urticating bristles) [18-22].

Nowadays, with the advancement of technological capabilities, new avenues have emerged for understanding the properties of venom. Venom is a significant natural biological resource, harboring potent compounds that hold great promise in targeted therapies. Each venom contains multiple components with potential therapeutic value, offering a diverse range of applications in the development of life-saving drugs, research tools, and also, environmentally friendly insecticides. The majority of published articles focus on medically important arthropods, exploring their venoms, compositions, biological activities, and the medical implications of envenomation or stings on humans. However, there has been relatively less emphasis on other vertebrate groups. In light of this, this review aims to encompass all venomous and poisonous arthropods, including biting and stinging families, while placing particular attention on their distribution in West Asia and the Middle East, with Iran as a focal point (Figure 1).

Figure 1.
Map of Middle East and West Asia (Prepared by Dehghani R)

Arthropods, like other wildlife, transcend political borders and are found in habitats worldwide based on their natural requirements. They exhibit remarkable adaptations and thrive in diverse environments. Consequently, studying these creatures in Iran extends beyond its geographical boundaries, encompassing neighboring countries that share common features despite having distinct climates.

Data collection: Sources and Methods

The study was conducted using the review method. Based on highly relevant keywords such as arthropod, venomous, poisonous, biting, sting, class, order, family, terrestrial, and aquatic, extensive searches were performed in electronic databases including PubMed, EMBASE, Google Scholar, Scopus, Web of Science, and CINAHL covering the period from 1978 to 2023. The most appropriate articles and books that satisfied the criteria regarding arthropod species in the Middle East and Iran were selected. A total of 324 carefully chosen articles and books were derived from approximately 500 sources. In the tables 1 and 2, biting and venomous animals belonging to the phylum Arthropoda, as well as their respective subphyla, classes, orders, and families, are presented along with information about their venom apparatus or sting structure. Additionally, their significance in terms of biting and stinging incidents and their contribution to injuries were separately determined. Finally, recommendations for methods to prevent and treatment their envenoming were provided.

Findings

The importance of biting, stinging, venomous, and poisonous arthropods

Among the animals, there is no group that has as many chemical defense mechanisms as arthropods. The study of arthropods' defense mechanisms, particularly against predators, has been extensively researched. Arthropods are classified into two categories based on their defense mechanisms: venomous, which actively inject their toxins using specialized structures, (like stingers), and poisonous, which passively release their toxins when handled, pressed, crushed or consumed [23-25].

There are two major types of defensive substances in arthropods; those produced by specific exocrine glands and substances that have essentially no glandular origin. These substances can be found in the blood, stomach, or other parts of the body, either internally or on the body's surface. Glandular secretions can be classified into two groups: the first group consists of injectable substances delivered through a stinger in scorpions, bees, and chelicerae in spiders. The second group contains non-injectable substances, such as rove beetles, blister beetles, and millipedes, which lack injecting organs [26-28]. Arthropods are distributed worldwide, but their species distribution and diversity are particularly high in tropical and subtropical regions [29].

Iran with an area of 1648,000 square kilometers is located in West Asia and the Middle East and has a tropical and subtropical climate that supports high diversity of arthropods. Venomous and poisonous arthropods of Iran, are in two sub-phylums; Chelicerata and Mandibulata. It is worth mentioning that this distribution is not exclusive to geography of Iran; other countries in the region also host similar arthropod species, although the proportions may vary (Figure 1, Table 1).

Subphylum Chelicerata

Chelicerata is the second largest group of arthropods with one class, Arachnida (Table 1). The subphylum Chelicerata has a pair of highly specialized organs, called chelicers instead of mandibles which is modified and seen as scissors or pincer-like. Many species in this group are venomous and are medically important in health and medicine.

Arachnida class

The class Arachnida includes the most important venomous members, such as scorpions, spiders, and ticks (Figure 2). Certain orders within this class, such as Solpugidae are predators, with the segmented abdomen and highly bigger chelicers than spiders. Unlike spiders, they do not possess venom, but because of their aggressive behavior, they cause fear and panic. Sulpogida bite in self-defense, which can cause tissue wounds, contamination of these wounds with soil microbial agents may cause severe infection.

Scorpiones order

Up to now, 2231 scorpion's species in 208 genera and 20 families have been documented worldwide [30,31]. Approximately 50 species are medically important, most of which are found in the Buthidae family. The most dangerous scorpion's species have been reported across regions including Africa, Middle East, South America, Central, and North America, and Asia [32,33]. Among arthropods, Scorpion stings and injuries are most frequently observed in Iran. The occurrence of scorpion sting accidents and the extent of damage in different parts of Iran depend on several factors such as the local way of life, socio-economic status, housing conditions, availability of healthcare facilities, and the specific scorpion species present in each geographical region. In Iran, 68 species, 19 genera belonging to 4 families have been

Table 1.
Venomous and poisonous arthropods present in Iran (prepared by Dehghani R)

Subphylum	Class	Order	family	common name	Venom delivery apparatus
Chelicerata	Arachnida	Scorpiones	Scorpionidae	scorpion	Stinger
			Hemiscorpidae		
			Buthidae		
			Diplocenteridae		
		Aranida	Theridiidae	Chelicerae	Chelicerae
			Sicariidae	Brown widow	
		Acari	Ixodidae	Hard tick	Hypostome
			Argasidae	Soft tick	
		Pseudoscorpiones	12 families	False scorpion	Pedipalps
		Solifugae	Galeodidae	Solifuge	Not venomous, but bite with Chelicers
			Karschiidae		
			Daesiidae		
			Glyppidae		
			Rhagodidae		
Mandibulata	Diplopodia	Spirostreptida	Cambalidae	Millipede	Dermal glands
	Chilopoda	Sclopendromorpha	Sclopendridae	Centipede	Forcipules
		Lithobiomorpha	Lithobiidae		

reported. The stings of Androctonus crassicauda (Olivier, 1807) and Hemiscorpius leptorus (Peters, 1861) species are particularly dangerous, with documented cases of mortality associated with their stings, especially in the southern region of Iran [34-37]. The venom of Androctonus species is neurotoxic, while that of Hemiscorpius species is cytotoxic. The sting of scorpions with neurotoxic venom causes severe pain and neuromuscular blocking activities by inhibition of nerve-mediated twitches while the sting of scorpions with cytotoxic venom led to local necrosis, including myotoxicity, kidney degenerative glomeruli and necrotic tubular, heart myocytolysis and intestinal edema of lamina propria, and villous necrosis [38,39,40,41,42]. Scorpions are distributed in a wide range of habitats, both inside and outside houses as well as in the fringes of villages or cities. They are particularly abundant in the eastern and western regions of Iran and their stings can be quite painful.

Currently, the primary method of treating scorpion stings in Iran involves the use of antivenom serum that is produced domestically [43-45]. To minimize the occurrence of scorpion stings in any region of Iran, it is essential to improve residential housing conditions and prevent the entry of these creatures into suburban areas. Also, capturing or repelling these animals can help to avoid or reduce the risk of being bitten by them [46,47].

Araneida order

Spiders are predatory arthropods that play a crucial role in controlling pests' populations and maintaining ecosystem balance. Nearly 40,000 species of spiders have been described worldwide, from which approximately 200 species posing a threat to humans. Spiders have a global distribution and some of them are able to survive even in urban environments, occasionally coming into contact with humans under certain conditions. However, spiders tend to living in the desert [48,49]. Most spiders are not considered harmful to humans due to their harmless venom or the small amount of injectable venom, their small chelicerae size, their lifestyle, and their non-invasive behavior [50]. Spider venoms can be classified into two main groups; neurotoxin and necrotoxin, based on their mechanism of action. Black widow spiders possess highly potent neurotoxic venom that affects the nervous system of insects and mammals. The second

class	Order	Family	Figure	References
Arachnida	Scorpionda	4 families		Prepared by Dehghani R
	Aranida	Theridiidae		Prepared by Dehghani R
		Sicariidae		Zamani et al., (2014) with permission
	Acarina	Ixodidae		Prepared by Dehghani R
		Argasidae		Prepared by Dehghani R

Arachnida	Pseduscorpionda	12 families		Cokendolpher et al., (2019) with permission
	Solifugae	5 families		Prepared by Dehghani R

Figure 2.
Stinging, biting, and venomous agents belong to orders of the Arachnida class. (Prepared by Dehghani R)

group includes brown widow spiders, whose venom is necrotic and causes skin lesions. This venom causes tissue injuries and cell apoptosis, and various symptoms such as nausea, vomiting, chills, fever, muscle pain, general purpura rash, hemolytic anemia, acute renal failure, shock, coma, and even death [51-53

In Iran, the species of Latrodectus from the Theridiidae family, commonly known as black widow spiders, have been frequently reported. Many of these spiders are medically important as they are capable of envenoming in humans. Latrodectus species can be found in most parts of the world, except for cold regions in Europe and Asia. Almost all species within this genus are highly important in medical and veterinary sciences. So far, about 30 species in the genus Latrodectus have been identified in the world from this, five species have been reported in Iran [54,55].

Black widow spider bites cause a range of clinical symptoms which is known as Latrodectism. The major component of its venom is α-Latrotoxin (α-LTX) [56]. The toxin is characterized by its ability to rapidly release acetylcholine from nerve terminals and endocrine cells in vertebrates. As a result, toxin causes systemic clinical complications in the victims and in severe cases disturbs the cardiovascular system, respiratory system, nervous system (including the peripheral and autonomic branches), skeletal and smooth muscles, gastrointestinal tract, urinary system as well as causing localized skin reactions or marks [57-61].

Brown widow spiders, specifically those belonging to the Loxosceles genus, are commonly referred to as recluse, violin, or fiddle-back spiders and belong to the Sicariidae family. All these species possess venom with necrotic properties, making their bites dangerous. Among the 117 species worldwide, only Loxosceles rufescens (Dufour 1820) has been identified in Iran. This species was initially reported in Tehran province and subsequently in Hormozgan and Fars provinces [62,63,64]. Brown spiders, known for their shy nature, typically inhabit quiet, dark, and isolated places. They do not exhibit aggressive behavior but may bite if provoked, trapped against the skin, or accidentally touched. However, multiple bites from these spiders are uncommon. Anti-venoms are available for Loxosceles spp. envenomation. The severity of reactions to their bites can vary depending on factors such as the amount of venom injected, the bite location, the age of the victim, and their overall health conditions.

Treatment for Loxosceles spp spider bites typically involves the administration of steroids, antibiotics, hyperbaric oxygen therapy, wound debridement, and scar repair. However, the effectiveness of treatment can vary from person to person [56, 61, 65]. Given the widespread distribution of medically significant spiders in Iran, accurate diagnosis is crucial for appropriate treatment and preventive measures. Incidences of bites by venomous animals like widow spiders tend to be more common in areas surrounded by natural

open spaces, such as the suburbs of cities and villages.

In the summer of 2017, in the city of Kashan in central Iran, a 48-year-old female cleaner was bitten by a spider while collecting garbage. The spider was later identified as a member of the Loxosceles sp (Araneae-Sicariidae). The initial symptoms she experienced included immediate irritation, itching, swelling, redness on her arm and numbness in three of her fingers. She also suffered from shortness of breath. After four days, her hand became edematous and painful, and she also experienced insomnia. Her condition worsened to the point where she lost the ability to move her fingers. Due to the severity of her condition, she was hospitalized for four days and received various treatments including normal saline, corticosteroids (dexamethasone), antibiotics, antihistamines, and analgesics. Additionally, she was administered a tetanus vaccine and tetabulin [60].

Acarina Order

Ticks are classified into two families: Ixodidae and Argasidae. The Ixodidae family consists of hard ticks, whereas the Argasidae family includes soft ticks. In Iran, there have been reports of 26 species of ticks belonging to both the Argasidae and Ixodidae families, which are distributed throughout the country. Ticks are considered dangerous obligate hematophagous (blood-feeding) arthropods and are the most important vectors of pathogens. While blood-feeding on a host's, they firmly attach themselves by producing cement that secures their hypostome in the host's skin [66-71]. Their salivary secretions are highly toxic, particularly in hard ticks. Depending on the species, tick saliva contains a complex mixture of various pharmacologically active compounds that play a role in regulating the secretion of salivary proteins and countering host defense mechanisms. The composition of tick saliva changes as the feeding process progresses and the tick encounters the dynamic host response. Furthermore, the precise composition may vary among different tick species. The common constituents found in tick saliva include anticoagulants, anesthetics, immunosuppressants, vasodilators, thrombin inhibitors, proteases and protease inhibitors, anti-inflammatory compounds, inhibitors of platelet aggregation, metalloproteases, and phospholipase A2. The specific antihemostatic agents differ among tick species and genera; however, they have not been thoroughly investigated or explored [70-72].

Moreover, ticks are capable of producing a variety of other molecules with diverse biological activities. These include components found in the cement cone, cardiotoxic factors, neurotoxins, various enzymes, and enzyme inhibitors. As a result, tick saliva can be harmful as it serves as a vehicle for transmitting a wide range of tick-borne pathogens into the host's bloodstream. These pathogens encompass viruses, bacteria, rickettsia, and protozoa, which have the potential to cause diseases such as Lyme disease, babesiosis, tick-borne encephalitis, Crimean-Congo Hemorrhagic Fever (CCHF), Tularemia, and Q fever in both humans and animals [71-75]. Due to their ability to infest multiple hosts, ticks can transmit a wide variety of diseases, posing significant challenges in the fields of medicine and veterinary medicine. Ticks secrete their salivary paralyzing neurotoxin into the hosts through their hypostome, numb the bite site, and can cause an acute, progressive, symmetrical, muscle paralysis, which can potentially be fatal. Early detection and prompt removal of ticks are crucial for facilitating faster recovery from tick paralysis. Although ticks can attach and enter their hypostome anywhere on the body, they tend to attach to the scalp due to its warmth, hair density, and suitability for hiding [73-75].

The presence of anticoagulants and other components in tick salivary secretions can cause redness, local skin hematoma, swelling and rashes, which are the most common signs of tick blood-feeding on humans. The symptoms may resemble the bites of other venomous animals, and can potentially result in secondary infections caused by opportunistic microorganisms. Therefore, it is important to learn about tick bites and prevention methods. It is necessary to adhere to health standard guidelines in animal shelters and residential areas for both humans and animals [69,76,77].

In 2006, a 48-year-old woman with head and neck edema, fever and imbalance visited a clinic in Tehran. She had traveled to the mountainous regions in early spring. During the physical examination, a small tick was found firmly attached to the head, which sent to the Razi Vaccine and Serum Research Institute after being removed. This tick was identified as the female Dermacentor marginatus (Sulzer 1776) (Acari: Ixodidae). After removing the tick, the patient recovered [78].

In 2019, a 71-year-old woman from northern Isfahan Province, referred to local health center because of a burning sensation, pain and a red bumps and hematoma on her neck without fever. The clinical examination detected an arthropod attached to the neck, which was identified as a female Hyalomma spp (Acari: Ixodidae) tick. Tick was completely engorged and measured 20 mm in length. The patient was discharged after prescription of cephalexin 500 mg every 6 hours and Ibuprofen 400 mg/orally every 8 hours. The patient underwent monitoring for the next 10 days for any symptoms of tick-born disease such as Crimean-Congo hemorrhagic fever [79].

Table 2.
Venomous arthropods present in Iran (Prepared by Dehghani R).

Subphylum	Class	Order	Family	common name	Venom delivery apparatus
		Hymenoptera	Vespidae	Wasp	Stinger (modified ovipositor)
			Formicidae	Fireant	
			Mutilidae	Velvet ant	
			Apidae	Bee	
		Hemiptera	Corixidae	Water boatman	Bite by proboscis or rostrum or piercing cylindrical beak mouthpart, needle-like
			Belostomatidae	Giant water bug	
			Notonectidae	Back swimmer	
			Nepidae	Water scorpion	
			Reduviidae	Assassin bug	
			Anthocoridae	Minute pirate bug	
			Nabidae	Damsel bug	
			Coreidae	Coreid bug	
			Pentatomidae	Stink bug	Thoracic glands
	Hexapoda	Coleoptera	Staphylinidae	Rove beetle	Haemolymph The release of Coelomic fluid or Haemolymph
			Meloidae	Blister beetle	
			Dermestidae	Skin beetle	Urticating bristles on larvae
			Oedemeridae	False blister beetle	Corporal fluid
		Lepidoptera	Saturniidae	Giant silkworm moth	Urticating bristles on larvae
			Thaumetopoeidae	Oak processinary moth	
			Lymantriidae	Tussock moth	
			Lasiocampidae	Snout moth	
		Diptera	Asilidae	Robber fly	Mandibles and Hypopharynx
			Sciomyzidae	Marsh fly	
			Tabanidae	Hourse fly	
			Cecidomyiidae	Gall midge	
		Neuroptera	Myrmeleontidae	Antlion	Maxillae/mandibles

Also, in 1999, tick paralysis was reported among 400 nomadic sheep flocks in Iran. Thirty of them were affected over three days of which 15 ewes died. They showed ascending posterior paralysis, sternal recumbency, an inability to raise the head from the ground, no response to pricking the skin with a needle, and finally, lateral recumbency, deep coma, and fully dilated pupils before death. Less than 15 female ticks of Ornithodoros lahorensis (Neumann 1908) (Acari: Argasidae) were found in the carcass of dead and sick animals. The affected ewes were treated subcutaneously with 0.2 mg/kg ivermectin. All affected ewes had fully recovered after 12 to 24 hours [80].

Pseudoscorpiones order

Until now, toxic components have been identified in three of the four venomous arachnid orders, which include scorpions, spiders, and ticks. However, no information is currently available on the venom and venom glands of the fourth group, pseudoscorpiones [81]. Pseudoscorpiones are very small arthropods, ranging from 0.5 to 5 mm in size. They are commonly

known as false scorpions or book scorpions, lacking a tail or stinger on their posterior end. Pseudoscorpiones have a widespread distribution all over the world, inhabiting various terrestrial habitats such as soil, leaf litter, caves, and coastal areas. They possess a unique venom delivery system in the chelal fingers of their pedipalps, which has evolved independently from that of scorpions and spiders. Additionally, some pseudoscorpiones possess another form of venom delivery called "lamina defensor," where the venom glands arise at the base of the venom tooth. They possess a pair of fingers called pedipalps, as well as one or two venomous glands that they use to immobilize their prey. However, in some pseudoscorpiones families, these venom glands are absent [82].

In 2006, Santos and colleagues [83] conducted research on the effect of Paratemnoides elongates (Banks 1895) venom, extracted from chelal hands on the rat cerebral cortex. The results indicated the possible antagonistic action of specific compounds in its venom to inhibit the binding of the excitatory neurotransmitter L-glutamate (L-Glu) to its specific sites and also decreases the GABA uptake. Additionally, in 2019, Krämer et al., successfully extracted pure venom compounds from pseudoscorpiones Chelifer cancroides (Linnaeus 1758) [84]. Studies have shown pseudoscorpion venoms contain peptides with antimicrobial properties [84]. Species of the pseudoscorpions order in Iran have received less attention due to their small size, hidden habits and habitats, and lack of significant economic importance. In 1918, the European scientist Redikorzev was the first who described and recorded them from Iran. He also described the new species, Chelifer Spinipalpis (Redikorzev 1918) [85]. Up to now, 68 species of this order in 12 families have been identified in various regions of Iran. However, there is no documented reports regarding their medical importance or their bite in Iran [86-89].

Solifugae order

Solifugae include 1000 known species) [90]. They are among the arthropods that play a crucial role in maintaining the ecological balance. Their anatomy consists of two primary divisions: the abdomen and the cephalothorax. Solifugae possess chelicerae that vary in strength across different species, facilitating the tearing of their prey) [91]. They can grow up to 12 to 15 cm in size, and have noticeably large pedipalps, which often are larger than their front legs. Although Solifugae tend to be more active at night, some species are active during the day) [92.93]. Their pedipalps have a variety of hairs and papillae that are various in different families, they play a key role as mechanical and chemical receptors [94,95]. Unlike spiders, they are not venomous. Only one case has been reported of a venomous Solifugae species from India, which caused the death of mice with its venom. However, it should be noted that no other reports have confirmed this claim.

In general, Solifugae species are not dangerous, and only in rare cases, such as when threatened by humans, they may become aggressive, make noise, and bite in self-defense. Due to their strong chelicerae, they can cause deep puncture wounds that may lead to contaminated wounds. Solifugae species primarily inhabit hot, dry, semi-arid, or desert areas. They tend to be more active in regions with spring and summer temperate climates [96-103].

A number of studies have confirmed the existence of several species of these animals in Iran [104-107]. However, they have been less studied in neighboring countries [99,108-111]. Gaining sufficient knowledge about the biology and behavior of these animals and seeking advice from experts can greatly reduce feeling of insecurity and unfounded fear (Figure 2).

Subphylum Mandibulata

Mandibulata is the largest and most divers subphylum of arthropods, comprises of three classes including Diplopoda (Millipedes or Millipede)., Chilopeda (Centipedes or Centipoda), (Table 1) and Hexapoda (insects), (Table 2) with a total of 14 orders, and 54 families. Mandibles are a unifying feature of the Mandibulata. Many species, within this subphylum are venomous and hold significant medical importance in the field of health and medicine (Table 1).

Diplopoda class

Diplo mean twofold and pods mean foot, a class of arthropods, commonly called Millipedes. Millipedes live in damp, dark places such as under rocks, or in rotten woods. They avoid light. They feed on decaying plants and other vegetation on the ground, and sometimes they also consume animal-based materials like earthworms, snails, and insects [112]. (The class Diplopoda consists of 16 orders and 140 families that are distributed worldwide. They are generally considered harmless. However, certain tropical species may pose a threat to humans when defending, [113-115]. Their medical importance arises from their ability to release an irritating defensive liquid through pores located along their sides. The defense mechanisms of millipedes can potentially harm humans, in some cases and resulting in erythematous, purpuric, and cyanotic lesions accompanied by local pain and paresthesia. Their toxin contains compounds such as benzoquinones, aldehydes, hydrocyanic acid, and various other substances [116].

Spirostreptida order

The Spirostreptida order has been reported in Iran. These diplopods do not have stings, but they possess secretory glands located in each of their body segments. From these glands, a toxic liquid may leak out, leading to the development of erythema and brown or black spots on the affected skin. It may take several months for these markings to disappear. The majority of injuries occur when individuals wear shoes without inspecting the insides, as millipedes often seek dark and quiet locations to take refuge and rest [117]. In response to enemies or threat stimuli, some species curling into a spiral shape, and their defensive apparatus releases irritant liquid toxic that damage human skin. This toxic contains hydrogen cyanide, benzoquinones, esters, phenols, and aldehydes which cause irritation, pain, and blisters at the site of skin contact and also pain and irritation in the eyes [118,119]. Millipedes cause acute inflammatory lesions without major effects. Immediate use of alcohol or ether on the site of contact is recommended, because they can dissolve toxins. As first aid, the eyes should be irrigated with water and the patient should be referred to an ophthalmologist. In severe cases injuries can lead to blindness [120,121].

Chilopoda class

Chilo means jaw and pods means foot. Centipedes or Chilopoda are dorsoventrally flattened with numerous segments, each having one pair of legs. They possess a pair of venomous front legs on the cranial segment that is modified into special apparatus equipped with toxin hooks, used for injecting venom into prey. They have a pair of long, jointed antennae consisting of 12 or more small segments on their heads. These animals have simple eyes. Centipedes mostly live in tropical countries, active and hunting at night and feeding on a variety of insects and other arthropods. The large species may also hunt mice. Some centipedes live in buildings and move quickly. About 1700 species of centipedes have been identified. Scolopendra gigantea (Linnaeus 1758) is up to 100 mm long and has 21 pairs of legs. The centipedes found in Iran and neighboring countries consist of 48 species, of which 17 species have been reported exclusively from Iran so far [122,123].

Sclopendromorpha and Lithobiomorpha orders

The species of these orders have been reported in Iran. They are the only species in which the bite by some of them in humans can lead to death. Scutigera (Cermatia) sp (Wood 1867) is one such species. It can grow up to 25 mm in length, and has 15 pairs of legs, long and narrow antennae and compound eyes. These creatures typically reside in buildings, move quickly, and feed on insects. The bite of Scolopendra valid (Lucas, 1840) species has been reported from Ahvaz, a city located in the southwest of Iran and the capital of Khuzestan province. It bites cause various clinical and laboratory manifestations including pain, skin inflammation, itching, hematuria, and hemoglobinuria. Some researchers have suggested that these species may contain substances such as 5-hydroxytryptamine, hemolytic phospholipase A2, and a cardiotoxic protein [123,124,125]. The venom proteins of these species are highly diverse, and the majority of them are dissimilar to proteins and peptides found in venoms of other animals. This underscores the distinctiveness of centipede venoms (Figure 3) [126,127,128].

Hexapoda (Insecta) class

The class Insecta is adapted to various environmental conditions, primarily inhabiting land, although some species can be found in both aquatic ecosystems and terrestrial habitats. Their head is composed of six fused segments, and their thorax consists of three fused segments with three pairs of jointed legs and two pairs of wings. Their abdomen consists of 11 or fewer segments and lacks appendages for movement. Insects possess compound and simple eyes. With more than 800,000 species, there are six orders of insects classified as venomous and biting species (Table 2).

Hymenoptera order

The order of Hymenoptera consists of four families: Apidae (bees), Vespidae (wasps and hornets), Mutillidae (velvet ants), and Formicidae (all ants, specifically, fire ants). These families are the most clinically important in this order and their sting have been reported in Iran. They produce venom, which they use it for defending their territory against predators, obtaining food, and ensuring survival. In general, members of these families are capable of stinging multiple times, with the exception of bees [129,130,131]. While most stings only cause minor issues, it's important to note that the majority of deaths resulting from Hymenoptera stings are due to immediate allergic reactions and anaphylaxis. The venom of Hymenoptera is a complex mixture of biologically active molecules, such as enzymes, amines, glycoproteins, and peptides. A variety of these compounds are allergens that can induce allergic reactions in victim's body [131,132].

In non-allergic individuals, massive envenomation and other complications can potentially result in death. For most mammals, the estimated lethal dose is approximately 20 stings per kilogram of body weight. However, it's important to note that the dose or number of Hymenoptera stings does not influence or prevent anaphylactic reactions since these reactions are

class	Order	Family	Figure	References
Diplopda	Spirostreptida	Cambalidae		Prepared by Dehghani R
Chilopoda	Sclopendromorpha	Sclopendridae		Prepared by Dehghani R

Figure3.
Biting and venomous agents belong to Diplopoda and Chilopoda classes (Prepared by Dehghani R)

not dose-dependent. After insect stings, four possible reactions have been observed including the local reactions, regional reactions, systemic anaphylactic reactions, and less frequently seen delayed-type hypersensitivity. The onset of life-threatening anaphylactic symptoms usually appears within 10 minutes after the sting. Prompt diagnosis and initiation of treatment are crucial for successfully managing anaphylactic reactions caused by Hymenoptera stings [133-135].

Insect stings, particularly from bees, can result in various complications such as pancreatitis, hemolysis, rhabdomyolysis, nephritis, and acute renal failure The death of the victim may be attributed to kidney failure or cardiac problems [136-143. In most cases, pain and discomfort resulting from hymenopteran stings resolve within a few hours even without [144-147

In the summer of 2020, a 3-year-old boy was attacked by a group of hornets (Hymenoptera -Vespidae). The insects stung him on his head, arms, back, and buttocks. Upon arrival at the hospital, he was conscious but lethargic, and approximately 30 painful sting sites in the form of hyperpigmented papular lesions (papular urticaria) were observed on his body. Although his head and neck did not show signs of redness (erythema), his limbs, neck, face, lips, and eyelids became increasingly swollen. Additionally, his urine output decreased (oliguria), and the urine test revealed proteinuria and hematuria. The patient initially experienced stress-induced hyperglycemia, and he was unable to open his eyes. His clinical condition progressed to multiple complications, including gross hematuria, intravascular hemolysis, and myoglobinuria, anemia, thrombocytopenia, rhabdomyolysis, acute renal failure, hepatocellular necrosis, epistaxis, and respiratory distress. Metabolic acidosis and respiratory alkalosis were confirmed through a blood gas test. An abdominal ultrasound examination detected a notable bloody fluid in both sides of the subphrenic region, Morrison and splenorenal spaces, as well as the pelvis. Despite supportive treatments, his condition worsened, he was admitted to the ICU due to critical illness. He received various interventions, including administration of normal saline, a diuretic agent (furosemide), insulin, corticosteroids (dexamethasone), dopamine, and a calcium supplement. In addition, sodium bicarbonate was administered to address urine alkalinization, and blood transfusion and plasmapheresis were performed twice. Intermittent hemodialysis was also performed five times to manage acute tubular necrosis (ATN) caused by hemolysis and rhabdomyolysis. The lesions were treated by applying topical corticosteroid, zinc oxide, and Aloe Vera gel. After two weeks of hospitalization, the papules at the sting sites became necrotic. The liver enzymes reached normal levels, urine output increased, and the functions of other organs returned to normal. After 19 days of hospitalization, the patient was discharged in relatively good general condition [148].

Apidae family

Bees normally live socially and become aggressive, attacking, and stinging in groups if threatened or if their nest is disturbed. Humans may be stung repeatedly by a large number of bees simultaneously,

which worsens the problem [149-153]. Honey bees can only sting once because they have barbs (hooks) on their stingers that cannot be pulled out from skin. Consequently, they lose their stinger after stinging and die due to the detachment of the apparatus from their abdomen.

Clinical symptoms of bee sting include erythema, swelling, and pain at the site of the sting. Apamin, is the main neurotoxin peptide found in honey bee venom. It has the ability to directly affect the central nervous system (CNS) by crossing the blood-brain barrier (BBB), increasing neuronal excitability, and potentially triggering seizures. Additionally, when experimentally injected into rats, it can affect K+ channels in cell membranes, leading to convulsions [23,154]. The second major component of bee venom is melittin, which is responsible for the hemolysis of red blood cells and the sensation of pain.

Vespidae family

Wasps defend their colony when disturbed or threated. They use their stings to temporarily paralyze prey for egg laying or kill it for food. Unlike honey bees, wasps are able to sting multiple times. The venom of wasps and bees is complex mixture of hyaluronidase, phosphatase acid and lysophospholipase, histamine, dopamine, norepinephrine, serotonin, and mast-cell degranulation protein. Two species of the Vespa genus from the Vespidae family have been reported in Iran, namely Vespa carbro (Linnaeus 1758) and Vespa Orientalis (Linnaeus, 1761) which are widely distributed [130,132,152,155].

Mutillidae family

Members or velvet ants are not invasive, they are usually stinging individually for defense. These arthropods are known as “cow killer ants” by the reputation of female sting that is so potent and painful, however, the venom is not highly toxic, and deaths from their stings have not been reported yet. Velvet ant is a parasitoid whose larvae live as parasites on immature stages of other insects, such as bees and eventually kill them. The sting is similar to bees and people who have severe allergies to the sting may show overreact to it, so contact and stings should be avoided [156-158]. Mutillidae contains about 230 genera and about 8000 species. They are distributed all over the world [159,160].

In 2014-2015, velvet ant stings caused by the Dentilla sp (Hymenoptera: Mutillidae) were reported among 49 individuals in Kashan, a city in central Iran. The majority of cases (72%) occurred in women carpet weaving workshops and inside houses, particularly in sitting rooms and bedrooms. The victims complained of severe and sharp pain, as well as redness and itching at the sting sites. In later stages, they present hemolysis and bruising manifested as brown spots. To reduce severe pain and itching in the treatment center, corticosteroids (dexamethasone), analgesics and antihistamines were prescribed Based on these findings, it was concluded that this arthropod is a domestic pest [161,162].

Formicidae family

Among these, fire ants have been identified as the primary cause of ant stings in the Qeshm Island, the largest Iranian island located in the Persian Gulf [163-168] The venom of bees and wasps is mostly composed of protein while the venom of fire ants is made up of 95% water-insoluble alkaloids [27,133,134,135]. However, it has been observed that anaphylactic shock resulting from fire ant stings exhibits similarities to anaphylactic reactions caused by bee stings.

Piperidine alkaloids are the most toxic agents in fire ant venom, causing local necrotic and hemolytic effects and being responsible for pain [27]. While some sources report a species count of 4200 along with 208 genera, it is estimated that the actual number of species may reach 6000 [169]. In Iran, 67 species from 21 different genera have been documented, with specimens collected from various provinces including Ilam, West Azerbaijan, and East Azerbaijan [170,171]. Bees, wasps, and ants are considered health pests as they frequently cause stings in both urban and rural environments. Their presence can disrupt normal outdoor activities in yards, parks, and school grounds. When provoked, they often invade residential areas, and tend to swarm, posing a threat to children and the elderly. In preparation for a swarm attack, these insects release alarm pheromones that signal other members within the colony (Figure. 4) [172,173,151,132].

Hemiptera order

The order Hemiptera contains numerous families, such as Corixidae, Notonectidae, Blastomidae, Nepidae, Pentatomidae, Reduviidae, Anthocoridae, Coreidae, and Nabidae. However, most of them are not medically important. Hemiptera, commonly known as bugs, derives its name from the Greek words "hetero-" meaning different and "ptera" meaning wings. Bugs have two pairs of wings, with the thin hind wings located under the front wings. As a result, the forewings are called Hemielytra. They possess mouthparts that resemble perforating and hypodermic needles, which enable them to extract subsurface fluids from plants and animal [174,175].

While most bug families are terrestrial, many also inhabit aquatic environments. Many bugs are vegetarian and consume plant nectar, making them

class	Order	Family	Figure	References
Hexapoda	Hymenoptera	Vespidae		Prepared by Dehghani R
		Formicidae		Shiran et al., (2013) with permission
		Mutilidae		Prepared by Dehghani R
		Apidae		Prepared by Dehghani R

Figure 4.
Stinging and venomous agents belong to Hymenoptera order (Prepared by Dehghani R)

significant pests. However, certain species are predators and are considered beneficial insects [176,177]. Several species of bugs are known to attack humans and livestock, feeding on their blood. In addition, some species are vectors for pathogens and associated with human disease [178,179]. warm regions. These insects vary in size, ranging from a few millimeters to a few centimeters. Bugs are mostly abundant in warm regions. Terrestrial species belonging to the families such as Reduviidae, Anthocoridae, Nabidae, and Coreidae pose a potential biting risk to humans [180-185]. One characteristics of terrestrial bug families is the presence of glands in both nymphal and adult stages. These glands typically produce defense chemicals known as allomones, which can vary between species but are often similar within closely related groups.

The presence of allomones is common among different species. Many Hemiptera species that inhabit soil possess special glands with ducts opening on both sides of the thorax. In defensive situations, these glands emit secretions with distinct odors. One

example is the Pentatomidae family, which has specialized secretory glands in their thorax. Contact with the fluid emitted by these glands can cause damage to human skin [186-192].

Most species belonging to aquatic bug families are predators and have the potential to harm humans and other animals [193]. The Corixidae family is characterized by an ovoid shape and a flattened gray body, enabling them to swim deep in the water for extended periods due to the air stored under their wings. While they may resemble Notonectidae, they differ in that they are not backswimmers. Bites from Corixidae have been reported on humans, as they feed on mosquito larvae and small aquatic insects [194,195].

Bugs belonging to the Notonectidae family are commonly known as backswimmers because they swim on their backs. They typically rest obliquely on the water with their hind legs spread open. These bugs can bite humans, and their bites often result in swelling at the bite sites. The pain caused by their bite is comparable to that of a bee sting and usually lasts for 2 to 3 hours. Their dorsal surface is generally ovoid in shape and light in color. These bugs are predatory in nature, feeding on small insects and fish. They may also attack larger animals, extracting blood and bodily fluids. These insects lay their eggs on aquatic plants [196,197].

Aquatic bugs belonging to the Blastomidae family are the largest species among their kind. They have ovate and flattened bodies, reaching lengths of 5 to 8 cm, and commonly referred to as giant water bugs. They inhabit stagnant waters such as pools and lakes and are attracted to light sources, earning them the name "electric light bugs." These bugs have a brown coloration. With their specialized hunting legs, they prey on insects, snails, and even small fish. Typically, they fly to water-rich areas for hunting purposes. Their bites can cause intense and excruciating pain, posing a particular threat to children [198-200].

Bugs belonging to the Nepidae family are predators. Their front legs are modified into hunting limbs, and they possess a pair of long breathing tubes called cerci, which are as long as their bodies. These bugs have a slow-moving nature and primarily feed on aquatic animals. If handled without caution, they may bite, causing significant pain. While they possess fully grown wings, they rarely engage in flight. These insects lay their eggs inside the tissues of aquatic plants [201-206].

The aggressive and annoying behavior of these bugs, as well as their tendency to bite, is mostly triggered by catching and squeezing them. While these bugs are not typically invasive, children, in particular, are prone to bites due to their curiosity (Figure. 5). Predaceous bugs produce venom that causes rapid paralysis and liquefaction. These venoms contain highly insecticidal properties and can induce paralysis or even death if injected into vertebrates. Predatory venoms have been found to contain disulfide-rich peptides, bioactive phospholipids, small molecules such as N, N-dimethylaniline and 1,2,5-trithiepane, as well as toxic enzymes like phospholipase A2 [207].

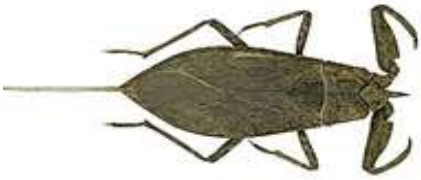
Coleoptera order

The Coleoptera order, which includes beetles and weevils, is the largest and most diverse insect order in the world. It continues to grow as new species are discovered (Figure 6). These insects exhibit a wide variety of body shapes, sizes, and colors. They are typically characterized by having two pairs of wings: a pair of modified hardened front wings called elytra, which serve as protective covers, and a second pair of membranous hind wings used for flying. Additionally, Coleoptera possess a hard outer exoskeleton, segmented antennae, and large compound eyes. Adult beetles and their larvae, known as grubs, have mouthparts adapted for chewing various materials such as other insects, fruits, nectar, leaves, fungi, dead animals, plants, and even hardwood.

Beetles play diverse and economically important roles in ecosystems due to their wide-ranging diet. In fact, beetles are known to feed on nearly every available food source in nature [208,209]. Some beetles, such as crop pests, cause damage to stored products by feeding on foodstuffs like grains and cereals. Many can also cause damage to wooden furniture, carpets, and stored food items in households. Additionally, some beetles can be problematic in gardens or agriculture as they attack plat flowers, fruits, leaves, and roots. Beetles inhabit in almost any ecosystem, ranging from the poles, deserts, lakes, ponds to underground habitats and mountain tops. Many species live in the nests of other animals and form symbiotic relationships with them.

The Coleoptera order is divided into four sub-orders: Adephaga, Archostemata, Myxophaga, and Polyphaga, comprising approximately 400,000 species. Only species belonging to the sub-order Polyphaga are poisonous. This sub-order consists of 17 superfamilies, with the most important ones being Staphylinoidea, Tenebrionoidea, and Bostrichoidea. Within these superfamilies, there are families that are both of importance to health and economy, as they can either cause harm or provide benefit to humans. These families include Staphylinidae, Meloidae, Oedemeridae, and Dermestidae.

The Staphylinidae family includes rove beetles, which are known for causing skin damage [210-212]. Also, small beetles in this family are attracted to light sources during nighttime and if they come into con-

class	Order	Family	Figure	References
Hexapoda	Aquatic bug	Belostomatidae		Jehamalar & Chandra, (2013) with permission
		Corixidae		Havemann et al., (2018) with permission
		Notonectidae		Prepared by Dehghani R
		Nepidae		Havemann et al., (2018) with permission
	Terrestrial bug	Reduviidae		Dioli et al., (2020) with permission
		Anthocoridae		Gil-Santana (2017) with permission

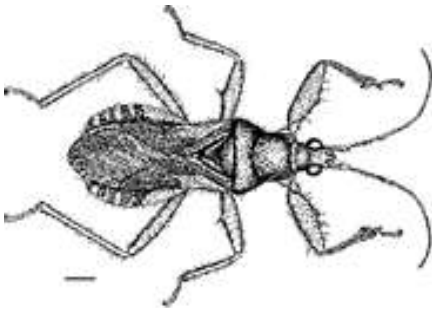
Terrestrial bug	Nabidae		Zhao et al., (2019) with permission
	Coreidae		Doughty., et al (2016) with permission
	Pentatomidae		Prepared by Dehghani R

Figure 5.
Biting and venomous agents belong to Heteroptera order (Prepared by Dehghani R)

tact with human skin, they secrete a defensive body fluid that can cause infectious blisters. It is important to note that these beetles do not bite and move slowly on the skin. However, during their movement, they release a potent poisonous compound called Paederin [213,214]. which leads to acute irritant dermatitis. This condition presents as large blisters accompanied by pain, burning sensations, and itching. There is no specific antidote or special treatment for these blisters. It is recommended not to touch or rupture them to prevent potential secondary infections. To alleviate the symptoms, immediate washing with water and soap is advised, along with the use of disinfectants and analgesic ointments. It should be noted that the healing time for these blisters is approximately 10 days or longer. While they can affect any part of the body, they are most commonly found on the face, hands, forearms, and legs [215].

In the northern region of Iran, there is an increasing prevalence of skin disorders attributed to the widespread presence of small beetles known as "Dracula" by the local population. The favorable environmental conditions, along with their high breeding and reproduction rates, have resulted in their wide distribution throughout the area. These skin problems are observed in individuals of all age groups and genders, affecting individuals from various social classes. However, it appears that the prevalence of skin disorders is particularly higher among young women [216-218].

In 2014, a 9 year old boy who had a trip to the North of Iran, was referred to the hospital due to signs of irritation on his neck skin, characterized by red plaques and blisters. He also complained of insomnia. Upon investigation, rove beetles (Coleoptera -Staphylinidae) were found in his resting place. A medical examination diagnosed him with Paederus dermatitis, which occurs as a result of contact with the hemolymph of certain rove beetles. Paederus dermatitis is a common in the northern region of Iran, primarily affecting the face, neck, and hands. Preventive measures for this condition include using mosquito nets, wearing long-sleeved clothing, and avoiding the use of fluorescent lamps. If beetles are observed on the skin, it is recommended to gently brush them away, as they do not bite but instead release a chemical toxin called Pederin, which can cause skin irritation and

blistering. The treatment for the boy included quickly washing the infected area, applying cold compresses and anti-itching calamine lotion, using topical steroids, and taking oral antibiotics, and antihistamines. His recovery duration was 27 days [219]. (Dehghani et al. 2014c).

The insects of the Meloidae family inhabit arid areas, temperate steppes, subtropical and tropical savannas, as well as vast open habitats. They are found in various parts of Iran. While these beetles do not bite, their body secretion, known as Cantharidin, is venomous and can cause blisters, irritation, and burns on the skin [220-223]. (Nikbakhtzadeh and Ebrahimi 2007; Serri et al. 2012; Moslemi and Pashai Rad 2015; Nezhad-Ghaderi et al. 2021). This family, also referred to as blisters beetles, is characterized by hardened shield-like forewings or elytra. It encompasses approximately 3000 species and 120 genera worldwide [224,225]. (Bologna and Pinto 2001; Fekrat and Awal 2015). They primarily feed on plant pollen, although some of them are predators (Figure. 6).

The insects of the family Oedemeridae are commonly known as false blister beetles. Many beetles, especially those with bright colors, possess special glands that enable them to produce chemical compounds for protection against bacterial, fungal, or predatory attacks. Adult beetles of this family possess Cantharidin in their body fluids, which serves as a defense mechanism [226]. (Abtahi et al. 2012). Members of this family have a tubular body shape and display vibrant colors. When these beetles are pinched or crushed against the skin, they can cause skin lesions and blisters. While they often reside on flowers during the day, they are attracted in significant numbers to sources of light such as parks, amusement parks, and swimming pools at night, posing a potential threat to individuals (Figure. 6) [227-230].

Within the Dermestidae beetle family, there have been reports of 12 genera and 123 species in Iran. The larvae and pupae of these beetles possess specialized hairs called hastisetae, which serve as their primary defense mechanism against invertebrate and potentially vertebrate predators [231,232]. These insects feed on a variety of organic materials and their hairs can significantly contaminate stored products and environments [153]. Exposure to hastisetae can lead to allergic reactions in humans, including skin rashes, asthma, conjunctivitis, and gastrointestinal inflammation. However, little is known about the exact mechanism of action of these reactions (Figure. 6) [233-235,230].

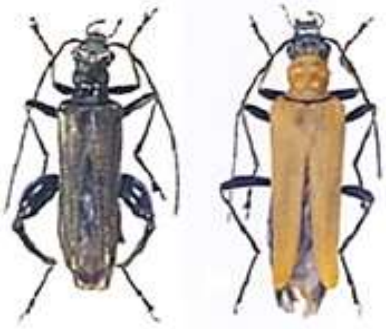
Lepidoptera order

Lepidopterism is a term used to describe various medical conditions in humans that typically involve cutaneous and systemic reactions resulting from contact with the larval hairs or adult scales of certain butterfly species. Butterflies are insects characterized by having four wings that are typically covered in scales (Figure. 6). The larvae (caterpillars) of most butterfly species, with a few exceptions, feed on a variety of plants. Some butterfly larvae, known as stinging caterpillars, are equipped with hollow quill-like hairs that serve as defensive weapons against their natural enemies. These hairs may be connected to poison sacs containing irritating chemicals, which can cause mild itching, severe local reactions, painful blistering, or even systemic issues such as intestinal disorders. Contact with adult butterflies can also lead to skin allergies and respiratory conditions due to their scales [236-239].

A group of leaf-eating butterflies feeds on the leaves of various trees, including forest shrubs like mountain pistachio, oak, and juniper (Arjan). Their larvae are covered with fur-like hairs that, when in direct contact with human skin, can cause allergic reactions such as dermatitis, urticaria, red nodules, itching, and burning sensations. Currently, six species from four butterfly families have been identified in Iran. The larvae of these butterflies often experience periodic population peaks or outbreaks every 7 to 10 years. Some of them live in groups on their host plants and have the ability to spin silk threads and create cocoons on the trees [240,241].

Allergic reactions can occur when human skin comes into contact with the setae (hairs) and pupae (cocoon) of butterflies, or when the scales and microscopic setae from the abdomen of adult insects are inhaled. These reactions typically start with itching, burning sensations, and the appearance of red papule-like lesions measuring 3 to 7 mm. In severe cases, it can even lead to shock. During years when their population increases, these butterflies can pose problems for forest dwellers or nomadic tribes in the region. Additionally, researchers who handle these butterflies without following proper safety measures have experienced lepidopterism [242].

Envenomation resulting from contact with caterpillars is a global health issue. Any direct or indirect contact with the caterpillar's urticating hairs can lead to clinical manifestations, including localized dermatitis and potentially life-threatening systemic effects. These problems arise due to the bioactive components present in the venom of these insects, which disrupt the functioning of various organ systems in the human body. The pathophysiology of this condition is not well understood, and currently, only symptomatic relief is provided by medical professionals since there is no effective treatment available. The health and economic impacts of this problem have been underesti-

class	Order	Family	Figure	References
Hexapoda	Coleoptera	Staphylinidae		Prepared by Dehghani R
		Meloidae		Prepared by Dehghani R
		Dermestidae		Prepared by Dehghani R
		Oedemeridae		Abtahi et al., (2012) with permission
	Lepidoptera	Saturniidae		Schowalter & Ring (2017) with permission

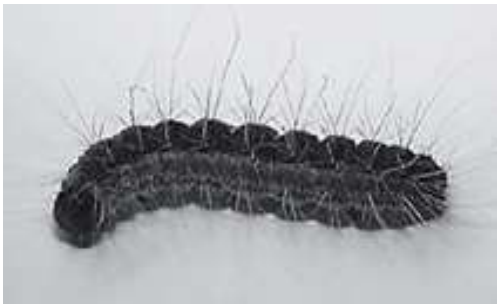
		Thaumetopoeidae		Gottschling., et al (2007) with permission
Hexapoda	Lepidoptera	Lymantriidae		Prepared by Dehghani R
		Lasiocampidae		Prepared by Dehghani R

Figure 6.
Venomous agents belong to Coleoptera and Lepidoptera orders (Prepared by Dehghani R)

mated, making it a growing concern for the future. Therefore, increasing awareness to prevent contact with these caterpillars is crucial [243-245].

Diptera order

The Diptera order, also known as true flies, encompasses a variety of flies and mosquitoes (Figure 7). Dipteran insects have mouthparts that are adapted to consume a wide range of foods, including a variety of materials such as pollen, plants, meat, feces or dung, and blood [246-249]. The Diptera order holds great medical significance as many diseases are transmitted to humans and other organisms by insects belonging to this order [250-254]. It is important to note that, despite the vast diversity and large number of species within the Diptera order, only a small percentage of them act as vectors responsible for transmitting pathogens to humans [255-258]. The Diptera order has three suborders: Brachycera, Nematocera, and Cyclorrhapha, each with numerous families. In the Brachycera suborder, three venomous families exist: Asilidae (robber flies), Tabanidae and Sciomyzidae. In the Nematocera suborder, only one venomous family, Cecidomyiidae is known. However, no venomous families have been reported in the Cyclorrhapha suborder [259].

The members of the Asilidae family, also known as robber flies, are venomous insects. Their venom contains numerous enzymes, including proteases, phosphatases, amylase, hydrolase, nucleases, and dehydrogenases. They inject their paralyzing venom into their prey while hunting, although their venom is comparatively weaker than that of spiders, scorpions, or Hymenoptera. Unlike other piercing flies that primarily feed on liquid, robber flies have independently evolved a venom delivery system. Adult robber flies produce venom in their thoracic glands and transmit it through ducts in the hypopharynx [260-262]. More than 150 species have been identified within the family of robber flies. They play a crucial role in maintaining the biological balance of insect populations because both the larvae and adults act as predators, feeding on various arthropods. Robber flies are typically active during the day (diurnal) and hunt their prey in sunny habitats. Some species within this family have developed adaptations that allow them to thrive in desert climates [263].

Tabanidae flies, commonly known as horseflies, are capable of producing paralyzing venom. In contrast to robber flies, the larvae of Tabanidae are ven-

omous predators, while the adults feed on flower nectar or blood. Tabanidae larvae are formidable hunters that are typically active in water, on aquatic plants, and among algae. When in contact with humans, they bite, causing pain, irritation, and itching. The venom is transmitted through a channel located near the tip of the mandible. This channel is connected to a gland inside the head, which is completely separate from the feeding duct. The Tabanidae larvae inject their venom through the lower channels of the mandible [259,264]. Iran, with diverse climates, is expected to harbor a significant number of unidentified species within the Tabanidae family, which are likely to be discovered in the future [265,266 35,54].

The larvae of the Sciomyzidae family possess a potent venom composed of neurotoxins, enzymes, and small peptide molecules. This venom originates from the salivary glands of the larvae [267-269]. The larvae utilize their venom for hunting snails. When a snail is envenomated, it experiences tremors and paralysis within 60 seconds. If the snail is not consumed by the larvae, it will eventually recover as the paralysis is reversible. The recovery time depends on the duration of the bite; for example, a 15-second bite can result in one hour of snail paralysis.

In Nematocera suborder, the larvae of the Cecidomyiidae family are venomous predators. They inject venom from their salivary glands into the bodies of aphids, causing temporary paralysis for a few minutes. In Iran, 61 species belonging to 33 genera have been identified, and they are distributed throughout the country from north to south [270,271].

Neuroptera order

Species belonging to the Neuroptera order can be distinguished by characteristics such as having antennae that are clavate and long, compared to the setaceous and short antennae of Odonata (Figure 7). Many Neuroptera flies are active during the night, while others feed on wild flowers during the day, contributing to pollination. Different species of Neuroptera flies also play a crucial role in biological control by hunting small insects like aphids. Within this order, 19 families have been identified [272]. The family Myrmeleontidae, commonly known as antlions, primarily inhabit land. The larvae of this family construct conical pits as traps in sandy and loose soil. When ants fall into these inverted cones, the antlions larvae capture and feed on them [273,274].

The family Myrmeleontidae comprises more than 200 genera and over 1500 described species worldwide. Adult antlions are commonly observed flying around lights, especially during spring and summer. They possess elongated bodies, resembling dragonflies. The larvae of antlions are aggressive and active predators, characterized by their robust physique [275,276]. The antlion larvae are aggressive and active predators with strong bodies [275, 276]. They possess three pairs of legs and a narrow neck. Their small flattened head has a huge pair of sickle-like mandibles with several sharp spines. The maxillae of antlion larvae resemble hypodermic syringes, allowing them to pierce their prey's body and extract fluids. Their jaws are capable of injecting venom that aids in digesting and dissolving the contents of the prey's body. Incidents of antlion larvae biting humans are rare [277]. There has been a reported case of a rare bite occurring on the finger and arm of an elderly woman from Queensland, Australia [278].

In Iran, 97 species of antlions have been identified across various provinces, including Baluchistan, Fars, Golestan, Hamedan, Hormozgan, Kermanshah, Khorasan, and Kurdistan [279-281].

Prevention and Treatment

Arthropods' stings and bites generally result in minor trauma. However, if they deliver venom, they can cause potentially severe local or systemic envenoming. Most venomous arthropods administer their venom through a stinger, chelicerae, pedipalps, or mandibles, while others release toxic compounds from their secretory glands upon direct contact with the victim. The treatment for an arthropod sting or bite depends on factors such as the type of venom or poison involved, the quantity injected, and the overall health condition of the victim. In individuals with a history of IgE-mediated systemic allergic reactions to insect venom, self-administration of epinephrine intramuscularly (IM) into the lateral thigh should be initiated immediately to prevent potential anaphylactic reactions. Also, Venom Immunotherapy Therapy (VIT) is typically recommended to prevent allergic reactions caused by certain Hymenoptera stings. Venom immunotherapy is a treatment rather than a preventive measure. The treatment involves gradually introducing small amounts of the venom into the body in order to desensitize the immune system and reduce or eliminate the allergic response. Over time, this helps to build tolerance and prevent severe reactions in case of future stings. The decision to initiate venom immunotherapy depends on the advice of an immunologist, taking into consideration factors such as the patient's age, cardiovascular health, and the risk of allergic reactions to the treatment. However, it should be noted that approximately 1 in 10 people who undergo venom immunotherapy may experience an allergic reaction to this treatment [282-286]. Although the treatment of most insect stings and bites is similar in several aspects, each treatment may also have specific features. In the following explanation,

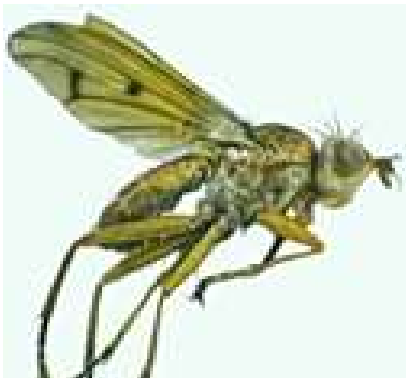
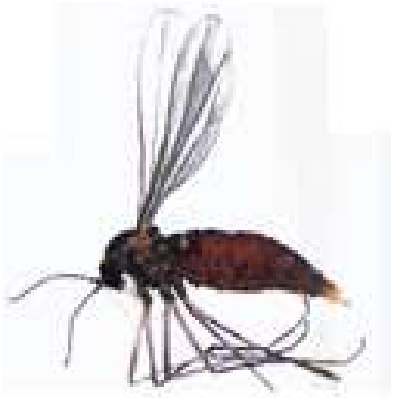
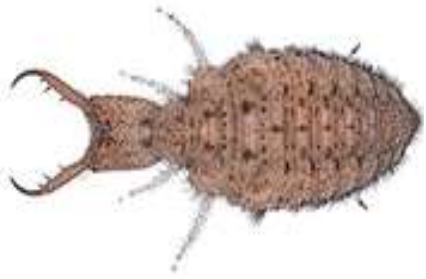
class	Order	Family	Figure	References
Diptera	Diptera	Asilidae		Mohammadi et al., (2021) with permission
		Tabanidae		Prepared by Dehghani R
		Tabanidae		Kazerani et al., (2017) with permission
		Cecidomyiidae		Schmid et al., 2018 with permission
	Neuroptera			Hajiesmaeilian et al., 2020 with permission "© Magnolia Press"

Figure 7.
Biting and venomous agents belong to Diptera and Neuroptera orders (Prepared by Dehghani R) "Neuroptera image used with permission from the copyright holder Zootaxa.

we will outline these aspects of treatment for different type of arthropods.

Scorpion venoms contain neurotoxic and cytotoxic components. The effects of scorpion stings can range from local envenoming to systemic envenoming. Approximately 50,000 cases of scorpion stings are recorded annually in Iran, mainly in the southwest region of the country. Hemiscorpius lepturus is responsible for the majority of envenoming cases and is the most prevalent species in Iran and its neighboring countries. The venom of H. lepturus primarily acts as a cytotoxic agent, exhibiting hemolytic, nephrotoxic, and hepatotoxic effects. It has been reported that the toxicity and clinical features of H. lepturus stings differ from those caused by other medically significant scorpion species [287]. The venom remains in the body, and its destructive effects manifest with a delay. Moreover, H. lepturus stings may not be easily recognizable due to their smaller size compared to other scorpions, such as the black scorpion (A. Crassicauda). In the first 24 hours, symptoms typically include minor itching and pain at the site of the sting, which many victims tend to ignore. Consequently, individuals affected by these stings often do not seek medical assistance until the main toxic symptoms become established. This delay in seeking treatment frequently results in longer hospital stays for patients in order to achieve full recovery.

Local symptoms; include cellulitis, severe inflammation, intense pain, gangrene, necrosis, swelling, and erythema at the sting site due to venom penetration into the dermis and adjacent tissues. The symptoms typically appear after 24 hours [287,288]. They can be managed with local or regional anesthesia, corticosteroids (e.g. hydrocortisone) to reduce inflammation, pain killers (e.g. acetaminophen or aspirin), and antihistamines (e.g Benadryl) [289,290,291]. In some cases, the venom may enter the blood and lymphatic systems, leading to the development of non-uniform ecchymotic patches with a diameter of up to 25 cm [287,292]. Over time, the gangrenous area may rupture, causing formation of lesions through the fatty tissue beneath the skin [287]. Extensive wounds often require skin grafts for proper healing [293-297].

Systemic symptoms; manifests as cardiotoxicity, acute kidney injury (AKI), hemoglobinuria, and CNS effects. Mental disorders can lead to cholinergic effects, such as nausea, vomiting, sweating, excessive salivation, priapism, bradycardia, hypotension, seizures, restlessness, headache, and confusion. In addition, individuals may experience adrenergic effects such as increased blood pressure, tachycardia, and heart failure [298,299]. In general, mild symptoms in adults may persist for 2 to 3 weeks without treatment [288]. The clinical treatment of systemic scorpion envenomation involves the essential use of antivenom as primary therapy [289,290,297]. In Iran, for the past 30 years, the most commonly used treatment for six common Iranian scorpions, including Androctonus crassicauda (Olivier 1807), Hottentotta (Buthotus) saulcyi (Simon, 1880), Hottentotta(Buthotus) schach (Birula 1905), Odontobuthus doriae (Thorell 1876), Mesobuthus eupeus (Koch 1839), and H. lepturus (Peters 1861), has been the administration of polyvalent antivenoms. These antivenoms are derived from equine hyperimmune serum and manufactured by the Razi Vaccine and Serum Research Institute in Karaj, Iran [292,300].

Other treatment for systemic scorpion envenomation, including standard heart failure treatment, involve the administration of inotropes along with diuretics (e.g. furosemide) to treat cardiogenic shock. Additionally, ACE inhibitors such as captopril, alpha-blockers (e.g., prazosin or doxazosin), and calcium channel blockers (e.g., nifedipine) are used to lower blood pressure [290,291,297].

In cases of low blood pressure, if necessary dobutamine, a medication used in the ICU to manage low blood pressure may be administrated. In more severe cases, excessive bronchial secretion can lead to pulmonary edema and respiratory failure. The treatment involves mechanical ventilation and the use of diuretics to manage respiratory failure [290,291,297,301, 302].

Spiders; the majority of spiders have venom that is relatively weak and often unable to cause substantial envenoming. Also, their chelicerae are usually too small to penetrate human skin. Most spider bites go unnoticed until clinical symptoms develop, and turn into local erythematous edema. Certain spiders, like brown recluse, can cause necrotic lesions, local edema, and ischemia at the bite site, leading to the gradual formation of an eschar and tissue necrosis. Other spiders, such as widow spiders, funnel web spiders, and wandering spiders, can cause systemic neurotoxic envenoming similar to scorpion stings. The clinical treatment of local venom effects is generally minor and involves routine wound care and tetanus prophylaxis. However, in severe cases with tissue necrosis around the bite site, special attention should be given the treating the necrotic tissue. In situations involving Loxosceles sp bites and systemic envenomation, specific antivenom, along with epinephrine, antihistamine, and steroids, may be necessary [288,303].

In the summer of 2017, in the city of Kashan in central Iran, a 48-year-old female cleaner was bitten by a spider while collecting garbage. The spider was later identified as a member of the Loxosceles sp (Araneae-Sicariidae). The initial symptoms she experienced included immediate irritation, itching, swelling, redness

on her arm and numbness in three of her fingers. She also suffered from shortness of breath. After four days, her hand became edematous and painful, and she also experienced insomnia. Her condition worsened to the point where she lost the ability to move her fingers. Due to the severity of her condition, she was hospitalized for four days and received various treatments including normal saline, corticosteroids (dexamethasone), antibiotics, antihistamines, and analgesics. Additionally, she was administered a tetanus vaccine and tetabulin [42].

Ticks; the first step in the treatment of tick bites is to remove the attacker's tick with fine-tipped tweezers to grasp the tick at the level of the skin to remove the tick, particularly the head and mouthparts. Then thoroughly clean the bite site with soap and water, and apply alcohol or an iodine scrub to prevent infection. A dose of antibiotics may need to prevent accruing of tick-borne diseases like Lyme disease. If symptoms of paralysis appear, respiratory function should be monitored closely. In severe cases, mechanical ventilation at the ICU level may be required [304-306]. It should be noted that, researchers have been studying various approaches to develop vaccines against ticks that involves identifying specific proteins or molecules found in ticks that play crucial roles in their life cycle or ability to transmit diseases, then isolate and purify these proteins and use them to create a vaccine [71].

Hymenopterian sting treatment options, the result of the most Hymenopteran stings are uncomplicated and limited to local inflammation in a small area. They typically present with focal edema, warmth and redness, which usually recover within a few days. However, in cases when multiple stings occur or in large local reactions (10 cm or more), more severe symptoms such as erythema, induration, increased warmth, intense pain, and longer persistence may be observed.

Systemic consequences resulting from immediate hypersensitivity reactions and anaphylaxis, can be fatal, and needs early medical intervention. Symptoms include difficulty breathing, wheezing, generalized urticaria, angioedema, flushing, and hypotension or even shock. The therapeutic approach includes prescribing a wide range of medicines along with necessary therapeutic measures based on the type of reactions to stings. Before any pharmacological treatment, the stinger should be removed via scraping with a credit card (or like that) rather than squeezing to avoid further venom injection. After that, ice packs should be applying to reduce pain. For uncomplicated local reactions, common prescriptions include non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen for pain relief, as well as H1/H2 blockers/antihistamines, such as diphenhydramine and normal saline. In patients with large local reactions, the same treatment is administrated along with glucocorticoids, such as prednisone, at doses of 40 to 50 mg/kg for 3-5 days [134,307].

Systemic reactions, such as anaphylaxis, present with generalized urticaria, angioedema/facial swelling, stridor, respiratory distress/wheezing due to bronchospasm, abdominal pain, nausea, vomiting and flushing. For treatment of systemic reactions, monitoring the function of respiratory system is crucial because the airway can be compromised within seconds to minutes. Therefore, early intubation may be necessary. For anaphylactic reactions, life-saving administration of epinephrine is essential. The recommended dose is 0.3-0.5 mg administered intramuscularly (IM), and it can be repeated every 5 to 15 minutes if needed. Corticosteroids (such as prednisone, methylprednisolone, or dexamethasone) should be given to reduce inflammation. Additionally, antihistamines such as diphenhydramine (Benadryl), should be administered to alleviate pruritus, erythema, and urticaria. Intravenous (IV) fluids (isotonic crystalloids) at doses of 10-20 ml/kg, should be provided immediately [134,307, 308].

Bees, wasps, Fire ants and Velvet ants, the stings of honeybees and wasps can be dangerous for allergic individuals. True envenoming is rare and typically requires hundreds of stings in adults. Unlike allergic reactions, only 5% of all deaths are caused directly by their venom. The first step in treating a bee sting is to remove the stinger as quickly as possible because it continues to pump venom into the skin even after detaching from the bee's body [309].

In most non-allergic patients, a single sting may only cause local inflammation, pain, redness, and swelling as the main clinical symptoms. However, multiple stings can lead to extensive swelling, which can result in hypovolemia, hemolysis, neurological disorders, myolysis, and renal failure. Immediate hypersensitivity and anaphylaxis reactions pose the most significant risk associated with hymenopteran stings and can be life-threatening. Within minutes after a sting, systemic symptoms such as tachycardia, flushing, abdominal colic, or diarrhea may appear. Without effective treatment, these symptoms can progress to hypotension, coma, and even death [307,309]. The standard clinical treatment involves administration of adrenaline, steroids, and antihistamines to counteract allergic reactions. For patients experiencing sting anaphylaxis, adrenaline (0.1%) is life-saving intervention given at doses of 0.5–1.0 ml for adults, and 0.01 ml/kg for children, administered intramuscularly (IM). In allergic and non-allergic individuals, local wound management and tetanus prophylaxis are applied if necessary [309]. Fire ant, (Hymenopteran) stings may cause serious health problems in highly sensitive in-

dividuals and causes nausea, vomiting, diarrhea, and even difficulty breathing and emergency hospilatisation. Generally, Fire ant stings are treated symptomatically with antihistamines (e.g., Benadryl) or a similar over-the-counter oral antihistamine, topical corticosteroids (e.g., hydrocortisone ointment), and cold compresses, which can effectively alleviate pain and reduce localized reactions [155,167,310].

Over-all, systemic analgesics such as ibuprofen or paracetamol (acetaminophen) are recommended for pain control, and intense itching of bite site [125,123]. Velvet ants; Symptoms of a velvet ant stings typically include minor local reactions such as pain, swelling, and redness, which are generally not life-threatening. The treatment involves removing the sting, if present, using a similar method to that used for removing a bee sting. The sting site should be cleaned with soap and water, and applying a cold compress to alleviate pain and reduce swelling. If necessary, over-the-counter pain relievers such as ibuprofen or acetaminophen can be used. To relieve itching, topically applying calamine lotion is effective. It is important to keep the sting site clean, in order to prevent skin infections. Additionally, using zinc oxide ointment can aid in healing the irritated skin. Furthermore, administration of an antihistamine such as Benadryl or Claritin can help counteract histamine reactions at the bite site [166,162,163].

Beetles and Miilipede; the Beetles of the families Staphylinidae, Meloidae, Oedemeridae, Dermestidae, and species of Millipede class, release their chemical toxin upon contact with the victim, therefore, before applying any medicinal treatment such as topical steroid ointments, it is recommended to wash the affected area with soap and water as soon as possible. Additionally, irrigation with alcohol or ether can be beneficial. In the case of contact with larvae of Lepidoptera, oral antihistamines and topical corticosteroids are commonly used for treatment [81,116,310]. For centipedes' bites, it is recommended to wash the bite site with soap and clean water and apply a cold compress.

Bugs; aquatic bugs are the primary biting insects in the Hemiptera order. Their bites are very painful, and if scratching occurs, the bite site may become infected. The treatment for aquatic bug bites is generally symptomatic [310].

Conclusion

This study highlights the widespread presence of venomous and poisonous arthropods across Iran and its neighboring countries, an area that has received insufficient attention regarding their significance in various scientific disciplines. It is suggested to entomologists and biologists to intensify their efforts in species identification, behavioral studies, and understanding the biology and dispersal of these organisms, as well as their implications for human health and the environment. Increased awareness and knowledge of these species will equip professionals to respond more effectively to natural and accidental incidents, particularly in emergency situations.

The study also provides a comprehensive list of biting and stinging venomous arthropods in Iran and the broader Middle East, emphasizing the need for physicians and healthcare staff to recognize the medical significance of these species, assess the severity of their bites or stings, and implement appropriate patient management strategies. Currently, the educational framework for medical staff regarding the risks associated with arthropod and other venomous animal bites is lacking. Therefore, it is crucial for health policymakers at both national and regional levels to prioritize these issues and integrate them into academic curricula to enhance preparedness and response capabilities in healthcare settings.

Authors' Contributions

R. Dehghani study conception and design. R. Dehghani, M. Dehghani and N. Mohammadzadeh were contributed in Material preparation and, data collection. The first draft of the manuscript was written by R. Dehghani and B. Fathi and all authors commented on previous versions of the manuscript. B. Fathi critically revised, edit and submitted the manuscript. Finally, all authors read and approved the final manuscript.

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Conflict of interest

The authors do not have conflict of interest.

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Eremurus spectabilis Root Extract: Evaluating Different Extraction Methods and Antimicrobial and Antioxidant Characteristics

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ABSTRACT

The current study aimed to investigate the potential antimicrobial and antioxidant activities of Eremurus spectabilis (*E. spectabilis*) in three extraction techniques. Three methods were selected to extract the aqueous, alcoholic, and hydroalcoholic extracts of *E. spectabilis*. The extraction yield was obtained from 10 g of *E. spectabilis* powder. The carbohydrate test was performed using the phenol sulfuric acid method. The Kjeldahl method was used in two replicates based on the AOAC 2550 standard to determine the protein content. The concentration of phenolic compounds was measured by the Folin-Ciocalteu assay. Based on the results, *E. spectabilis* had 70.33% w/w carbohydrates and 7.1% w/w proteins. The extraction percentages for the aqueous, alcoholic, and hydroalcoholic extracts of *E. spectabilis* were 50%, 10%, and 25%, respectively. The results showed that the aqueous extraction method was the most efficient. The total phenol amount for *E. spectabilis aqueous* extract was 150.04 mg/g. The antioxidant property of the *E. spectabilis* aqueous extract was determined to be 50.71% None of the concentrations of the aqueous extract did not have antimicrobial properties against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. These findings demonstrates the need for further studies on other pathogens and using different concentrations.

Keywords

Antimicrobial, Antioxidant, Eremurus spectabilis, Hydroalcoholic extraction, Alcoholic extraction

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Number of Tables: 1
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Abbreviations

E. spectabilis: Eremurus spectabilis
E. coli: Escherichia coli

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Introduction

Natural antimicrobial compounds have gained attention due to their compatibility with the body, lower toxicity, and fewer side effects [1, 2]. These compounds, often obtained from plants, animals, or microbial sources, prevent the growth and reproduction of pathogenic microorganisms by various mechanisms [3, 4]. Among these mechanisms, we can mention the destruction of the cell membrane, inhibiting the production of essential proteins, and disrupting the cellular metabolism [5, 6]. Meanwhile, synthetic and chemical antimicrobial compounds, although effective in some cases, are associated with diverse problems, such as increased microbial resistance, high toxicity for human cells, and environmental pollution [7]. The widespread use of these chemical compounds not only reduces the effectiveness of medications over time but also causes irreparable side effects due to their accumulation in the human body and environment [8, 9]. Therefore, natural antimicrobial compounds are valuable and important as a safer and more effective alternative to fight infections, especially when drug resistance has become a serious problem [10, 11].

As a perennial herbaceous plant native to Central Asia and parts of the Middle East, *E. spectabilis* is often referred to as either foxtail lily or desert candle [12]. The plant belongs to the family Asphodelaceae and has been used for medical purposes for a long time. Various bioactive compounds, including glycosides, flavonoids, saponins, and other secondary metabolites, are present in *E. spectabilis* roots [13, 14]. Some of these compounds are highly potent antimicrobial and antioxidant compounds [15], making the plant an ideal subject of study for potential therapeutic applications because of its significant antimicrobial and antioxidant properties [16, 17].

Since the emergence of antibiotic-resistant microorganisms in the past few decades, interest in natural antioxidants and antimicrobials has surged due to growing concerns about the side effects of synthetic compounds and the growing prevalence of side effects caused by synthetic compounds [18-21]. A range of natural products, such as those derived from plants, offer an alternative to traditional medications because they are biocompatible, have a low level of toxicity, and are effective against a wide range of bacteria and viruses [22, 23]. The ethnobotanical and historical relevance of *E. spectabilis* makes it an attractive candidate for further studying its root extracts and investigating its potential medicinal implications [24, 25].

Several extraction methods could be used to extract bioactive compounds from *E. spectabilis* roots; however, it is extremely significant that these methods are compared to determine the most effective one [26]. Some considerations, such as the extraction yield, compound's stability, environmental impact, and operation efficiency are all critical [27]. A systematic evaluation of these methods will greatly help researchers optimize the extraction process, providing root extracts with the most effective antimicrobial and antioxidant properties.

It has been established that the antimicrobial properties of the roots of *E. spectabilis* are particularly important in dealing with the bacteria resistant to antibiotics [28]. According to preliminary studies, these extracts may show a significant activity against various bacterial and fungal pathogens, including those associated with Candida albicans [29, 30]. In addition, the antioxidant properties of the extracts make them suitable for preventing diseases related to oxidative stress [31]. These diseases include cardiovascular diseases, neurodegenerative disorders, and some types of cancer due to their antioxidant properties.

E. spectabilis is a promising source of natural antimicrobial and antioxidant agents [32]. Selecting an extraction method considering the bioactive compounds of a plant is crucial to maximize its potential. Therefore, the current study examined the potential antimicrobial and antioxidant activities of *E. spectabilis* extract.

Results

Chemical properties of E. spectabilis

According to our results, *E. spectabilis* had 70.33% w/w carbohydrates and 7.1% w/w proteins.

Extraction yield

The yield of extraction from *E. spectabilis* root by aqueous, alcoholic, and hydroalcoholic solvents are shown in Figure 1. The extraction yield for the aqueous, alcoholic, and hydroalcoholic extracts of *E. spectabilis* was 50%, 10%, and 25%, respectively (Table 1). The results showed that the aqueous extraction method was the most efficient.

TPC content

As it was shown in Table 1, the TPC content for *E. spectabilis* aqueous extract was 150.04 mg/g.

Antioxidant properties

The RSC% of *E. spectabilis* aqueous extract was equal to 50.71% (Table 1).

Antimicrobial property

None of the concentrations of the aqueous extract had antimicrobial properties against any of the tested

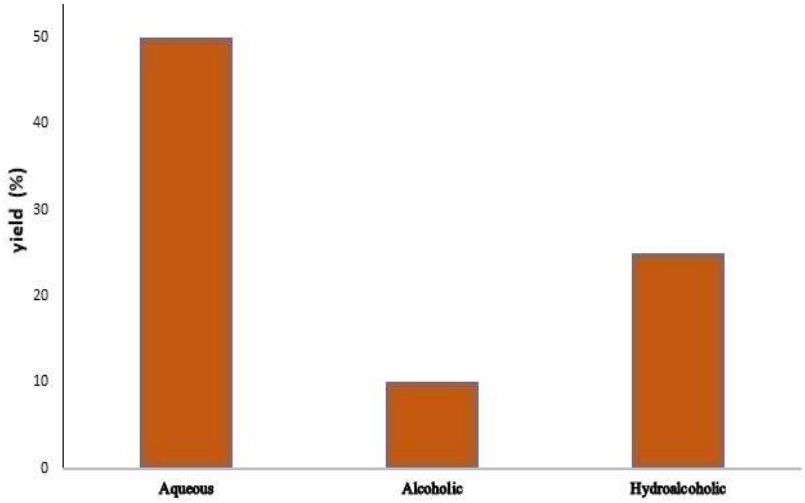

Figure 1.
Extraction yield of E. spectabilis root extracts by different solvents

Table 1.
Chemical properties of E. spectabilis root extracts by different methods.

Extraction method	Extraction yield	(mg/g)TPC	RSC (%)
Aqueous	50	150.04	50.71
Alcoholic	10	-	-
Hydroalcoholic	25	-	-

microorganisms, and no growth inhibition zone was observed. The diameter of the growth inhibition zones of *S. aureus* (ATCC:25923), *E. coli* (NCTC:12900), and *Pseudomonas aeruginosa* (ATCC:27853) for the gentamicin antibiotic disc were 17, 16, and 20 mm, respectively.

Discussion

The results of the present study showed that the main components of *E. spectabilis* were carbohydrate (70.33% w/w) and proteins (7.1% w/w). According to the current study, *E. spectabilis* is a good source of carbohydrates and proteins. The findings of this research correspond with those reported by Salehi *et al.* (2022) regarding the root gum of *E. spectabilis.* The researchers determined that the root gum powder of Eremurus luteus contained an average moisture content of 6.27% (w.b.), 4.17% (d.b.) ash, 6.22% (d.b.) protein, 86.45% (d.b.) carbohydrate, and 8.6% (d.b.) uronic acids [33]. In the present study, the highest extraction percentage was for the aqueous extract (50%), while the lowest was for the alcoholic extract (10%). Phenolic compounds in the aqueous extract of *E. spectabilis* were 150.04 mg/g. A recent study reported that *E. spectabilis* extracted by methanol, ethanol, and aqueous media had a total phenolic content in the range of 31.7-92.15 mg GAE/g and antioxidant activity in the range of 72.01-81.21 mg AAE/g [16].

In addition to flavonoids, phenolic compounds, and saponins, *E. spectabilis* extract has many medicinal properties [12]. A powerful antioxidant is vital for fighting free radicals, leading to preventing cells from being damaged. A high phenol composition gives *E. spectabilis* extracts a strong antioxidant activity [34]. In addition to helping prevent chronic diseases, such as cancer, arthritis, and cardiomyopathies, antioxidant activity may also help treat neurological disorders [23-35].

It is also noteworthy that *E. spectabilis* extract possesses antimicrobial properties that are important for combating various pathogenic microorganisms. Bircan and Kırbağ (2015) reported that zones of inhibition were seen for *S. aureus* (12 mm), *E. coli* (14 mm), and *C. albicans* (9 mm), and *Epidermophyton spp(8mm),* when *E. spectabilis* extract was used [15]. In contrast, Karaman *et al.* (2011) revealed that the 1% concentrations of the methanol, ethanol, and aqueous extracts of *E. spectabilis* showed no inhibitive effect on Yersinia enterocolitica and Pseudomonas aeruginosa [16]. The Eremurus extract has been shown to work as a natural alternative to synthetic antibiotics by exerting inhibitory effects against various bacteria and fungi, suggesting that *E. spectabilis* may be a valuable alternative in the future [18]. Considering that antibiotic resistance is rising, alternative treatments have become more and more necessary, especially in the light of the increasing need for alternative treatments. It has been shown that using the extracts of *E. spectabilis* could be beneficial to animals in a variety of ways, including the prevention of infections and the reduction of oxidative stress-related conditions. Compared to traditional medications, herbal agents are considered safer alternatives with fewer side effects due to the natural origin of their ingredients and their bioactive components [36]. The benefits that will be gained from *E. spectabilis* are on both human and animal sides in terms of enhancing health.

Studies have shown that the main volatile components of the *E. spectabilis* root are carone (terpenoid), carvacrol (monoterpenoid phenolic compound), pentane, 2-methyl (E) caryophyllene (natural bicyclic sesquiterpene), valencene, cadalene, and acetic acid. These volatile components contribute to the antioxidant and antibacterial activity in the *E. spectabilis* root as a defense mechanism against insects, fungi, and

other environmental stresses. On the other hand, glucomannans are water-soluble bioactive polysaccharides in the root of *E. spectabilis* and contribute to the antioxidant activity [37].

Gram-negative organisms are generally believed to be less sensitive to antimicrobial components due to the outer lipopolysaccharide membrane surrounding their cell wall, which provides surface hydrophilicity, preventing access to the antimicrobial components of a predominantly hydrophobic nature. In the current study, the aqueous extract of *E. spectabilis* in the used concentrations did not have antimicrobial properties against any of the tested microorganisms. However, Tuzko *et al.* (2017) concluded that *E. spectabilis* has antimicrobial activity against Gram-negative (E. coli) and Gram-positive (B. subtilis) organisms [17]. Its antimicrobial activity can be attributed to the phenolic compounds, essential oils, and volatile components. It has been reported that n-octane and n-decane, the main components of *E. spectabilis* essential oil, are responsible for the antimicrobial activity due to their hydrophobic nature. Kanani and Mohammadi Sani (2015) showed that the roots of *E. spectabilis* can prevent the growth of Gram-positive and Gram-negative bacteria [38].

Tuzcu *et al.* (2017) examined the antioxidant properties, antimicrobial effects, anticancer properties, as well as the apoptotic and anticancer properties of the aqueous and organic extracts from *E. spectabilis* leaves and roots [17]. In this study, the Folin-Ciocalteu method was used to assess the total content of phenols in these extracts and revealed that the extracts possess significant antioxidant potential. In addition, DPPH radical scavenging assays and lipid peroxidation assays were conducted as further assessments, demonstrating this plant's potent free radical-neutralizing properties. In addition, the antimicrobial efficacy of 500 µg/ml of the extracts of *E. spectabilis* was assessed through disk diffusion and revealed that the extracts were effective against *Listeria monocytogenes*, *Saccharomyces cerevisiae*, *S. aureus*, and *E. coli*. However, in our study, 100 µg/ml concentrations were used. Among the different extracts, the acetone extract of leaves exhibited the highest phenolic and flavonoid content. It also had an antioxidant activity measured at 3703.25 µg ascorbic acid/g dry weight. According to these findings, *E. spectabilis* is a promising natural resource that can be utilized to develop new therapeutic agents for both veterinary and medical uses.

Some limitations were identified in the present study that examined the antimicrobial and antioxidant potential of *E. spectabilis* extract. The extract used in the study may not have had sufficient antimicrobial effects due to its low concentration. The extract may have stronger antimicrobial properties at higher concentrations, which would make it possible to have a greater effect at higher levels. To find out the optimal dosage of the extract for effectively inhibiting microorganisms, further research needs to be conducted using a variety of concentrations of the extract. Furthermore, we tested the extract against a limited range of microorganisms to determine its effectiveness. In order to better understand the extract's antimicrobial properties, it is important to increase the spectrum of microorganisms tested. This would enable us to understand the extract's antimicrobial properties. By extending these tests to a broader range of pathogens, we might be able to identify different types of microbes that are more susceptible to the bioactive compounds of *E. spectabilis*.

Although the current study has some limitations, its results open up a wide range of interesting areas for future research. *E. spectabilis* extract has synergistic effects when combined with other natural antimicrobial agents. In this way, multiple extracts can be used in conjunction to produce more powerful antimicrobial and antioxidant effects resulting from their combined bioactive properties.

The aqueous extract of *E. spectabilis*, despite promising bioactive properties, failed to show any antimicrobial activity against certain bacteria. As a result, it is clear that the extract, in its current form and concentration, is not effective at inhibiting microbial growth. Exploring different extraction methods, higher concentrations, or combining different antimicrobial agents would be beneficial. This will enable us to fully comprehend the potential of *E. spectabilis* in future. Furthermore, a full investigation into the antioxidant properties of the material is required.

Materials & Methods

Materials

The roots of *E. spectabilis* were collected from the heights of the Binaloud mountain range (Razavi Khorasan, Iran) and were washed with water for deflowering and soil removal. Then, they were dried on a cotton cloth in the shade and powdered in a semi-industrial mill. Next, using a sieve with a mesh size of 100 mesh , the powders were sieved and made the same size. The resulting powder was kept in a cool place and away from sunlight until use. DPPH, ethanol, and other chemicals, including culture media, were obtained from Merck. Bacterial strains *S. aureus* (ATCC:25923), *E. coli* (NCTC:12900), and *P. aeruginosa* (ATCC:27853) were obtained from the Food Hygiene Department, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Extraction techniques

Three methods were used to extract *E. spectabilis* gum. In the aqueous extract method, 3% W/V suspension was prepared and homogenized on a magnetic stirrer for one hour. The suspension was heated in a bain-marie at 80°C-95°C for 15 min and smoothed with a linen

cloth to further dissolve. A centrifuge operating at 4500 g (Universal PRP, Iran) was employed at a temperature of 20°C for 10 min to purify the extract. The extract obtained by the extraction process was dried in a fan-assisted oven at a temperature of 40°C. Subsequently, it was ground and filtered through a 100-mesh sieve [39].

An ethanolic extract was prepared by mixing 10 g of *E. spectabilis* root powder with 150 ml of ethanol and stirring in a magnetic stirrer at 150 rpm for 2 h at room temperature (6). The resulting mixture was macerated at room temperature for 24 h and filtered with a 100-mesh sieve. The resulting solution was dried under a low-pressure evaporator at 4°C and kept in the dark.

In the third method, a hydroalcoholic extract was prepared using 10 g of the *E. spectabilis* root powder mixed with 150 ml of the ethanol-water mixture in a ratio of 50:50. The rest of the steps were carried out according to the second method, and the hydroalcoholic extract of E. spectabilis was prepared [17].

Extraction yield

The extraction yield was obtained from 10 g of *E. spectabilis* powder. First, the plate for drying the extract solution was weighed, and the weight difference between the empty plate and the plate containing the dried extract was calculated in all three samples.

Eremurus spectabilis properties

Color

To determine the color, 0.1 g of three aqueous, alcoholic, and hydroalcoholic extracts were dissolved in 6 ml of distilled water, and the absorbance of the sample was measured at 420 nm [40, 41] by a spectrophotometer (UV-VIS single beam spectrophotometer, UNICO, USA).

Total carbohydrates

The carbohydrate test was performed using the phenol sulfuric acid method. A volume of 2 ml of carbohydrate solution was mixed with 1 ml of 5% phenol aqueous solution in a test tube. Afterwards, 5 ml of concentrated sulfuric acid was quickly added to the mixture. The mixture was set for 20 min in a water bath at 30°C. Next, the absorption at 490 nm was recorded by a spectrophotometer. In the end, fructose was used as a standard, and the amount of total sugar was determined based on the absorption standard curve of similar solutions [42].

Protein amount

The Kjeldahl method was used in two replicates based on the AOAC 2550 standard to determine the protein content [43].

Total Phenolic Compounds (TPC)

The concentration of phenolic compounds with potential antioxidant activities can be measured by the Folin-Ciocalteu assay and expressed in gallic acid. A volume of 0.5 ml of the extract (25 ml/250 g) was mixed with 2.5 ml of 10% Folin-Ciocalteu and stirred for 5 min. Next, 2 ml of 5% sodium carbonate solution was added and it was kept for 30 min in a dark place at room temperature. The absorbance of the samples was measured at 760 nm by a spectrophotometer. The TPC content in the extract was measured and reported using a standard curve based on the micrograms of the gallic acid per gram of extract [44].

Antioxidant property

The DPPH radical scavenging method was used to evaluate the antioxidant activity [45]. A volume of 500 µl of the extract was mixed with 4 moles of the methanolic solution of DPPH 0.08 mmol/l and was placed in a bain-marie at 30°C for 30 min. The absorbance of the sample was measured at 517 nm. Radical scavenging capacity was determined using equation 1:

$$RSC\ (\%) = (A_{blank} - A_{sample}/A_{blank}) \times 100 \quad (1)$$

Antimicrobial properties

Mueller Hinton agar culture medium was used to investigate the antibacterial properties of the aqueous extract of E. spectabilis using the disc diffusion method. In this method, from the 24-hour culture of the bacterial strains *S. aureus* (ATCC:25923), *E. coli* (NCTC:12900), and *P. aeruginosa* (ATCC:27853) in Mueller Hinton agar medium, the turbidity liquid equivalent to 0.5 McFarland (1.5×108 CFU/ml) was prepared. After diluting, a suspension with a concentration of 1×104 CFU/ml was obtained and cultured in Mueller Hinton agar and placed at a certain distance from each other and the edge of the plate on the agar medium. A volume of 100 µl of 30%, 40%, and 50% extract concentrations in dimethyl sulfoxide solution was added to the discs and incubated at 37°C. Sterile distilled water was used as a negative control, and gentamicin antibiotic disc was used as a positive control. After 24 h of incubation, the diameter of the zone (Figure 2) was determined [46].

Data availability

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

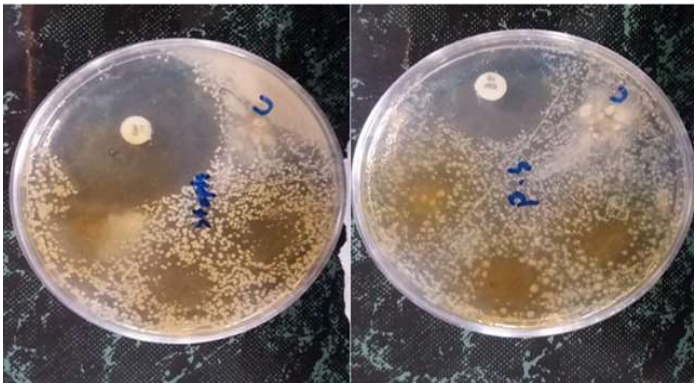

Figure 2.
The measurement of the diameter of the zone of growth inhibition for the microorganisms *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Authors' Contributions

Conceptualization: Razieh Niazmand; Methodology: Elham Merrikhi Ardebili; Formal analysis and investigation: Elham Merrikhi Ardebili & Razieh Niazmand; Writing - original draft preparation: Elham Merrikhi Ardebili; Writing - review and editing: Elham Merrikhi Ardebili & Razieh Niazmand & Abdollah Jamshidi]; Funding acquisition: Abdollah Jamshidi & Razieh Niazmand; Supervision: Razieh Niazmand & Abdollah Jamshidi. All authors checked and approved the final version of the manuscript for publication in the present journal.

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Conflict of interest

The authors declare that there is no conflict of the interest

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Effects of Vernonia amygdalina Extract on the Modulation of Liver Antioxidant Enzymes, Cytokines, Adipokines, DNA Biomarkers, and Growth in Aflatoxin B1-Exposed Broiler Chickens

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ABSTRACT

Aflatoxicosis has emerged as a notable factor that hampers the well-being and productivity of broilers in recent times. This study examined *V. amygdalina* leaf extract's protective effects against aflatoxin B1-induced hepatoxicity and growth suppression in broiler chickens. 240 Cobb500 day-old chicks were divided into 4 groups: CONT (uncontaminated diet), AFTB1 (0.5 ppm aflatoxin B1 contamination), VE1AF (0.5 ppm aflatoxin B1 + 1 g *V. amygdalina* leaf powder/liter of water), and VE2AF (0.5 ppm aflatoxin B1 + 2 g *V. amygdalina* leaf powder/liter of water) in a completely randomized design. Liver catalase, glutathione peroxidase, and superoxide dismutase levels exhibited a marked reduction in the AFTB1 group compared to CONT and VE2AF groups ($p < 0.05$). Malondialdehyde levels, indicative of lipid peroxidation, were markedly increased in the AFTB1 group compared to all other groups ($p < 0.05$). The levels of pro-inflammatory cytokines (TNF-α, IL-6, and IFN-γ) exhibited a marked increase in the AFTB1 group compared to CONT and VE2AF groups ($p < 0.05$). Additionally, anti-inflammatory cytokines, adipokines, and oxidative DNA damage biomarkers varied significantly among treatment groups ($p < 0.05$). Both 1 g and 2 g of *V. amygdalina* leaf powder per liter of water effectively countered the detrimental effects of aflatoxin B1 on liver health in broiler chickens.

Keywords

Aflatoxicosis, Hepatoxicity mitigation, Phytochemicals, Poultry

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Abbreviations

AFB1: Aflatoxin B1
DNA: Deoxyribonucleic Acid
TNF-α: Tumor necrosis factor α
IL-6: Interleukin 6
IFN-γ: Interferon Gamma
IL-1β: Interleukin 1 Beta

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Introduction

Aflatoxicosis has recently been identified as a significant factor limiting the well-being and physiological condition of broilers [1]. Aflatoxins, which are secondary metabolic intermediates or by-products produced by certain subtypes or strains of Aspergillus fungi, pose substantial health risks to both human and animal populations globally [2]. Among these mycotoxins, aflatoxin B1 (AFB1) emerges as one of the most potent and prevalent contaminants in agricultural commodities, particularly in regions characterized by warm and humid climates such as the tropics [3]. Chronic exposure to AFB1 through contaminated feedstuffs has been linked to various adverse effects on animal health, with the liver serving as the primary target organ due to its pivotal role in detoxification and metabolism [4].

Aflatoxicosis, arising from exposure to aflatoxins, primarily manifests as hepatic damage, culminating in hepatotoxicity and the potential development of hepatocellular carcinoma and growth depression [1]. In poultry production, broiler chickens exhibit heightened susceptibility to aflatoxicosis owing to their elevated metabolic rate and rapid growth, rendering them particularly vulnerable to feed-borne mycotoxin contamination [5].

To mitigate the adverse effects of aflatoxicosis in broiler chickens, researchers have explored various strategies, among them, the utilization of dietary supplements with potential ameliorative properties [6]. One such promising candidate is the extract obtained from the leaves of *Vernonia amygdalina* using water, a plant indigenous to tropical regions renowned for its pharmacological attributes, including antioxidant and hepatoprotective effects [7]. Despite its traditional application and promising biological activities, the efficacy of bitter leaf extract as an ameliorative agent against aflatoxicosis in poultry remains largely unexplored, particularly concerning AFB1-induced hepatotoxicity.

Therefore, the current investigation aimed to assess *Vernonia amygdalina* leaf aqueous extract as a novel ameliorative agent for hepatoxicity in AFB1-exposed broiler chickens by evaluating its impact on liver function and biochemical markers of hepatic injury and growth.

Abbreviations - cont'd

8-OHdG: 8-hydroxy-2' –deoxyguanosine (ng/ml)
HPLC: High-performance liquid chromatography
CONT: No aflatoxin B1 contamination; no administration of Vernonia amygdalina extract.
AFB1: 0.5 ppm aflatoxin B1 contamination.
VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g Vernonia amygdalina leaf powder per liter of water.
VE2AF: 0.5 ppm aflatoxin B1 contamination + 2 g Vernonia amygdalina leaf powder per liter of water.
CAT: Catalase
SOD: Superoxide dismutase
GPx: Glutathione peroxidase

Results

Antioxidant enzymes and malondialdehyde in the liver

A significant decrease in catalase levels was observed in the liver of the AFTB1 treatment group compared to the CONT, VE1AF, and VE2AF groups ($p < 0.05$). Likewise, the concentration of liver glutathione peroxidase and superoxide dismutase in the broiler chickens exposed to AFTB1 treatment significantly decreased ($p < 0.05$) compared to the CONT and VE2AF. Conversely, the quantity of these enzymes in the CONT, VE1AF, and VE2AF exhibited no significant difference ($p > 0.05$) (Table 1). Moreover, the liver malondialdehyde levels in the birds subjected to AFTB1 were notably reduced ($p < 0.05$) compared to the other treatment groups.

Inflammatory cytokines in the liver

The concentrations of tumor necrosis factor α, interleukin 6, and interferon gamma in the liver were notably elevated (p <0.05) in the AFTB1 group compared to both the CONT and VE2AF groups (Table 2). Furthermore, the concentrations of these cytokines in the AFTB1 treatment group exhibited no significant difference ($p > 0.05$) compared to those in the VE1AF group. The presence of interleukin 1 Beta in the liver displayed a trend ($p = 0.09$) toward being influenced by the treatment.

Anti-inflammatory cytokine, adipokine, and oxidative DNA damage biomarkers in liver

Table 3 displays the levels of anti-inflammatory cytokines, adipokines, and oxidative DNA damage biomarkers in the livers of broilers exposed to Aflatoxin B1 and administered *Vernonia amygdalina* aqueous leaf extract. The liver adiponectin concentration of birds from the AFTB1 group was notably reduced ($p < 0.05$) compared to those in the CONT, VE1AF, and VE2AF groups. In a similar vein, liver tissue leptin levels of the AFTB1 group were significantly decreased ($p < 0.05$) compared to the CONT group. In contrast, leptin levels in the VE1AF and VE2AF showed no significant difference ($p < 0.05$) compared to the CONT. Moreover, the liver 8-hydroxy-2'-deoxyguanosine levels of AFTB1 were elevated ($p < 0.05$) compared to those in the CONT, while the levels in the VE1AF and VE2AF groups showed no significant difference ($p < 0.05$) compared to the CONT group.

Table 1.
Antioxidant enzymes and malondialdehyde in the liver of broiler chickens exposed to Aflatoxin B1 and administered *Vernonia amygdalina* aqueous leaf extract

Parameters	CONT	AFTB1	VE1AF	VE2AF	SEM	P value
Catalase (ng/mg)	18.52a	12.88b	17.56a	17.57a	0.78	0.01
Glutathione peroxidase (ng/mg)	18.51a	14.09b	16.53ab	18.39a	0.66	0.02
Superoxide dismutase (ng/mg)	68.42a	53.10b	59.57ab	70.23a	2.52	0.02
Malondialdehyde (u/mg)	2.58b	3.73a	2.47b	2.60b	0.19	0.03

Within the same row, differing superscript letters indicate significant differences ($p < 0.05$). CONT: Control; AFTB1: 0.5 ppm aflatoxin contamination; VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g *Vernonia amygdalina* leaf powder/liter of water; VE2AF: 0.5 ppm aflatoxin B_1 contamination + 2 g *Vernonia amygdalina* leaf powder/liter of water.

Table 2.
Pro-inflammatory cytokines in the liver of broiler chickens exposed to Aflatoxin B1 and administered *Vernonia amygdalina* aqueous leaf extract.

Parameters	CONT	AFTB1	VE1AF	VE2AF	SEM	P value
Tumor necrosis factor α (pg/ml)	6.97b	9.11a	8.08ab	6.95b	0.33	0.02
Interleukin 6 (pg/ml)	9.03b	11.39a	10.26ab	9.53b	0.35	0.04
Interleukin 1 Beta (pg/ml)	11.79	15.16	13.56	12.11	0.55	0.09
Interferon Gamma (pg/ml)	1.31b	1.56a	1.45ab	1.36b	0.03	0.04

Within the same row, differing superscript letters indicate significant differences ($p < 0.05$). CONT: Control; AFTB1: 0.5 ppm aflatoxin contamination; VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g *Vernonia amygdalina* leaf powder/liter of water; VE2AF: 0.5 ppm aflatoxin B1 contamination + 2 g *Vernonia amygdalina* leaf powder/liter of water.

Table 3.
Anti-inflammatory cytokine, adipokine, and oxidative DNA damage biomarkers in the liver of broiler chickens exposed to Aflatoxin B1 administered *Vernonia amygdalina* aqueous leaf extract.

Parameters	CONT	AFTB1	VE1AF	VE2AF	SEM	P value
Adiponectin (ng/ml)	12.45a	8.66b	11.45a	11.70a	0.53	0.03
Leptin (ng/ml)	1.55a	1.29b	1.44ab	1.52ab	0.03	0.04
8-hydroxy-2' –deoxyguanosine (ng/ml)	12.40b	16.32a	14.24ab	12.70b	0.59	0.04

Within the same row, differing superscript letters indicate significant differences ($P < 0.05$). CONT: Control; AFTB1: 0.5 ppm aflatoxin contamination; VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g *Vernonia amygdalina* leaf powder/liter of water; VE2AF: 0.5 ppm aflatoxin B1 contamination + 2 g *Vernonia amygdalina* leaf powder/liter of water.

The Relative Growth Rate

The Relative Growth Rate (RGR) of broiler chickens exposed to Aflatoxin B1 (AFB1) contamination and treated with *Vernonia amygdalina* aqueous leaf extract showed variation among the different experimental groups (Figure 1). Broilers in the CONT had a similar ($p < 0.05$) RGR to the VE1AF (0.5 ppm AFB1 + 1 g/L *V. amygdalina*) and VE2AF 0.5 ppm AFB1 + 2 g/L *V. amygdalina*). The broiler chickens in the AFTB1 group (0.5 ppm AFB1 contamination without supplementation) had the lowest RGR compared to those in CONT, VE1AF, and VE2AF.

Discussion

Catalase is an important antioxidant enzyme responsible for catalyzing the breaking down of hydrogen peroxide into oxygen and water, thereby shielding cells from oxidative harm. The notable decrease observed in liver catalase presence within the AFTB1

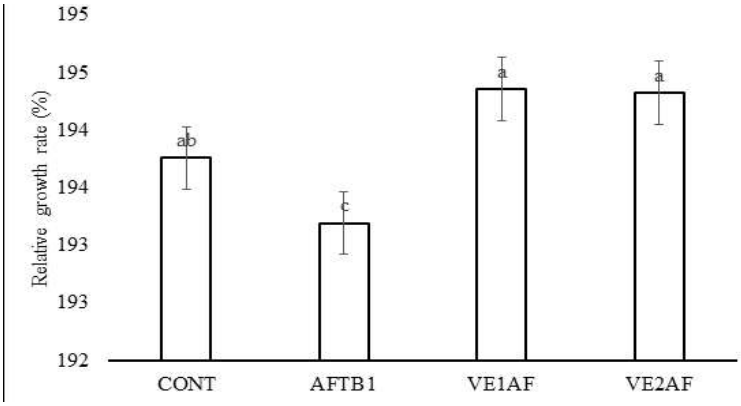

Figure 1.
Relative growth rate of broiler chickens exposed to Aflatoxin B1 and administered *Vernonia amygdalina* aqueous leaf extract.
CONT: Control; AFTB1: 0.5 ppm aflatoxin contamination; VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g V*ernonia amygdalina* leaf powder/litre of water; VE2AF: 0.5 ppm aflatoxin B1 contamination + 2 g *Vernonia amygdalina* leaf powder/litre of water.

group, as opposed to both the CONT and Vernonia amygdalina-treated groups, implies that exposure to AFB1 could potentially compromise antioxidant defense mechanisms, likely as a result of heightened oxidative stress. AFB1 is known to stimulate the reactive oxygen species (ROS) production within the liver [1, 2], which can overwhelm the antioxidant capacity of catalase, leading to its depletion [8]. However, the administration of Vernonia amygdalina aqueous leaf extract may help alleviate this oxidative stress by providing additional antioxidant compounds that can scavenge ROS and support the activity of catalase [9]. Glutathione peroxidase and superoxide dismutase play crucial roles as enzymes with antioxidant properties that scavenge reactive ROS and shield cells from oxidative harm. The significant decrease in liver glutathione peroxidase and liver superoxide dismutase levels in the AFTB1 compared to the control (CONT) and VE2AF groups suggests a compromised antioxidant defense system in response to AFB1 exposure. This could be attributed to the depletion of antioxidant reserves or inhibition of enzyme activity by AFB1-induced oxidative stress. Interestingly, the resemblance in enzyme levels between the AFTB1 and VE1AF groups suggests that administration of Vernonia amygdalina aqueous leaf extract, particularly at the lower concentration, may partially mitigate the decrease in glutathione peroxidase and superoxide dismutase levels induced by AFB1 [10]. Vernonia amygdalina is known to contain various active compounds including flavonoids, alkaloids, and phenolic compounds, which possess potent antioxidant properties. These compounds may enhance the antioxidant capacity of the liver and help replenish the levels of glutathione peroxidase and superoxide dismutase in the presence of AFB1-induced oxidative stress [7]. These bioactive compounds present in Vernonia amygdalina leaf extract could scavenge free radicals and reactive oxygen species generated by aflatoxin B1 exposure, and could also trigger the production or activation of antioxidant enzymes like catalase, glutathione peroxidase, and superoxide dismutase in the liver mediated through stimulation of transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), which governs the expression of antioxidant enzymes and other protective genes within the cell [11; 12].

Malondialdehyde (MDA) is a marker of lipid peroxidation and oxidative damage. The unexpected decrease in liver MDA levels in the AFTB1 compared to the other groups is intriguing. One possible explanation could be the activation of compensatory antioxidant mechanisms in response to AFB1-induced oxidative stress [13]. It is plausible that the administration of Vernonia amygdalina aqueous leaf extract, particularly at higher concentrations, may stimulate the synthesis or enhance the activity of antioxidant enzymes and other defense mechanisms, leading to the reduction of lipid peroxidation and MDA formation in the liver [14]. Additionally, Vernonia amygdalina contains bioactive substances including sesquiterpene lactones and flavonoids, which have been documented to exhibit both anti-inflammatory and antioxidant characteristics. These compounds may directly scavenge free radicals and inhibit lipid peroxidation, contributing to the observed decrease in liver MDA levels [15].

Tumor Necrosis Factor α (TNF-α), Interleukin 6 (IL-6), and Interferon Gamma (IFN-γ) are cytokines known for their pro-inflammatory effects that play crucial roles in facilitating inflammatory reactions [16]. The notably elevated levels of these cytokines in the livers of broilers in the AFTB1, in comparison to the CONT and VE2AF groups, suggest a robust inflammatory reaction triggered by Aflatoxin B1 contamination. Aflatoxin B1 is known to trigger inflammation in the liver by activating NF-κB and other inflammatory signaling pathways [17]. The observed elevation in TNF-α, IL-6, and IFN-γ presence corroborates previous findings linking Aflatoxin B1 exposure to hepatic inflammation. Administration of Vernonia amygdalina aqueous leaf extract appeared to counteract the rise in pro-inflammatory cytokine levels caused by Aflatoxin B1 contamination [6]. The concentrations of TNF-α, IL-6, and IFN-γ in the CONT were similar to those in the VE1AF group, suggesting a potential safeguarding impact of Ver-

nonia amygdalina against inflammation triggered by Aflatoxin B1. Vernonia amygdalina contains various bioactive compounds, such as flavonoids, alkaloids, and phenolic compounds, known for their anti-inflammatory properties. These compounds may modulate inflammatory signaling pathways and attenuate the production of pro-inflammatory cytokines in response to AFB1 exposure [7].

While not reaching statistical significance ($p = 0.09$), there was an observed trend indicating a potential impact of treatment on liver Interleukin 1 Beta (IL-1β) levels. Interleukin 1 Beta is another crucial pro-inflammatory cytokine known for its involvement in regulating immune responses and inflammatory pathways. The observed trend suggests a potential influence of AFB1 contamination and Vernonia amygdalina administration on IL-1β levels, warranting further investigation to elucidate its significance.

Adiponectin is known for its anti-inflammatory and insulin-sensitizing properties [18]. The significant decrease in liver adiponectin levels in the AFTB1 treatment group suggests a disruption in adiponectin signaling induced by AFB1 contamination. This disruption could contribute to metabolic dysfunction and inflammation in the liver [19]. Vernonia amygdalina is rich in bioactive compounds, which have demonstrated anti-inflammatory properties. These compounds may help restore adiponectin levels by modulating inflammatory pathways and improving insulin sensitivity [20]. Additionally, Vernonia amygdalina has been reported to enhance glucose metabolism and insulin sensitivity in various animal models [21], further supporting its potential to counteract the effects of Aflatoxin B1-induced adiponectin dysregulation.

Leptin plays a vital function in regulating energy equilibrium and metabolism [22]. A notable decrease in liver leptin levels in the AFTB1 treatment group suggests a disruption in leptin signaling induced by AFB1 contamination. Leptin resistance and decreased leptin levels are commonly observed in liver diseases, including those induced by aflatoxicosis [23]. Vernonia amygdalina contains bioactive compounds like flavonoids and sesquiterpene lactones, which have demonstrated the ability to regulate leptin signaling and enhance leptin sensitivity. [24]. These compounds may help restore leptin levels and mitigate the adverse effects of AFB1 on energy metabolism and lipid homeostasis in the liver.

8-Hydroxydeoxyguanosine is a biomarker for oxidative DNA damage, reflecting elevated hepatic oxidative stress [25]. The significant increase in liver 8-OHdG levels in the AFTB1 group indicates oxidative DNA damage induced by AFB1 contamination. Vernonia amygdalina is abundant in antioxidant compounds like phenolic acids, flavonoids, and tannins, which exhibit strong capabilities in scavenging free radicals. These compounds may help mitigate oxidative stress and prevent DNA damage by neutralizing reactive oxygen species (ROS) generated by AFB1 exposure [26]. Moreover, Vernonia amygdalina has been shown to boost the activity of endogenous antioxidant enzymes like superoxide dismutase and catalase [27], thereby reinforcing its protective effects against AFB1-induced oxidative DNA damage. Vernonia amygdalina leaf extract comprises a diverse array of bioactive compounds encompassing alkaloids, flavonoids, phenolics, and terpenoids, which jointly contribute to its potential to prevent hepatic DNA damage in broilers exposed to aflatoxin B1 by scavenging free radicals, inhibiting lipid peroxidation, and reducing inflammation, thereby mitigating oxidative stress [17]. Additionally, some compounds may chelate metal ions involved in reactive oxygen species generation, further reducing oxidative damage. These bioactive compounds may also directly interact with DNA, stabilizing its structure and preventing damage [28]. Together, these mechanisms help protect DNA molecules in the liver from the harmful effects of aflatoxin B1 exposure, making Vernonia amygdalina leaf extract a promising candidate for preventing hepatotoxicity and carcinogenesis in broiler chickens.

Vernonia amygdalina leaf extract demonstrates potential as a protective agent against aflatoxin-induced hepatoxicity in broiler chickens. Supplementation with the extract effectively modulates antioxidant enzyme activity, pro-inflammatory cytokine levels, and oxidative DNA damage biomarkers, mitigating liver damage associated with aflatoxin exposure. However, further research is needed to optimize dosage regimens and evaluate long-term effects in commercial poultry production. Incorporating Vernonia amygdalina supplementation may offer a promising strategy to enhance liver health and welfare in broiler production systems.

Broilers in the AFTB1 group exhibited the lowest RGR, which aligns with previous studies demonstrating that aflatoxins negatively impact growth performance in poultry [5]. Aflatoxin B1, the most toxic aflatoxin, exerts its adverse effects primarily through hepatic dysfunction, oxidative stress, and immunosuppression [2]. Upon ingestion, AFB1 undergoes bioactivation in the liver via cytochrome P450 enzymes, leading to the formation of the reactive metabolite Aflatoxin B1-8,9-epoxide (AFBO), which forms adducts with DNA and proteins, thereby impairing cellular function and protein synthesis [29]. Consequently, broilers exposed to AFB1 suffer from reduced feed intake, poor nutrient absorption, hepatic damage, and suppressed immune respons-

es, all of which contribute to growth retardation [2, 30]. The significant reduction in RGR observed in the AFTB1 group compared to the CONT group confirms the growth-depressing effect of aflatoxin B1 contamination [2, 30]. A key observation in this study was the restorative effect of *V. amygdalina* supplementation. Broilers in the VE1AF (0.5 ppm AFB1 + 1 g/L *V. amygdalina*) and VE2AF (0.5 ppm AFB1 + 2 g/L *V. amygdalina*) groups exhibited improved RGR compared to the AFTB1 group, suggesting that *V. amygdalina* may serve as a natural detoxifier against aflatoxin-induced growth suppression. The ameliorative effect of *V. amygdalina* can be attributed to its rich phytochemical profile, which includes flavonoids, alkaloids, phenols, saponins, tannins, and terpenoids [6, 31]. The observation that the VE2AF group had the highest relative growth rate (RGR) indicates that a higher dosage (2 g/L) of *V. amygdalina* offered a more significant protective effect compared to the lower dosage (1 g/L). This dose-dependent response aligns with previous research showing that greater concentrations of *V. amygdalina* extract lead to improved antioxidant capacity and metabolic function in animals exposed to toxins [6].

Materials & Methods

Ethical Approval and Experimental Site

The protocol for animal care and use received approval from the Animal Welfare and Use Committee in the Department of Animal Science at Adekunle Ajasin University, located in Akungba Akoko, Nigeria [2]. The research was conducted at the Avian Experimental Unit located within the Teaching and Research Farm at Adekunle Ajasin University, Akungba Akoko, Nigeria [2]. The feeding trial took place during the dry season, specifically between December 2023 and January 2024.

Preparation of Vernonia amygdalina Leaf powder

Fresh Vernonia amygdalina leaves were harvested from their parent plants, within the premises of the Crop Production Unit of Adekunle Ajasin University Teaching and Research Farm, Akungba Akoko, Nigeria. The leaves were washed with clean water, drained, sliced into smaller segments, and laid out on polythene sheets to air-dry under shade for a period of 7 days. Following drying, the leaves were finely ground into Vernonia amygdalina leaf powder using an electric blender, and subsequently stored in an airtight container until required for use.

Aflatoxin B1, Experimental Diet Composition, and Aqueous Extract Preparation

Aflatoxin B1 (AFB1) was produced by growing Aspergillus flavus on coarse maize meal and quantified for AFB1 levels utilizing a high-performance liquid chromatography (HPLC) system, comprising a model 600 pump, model 470 scanning fluorescence detector, 717 autosampler, and in-line degasser [32]. Standard diets for the starter (1-21 days) and finisher (22-42 days) stages of broiler production were formulated [30, 33] and are presented in Table 4. Each phase's diets were divided into four equivalent shares (named treatments 1 to 4). The first portion remained uncontaminated with aflatoxin B1, while the remaining portions (2, 3, and 4) were contaminated with aflatoxin B1 to a level of 0.5 ppm, following the procedures described by Olarotimi et al. [6].

One gram (1g) of *Vernonia amygdalina* leaf powder was immersed in one liter of warm water (70°C) for 24 hours, and this procedure was repeated daily. The preparation was subsequently filtered using a muslin cloth to segregate debris from the filtrate, resulting in the production of 1g/liter *Vernonia amygdalina* aqueous extract (VE1), which was stored in clean containers. The same procedure was employed to produce 2g/liter *Vernonia amygdalina* aqueous extract (VE2).

Experimental Birds and Treatments

The present investigation was conducted using 240 one-day-old chicks (mixed sex) from the Cobb 500 broiler breed. The day-old broiler chicks were randomly allocated into four distinct experimental treatment groups, with each group comprising 60 birds (6 replicates per treatment, with 10 birds per replicate). Birds in the first treatment group (CONT) were fed an uncontaminated diet, while those in the remaining treatment groups (AFB1, VE1AF, and VE2AF) were exposed to 0.5 ppm of aflatoxin B1 in their feed. Additionally, birds in treatment groups 3 and 4 were orally administered VE1 and VE2, respectively. The treatment details are summarized as follows:

- CONT: No aflatoxin B1 contamination; no administration of Vernonia amygdalina extract.
- AFB1: 0.5 ppm aflatoxin B1 contamination.
- VE1AF: 0.5 ppm aflatoxin B1 contamination + 1 g *Vernonia amygdalina* leaf powder per liter of water.
- VE2AF: 0.5 ppm aflatoxin B1 contamination + 2 g *Vernonia amygdalina* leaf powder per liter of water.

The ambient conditions within the experimental enclosure were meticulously regulated throughout the observation period. For the initial 7 days of the feeding experiment, the temperature in the experimental enclosure was upheld at 31 ± 2°C. Subsequently, from day 8 to day 27 of the study, the ambient temperature was gradually decreased by 2°C per week. During the final phase of the rearing period, from day 28 to day 42, the broiler chickens were subjected to the natural ambient temperature of their environment.

Additionally, at the beginning of the feeding trial, the illumination in the experimental enclosure followed a daily pattern comprising six hours of darkness over the course of each 24-hour period. The illumination regimen remained consistent for up to 3 days before culling.

Sample collection and analysis

On the 42nd day of the trial, eighteen birds were chosen randomly from each treatment (one bird per replicate) and euthanized. Following euthanasia, the carcasses underwent spray-washing and were subsequently cooled for 30 minutes at 2°C. The livers of the selected broiler chickens (three per replicate) were then excised for hepatotoxicity studies immediately after slaughter. The liver tissue was collected using a sharp, sterile knife and rinsed with a cold phosphate-buffered solution to eliminate any blood or debris.

Liver tissue homogenates were prepared by homogenizing 20% liver samples from each broiler chicken at 4°C in 0.15 M KCl solution. Subsequently, the homogenates underwent centrifugation at 12,000 rpm for 45 minutes at 0 to 4°C to obtain the supernatant, as described by Venkatanarayana et al. [33]. The supernatants were subjected to a variety of analyses as outlined hereafter:

Malondialdehyde (MDA), glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) levels were quantified according to methods previously described by Jimoh [34]. To measure SOD activity, 2.1 ml of 50 mM buffer, 0.02 ml of enzyme source, and 0.86 ml of distilled water were combined to create a reaction mixture. Adding 0.02 ml of 10 mM pyrogallol initiated the reaction, and the change in absorbance was monitored at 420 nm. In a conventional assay setup

with three milliliters, one unit of SOD activity is equivalent to the quantity of enzyme required to reduce the auto-oxidation of pyrogallol by fifty percent. SOD's specific activity is measured in units per minute per protein nanogram. The test setup consisted of 1.0 ml of 0.059 M H_2O_2 and 1.9 ml of 0.05 M buffer at pH 7.0 to assess catalase activity. The enzyme source was added in an amount of 0.1 ml to initiate the reaction. For five minutes, the absorbance drop was observed at 240 nm at one-minute intervals. The amount of H_2O_2 that is broken down into nanomoles per minute per nanogram of protein is the catalase activity. A reaction mixture containing 0.5 ml of 0.4 M buffer at pH 7.0, 0.2 ml of enzyme source, 0.2 ml of 2 mM GSH, and 0.1 ml of 0.2 mM H_2O_2 was created to assess glutathione peroxidase activity. After that, this combination was incubated for ten minutes at room temperature. Additionally, a control tube was created with all the reagents removed, save for the enzyme source. After adding 0.5 ml of 10% TCA and centrifuging for five minutes at 4000 rpm, the reaction was stopped. Next, the amount of glutathione (GSH) in 0.5 milliliters of the supernatant was calculated. Per milligrams of protein, glutathione peroxidase activity is reported as micrograms of GSH used per minute. Preparing a reaction mixture with a total volume of 3.0 ml, comprising 1.0 ml of serum and 1.0 ml of TCA (0.67%), was necessary for the lipid peroxidation assay. After that, each test tube spent 45 minutes submerged in boiling water. The tubes were then placed in a cold bath and centrifuged for ten minutes at 2500 rpm. A measurement of the optical density of the supernatant at 532 nm was used to calculate the amount of malondialdehyde (MDA) generated in each sample.

Utilizing a Rat IL-6 ELISA kit from Elabscience Biotechnology Inc., USA, interleukin 6 (IL-6) levels were determined. The levels of tumor necrosis factor-alpha (TNF-α) were measured with an ELISA kit from Elabscience Biotechnology Inc. in the United States. Using the Quantikine Human IFN-γ Immunoassay from R&D Systems, Minneapolis, MN, USA, interferon-gamma (IFN-γ) levels were ascertained. The Human IL-1 ELISA MAX Deluxe kit from BioLegend, San Diego, CA, USA, was used to measure the quantities of interleukin-1 beta (IL-1β). The lipHsp70 ELISA technique, as reported by Breuninger et al. [35], was utilized to measure the amounts of heat shock protein (HSP 70) present in the liver. An ELISA kit from Millipore, USA, was used to measure the levels of serum adiponectin. The method outlined by Zhang et al. [36] was used to assess the amounts of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Utilizing the Human Leptin ELISA kit from Abcam, Shanghai, China, the concentration of leptin was ascertained. The Human NFKB-p65 ELISA Kit from Elabscience, USA, was used to measure the levels of Nuclear Factor Kappa B-p65 (NFKB-p65).

At the beginning of the feeding trial (Day 1) and at the conclusion of the study (Day 42), the body weights of the broiler chicks were measured and documented. The Relative Growth Rate (RGR) was computed based on the formula previously outlined by Oloruntola et al. [2]:

$$RGR = [(wt2 - wt1) / ((wt1 + wt2) / 2)] * 100$$

Where Wt_1 represents the initial body weight of the broiler chicks before the commencement of the experiment, and Wt_2 corresponds to their final weight at the end of the study.

Table 4.
Components of the test diets

Ingredients (%)	Starter Phase (1- 28 days)	Finisher phase (28 – 42 days)
Maize	51.35	56.35
Maize bran	3.00	5.92
Rice bran	0.00	2.00
Fish meal	3.00	2.90
Soybean meal	37.00	26.95
Bone meal	3.00	3.10
Premix	0.31	0.31
Limestone	0.49	0.50
Salt	0.31	0.32
Lysine	0.24	0.25
Methionine	0.30	0.30
Soy oil	1.00	1.10
Composition (%)		
Metabolizable energy (Kcal/kg)	2920	3051
Available phosphorus	0.58	0.36
Calcium	0.94	0.74
Crude fibre	3.52	3.57
Crude fat	4.23	2.38
Crude protein	22.00	19.00
Methionine	0.48	0.45
Lysine	1.25	1.04

Statistical data analysis

SPSS v.20 software was used to do an analysis of variance (ANOVA) on the data. The Duncan multiple range test, which was included in the same program, was then used to see if the treatment means differed significantly from one another.

Authors' Contributions

O.D.O., S.A.A., and F.S.O. conceived and planned the experiments. F.O.S., D.A.O., and E.K.A. produced the aflatoxin B1 cultured maize. O.J.O., A.B.F., O.E.A., and O.A.A. contributed to the preparation of samples for analysis. O.D.O., F.O.S., D.A.O., and E.K.A. contributed to the laboratory analysis of samples. All authors carried out the experiments and contributed to the interpretation of the results. O.D.O. took the lead in writing the manuscript, while E.K.A. revised the first draft. All authors reviewed the final manuscript.

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Conflict of interest

The authors declare that there is no conflict of the interest

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Tylosin Residue in Chicken: Detection with ELISA, Four Plate Test, HPLC, Effect of Heat Treatment and implications for Human Health

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ABSTRACT

Tylosin residues (TR) in chicken meat pose potential health risks to consumers. This study aimed to detect and quantify TR in chicken tissues from Ikpa slaughterhouse, Nsukka and evaluate the effect of heat treatment on TR concentrations. Sixty randomly sampled chicken were processed, and their muscle, liver, and kidney tissues were collected and tested for TR at raw and after ten, fifteen , and twenty minutes of cooking and microwaving using enzyme-linked immunosorbent assay (ELISA), four plate test (FPT), and high-performance liquid chromatography (HPLC). Of the 180 tissues, 93 (51.7%) were positive for TR. The prevalence of TR was 40% in muscles, 55% in liver, and 60% were in kidney samples with ELISA. Six liver samples exceeded the maximum residue level (MRL) of100 µg/kg. Cooking and microwaving reduced TR concentrations by 97-100% in muscle and liver tissues using HPLC. The mean inhibition zones decreased by 87-100% after cooking and microwaving using FPT. Chicken at Ikpa slaughterhouse, Nsukka have TR even in concentrations above the MRL but were significantly eliminated ($p < 0.05$) after 20 minutes of heat treatments (cooking or microwaving). Hence, mitigating the health risks associated with TR in meat requires regular screening and quantification, public awareness campaigns targeting consumers of raw or improperly cooked chicken, strict policies on antibiotics use in poultry, and enhanced meat handling and processing practices in food industry.

Keywords

Tylosin Residue Analysis, Chicken, Cooking, Microwaving, ELISA, Four Plate Test, HPLC, Food Safety

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Abbreviations

ELISA : enzyme-linked immunosorbent assay
FPT : four-plate test
HPLC : high-performance liquid chromatography
MRL : maximum residue level
CRD : chronic respiratory disease

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Introduction

Chicken is the second most widely produced, exported, and consumed meat worldwide [1, 2]. It has contributed significantly to the supply of about 40% of protein needed as demanded by the increasing human population globally [3]. However, the modern-day integrated and intensive production system has been associated with the unwarranted use and misuse of antimicrobials in preventing disease occurrences and as growth promoters for poultry in compounded animal feed [4]. Tylosin is a 16-membered macrolide approved for therapy for a variety of infectious disease agents, including Mycoplasma gallisepticum and M. synoviae, which causes chronic respiratory disease (CRD) in poultry. They are metabolized in the liver, where the highest tissue concentrations are found, especially in chickens and turkeys [5]. TR should not be detected in the edible tissue of treated birds and other products of animal origin in concentrations exceeding the recommended MRL of 100µg/kg [6]. Hence, the recommendation that chickens should not be slaughtered for human consumption 6 days after the last oral tylosin administration [7]. The excess of the residues in meat above MRL could pose high toxicological, microbiologically, or immuno-pathological damage to the consumers of contaminated meat hence the need to effectively prevent and reduce the TR occurrence in meat for human consumption.

Chicken is usually cooked or roasted ("suya") with or without food additives to increase taste, shell life, digestibility, and other sensory properties thereby making them appetizing to the consumers [8]. Nevertheless, the knowledge of the reductive impact of cooking or any other thermal processing methods on TR in heat-and-serve meat is still very limited [9]. There has been a report of an overall reduction rate of TR in meat by 35.3% and 79.9% after 2 and 30 minutes of microwaving and boiling respectively [10]. Meanwhile, other factors, including the concentration of TR in raw meat before processing have been reported to influence the rate of TR reduction in meat [11].

The use of ELISA kit has been reported to have good performance in the analysis of antimicrobial residues like Tylosin in meat as it has the advantages of specificity and sensitivity [12]. It allows the analysis of a large number of samples per kit in a few hrs without the requirement of sophisticated instrumentation unlike the Four Plate Test (FPT) [13]. HPLC on the other hand, quantifies the concentration of the residue in meat unlike FPT [14]. It has more prospects of repeatability, selectivity, resolution, high recovery, and ease of application compared to others [15, 16]. FPT, a microbiological assay, offers a simple and cost effective approach, but may be less sensitive and specific compared to ELISA and HPLC. However, Enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), and other chromatography-mass spectrometry types are generally used in diverse analytical techniques for the detection of antimicrobial residues in foods of animal origin [12, 17, 18]. The respective strength of the three methods was leveraged in this study to ensure a comprehensive and accurate assessment of TR in chicken. Tylosin is indiscriminately used by farmers in the study area while data on the contamination rates in the processed chicken are unavailable in the literature to the best of our knowledge. Hence it has become necessary to constantly monitor and periodically assess the risks of exposure to TR associated with misuse or abuse in poultry production and take apt action to ensure meat safety [19]. Moreover, the detection rates with different tests and effects of thermal meat processing could be validated to guarantee consumers' confidence and meat safety. The study therefore evaluates the concentration of TR in chicken sold to consumers in Nsukka, Nigeria and the impact of heat treatment using ELISA, Four Plate test and HPLC for informed evidence-based strategies for ensuring food safety and mitigating the risk associated with TR and antimicrobial resistance in southeast, Nigeria.

Results

Detection of TR in muscles, kidney, and liver tissues of slaughtered chicken using using ELISA

Out of 180 tissues of sampled birds, a total of 93 (51.7%) was positive for TR, with 24 (40%), 33 (55%), and 36 (60%) of the detected proportion in muscles, liver, and kidney respectively while 6 out of 33 (18%) positive liver samples were above the MRL of 100 µg/kg. However, there was no association between the type of tissue and the occurrence of TR ($x^2 = 5.206$; P = 0.0741) (Table 1).

Effect of cooking and microwaving on TR in muscles and liver tissues of chicken using FPT

The effect of cooking at different times (10, 15, and 20) minutes revealed reduction rates of TR concentration at 28.2%, 64.1%, and 100% with the decreasing mean inhibition zone from 9.75mm to (7, 3.5, and 0 mm) respectively in muscle tissues, and 22.2%, 53.3%, and 100% with the decreasing mean inhibition zone from 11.25mm to (8.75, 5.25, and 0 mm) in the same order of cooking in the liver tissue (Table 2).

On the other hand, the impact of microwaving revealed a 29.5%, 46.3%, and 100% reduction rate after

Table 1.
Detection of Tylosin residue in raw chicken tissues from Ikpa slaughter, Nsukka, using ELISA test

Tissue (60 each)	Frequency (concentration (µg/kg)		Proportion detected (%)	Above MRL 100 µg/kg (%)
	Undetected (≤ 2.0)	Detected (> 2.0)		
Muscle	36	24	40	0 (0)
Liver	27	33	55	6 (18.0)
Kidney	24	36	60	0 (0)
Total	87	93	51.7	6 (6.5)

($x^2 = 5.206$; P = 0.0741). MRL (Maximum Residue Level) WHO (2004)

Table 2.
Effect of cooking versus microwaving on TR concentrations in chicken tissues using FPT and HPLC

Tissues	Mean inhibition zone raw chicken (mm)	Mean inhibition zone (mm) and reduction rate (%) after 10-20 (mins) cooking			Mean inhibition zone (mm) and reduction rate (%) after 10- 20 (mins) of microwaving		
		10	15	20	10	15	20
FPT	Muscle	7.0	3.5	0	6.7	5.1	0
		(28.2)	(64.1)	(100)	(29.5)	(46.3)	(100)
FPT	Liver	8.75	5.25	0	7.75	5.1	1.5
		(22.2)	(53.3)	(100)	(32.6)	(55. 7)	(87)
HPLC	Muscle	16.4	9.6	0.4	22.2	5	0
		(48.1)	(69.6)	(98.7)	(29.7)	(84.2)	(100)
HPLC	Liver	31.8	15.8	0.6	36.6	16.8	1.4
		(33.2)	(66.8)	(99)	(23.1)	(64.7)	(97.1)

10, 15, and 20 mins with the decreasing mean inhibition zones from 9.5mm to (7.75, 5.1, and 1.5 mm), respectively, in the muscle tissue and 32.62%, 55.7% and 87% with decreasing mean inhibition zones of (6.7, 5.1, and 0 mm) in the same order in the liver tissue (Table 2).

Effect of cooking and microwaving on TR in muscles and liver tissues of chicken using HPLC

The use of HPLC revealed a reduction rate of 48.1%, 69.6%, and 98.7% in the TR concentration from 31.6 µg/kg to (16.4, 9.6, and 0 µg/kg) respectively after 10, 15, and 20 minutes of cooking in the muscle tissue in the same order, while 33.2%, 66.8% and 99% reduction in concentration from the initial 47.6 µg/kg to (31.8, 15.8, and 0.6 µg/kg) in the same order of cooking were revealed for the liver tissue. Significant differences exist between contamination rates in raw and cooking of both muscle and liver tissue at 20 mins ($p < 0.05$) (Table 2) (Figs. 1 and 2).

Moreover, the effect of microwaving on TR con-

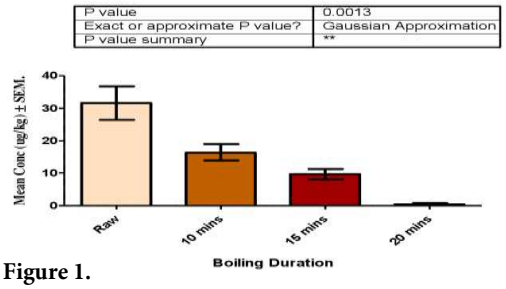

Figure 1.
Mean concentration of Tylosin residue in raw muscle tissue and after different time of cooking.

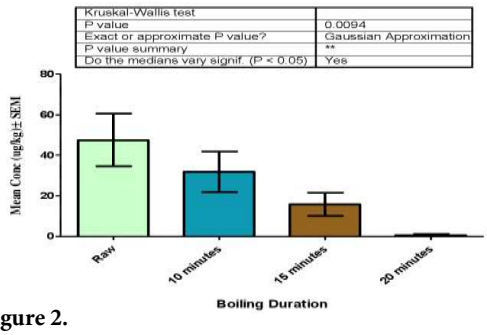

Figure 2.
Mean concentration of Tylosin residue in raw liver tissue and after different time of cooking.

centration reduction using HPLC also revealed 29.7%, 84.2%, and 100% reduction rate with corresponding values from 31.6 µg/kg to (22.2, 5 and 0 µg/kg) after 10, 15, and 20 mins of microwaving respectively in the muscles tissue and 23.1%, 64.7% and 97.1% with corresponding values from 47.6 µg/kg to 36.6, 16.8 and 1.4 µg/kg in the liver tissues in the same order (Table 2). Statistically significant differences exist between the raw and after 20 mins microwaving of both muscle and liver tissues ($p < 0.05$) (Figs. 3 and 4).

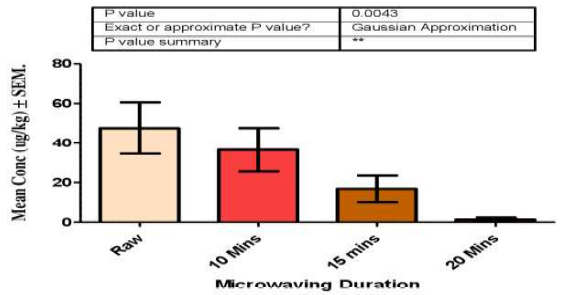

Figure 3.
Mean concentration of Tylosin in raw liver tissue and after different time of microwaving.

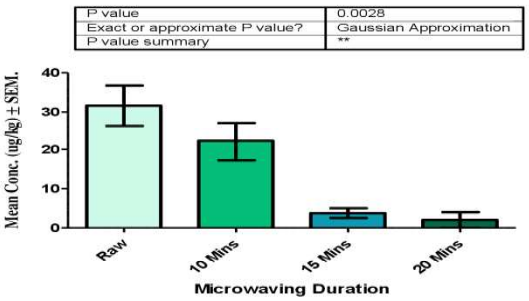

Figure 4.
Mean concentration of Tylosin in raw muscle tissue and after different time of microwaving.

Discussion

The contamination rate of TR in slaughtered chicken at 51.7% is of public health concern, especially with the values above the MRL in the liver tissues. Humans especially the consumers of such muscle parts and liver of contaminated chicken are at the risk of the many chronic health challenges which have been associated with the TR accumulation in the body system [23]. The residue is known to interrupt the colonization barrier of the gastrointestinal tract in humans because of its antibiotic activities against bacterial strains in the human colonic flora [23, 24]. This could lead to antibiotic resistance development and resistant gene transfer especially when such contaminated chicken is not properly processed via cooking or other thermal heat methods before consumption [25]. The effect is equally of economic consequences with regards to resistant or difficult-to-treat infections, high cost, and longer duration of medication or stay at the hospitals [26]. The health implications of the findings in this study could involve a wider range of consumer populations as the meat from Ikpa slaughterhouse, Nsukka, Enugu State, Nigeria are usually transported to the neighboring states of the country including Kogi and Benue. The chicken meat are processed, and consumed in the form of pepper soup by the street meat vendors or as roadside ready-to-eat meat 'suya' joints [27]. The accumulation of TR in chicken as revealed in the study could be a result of constant abuse of the drug in poultry production in Nsukka ranging from wrong dosage, wrong route of administration, non-adherence to the withdrawal minimum period of 3 days before slaughtering[11, 28]. There is enough evidence that the majority of the farmers are ignorant of the consequences of antibiotic misuse or abuse in poultry in the Nsukka area in particular and Nigeria at large [29, 30].

The detected concentration (51.7%) of the TR using the ELISA test, serves as a true reflection of the contamination rate in the study area since it screened tylosin, specifically not macrolids. The observed rate is higher than 6.3% in meat samples in Croatia using the same ELISA test [31]. Furthermore, the detection rate of TR at 40% in the muscle in this study was slightly lower than 50.6 % in chicken breast meats as reported in Oman using the ELISA method [12]. The differences may be a reflection of differences in the level of exposure of poultry to tylosin or abuse by farmers in developing countries, where farmers easily assess drugs over the counter without prescription, compared to the developed countries where restrictions on drug use are fully implemented. The maximum Residue Limit (MRL) is the maximum concentration of drug residue like tylosin permitted in food products including chicken. It was established in accordance with international standards to guarantee food products safety for human consumption and to protect public and environmental health. The higher distribution of the residue in the liver tissues with values above the MRL was in agreement with the report of Pavlov et al. (2008) that recorded higher residues in kidney and liver tissues, and this could be attributed to the biotransformation and detoxification actions of the liver with slower depletion rate when compared with the muscle tissues [32]. This further agrees with the JECFA (2006) report of positive meat samples with higher TR in the liver and kidney than in muscle tissues [33]. However, the estimated daily intake of muscle tissues is more than that of liver and kidney, thereby making muscle tissues a more important risk assessment point for TR effect in humans. The 18% rate of TR exceeding MRL as found in the study, was lower than 47.83% in

chicken meat with the use of high-performance liquid chromatography method [34]. Meanwhile, the range of tylosin concentration in the study (31.6-47.7) µg/kg was within the average amount of tylosin (38.8) µg/kg reported in China even though it was in swine and bovine tissue samples (muscle, liver, kidney) using LC-MS/MS [35]. On the other hand, very high values (105.4-109.2) µg/kg in 2 (0.6%) of 300 chicken meats have been reported using the HPLC method [36].

The effect of the heat treatments on the TR, as detected using FPT, has revealed total elimination in both the muscle and liver tissues after boiling for 20 minutes compared to 87% and 100% in muscle and liver tissues after 20 minutes of microwaving. This was in agreement with the effect of the cooking process that significantly reduced the TR in both thermal processing procedures with negative correlations between the length of cooking time and the reduction percentage of tylosin using HPLC [10]. However, it disagrees with the overall effect of cooking time on TR reduction in meat, which was reported to significantly decreased after cooking but not microwaving [37]. This may be because of the longer microwaving time of 20 minutes in this study, as opposed to the two minutes in the reported study. A lower reduction rate of 35.3% has also been reported for TR after two minutes of meat microwaving [11]. Limited education and training, easy access to antibiotics in the country, absence of regulations and enforcement of antibiotic use by poultry farmers and lack of oversight by veterinarian may have contributed to the misuse or overuse of tylosin.

Limited monitoring and surveillance of antibiotic residues in poultry products may also have played a role in addition to economic pressure to maximize production and make profit. The reductive effects of cooking and microwaving were further confirmed with the detection of the TR concentration in the tissues using HPLC, which also recorded similar reduction rates with significant impact after microwaving at 20 minutes [38]. The use of both tests has shown that raw muscles and liver with a high concentration of TR have a time-dependent reduction rate when cooked or microwaved. The 100% significant reductions in the concentration of TR for both muscle and liver tissues over time between the raw, cooked, and microwaved chicken were in agreement with work done by Salaramoli et al. (2015), who also recorded a significant reduction in TR in chicken meatballs subjected to microwaving and boiling treatments [10]. Furthermore, other similar studies have reported a 90-100% reduction of antibiotic residues including that of ciprofloxacin, oxytetracycline, and sulfamethazine in meat tissues using HPLC [11, 39]. It has been reported that the residue levels in meat tissues following heat treatment of different cooking methods and time reduced in the tissues but increased in the juiced water, however, the level in the juiced water was not checked in this study and can be a limitation.

As a result of abuse or misuse of Tylosin, and not complying with the withdrawal period in treated poultry, TR was found in slaughtered chicken tissues from Ikpa slaughterhouse even in concentrations above the MRL, hence it constitutes a health risk to the consumers. Awareness campaigns on the health implications of TR in ready-to-eat meat, judicious use of antibiotics, adherence to withdrawal time before slaughter, and the use of probiotics as alternatives in poultry production have become inevitable. It has been shown that 20 minutes of cooking or microwaving significantly eliminates TR in meat. Therefore, adequate heat application on meat, either by cooking or microwaving, should be encouraged, especially for ready-to-eat meat products. Further studies to check the concentration of the TR in the juice (broth) of the cooked chicken should be encouraged since consumers also drink the juice of cooked meat in different forms.

Materials & Methods

Ethical approval

The protocol for the research project was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, with reference no: FVM-UNN-IACUC-2024-03/147.

Study area and sample collection

The study area is Nsukka, while the sample collection site was Ikpa slaughterhouse (Fig. 5) [20]. The Ikpa slaughterhouse was visited twice a week, and on each visit, a 1 in 4 systematic random sampling technique was used to select three out of 12 to 15 birds from poultry retailers that bring birds for slaughter from different towns within Nsukka environs, including the university community. Two birds were selected using a simple random sampling technique from each selected retailer. In each visit, six birds were purchased, i.e., 12 birds per week for 5 weeks. A total of 60 birds were purchased and slaughtered; the breast muscles, kidney, and liver tissues were harvested. A total of 180 tissue specimens were collected using sterile universal bottles and transported in cold conditions, which were maintained with ice blocks to the Department of Veterinary Public Health and Preventive Medicine, University of Nigeria, Nsukka laboratory for analysis.

Sample preparation for TR detection

Two grams of each of the harvested tissues was weighed, macerated, and emulsified with an equal volume of distilled water in a 1:1 ratio and introduced into centrifuge tubes. The tubes were centrifuged at 5000 rpm for 10 mins, and the supernatant was decanted while the required quantity of the solution was used for Tylosin detection.

Detection of TR with ELISA test

The microtiter plates of the ELISA test kit and the reagents were sourced from Shenzhen Lvshiyuan Biotechnology Company Lim-

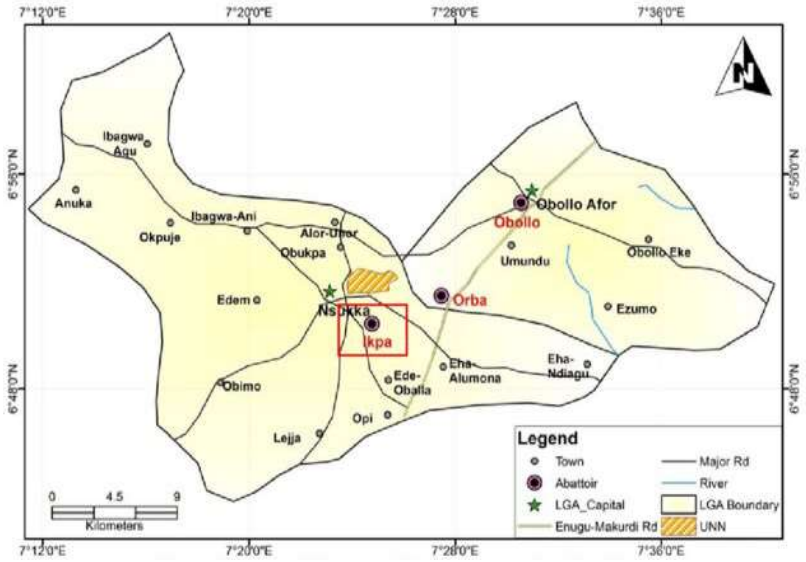

Figure 5
Map of Nsukka urban with blue rectangle showing the study site (Ikpa slaughter). Source: Nwankwo *et al.* (2021).

ited, Shenzhen, China. The microtiter plates, and the reagents were adjusted to room temperature before use. The lyophilized conjugate was reconstituted first with 1ml of conjugate diluents, vortexed, and diluted with the same conjugate diluents at a 1:10 ratio. The standards and the control were reconstituted with 1 ml of deionized water. The wash buffer (5X) concentrate was diluted at a ratio of 1:5. For each plate, a working scheme was prepared, and the standards and samples were run in duplicates as previously reported [21] and briefly described. Twelve strips, each containing 8 wells, were fixed on the plate. Each of the six provided 50 µl standard solutions (0, 2, 6, 18, 54, and 162 ng/kg) was added in duplicate wells according to the working scheme. 50 µl of each tissue extract sample was added in duplicate wells following the standards according to the working scheme. The antibody conjugate (50 µl) was added to each of the wells. The plate (wells) was covered with paraffin tape, and the content was mixed by circular motion on the bench for several seconds and then incubated at 30oC for 30 seconds. It was tapped from time to time to remove bubbles. The microtiter wells were further washed with a wash solution five times and tapped to remove bubbles completely. Solution A (50 µl) color was added, followed by solution B color immediately and mixed thoroughly by shaking. The microtitre plate was incubated at 37oC for 10 minutes. Stop solution (50 µl) was then added. The absorbance was read at 450 nm wavelength within 5 min of adding the Stop solution.

Thermal treatments of ELISA-positive samples

Each positive sample with a high concentration of tylosin after the ELISA test was divided into two parts by weight, and then subjected to different processing methods (boiling and microwaving). Twenty-gram sample each was placed into a strainer, immersed in a 10 ml water bath preheated to 100oC and cooked for 10, 15, and 20 min, and allowed to cool before extraction while the same quantity of sample was placed in a glass tray and microwaved at 450 W for 10, 15, and 20 min and allowed to cool before extraction.

Analysis of raw and heat-treated meat samples using FPT and HPLC

All the raw and heat-treated samples were subjected to FPT and HPLC analysis to determine the residue level using modified methods as previously reported [21, 22].

Four Plate Test: Briefly, two grams of each organ were macerated with an equal volume of sterile water in porcelain mortar and pestle, centrifuged at 3000 revolutions per minute (rpm) in a test tube for 10 minutes after which the supernatant was decanted and stored for analysis. Three batches of Mueller Hinton agar were prepared according to the manufacturer's recommendations and autoclaved. After cooling to 45 – 50oC, they were adjusted to pH 6, 7.2, and 8 using NaOH (base) and HCL (acid). Ten milliliters of the media was poured on sterile Petri dishes and allowed to solidify. Each plate with pH 6, 7.2, and 8 was seeded with a ready-to-use suspension of Bacillus subtilis (Merck Darmstadt, Germany), and another media at pH 8 was seeded with 24-hour culture of Micrococcus luteus bacterial suspension (ATCCR 10240). Four wells were bored, and 80 µl of each tissue extract was inoculated into each of the wells and the fourth well was inoculated with tissue extract from oxytetracycline treated bird as positive control. After, the plates were incubated for 24 hours at 37°C. They were observed for the clear zone of inhibition with an annular diameter ≥ 2 mm, which indicated a positive test for antimicrobial residues.

HPLC-based test: Briefly, tylosin stock solution was obtained from Sigma (St Louis, MO, USA). One mg/ml tylosin was prepared by dissolving 10 mg tylosin tartrate in 10 ml methanol and stored at -18oC. Working standard solutions for the calibration curve were prepared by appropriate diluting of the stock solution, using a dilution factor. The kidney, liver, and muscle samples that were tested and confirmed to be free of macrolide antibiotic residues (control) were used as blanks for the preparation of matrix-matched calibration curves. For fortification, standard solutions were prepared by dissolving a standard substance in methanol at concentrations 40, 20, 10, 5, and 2.5 mg/ml. Two grams each of the kidney, liver, and muscle samples of birds were weighed and macerated with mortar and pestle. 2 ml of distilled water was added, followed by 10 ml HPLC grade acetonitrile. It was then mixed with a vortex mixer to homogenize for 1 minute. Then, the sample was centrifuged for 15 minutes at 3,000 rpm. The clear extracted solvent layer was then collected using disposable pasture pipettes and diluted to 50 ml with distilled water. The SPE Cartridges Bond Elute C18 500 mg/3ml were activated with 2 ml of methanol and 5 ml of distilled water. The cartridge was washed with 20 ml of distilled water and allowed to dry. The extracted sample solution was loaded and allowed to elute from the cartridge with 3 ml of HPLC-grade methanol. The solution was passed through 0.45 µm membrane filter The samples were manually injected into the HPLC. Chromatographic analysis was performed with isocratic elution on Zorbax Eclipse XDB - C18 (150 x 4.6mm, 5 µm) analytical column at 30oC. The mobile phase composed of HPLC grade acetonitrile and water (90:10), at the flow rate of 1.00 ml/min, 20µl was injected. The chromatogram was monitored at wavelength 292 nm.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism statistical software version 5.02. The Chi-square ($\chi 2$) test was used to evaluate the association between TR concentrations with the tissue types. Kruskal–Wallis test was used to compare differences between the mean concentration of the raw values and each of the different cooking and microwaving times. p-value < 0.05 was considered statistically significant.

Authors' Contributions

S.O.O., and E.V.E. conceived and planned the experiments. S.O.O. carried out the experiments. E.V.E., J.A.N., and A.O.A supervised the work. I.O.N, S.O.O and E.V.E. contributed to sample preparation and the interpretation of the results. I.O.N. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of the interest

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Cimetidine-induced Male Reproductive Defects in Piroxicam-induced Gastric Ulcerated Wistar Rats and Their Amelioration by Melatonin

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ABSTRACT

Cimetidine is a known reproductive toxicant due to its adverse effects on testicular function. Melatonin is an antioxidant that has a role in mitigating any disorders that could affect spermatogenesis. Therefore, this study evaluated the ameliorative potential of melatonin on the adverse reproductive effects of cimetidine in piroxicam-induced gastric ulcerated male rats. Thirty rats were randomly divided into six groups (n=5), including normal control (distilled water), ulcerated and non-treated, ulcerated and treated with cimetidine (200mg/kg day), ulcerated and treated with cimetidine and melatonin (10mg/kg), treated with cimetidine and melatonin without ulceration, and ulcerated rats treated with only melatonin. All treatments were done orally per day for 14 days. On the 15th day, rats were sacrificed, and blood samples were collected for hormone and antioxidant assays. Then, the sperm parameters were analyzed according to standard procedures. Co-administration of melatonin to piroxicam-ulcerated rats treated with cimetidine showed a significant increase ($p < 0.05$) in sperm count and epididymal glutathione peroxidase compared to piroxicam-ulcerated rats treated with only cimetidine. Melatonin increases the serum level of Testosterone, FSH, and LH. Melatonin ameliorates the adverse reproductive effects of cimetidine through its antioxidant properties. Therefore, co-administration of melatonin with cimetidine in male ulcer patients is recommended.

Keywords

Antioxidants, Cimetidine, Gastric ulcer, Melatonin, Sperm parameters.

Number of Figures: 4
Number of Tables: 2
Number of References:: 36
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Abbreviations

GU: Gastric ulceration
FDA: Federal Drug Agency
DHT: Dihydrotestosterone
NF: Nuclear Factor
COX-2: Cyclooxygenase-2
NO: Nitric oxide

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Introduction

Gastric ulceration (GU) is the most prevalent gastrointestinal tract condition common among men and animals, with a global mortality rate of 1 out of every 1,000 complications every year [1]. It is a disruption or wound on the epithelial lining of the gastrointestinal tract. It is a benign injury to the mucosal wall of the intestine caused by the action of excessive stomach acid and pepsin on the mucosal epithelium, which can be acute or chronic in duration [2]. Helicobacter pylori infection and the use of nonsteroidal anti-inflammatory drugs are the most common predisposing factors for GUs [3].

Cimetidine is an FDA-approved drug that reduces gastric acid secretion, relieving symptoms such as peptic ulcers, gastroesophageal reflux, and hypersecretory disorders [4]. It works as an H2 receptor antagonist, blocking the action of histamine and inhibiting gastric acid secretion.

In addition to its primary action in reducing gastric acid secretion, cimetidine competitively blocks dihydrotestosterone (DHT) receptors in various tissues, such as the pituitary gland, hypothalamus, and accessory glands, including the prostate and seminal vesicles, thereby impacting male hormone function and causing side effects such as loss of libido and impotence [5]. Cimetidine affects testicular function, reduces testicular weight, alters tubular structure, and causes the loss of germ cells [6]. It primarily targets peritubular cells and induces apoptosis. It can also cause testicular vascular atrophy and damage the testicular microvasculature.

Abbreviations - cont'd

iNOS: Inducible Nitric Oxide synthase
ROS: Reactive Oxygen Species
UERC/FVM: University Ethical Review Committee/Faculty of Veterinary Medicine
DW: Distilled water
ML: Melatonin
U: Ulcerated
UCM: Ulcerated treated with Cimetidine
UCM+ML: Ulcerated treated with Cimetidine and Melatonin
UML: Ulcerated and treated with melatonin
CM+ML: treated with cimetidine and melatonin
SOD: Superoxide Dismutase
CAT: Catalase, GPx: Glutathione Peroxidase
ALP: Alkaline Phosphatase
LDH: Lactose Dehydrogenase
ELISA: Enzyme-Linked Immunosorbent Assay
H&E: Hematoxylin and Eosin
ANOVA: Analysis of Variance
BW: Body weight
MBW: Mean Body Weight
ASM: Abnormal Sperm Morphology
StAR: Steroidogenic Acute Regulatory Protein Factor
SF1: Steroidogenic factor 1.

Furthermore, cimetidine interferes with the nuclear factor (NF)-κB pathway, impacting inflammation, immune responses, and apoptosis [7]. It affects the expression of cyclooxygenase (COX)-2 and inducible nitric oxide (NO) synthase (iNOS), which are involved in the inflammatory process and produce NO and reactive oxygen species, which in turn cause cell death and lipid peroxidation [8].

Melatonin is a hormone produced in the pineal glands of all mammals and is chemically named N-acetyl-5-methoxytryptamine. It is responsible for regulating the circadian rhythm sleep-wake cycle of mammals. Melatonin plays various additional roles, including its ability to mitigate inflammation, inhibit cell proliferation, and promote apoptosis. Furthermore, it modulates the immune system and plays a pivotal role in regulating glucose levels [9].

Melatonin functions in the release of gonadotropins from the anterior pituitary gland and gonads [10]. The antioxidant capacity of melatonin is crucial for preserving testicular function and preventing conditions that impair spermatogenesis. This is due to its ability to reduce the concentration of free radicals (ROS and NO), which can result in oxidative stress and destruction of the testicular epithelium, which is responsible for spermatogenesis. It serves as an antioxidant to improve sperm cell motility and membrane integrity. It also serves as a scavenger for the presence of ROS and NO in spermatocytes [9, 11]. This study was designed to evaluate the corrective potential of melatonin on the adverse reproductive effects of cimetidine in piroxicam-induced gastric ulcerated male rats.

Results

Body weight and relative organ weight

Melatonin increases the percentage of body weight. A higher percentage increase in BW was observed in the rats treated with melatonin (UCM+ML, CM+ML, and UML). The highest increase was found in the UML group (Table 1). Figure 1 shows the effects of melatonin coadministration on the relative organ weights of piroxicam-treated male Wistar rats. There was no significant difference in the relative weight of the right testis, left testis, epididymis, prostate gland, pituitary gland, or seminal vesicles among the experimental groups.

Sperm parameters

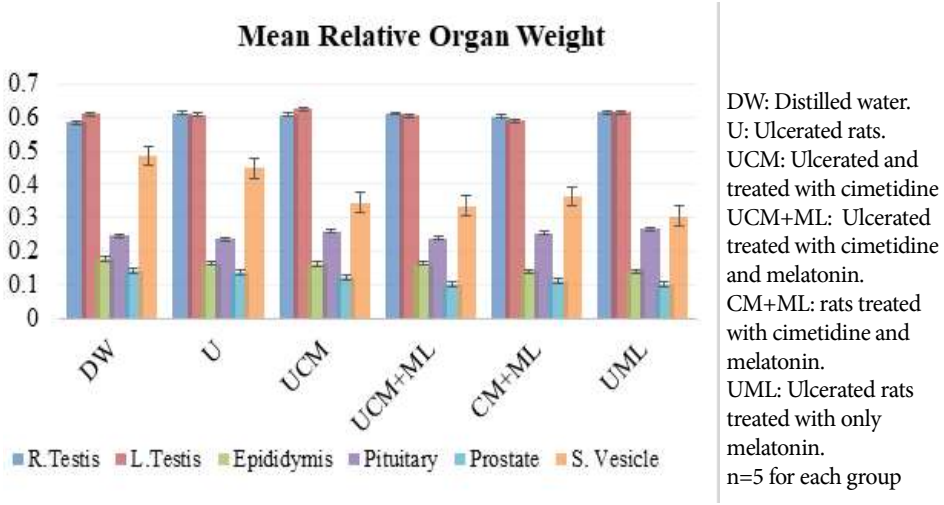
Melatonin improves the sperm parameters of piroxicam-ulcerated rats treated with cimetidine.

Cimetidine treatment of piroxicam-treated rats resulted in a significant ($p < 0.05$) reduction in sperm count and motility compared with those of ulcerat-

Table 1.
Melatonin increased the percentage of body weight

GROUPS	MBW day 1 (g)	MBW day 8 (g)	%MBW day 8	MBW day 14 (g)	%MBW day 14
DW	182.50	188.00	3.01	184.75	1.22
U	200.40	203.40	1.50	206.20	2.81
UCM	195.60	198.60	1.53	195.80	0.10
UCM+ML	186.20	195.00	4.73	192.40	3.22
CM+ML	205.50	212.00	3.16	212.00	3.07
UML	182.40	180.40	-1.10	189.80	3.90

n=5 for all the groups.
Keywords: MBW- Mean body weight, DW-Distilled water (control group), U-Ulcerated rats, UCM-Ulcerated rats administered cimetidine, UCM+ML-Ulcerated rats administered cimetidine and melatonin, CM+ML-Rats administered cimetidine and melatonin, UML-Ulcerated rats administered melatonin.



DW: Distilled water.
U: Ulcerated rats.
UCM: Ulcerated and treated with cimetidine
UCM+ML: Ulcerated treated with cimetidine and melatonin.
CM+ML: rats treated with cimetidine and melatonin.
UML: Ulcerated rats treated with only melatonin.
n=5 for each group

Figure 1.
Changes in the relative weights of the reproductive organs and pituitary following the coadministration of melatonin to ulcerated rats treated with cimetidine.

ed rats not given cimetidine. Compared with that in piroxicam-ulcerated rats treated with only cimetidine (UCM+ML), the sperm count in piroxicam-ulcerated rats treated with cimetidine (UCM) was significantly greater ($p < 0.05$) than that in piroxicam-ulcerated rats treated with only cimetidine (UCM). There were no significant changes in the percentage of sperm with abnormal morphology, as shown in Table 2.

Antioxidant status in the pituitary, epididymis, and testis of piroxicam-ulcerated rats treated with cimetidine and melatonin

The effects of melatonin on the antioxidant profiles of the pituitary, epididymis, and testis of piroxicam-ulcerated rats treated with cimetidine are shown in Figure 2. The pituitary glutathione peroxidase (GPX) level was significantly lower ($p < 0.05$) in the ulcerated group (U), ulcerated rats treated with cimetidine (UCM), and ulcerated rats treated with cimetidine and melatonin (CM+ML) than in the rats treated with distilled water (DW). Compared with rats treated with distilled water (DW), ulcerated rats treated with only melatonin (UML) presented a significant ($p < 0.05$) increase in pituitary catalase activity, as did ulcerated rats without any treatment (U). There was no significant difference in the pituitary SOD level between the treated groups and the control group.

Compared with ulcerated rats treated with only

Table 2.
Melatonin improves the sperm parameters of piroxicam-treated rats treated with cimetidine

Parameters	DW	U	UCM	UCM+ML	CM+ML	UML
Sperm motility [%]	83.75 ± 11.09	70.00 ± 7.07	64.00 ± 8.94[b]	66.00 ± 5.47[a]	70.00 ± 0.00	62.00 ± 4.47[b]
Sperm count [x106 cells/ml]	138.00 ± 18.26	112.80 ± 9.757[a]	103.40 ± 11.22[b]	110.40 ± 13.58[a,c]	108.80 ± 9.935[a]	101.40 ± 11.08[b]
Sperm viability %	96.80 ± 1.64	94.20 ± 5.36	96.20 ± 1.64	96.20 ± 1.64	95.20± 3.27	76.20 ± 38.44
% ASM	12.17 ± 0.57	13.00 ± 1.45	13.45 ± 1.17	13.17 ± 0.83	13.05±0.82	13.96 ± 0.56

ASM: Abnormal sperm morphology, a $p < 0.05$ significant reduction compared with DW, b $p < 0.05$ significant decrease in comparison with U, c $p < 0.05$ significant difference compared with UCM

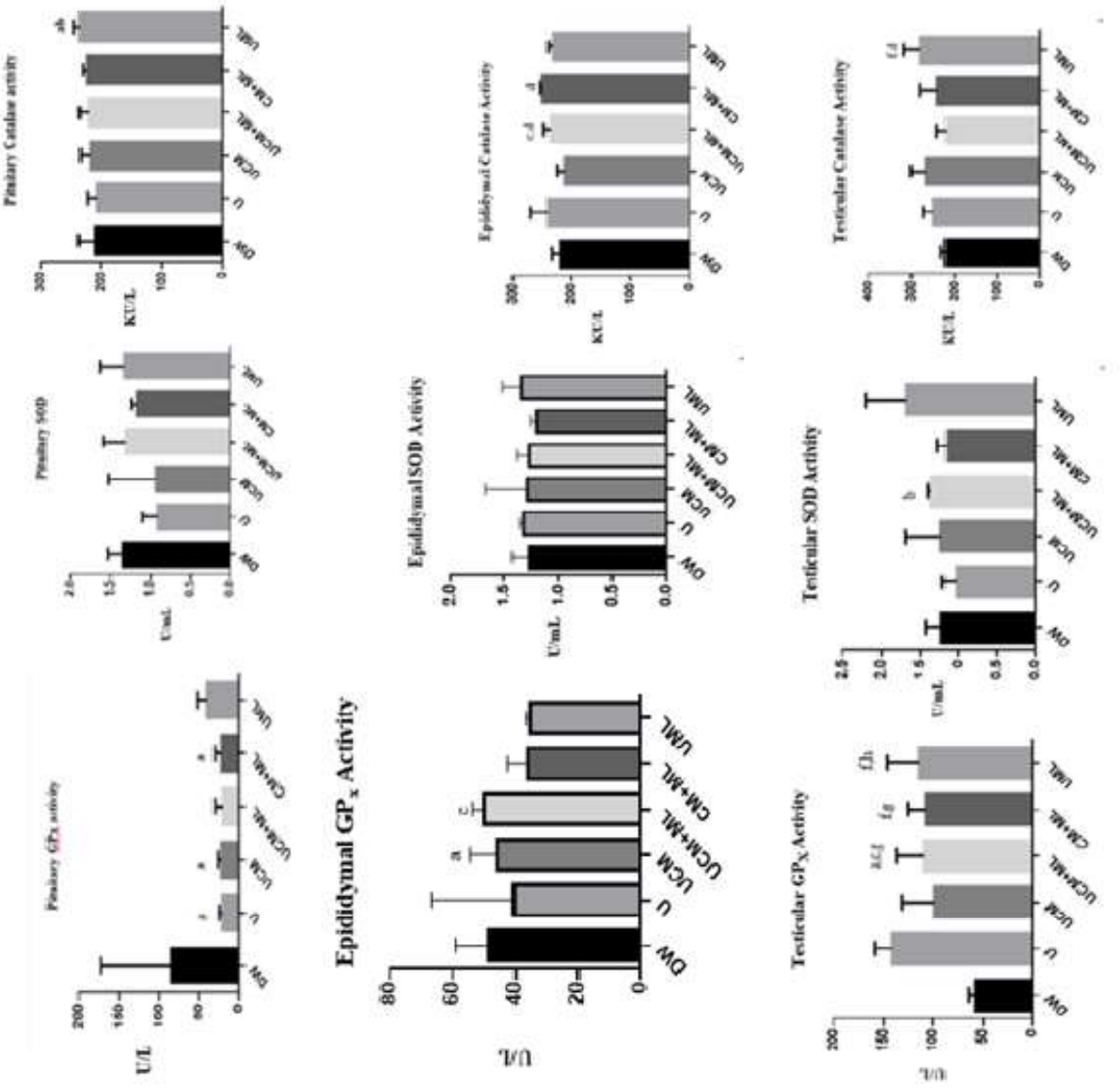


Figure 2.
Melatonin improves the antioxidant status in the pituitary, epididymis, and testis of ulcerated rats treated with cimetidine
DW-Distilled Water [control group], U-Ulcerated rats, UCM-Ulcerated rats administered cimetidine, UCM + ML-Ulcerated rats administered cimetidine and melatonin, CM + ML-Ulcerated rats administered cimetidine and melatonin, UML-Ulcerated rats administered melatonin, GPx-Glutathione peroxidase, CAT-Catalase, SOD-Superoxide dismutase.
a $p < 0.05$ significant difference compared with DW, b $p < 0.05$ significant difference compared with U, c $p < 0.05$ significant difference compared with UCM, d $p < 0.01$ significant difference compared with UCM, e $p < 0.05$ significant difference compared with UML, f $p < 0.01$ significant difference compared with DW, g $p < 0.01$ significant difference compared with U, h $p < 0.01$ significant difference compared with U.

cimetidine, ulcerated rats treated with cimetidine and melatonin presented a significant increase ($p < 0.05$) in the level of epididymal glutathione peroxidase, whereas the SOD level was not significantly affected by melatonin coadministration. Coadministration of melatonin to ulcerated rats treated with cimetidine caused a significant increase ($p < 0.05$) in epididymal catalase activity (UCM vs UCM+ML). Coadministration of melatonin to ulcerated rats treated with cimetidine caused a significant increase ($p < 0.05$) in testicular glutathione peroxidase (UCM+ML vs UCM). There was also a significant increase ($p < 0.05$) in testicular SOD activity in ulcerated rats treated with cimetidine and melatonin (UCM+ML) compared with untreated ulcerated rats (U). Melatonin administration to ulcerated rats (UML) caused a significant increase ($p < 0.05$) in testicular catalase activity compared with that in ulcerated rats treated with melatonin (UCM).

Testicular alkaline phosphatase and lactose dehydrogenase levels

Melatonin increases testicular alkaline phosphatase and lactose dehydrogenase. Melatonin administration to piroxicam-treated rats treated with cimetidine caused a significant increase ($p < 0.05$) in the testicular levels of alkaline phosphatase and lactose dehydrogenase, as shown in Figure 3.

Histopathological changes in the testes and epididymides of ulcerated Wistar rats treated with cimetidine and melatonin

The ductus epididymides of piroxicam-ulcerated rats treated with cimetidine presented empty pockets characterized by complete absence to reduce sperm activity, as shown in Figure 4. Coadministration of melatonin to piroxicam-ulcerated rats treated with cimetidine (UCM+ML) caused the epididymis to be filled with a few sperm cells. The epididymides of piroxicam-ulcerated rats treated with only melatonin (UML) presented with sperm-filled ductus. Melatonin reversed the marked cellular degeneration and reduced spermatogenic activity observed in the photomicrograph of the testes of piroxicam-treated rats treated with cimetidine.

Discussion

Male reproduction is a complex process subjected to hormonal interplay regulated by the hypothalamic-pituitary-gonadal axis. Certain antiulcer drugs negatively impact the male reproductive system and result in infertility and subfertility in male species [22]. One of these drugs is cimetidine, an H2-receptor antagonist that is commonly used as an antiulcer drug [23]. Melatonin involves multiple mechanisms to control cellular physiology, including via membrane receptors, nuclear binding sites, and interaction with cytosolic molecules. Melatonin acts through receptor and non-receptor pathways in testicular tissues, where it scavenges reactive oxygen species and increases the antioxidant body systems. Several studies have shown that sperm parameters (concentration, motility, and morphology) were positively correlated with exogenous administration of melatonin [24]. Several studies have shown that melatonin effectively ameliorates infertility in male humans and invariably in animals

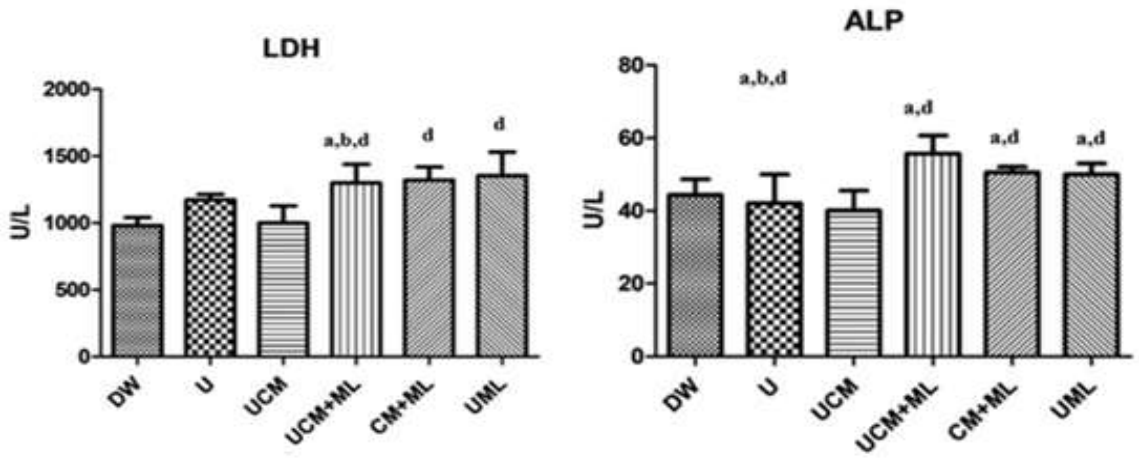


Figure 3.
Testicular levels of lactose dehydrogenase and alkaline phosphatase
DW-Distilled Water [control group], U-Ulcerated rats, UCM-Ulcerated rats treated with cimetidine, UCM+ML-Ulcerated rats treated with cimetidine and melatonin, CM+ML-Rats treated with cimetidine and melatonin, UML-Ulcerated rats treated with melatonin, LDH-Lactose dehydrogenase, ALP-Alkaline phosphatase.
a $p < 0.05$, significant reduction compared with DW; b $p < 0.05$, significant difference compared with U; c $p < 0.05$, significant difference compared with UCM; d $p < 0.01$, significant difference compared with UCM.

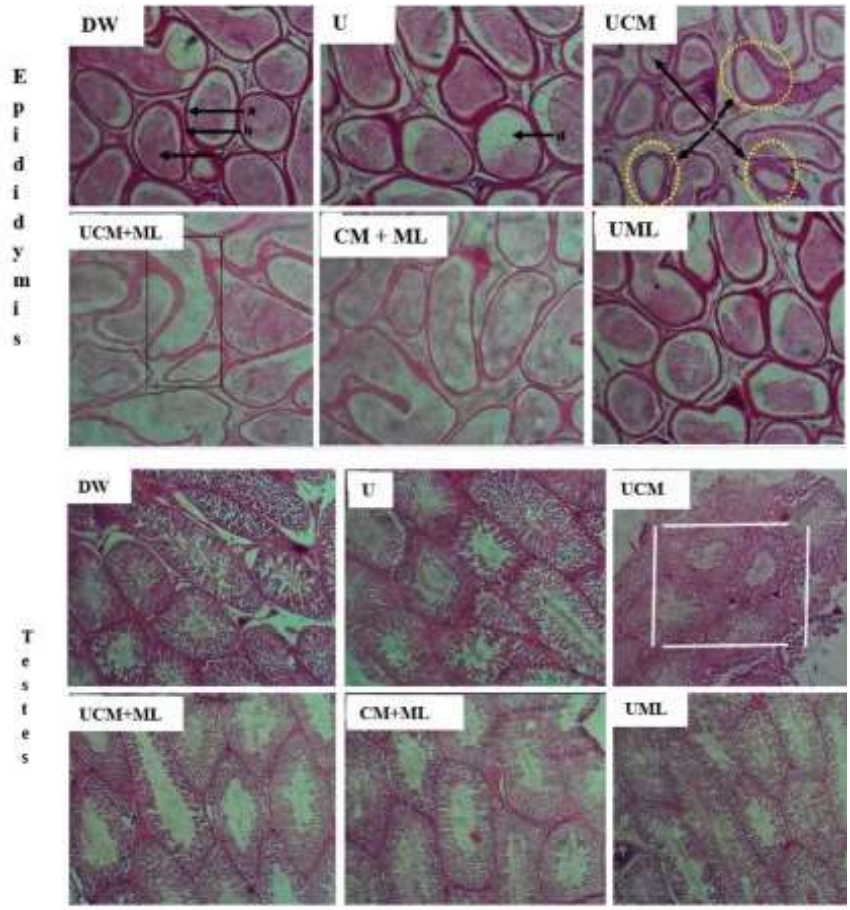


Figure 4.
Changes in the relative weights of the reproductive organs and pituitary following the coadministration of melatonin to ulcerated rats treated with cimetidine.

based on its antioxidant properties and ability to regulate the circadian rhythm [25, 26]. The antioxidative properties of melatonin have been shown to ameliorate the toxic effects of tramadol, including hepatoxicity, cholecystitis, and cholelithiasis, either by acting directly as an antioxidant or indirectly by increasing the concentration of other antioxidants in the body [14]. Because melatonin is lipophilic and hydrophilic, it is capable of crossing cell membranes, including the blood-testis barrier, thereby exerting its antioxidative influence on the reproductive system. Alcoholic patients exhibit a reduction in the endogenous melatonin blood concentration, resulting in stress-related disorders and other alcohol-related diseases. However, treatment with exogenous melatonin has been shown to mitigate these effects [27].

The findings of our study showed that melatonin consumption significantly improved feed consumption and invariably increased body weight.

The analysis of the mean body weight gain across all groups revealed an increase on days 8 and 14 compared with the mean body weight on day 1, except in the UML group, which later experienced a percentage increase in body weight on day 14. It was expected that there would be an increase in the body weight of rapidly growing rats administered the right feed in the right quantity and quality. Another study revealed that melatonin attenuated body weight gain in old female mice [28]. Moreover, the absence of melatonin led to overweight in rats [29]. The rat group with the lowest growth rate, as measured by the percentage increase in mean weight, was ulcerated and treated with cimetidine (UCM); on day 14, the percentage increase was 0.1%. In contrast, the three groups with the highest growth rates were the UML group [ulcerated rats administered melatonin – 3.9%], the UCM+ML group (ulcerated rats administered cimetidine and melatonin – 3.22%), and the CM+ML group (rats administered cimetidine and melatonin – 3.07%).

Cimetidine, an H2-receptor blocker, has been shown to cause oxidative stress in sperm cells, resulting in abnormalities in vital sperm parameters such as sperm morphology, sperm motility, and sperm count [23]. There was a significant reduction in the sperm motility of the ulcerated rats in the cimetidine (UCM) group, the ulcerated rats in the cimetidine and melatonin (UCM+ML) group, and the ulcerated rats in the melatonin (UML) group. There was a significant reduction in the sperm count in the U, UCM, UCM+ML, CM+ML, and UML groups.

LH, testosterone, and FSH are the three primary endocrine hormones that control testicular functions in mammals [19, 30]. LH in the pituitary activates the Leydig cells of the testes to produce testosterone, which then works with FSH to stimulate Sertoli cells and aids in initiating spermatogenesis. This promotes the progression of germ cells into spermatozoa and the nourishing of developing sperm cells [19]. In our study, the LH levels of ulcerated rats treated with melatonin and cimetidine (UCM+ML), rats treated with cimetidine and melatonin (CM+ML) and ulcerated rats treated with melatonin only (UML) were greater than those of unadministered melatonin. These findings indicate that melatonin aids in improving LH levels and thus aids in enhancing spermatogenesis.

This mechanism involves the suppression of Leydig cell apoptosis through the targeted action of melatonin on the mitochondrial apoptotic Bax/Bcl2 pathway, as indicated by signal transduction analysis [31]. Studies have shown that melatonin can improve spermatogenesis by mediating the upregulation of genes linked to testosterone synthesis, such as steroidogenic acute regulatory protein factor (StAR), steroidogenic factor 1 [SF1], and the transcription factor GATA-4, in Leydig cells [31].

The antioxidative property of melatonin is based on its ability to directly scavenge free radicals in body tissues [32] or indirectly scavenge free radicals by stimulating the activities of antioxidative enzymes. Superoxide dismutase (SOD) is the first line of antioxidant defense in the body and catalyzes the dismutation of O2- to H_2O_2. Catalase then abates oxidative stress by neutralizing H_2O_2 to H_2O and O2 [33]. SOD is considered the most important antioxidant enzyme in spermatozoa, where it plays a key role in sperm motility [34]. GPx maintains cellular lipid integrity by catalyzing the reaction of hydrogen peroxide with glutathione to form glutathione disulfide [33].

The antioxidant enzyme catalase (CAT) level in the pituitary gland was greater in ulcerated rats treated with melatonin (UML) than in the other groups. These findings reveal the role of melatonin in modulating CAT for antioxidative action. Catalase is an antioxidant present in almost all tissues, especially tissues that use oxygen, and it catalyzes the decomposition of H_2O_2 to H_2O and O_2. The presence of catalase in the pituitary is because it can cross the blood-brain barrier, where it exhibits antioxidative properties [35].

Compared with those in the other groups, the level of GPx in the epididymis of ulcerated rats treated with cimetidine and melatonin (UCM+ML) was greater. The antioxidative properties of catalase in the epididymis were greater in the UCM+ML group [ulcerated rats treated with cimetidine and melatonin] and CM+ML group [rats treated with cimetidine and melatonin]. These findings indicate that melatonin plays a major role in alleviating the effects of oxidative stress present in the epididymis.

In the testes, the GPx level was higher in the rats co-administered melatonin, i.e., UCM+ML (ulcerated rats administered cimetidine and melatonin), CM+ML (rats administered cimetidine and melatonin) and UML (ulcerated rats administered melatonin only), but it was lower in the rats treated with cimetidine. This result is consistent with studies showing decreased levels of antioxidant enzymes in testicular and epididymal tissues due to oxidative stress generated by cimetidine [23]. The rats in the UML group presented the highest GPx activity; this finding highlights the antioxidative properties of melatonin in modulating glutathione peroxidase activity. SOD, CAT, and LDH levels in the testes were greater in ulcerated rats coadministered melatonin (UML) than in the other groups, further revealing the antioxidative properties of melatonin through the modulation of SOD, CAT, and LDH in the testes. Alkaline phosphatase is an important enzyme needed for the biosynthesis of macromolecules, as well as for detoxification and the regulation of metabolic processes [33]. The alkaline phosphatase (ALP) level in the testes was greater in non-ulcerated rats concurrently administered cimetidine and melatonin (CM+ML) than in any other group.

In conclusion, the administration of cimetidine to piroxicam ulcerated rats induced oxidative stress in the epididymis, pituitary gland, and testis while also reducing the epididymal sperm count and motility. Cimetidine also disrupts the histological structure of the epididymis and testis. Melatonin coadministration reversed the alterations in the antioxidant system and improved the sperm parameters and histoarchitecture of the epididymis and testis. Therefore, melatonin coadministration with cimetidine is recommended for male ulcer patients who are in active reproductive life.

Materials & Methods

Chemicals and consumables

The following chemicals were purchased: Piroxicam capsule (Wintech Pharmaceuticals, Plot No. 45--46, Stice, Musalgaon, Nashik, Maharashtra, India, 422112. Mfg Llc. No. NKD-66, NAFDAC reg No. B4-7357), and cimetidine tablets (Manufactured by New Divine Favour Pharmaceutical Industries LTD, Anambra State, Nigeria. BN: NDF/NCC/0037, NAFDAC Reg. No: A4-218), melatonin powder (Glentham Life Sciences, UK. Batch number: 001ZDK), ethanol, diethyl ether, eosin/nigrosine stains, wells and agar stains, phosphate buffer solution, and ethylene diamine tetra acetic acid (EDTA). The following kits were also used: a Testosterone ELISA kit (Calbiotech, Catalogue no: TE187S, USA), Luteinizing Hormone (LH) ELISA kit (Calbiotech, Catalogue no: LH231F, USA), Follicle Stimulating Hormone (FSH) ELISA kit (Calbiotech, Catalogue no: FS046F, USA), Rat PRL (Prolactin) ELISA Kit (Elabscience, Catalogue No: E-EL-R3006, USA), SOD Fortress diagnostic kit (Fortress Diagnostics Limited, UK, product code: BXC0173), CAT Fortress diagnostic kit (Fortress Diagnostics Limited, UK, product code: BXC0173), GPx Fortress diagnostic kit (Fortress Diagnostics Limited, product code: BXC0551), ALP Fortress diagnostic kit (Fortress Diagnostics Limited, UK, product code: BXC0183A), LDH Biorex diagnostic kit (Biorex Diagnostic Limited, UK product code: BXC0242), ELISA plate reader (UV/VIS 752, Pec Medical, USA), and Electronic balance (Golden-Mettler®, USA).

Animal Care

Thirty (30) adult male Wistar rats were housed in a perforated

plastic cage with wood bedding at an optimum room temperature of 24°C +/- 2°C to acclimatize the Wistar male rats to laboratory conditions. The rats were obtained from the Biochemistry Department Faculty of the Faculty of Life Sciences, University of Ilorin. The rats were fed commercial pelletized grower feed (Vital® feeds), and portable water was provided ad libitum. This study was approved, and ethical approval no (UERC/FVM/15/32TA010) was assigned by the faculty of veterinary medicine ethical review committee.

Experimental design

Preliminary ulcer induction

Three rats were randomly chosen for the preliminary study of ulcer induction. The mice were administered 30 mg/kg of piroxicam and then sacrificed after 14 days. Ulcer indices were then measured, and it was confirmed that piroxicam could induce ulcers in rats treated with 30 mg/kg piroxicam [12].

Dosing protocol

The rats were randomly assigned to six groups (five rats each). The first group served as a control and was administered distilled water only (DW 3 ml/kg BW). Group 2 (U) was ulcerated with piroxicam at 30 mg/kg BW. Group 3 (UCM) was ulcerated with 30 mg/kg piroxicam and treated with 200 mg/kg cimetidine [13]. Groups 4 (UCM+ML) and 5 (CM+ML) were treated with 200 mg/kg cimetidine and 10 mg/kg melatonin [14], but only group 4 was ulcerated with 30 mg/kg piroxicam. Group 6 (UML) was ulcerated with 30 mg/kg piroxicam and treated with only melatonin (10 mg/kg BW) [15]. The duration of this treatment lasted for fourteen days.

Sperm analysis

Sperm collection: The epididymis was excised, and the caudal epididymis was squeezed as described by [16] and then placed in a Petri dish. Before the evaluation of the sperm parameters, the diluted sperm were incubated for 10 minutes at 32 °C in physiological saline.

Sperm parameter evaluation: Sperm motility, the live–dead ratio, the sperm count, the sperm volume, and morphology were determined on the basis of the principles of [17] and [18]. Sperm motility was assessed with a microscope [Olympus®] at 40x magnification. Motile and nonmotile spermatozoa were counted. Sperm motility was determined on the basis of the percentage of motile to total spermatozoa counted [18]. The epididymal sperm count was determined with the aid of a haemocytometer. Five milliliters [5 mL] of the diluted sperm was placed on the central square of the Neubauer counting chamber. The sperm cells were counted by viewing [at 40× magnification]. The 5 squares of the Neubauer counting chamber were observed under a microscope. The result was expressed as a 1 million/1 ml sample size [18]. The sperm morphology was determined by smearing the sperm on a glass slide and allowing it to air dry overnight. The sperm were further stained with 1% eosin-Y/5% nigrosine. The specimen was then viewed under a microscope at 100x magnification for the observation of spermatozoa with abnormal morphology, such as headless tails, looped tails, rudimentary tails, curved tails, curved midpieces, and bent midpieces [18].

Blood collection and serum preparation

Blood was drawn into an EDTA-filled sample bottle from experimental rats that had been anesthetized with a combination of xylazine and ketamine via their orbital sinus. The collected blood was kept undisturbed for half an hour on the laboratory bench. After that, the mixture was centrifuged at 2000 × g for 10 minutes. The supernatant was collected with a pipette and kept at -4 °C until use.

Tissue sample preparation

The tissue samples were placed into a table mortar and pounded with a pestle to form a homogenate before they were subsequently placed in plain sample bottles and spun at 5000 rpm for 30 min via a centrifuge [Axiom Medical Ltd., UK). The supernatants were dispensed into Eppendorf tubes via a Pasteur pipette and then analysed for antioxidant levels.

The antioxidant levels of superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx] in the epididymis, testes, and pituitary gland were assessed. SOD, CAT, and GPx were analysed by using a SOD Fortress diagnostic kit [Fortress Diagnostics Limited, UK; product code: BXC0173], a CAT Fortress diagnostic kit [product code: BXC0173], and a GPx Fortress diagnostic kit [product code: BXC0551], respectively, following the guidelines of the kit manufacturers.

An alkaline phosphatase [ALP] Fortress diagnostic kit [produced by Fortress Diagnostics Limited, UK; product code: BXC0183A] was used to measure ALP. The rate at which p-nitrophenol is formed through the reaction of alkaline phosphatase and p-nitrophenyl phosphate is closely correlated with alkaline phosphatase levels.

A lactate dehydrogenase [LDH] Biorex diagnostic kit [Biorex Diagnostic Limited, UK product code: BXC0242] was used to measure lactate dehydrogenase.

Hormone assay

Serum levels of testosterone, follicle-stimulating hormone, luteinizing hormone, and prolactin were measured via an enzyme-linked immunosorbent assay (ELISA) kit that is specific for each hormone to be assayed [19]. The ELISA kit manual was strictly adhered to during the procedure. Briefly, the ELISA principle is based on the following antigen-antibody response: the antigen, which is the blood sample, and the antibody, which is the precoated microwell for the specific ELISA hormone kit. Serum was added with the aid of an automatic pipette into the precoated microwells and then allowed to rest for an hour for the antibody-antigen reaction to take place before the plate was loaded into the automatic plate reader.

Histopathology

For histopathological changes, the testes and epididymides were first fixed in 10% buffered formaldehyde, dehydrated with increasing ethanol concentrations, and embedded in molten paraffin wax. Thin sections of the paraffin-embedded tissue were cut, collected on glass slides coated with glycerin egg albumin, and labelled. These sections were dried, stained with hematoxylin and eosin [H&E], dehydrated, and cleaned. A glass coverslip was placed over the tissue sections with a mounting medium, and the slides were examined under a light microscope and photographed to identify any histopathological changes [20].

Body weight and organ weight determination

The mean body weight was determined via a Metler balance on days 1, 8, and 14 across all groups, and the percentage mean body weight was calculated.

The mean organ weights of the right testis, left testis, left epididymis, liver, spleen, kidney, pituitary gland, prostate gland, and

seminal vesicle of the experimental rats were also measured, and the relative organ weights in terms of body weight were also measured [21].

Statistical analysis

The body weight data are expressed as percentages, while the relative organ weights are presented as simple bar charts. Other data are presented as the means ± standard deviations (means ± SDss). ANOVA was used to analyse the data, while the significance level was evaluated via Tukey's multiple comparisons test with the aid of GraphPad Prism® [version 8.3.0, GraphPad® Software, www.graphpad.com]. The values were deemed statistically significant at $p < 0.05$.

Authors' Contributions

BA and ACO: conceptual development and Design. BA, AA, and ACO: Methodology and literature search. ACO, OAO: dosing of the rats and daily management of rats. BA, ACO, AGJ, AA, IAO, and OAO: rat sacrifice, sample collection and sample processing. BA, ACO and AGJ: data analysis and manuscript drafting. AA, BA and OAO: Manuscript review and final copy.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Anatomical and Radiographical Studies of the Skull in Adult European Badger (*Meles meles*)

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ABSTRACT

The European badger (*Meles meles*) is a powerful animal native to Europe and parts of West Asia. The skull imparts the phylogenetic morphology to the skulls of animal species and functions as a safeguard for vital organs, including the brain and eyes. The aim of our study was to improve the current data by providing a comprehensive investigation of the morphology and dimensions of the cranium and mandible of badgers. This information enhances comprehension of radiological and surgical attributes. We conducted anatomical studies and measured the morphometric features. We received the skulls from naturally occurring carcasses. We employed unpaired t-test for statistical analysis. This study demonstrated that the anatomical features of the skull exhibit no discernible differences between badgers and other wild carnivores, such as tiger and wolf. The presence of a twin jugular foramen in the skull of badgers distinguishes them as a distinct characteristic not found in any other carnivorous animals. Moreover, radiographical studies showed two distinct sinuses and a cavity in the skull of badger which is different from dogs. Males and females differ significantly in some morphometric traits, which is entirely consistent with the behavioral and nutritional traits of the animal. Modern imaging techniques, such as CT scans, are necessary for more thorough studies on the skulls of wild carnivores.

Keywords

European badger, Skull, Morphology, Morphometry, Radiography.

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Abbreviations

CT: Computed Tomography

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Introduction

European badger is classified taxonomically as a member of the Mammalia class, Carnivora order, and Mustelidae family. Globally, this animal's habitat spans Europe, Asia, and Africa. Forests and steppe areas with soft soils are the best environments for badgers [1]. They are renowned for their distinctive black and white stripes and robust bodies, and they utilize their powerful front paws to excavate food and construct their unique burrows known as 'setts' [1]. The cranium determines the form and shape of the head, as well as creating a defensive bed for the enamel, brain, eyes, and ears [3]. The difference in shape between the skull and mandible simultaneously impacts the cross-sectional area of the masticatory muscle tissues and biting force [4]. To perform their treatment procedures in zoos and natural world, veterinarians and animal surgeons must have basic anatomical information about animals [21]. In addition, craniometry is the inspiration for medical and surgical practices. Furthermore, the exceptional openings in the skull have significant therapeutic relevance in the administration of local anesthetic in the head [5].

The Mustelidae family's skull anatomy has been the subject of numerous studies. In 2022, Martonos et al. conducted a comprehensive study on the middle ear ossicles in badgers. Zagrai et al. in 2019 compared skull morphology in badgers and otters. Taraska et al. in 2016 and Gálvez-López in 2022 conducted extensive studies on the corneal shape and craniometric parameters in mink species. In 2012, Suzuki et al. performed comprehensive research on the morphological characteristics of the cranium and lower jaw in weasels. He et al. performed osteological studies on the skull and mandible of ferrets in 2002. Mustelidae skull scale increases from weasels to stoats, minks, polecats, pine martens, and otters. Overall, the skull anatomy of Mustelidae adapts to their carnivorous diet, featuring specialized teeth and a form that facilitates efficient hunting and consumption of prey. The width of the zygomatic arch and the height of the sagittal crest are important variables for distinguishing mustelid species [6].

The primary objective of this study was to examine and analyze the anatomical characteristics of the skull and mandible, with a particular emphasis on their morphometric, morphological, and radiological properties. Table 1 and Figure 1 provide the meanings of the abbreviations used for these measurements. We thoroughly compared these traits in adult badgers and other carnivores, as well as between genders. The main goal of this study was to explain the macroscopic anatomy and radiology of cranial bones. We enhance the existing knowledge based on this topic by conducting anatomical comparisons with other species. The results of this study also contribute to the expansion of data on veterinarians who treat unusual animals. The specified morphometric and morphological specifications improve comprehension of radiological and surgical attributes. These findings can aid in the identification of wild species found in various natural environments.

Table 1.
Morphometric indices of skull of the badger

DLCS	**Dorsal length of craniuim of skull**
GL	Greatest length of skull
WCS	Width of the cranium of skull
ZW	Zygomatic arch width
WMOA	Width between the medial eye angles (Canthus)
DLFS	Dorsal length of the facial part of skull
BLCS	Basal length of the cranial part of skull
CBL	Condylo-basal Length of skull
RWHP	Rostral width of hard palate
CWHP	Caudal Width of hard palate
LHP	Length of the hard palate
LM	Maximum Length of mandible
WM	Maximum Width of mandible
HRM	Maximum High of ramus of mandible

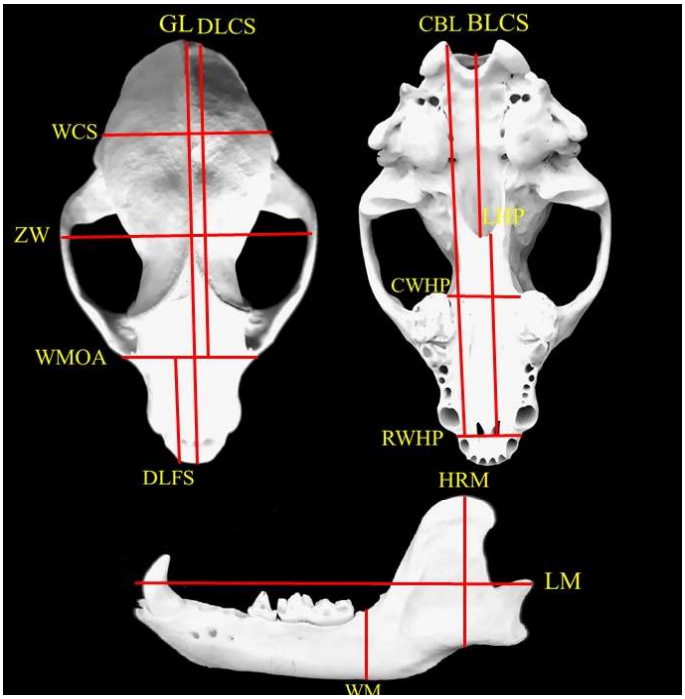

Figure 1.
Illustration of morphometric measurements of skull of the Badger.

Results

The frontal, nasal, and parietal bones were observed from a dorsal perspective. An important feature that stood out was a large external sagittal crest, clearly visible as a continuous, unbroken line. The prominent temporal lines emerged from the rostral of this crest and were connected to the zygomatic process of the frontal bones, forming the caudal border of the orbit. The parietal bones were observed as two asymmetrical triangles. These are the factors that led to the limited development of the frontal bones. The temporal fossa was a curved area that extends across the external sagittal crest. The supraorbital groove and supraorbital foramen were not observed (Figure 2).

The nuchal crest was highly noticeable at the posterior end of the external sagittal crest. The interparietal bone was integrated into this line and did not exist separately. The neuro-cranium exhibited significant elongation, measuring twice the length of the facial part of the skull. Consequently, the maxillary and nasal bones were limited and small (Figure 2). The ventrolateral view revealed the presence of several foramina in the pterygopalatine fossa located at the back of the orbit on the wing of the presphenoid bone. These foramina included the ethmoidal, optic, round, and rostral alar (Figure 3).

The zygomatic process of the temporal bone was strongly connected to the zygomatic bone's temporal process, causing the zygomatic arch to seem curved when viewed from the lateral view. Despite having a relatively large diameter, the external acoustic meatus had a low wall height. The articular tubercle and retro-articular process were noticed, but the retro-articular foramen was not readily visible. Furthermore, the mastoid process exhibited significant development in the posterior region of the external acoustic meatus. This view allowed the observation of a sharp Hamulus process. The frontal process of the zygomatic bone and the zygomatic process of the frontal bone were situated at a considerable distance from each other near the posterior border of the orbit. The infra-orbital foramen, clearly visible and of significant size, was situated over the alveolus of the third and fourth premolar teeth (Figure 4).

The ventral surface of the basi-occipital and basi-sphenoid bones had a short muscle tubercle. However, the border between the two bones was clear. The jugular foramen was observed in double form laterally to the basi-occipital bone. The boundary between the extensive tympanic bulla and

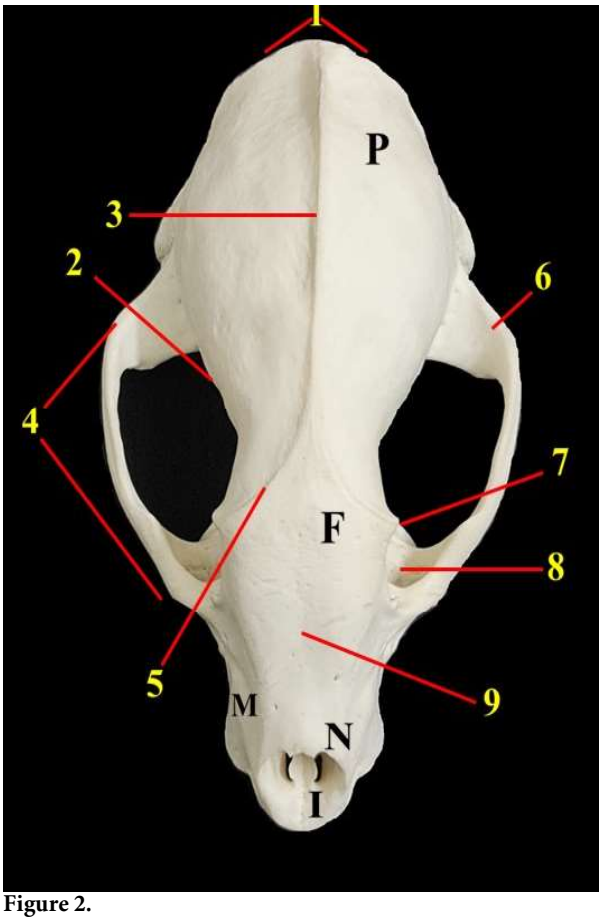

Figure 2.
Dorsal view of skull of the badger.
P) Parietal bone, F) Frontal bone, N) Nasal bone, M) Maxillary bone, I) Incisive bone, 1) Nuchal crest,2) Temporal fossa, 3) External sagittal crest, 4) Zygomatic arch, 5) Temporal line, 6) Zygomatic process of frontal bone, 7) Zygomatic process of frontal bone 8) Orbit 9) Inter-frontal suture.

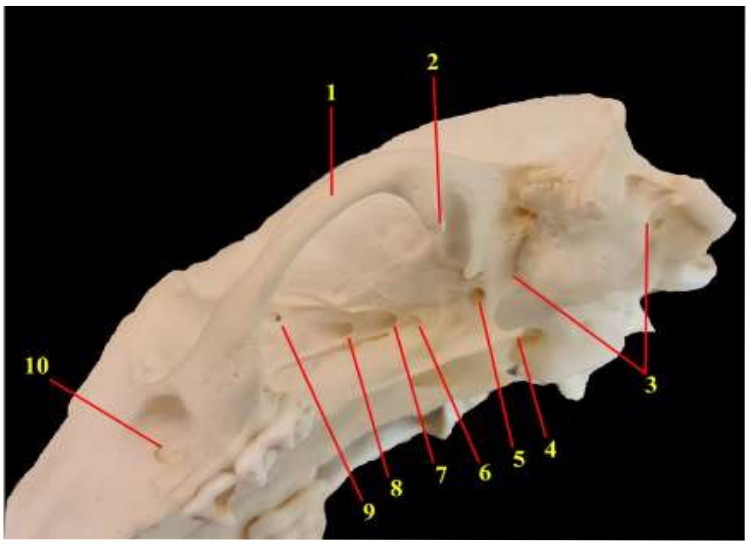

Figure 3.
Ventrolateral view of skull of the badger.
1) Zygomatic bone, 2) Condyloid process, 3) Tympanic bulla, 4) Hamulus pterygoideus, 5) Caudal alar foramen, 6) Rostral alar foramen 7) Orbital foramen 8)Optic foramen 9)Ethmoidal foramen 10)Infraorbital foramen.

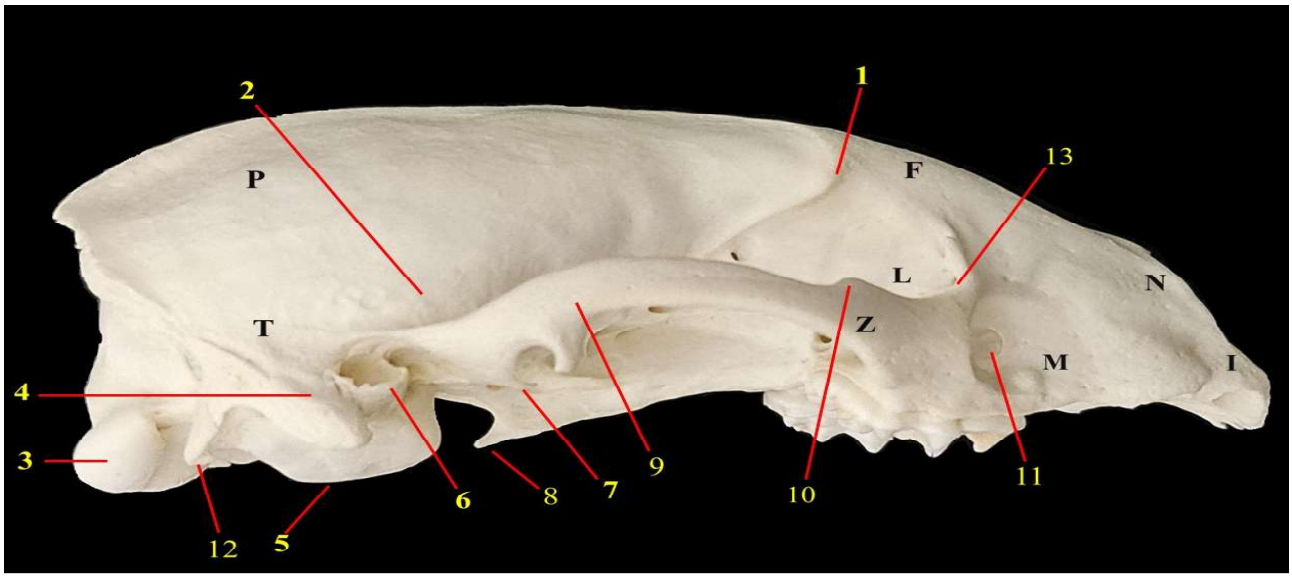

Figure 4.
Lateral view of skull of the badger.
T) Temporal bone, P) Parietal bone, F) Frontal bone, N) Nasal bone, M) Maxillary bone, L) Lacrimal bone I) Incisive bone, Z) Zygomatic bone, 1) Zygomatic process of frontal bone, 2) Temporal fossa, 3) Occipital condyle 4) Mastoid process 5, Tympanic bulla 6) External acoustic meatus, 7) Retro-articular process, 8) Hamulus pterygoideus, 9) Zygomatic process of temporal bone, 10) Frontal process of zygomatic bone, 11) Infra-orbital foramen, 12) Jugular process, 13) Lacrimal foramen.

the basi-sphenoid of the carotid foramen was distinct and clear. Despite being covered by tympanic bulla, the spinous and oval foramens were observable. Furthermore, the muscular process was observed to be very small and needle-shaped. The major palatine foramens were small and located medial to the fourth premolars in the hard palate area. These two were located along the two palatine grooves. Therefore, the horizontal part of the palatine bone was roughly large and constituted the largest portion of the hard palate. The incisive bones constituted a minor portion of the hard palate, and the prominence of palatine fissures was obvious (Figure 5).

The rostral view revealed a long, robust, single bone that extended into the nasal cavity at the front and connected with the premaxilla, presphenoid, and maxilla bones. Furthermore, a deep groove was visible between its edges. The concha bones in the nasal cavity were attached to its lateral walls as delicate, scroll-like, complex bony plates (Figure 6). The nuchal surface was somewhat triangular. The shape of the foramen magnum was rounded, and its diameter was recorded to be 1.5 cm. The nuchal crest at the dorsal part of the squamous occipital bone was bow-shaped and very promi-

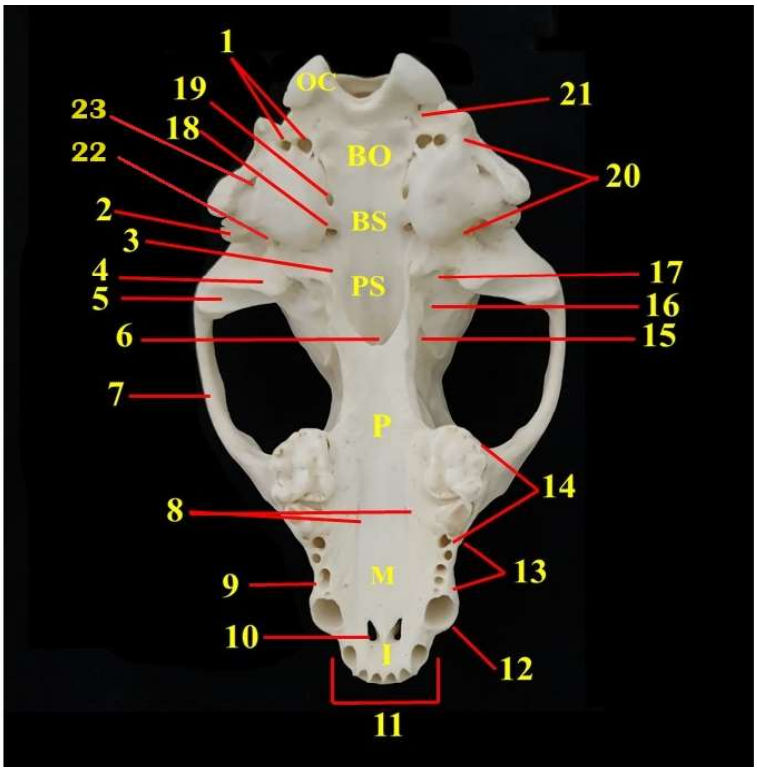

Figure 5.
Ventral view of skull of the badger.
Oc) Occipital condyle, Bo) Basi-occipital, Bs) Basi-sphenoid bone, Ps) Pre-sphenoid bone, P) Parietal bone, M) Maxillary bone, I) Incisive bone, 1) Jugular foramen, 2) Muscular process, 3) hamulus pterygoideus, 4) Retro-articular process, 5) Mandibular fossa, 6) Choana, 7) Zygomatic arch, 8) Minor palatine foramen, 9) Major palatine foramen, 10) P1 tooth 11) Palatine fissure, 12) Incisor teeth, 13) Canine tooth, 14) Premolar teeth, 15) Molar teeth, 16) Rostral alar foramen, 17) Caudal alar foramen, 18) Oval foramen, 19) Carotid foramen, 20) Tympanic bulla, 21)Hypoglossal canal, 22)Retroarticular foramen, 23)Stilomastoid foramen.

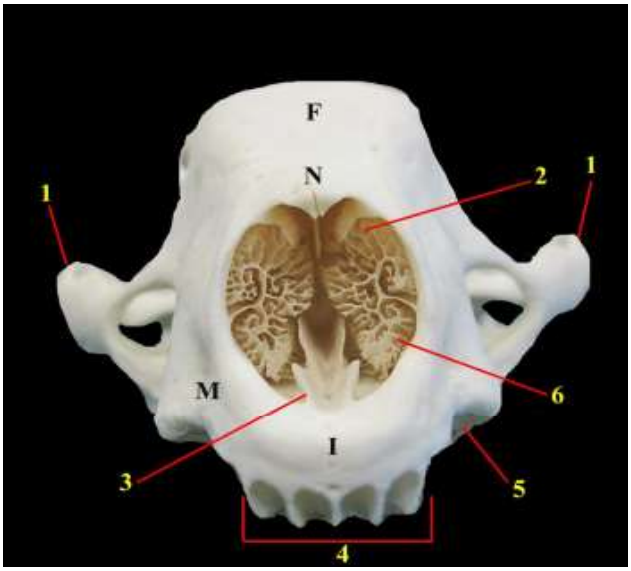

Figure 6.
Rostral view of skull of the Badger.
M)Maxillary bone F) Frontal bone. I) Incisive bone, N) Nasal bone, 1) Zygomatic arch, 2) Dorsal concha. 3)Vomer bone, 4) Incisor teeth alveoli, 5) Canine tooth alveolus, 6) Ventral concha.

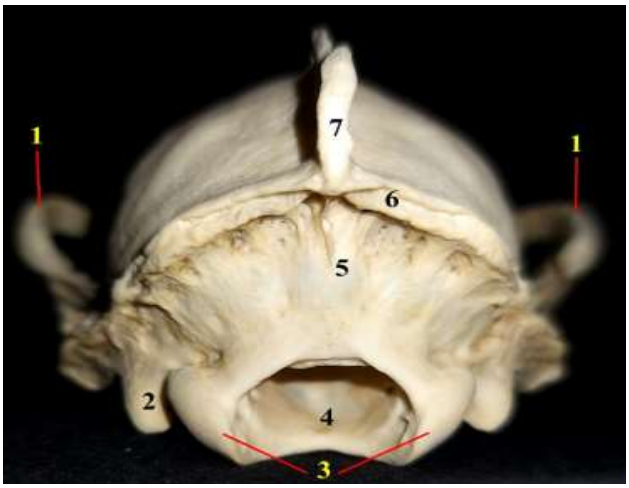

Figure 7.
Caudal view of the skull of the badger.
1) Zygomatic process of temporal bone, 2) Jugular process, 3) Occipital condyles, 4) Magnum foramen, 5) Squamous part of occipital bone, 6) Nuchal crest, 7) External sagittal crest.

nent, and the rounded mastoid process was very well developed and fused with the ventral end of the crest on each side. The jugular processes of the badger were small and pointed (Figure 7).

The mandibular body exhibited a robust and trapezoidal shape, with a medial flattening that formed the mandibular symphysis. Furthermore, the ventral border appeared nearly linear. There were three mental foramina located on the side surface of the front half of the mandibular body, positioned behind the sockets of the premolar teeth. The rostral, middle, and caudal mental foramina were referred to, and the middle foramen was larger in all cases. The mandible's ramus has a triangular shape and consists of three distinct processes. The coronoid process had a prominent and broad structure, constituting the upper half of the ramus. The angular process was small and positioned near the tail end. The condylar process, which is fashioned like a rod, was formed horizontally and connects with the mandibular fossa. A masseteric fossa was present on the lateral aspect of the coronoid process. The object had a triangle shape and provided a point of attachment for the masseteric muscle. The muscle's range of motion was restricted by the coronoid crest at the front and the condyloid crest at the back. The inner surface of the coronoid process had a minor degree of roughness, specifically designed to accommodate the insertion of the temporal muscle, located directly above the mandibular foramen (Figure 9).

Discussion

From a general standpoint, the badger's skull is more elongated than that of other carnivores, and the rostral area is not very wide. Dolichocephalic dogs also exhibit this feature, setting them apart from mongooses and lynxes that have a wide muzzle. The wideness of rostrum enhances the biting strength of the jaws [7]. Badger is a nocturnal and shy animal, and typically uses smaller animals and plants. On the other hand, the eyesight of this animal is weak, which limits its hunting abilities. As a result, the narrowness of this area is consistent with the animal's behavioral and nutritional characteristics.

The badger, similar to other wild carnivores, such as the tiger, wolf, cheetah, and lynx, does not possess the interparietal bone. Domestic cats and dogs also possess this bone (8, 9). With the exception of the marten and Egyptian mongoose, most carnivores have a noticeable external sagittal crest and pronounced temporal lines that extend to the posterior border of the orbit. These observations are also noted in the present investigation [10]. We did not observe the inter-incisive canal in our findings. However, this canal is present in brown bears, ferrets, and dogs. [11].

This study found that the orbital ligament completes the orbit, which is not entirely bony. All carnivores, except mongoose, have the same characteristic [5]. Four foramina, called ethmoidal, optic, orbital, and rostral alar, were situated on the posterior border of the orbit. These openings can also be seen in other carnivorous animals. However, the alar foramen is not present in lynx and cheetah [7]. The badger lacked the facial part of the lacrimal bone, but its orbital part was larger than that of a dog or domestic cat. The mastoid process of badger was very well-developed and distinct, similar to that of other carnivores. However,

This

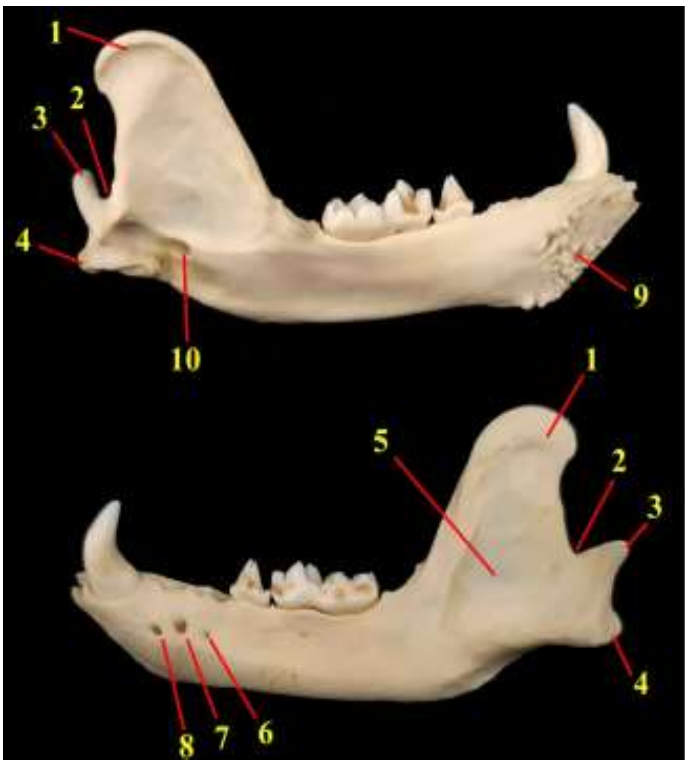

Figure 8.
Lateral and Medial view of mandible of the badger.
1)Coronoid process, 2) Mandibular notch, 3) Condylar process, 4) Angular process, 5) Masseteric fossa, 6) Caudal mental foramen, 7) Middle mental foramen 8) Rostral mental foramen 9) Mandibular symphysis 10) Mandibular canal

it seems that in badgers, it is relatively larger than in other species [10]. Research indicated that the infra-orbital foramen, which is both large and round in shape, is positioned above the alveolus of the fourth premolar tooth. Therefore, these data are highly helpful for tracking the infra-orbital nerve and are crucial for the process of desensitizing the skin on the upper lip, nostril, and cheek [12].

The badger's tympanic bulla was relatively large, similar to previous reports in lions, cats, dogs, and tigers. Although it was small in brown bears. A short jugular process is seen in all the carnivores. In the badger, this process was distinct and well-developed [11, 13, 14]. The important point was the presence of a double jugular foramen in the skull of the badger. This state has been reported only in human, macaca, and rats, and these two openings are called anterior and posterior jugular foramens. It seems that due to the location of these two openings in the badger, they are called medial and lateral jugular foramens, which are separated by an inter-jugular septum [15]. Furthermore, the retro-articular and stilo-mastoid foramina were indistinguishable on the ventral surface of the mongoose skull.

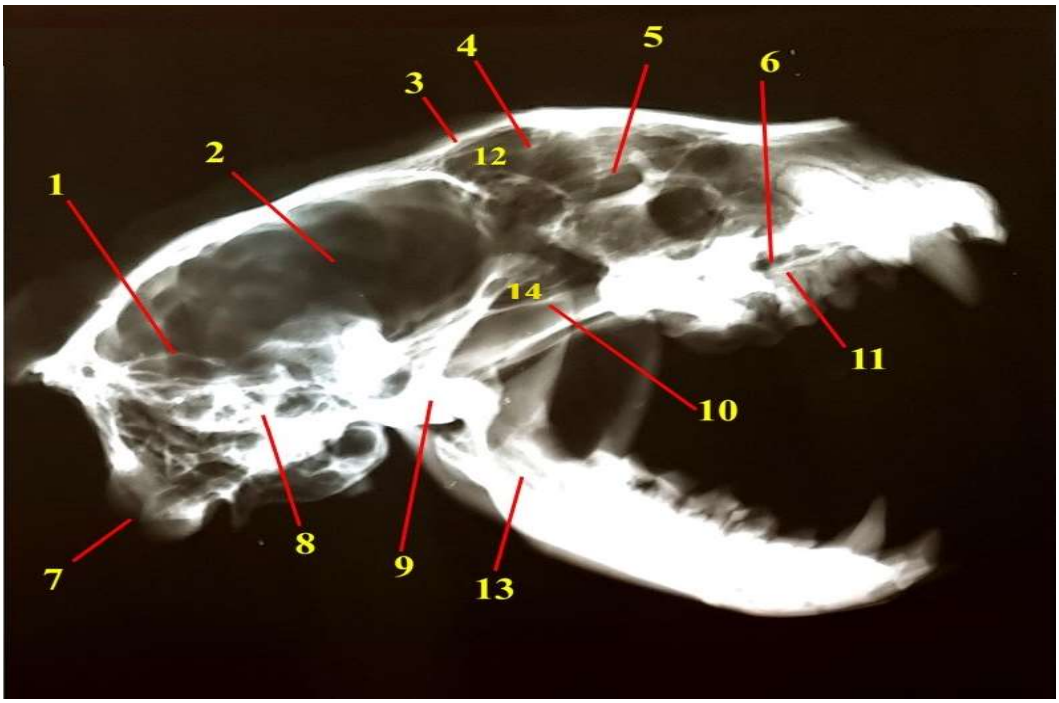

Figure 9.
Lateral radiograph of skull of the badger.
1) Tentorium cerebrali osseum, 2) Facies crebralis, 3) Frontal bone, 4) Cribriform plate, 5) Orbit, 6) Recessus maxillaris, 7) Occipital condyle, 8) Petrous part of temporal bone, 9) Temporomandibular joint, 10) Zygomatic arch, 11) Hard plate, 12) Frontal sinus, 13) Mandibular canal, 14) Sphenoid sinus.

Both of these foramina are present in the skulls of canines and ursids, but were not detected in the mongoose skull [5]. The ventral view did not reveal the border between the palatine bone and the maxilla. However, the presence of distinct major palatine foramina partially defines the large extent of the palatine. According to the previous studies, this feature seems to be consistent with mongooses, tiger, and lions, while different from canines and bears [14].

On the lateral surface of the mandible ramus, there were three mental foramina (rostral, middle, and caudal); the tiger, lynx, and dog showed a similar result. Nevertheless, the mongoose, leopard, and lion have two foramina, while the Persian cat and maned wolf only have one foramen. One can perform a mandibular nerve block in the mental zone by administering local anesthetic medications into the mandibular canal through three mental foramens. This will ensure the complete anesthesia of the lower incisors, premolar, and lower lip on the same side in cases of lower lip damage, dental extraction, and tooth traumas [14, 16]. In males, the rostral and middle mandibular foramens are large and similar, whereas in females, only the middle opening is large. It seems that the large skull in males increases the need for nerve and blood supply.

Table 2.
Mean and standard deviation of morphometric characteristics of skull of the badger
(* significant difference $p \leq 0.05$).

	Male (n= 5)	Female (n= 5)	P value
DLCS (mm)	88.29 ± 4.34	78.50 ± 2.65	0.032*
GL (mm)	112.23 ± 5.56	101.20 ±6.21	0.041*
WCS (mm)	43.63 ± 2.96	37.75 ± 3.30	0.072
ZW (mm)	62.33 ± 4.35	59.80 ± 3.97	0.086
WMOA (mm)	27.24 ± 2.66	23.42 ± 2.54	0.067
DLFS (mm)	23.85 ± 1.71	19.04 ± 2.23	0.042*
BLCS (mm)	48.06 ± 5.25	43.70 ± 3.88	0.039*
CBL (mm)	106.64 ± 6.74	87.84 ± 5.05	0.011*
RWHP (mm)	17.05± 2.88	14.21 ± 3.60	0.025*
CWHP (mm)	25.17 ± 3.62	20.93 ± 3.75	0.176
LHP (mm)	56.10 ± 4.02	45.95 ± 6.12	0.040*
LM (mm)	90.95 ± 6.03	75.96 ± 4.54	0.012*
WM (mm)	16.09 ± 3.40	12.50 ± 3.09	0.102
HRM (mm)	41.14 ± 4.54	34.55 ± 4.24	0.086

An examination of golden jackal skulls revealed distinct morphological variations between females and males, which contrasts with the findings of the current study [17]. Although males and females have identical appearances, there are numerous notable distinctions in the measurement of morphometric variables between the two genders. The SL index, indicating the maximum width of the skull in relation to its length (ZW/GL×100%), was 55.3% and 58.4% in males and females, respectively (Table 2 and Figure 1). In ferrets, the average index is 61.5 for males and 58.8 for females. In mongoose, the index is 54.8 for males and 56.8 for females. The index in cheetahs is consistently 83.86, irrespective of gender [5, 18]. In the study of Japanese weasels, the CBL (Condylo-basal Length of the skull) index reached a maximum of 60 mm. We measured this index at 106 mm in male badgers [19]. According to Taraska et al., increasing the length of the skull in Mustelidae species reduces the level of wildness and hunting power, which is in agreement with the present study [20] (Figure 10).

The radiographical studies showed two distinct sinuses and a cavity in the skull of the badger. These observations are apparent in mongooses, cats, and foxes, as they possess sphenoid and frontal sinuses. The recessus maxillaris does not qualify as a real sinus due to its formation between distinct bones rather than between the two plates of the maxilla [21].

This study examined the anatomical characteristics and measurements of the skulls of male and female badgers. These characteristics are consistent with the behavioral and nutritional characteristics of the animal, and they are more similar to those of wild carnivores. Furthermore, except in minor cases, such as the size of the mandible foramens, there was no clear difference between the skull anatomy in the two genders. In the current study, we conducted a thorough anatomical examination of the badger's skull. However, to obtain additional information, the use of advanced diagnostic imaging techniques, such as CT scans, is necessary.

Materials & Methods

This research focused on the skulls of ten adult European badgers, which included samples from both freshly obtained and museum collections. There were five male and five female badgers. The new samples were retrieved from naturally dying animals found in the wild. The Environment Organization sent us these cases over a three-year period from various regions of Semnan, Iran, and we then brought them to our center (Department of Anatomy, School of Veterinary Medicine). In the animal museum, we also used the complete skeletons of the Indian Gray Badgers. We selected them based on their

apparent good health and lack of bone-related ailments. Badgers typically display their complete set of teeth once they reach the age of ten months or older. To confirm their complete growth, we examined the teeth and dental formula of specimens (I3/3, C1/1, P4/4, M1/2=38) (Yam tav).

We cut the heads at a specific joint and then treated them in the laboratory to prepare their skeletons. A previous study (Zagrai) reported that this process involved boiling. We removed the bones from the soft parts of the body, removed the fat, whitened, and dried them. We conducted morphometric examinations and used a digital measuring tool (INSIZE, USA, Model: 1502S-1205) to obtain significant measurements of the anatomical characteristics. To describe, identify, and obtain approval, we adhered to the guidelines set by Nomina Anatomica Veterinaria. We recorded the results using a Canon Legria HF R16E digital camera from Canon Inc., Japan. An X-ray machine, called TUR 800 D-1, was used to perform the radiological study (Rontgenbelichtungsautomat–20029). Radiographs were obtained in dorsal-ventral and left lateral projections with a focus film distance of 120 cm, a kilovolt peak of 65 kV, and milliampere-seconds of 10 mAs.

We used descriptive statistics to describe the measurements. Moreover, we utilized the unpaired t-test to study the differences between the male and female skulls in terms of their measurements and proportions. The differences were significant if P-value < 0.05.

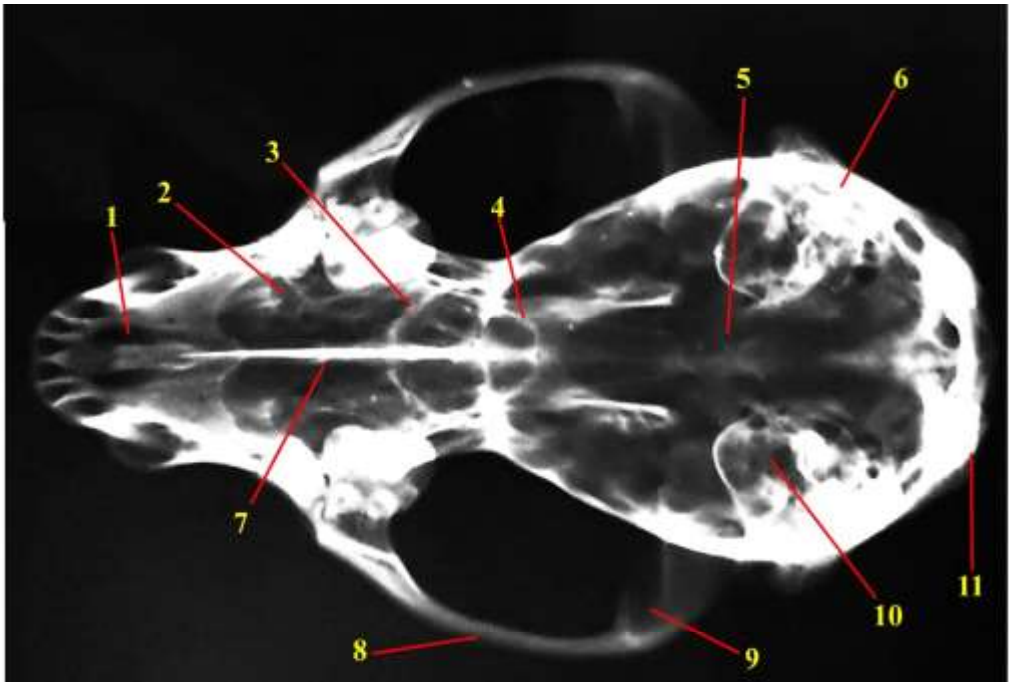

Figure 10.
Dorso-ventral radiograph of skull of badger.
1) Palatine fissure, 2) Maxillary recess, 3) Cribriform plate, 4) Naso-frontal suture, 5) Facies cerebralis, 6) Petrous part of temporal bone, 7) Inter-palatal suture, 8) Zygomatic arch, 9) Mandibular fossa, 10) Tympanic bulla, 11) Occipital condyle.

Authors' Contributions

A. and B. conceived and planned the experiments. A. and C carried out the experiments. C. contributed to sample preparation. A.C. and D. writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

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Development of the Respiratory Tract in Red Sokoto Goat (Capra Hircus): Histological Perspective

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ABSTRACT

The foetal development of the respiratory tract in the red Sokoto goat was investigated in this study using morphological techniques. Sections of the respiratory tract were obtained from the foetuses of 40 apparently healthy red Sokoto goats that were grouped into the first term, early 2nd term, late 2nd term, and 3rd term (n = 10/group). Laryngeal glands formed in the early 2nd term and also secreted neutral mucin within the same period, while apical budding and proliferation of the naïve epithelium led to the formation of stratified squamous epithelium just at the beginning of the 3rd term. The trachea consisted of a bi-stratified epithelium at foetal days (FD) 53 and later became ciliated pseudostratified columnar epithelium during the early 3rd term. At FD 102, the glandular epithelia contained bluish-stained areas, while the glandular lumina contained acidic mucins. The lungs of red Sokoto goats were at the pseudo-glandular stage at FD 54, canaliculi stage between FDs 71-76, terminal sac stage between FDs 76 – 104, and alveolar stage from FD 129. The structural changes in the respiratory tract of this breed are essential changes needed for neo-natal and post-natal functions. The lungs were structurally mature in the 3rd term and could support the animal even in preterm kids.

Keywords

larynx, trachea, lungs, foetal development, red Sokoto goats

Number of Figures: 5
Number of Tables: 0
Number of References:: 33
Number of Pages: 10

Abbreviations

FD(s): foetal day(s)

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Introduction

Goats are one of the most valuable livestock species present in many parts of the world. The Red Sokoto goat (RSG) is the most abundant breed of goat in Nigeria, particularly in Northern Nigeria [1]. This breed is also present in the Northern part of the Niger Republic and Cameroon [1]. RSG possesses a characteristically dark red coat colour with occasional occurrence of lighter coat colour. Several ecotypes of Red Sokoto goats, possessing varied coat colours such as dark red, light red, brown, light brown, black, and variegated, have also been reported [2, 3]. The goat breed has unique adaptive traits that make them most suited to the harsh environmental conditions of the tropics and are highly valued for their meat and skin.

The respiratory system has two functional parts: the conducting part which conveys, moistens, and warms the air passing to the lung, and the respiratory part, where gaseous exchange occurs. The upper respiratory tract is made up of the nasal cavity, oral cavity, pharynx, and their associated structures, while the lower respiratory tract consists of the trachea, bronchi, bronchioles, and alveoli. The respiratory system develops from the primitive gut tube which is an endodermal structure that forms during the lateral folding of the embryo [4]. The expression of Nkx2-1 (Titf1) in the ventral wall of the foregut marked the earliest signal for the development of the respiratory system and results in the specification of future trachea and lungs locations [5]. An out-pocketing of the proximal part of the foregut gives rise to the respiratory diverticulum which later bifurcates into two buds that eventually become the left and right primary bronchi [4]. After the lung buds form, the trachea bud appears and undergoes further morphological transformations, including tube separation, elongation, and diameter expansion [5]. The initial lung bud comprises of an endodermal epithelium that is surrounded by splanchnic mesoderm-derived mesenchyme [6]. Further lung development involves controlled cross-signalling between the epithelium, mesenchyme, and mesothelium [6, 7]. The Wnt signalling is indispensable for embryonic lung progenitor proliferation [8]. The maturation of fetal lungs for normal post-natal life also depends on gene expression [9]. The transcription factors, TTF-1 (thyroid transcription factor 1), CCAAT-binding proteins (or C/EBPα), FoxA2 (Foxhead box protein A2), and proteins such as Hopx, and Hdac2 are key regulators of gene expression needed for the modulation of lung maturation [9-11].

The timing and the pattern of epithelial differentiation during gestation varies among species. There are five morphological phases of development for most mammal lungs, namely the embryonic phase, pseudo-glandular phase, canalicular phase, terminal sac phase, and alveolar phase. In fetal bovine lungs, the typical features of the pseudo-glandular, canalicular, and alveolar stages of lungs were observed from days 84 – 98, 154 – 164, and 224 – 266 of gestation, respectively [12]. In sheep, the alveolar phase of lung development is established 4 weeks before birth [13]. The timing and pattern of development of the various respiratory tissues of caprine have not been reported. Thus, this study aims to investigate the morphological development of the larynx, trachea, and lungs in the fetuses of red Sokoto goats using histological and histochemical techniques.

Results

Histology of the larynx

The laryngeal walls consist of a modified mucosal layer, skeletal muscles, and cartilages. The laryngeal mucosa exhibited two bilateral folds (vocal folds/cords) and two upper pairs of folds known as vestibular folds (false vocal cords) (Figure 1). At FD 53, the forming epithelium of the laryngeal mucosa showed somewhat bi-stratified epithelial cells. The lamina propria mucosae was a mesenchymal tissue devoid of glands. Areas of naïve skeletal muscles were observed (Figures 1A, 1B). At FD 71 the vocal cord areas consisted of an epithelium with a basal area and an apical area. The basal epithelial area contained a single layer of basal cells that rested on a basal membrane, while the apical aspect of the epithelium was lined by stratified epithelial cells containing squamous to round nuclei. The subjacent areas showed lamina propria mucosae with glands and bundles of forming skeletal muscles (vocalis muscles). Vestibular folds were observed (Figure 1C). At FD 76, there were apical buddings of the stratified epithelium. The lamina propria mucosae contained glands which increased in population at FD 98 (Figure 1D). At FD 98, the nuclei of the topmost layer of the epithelium were more squamous than round. At FD 104, the laryngeal mucosa was largely lined by a well-defined stratified squamous epithelium. The population of glands in the submucosa increased while thick bundles of skeletal muscles were key constituents of the laryngeal wall (Figures 1E, 1F).

PAS-Alcian blue histochemistry of larynx

Following PAS-Alcian blue staining, there were no goblet cell areas at FD 99 of development. However, the observed glandular lumina contained magenta-stained secretions (Figure 2A). At FD 104, few areas of bluish-stained goblet cells were observed in the epithelium, while more magenta-stained glandu-

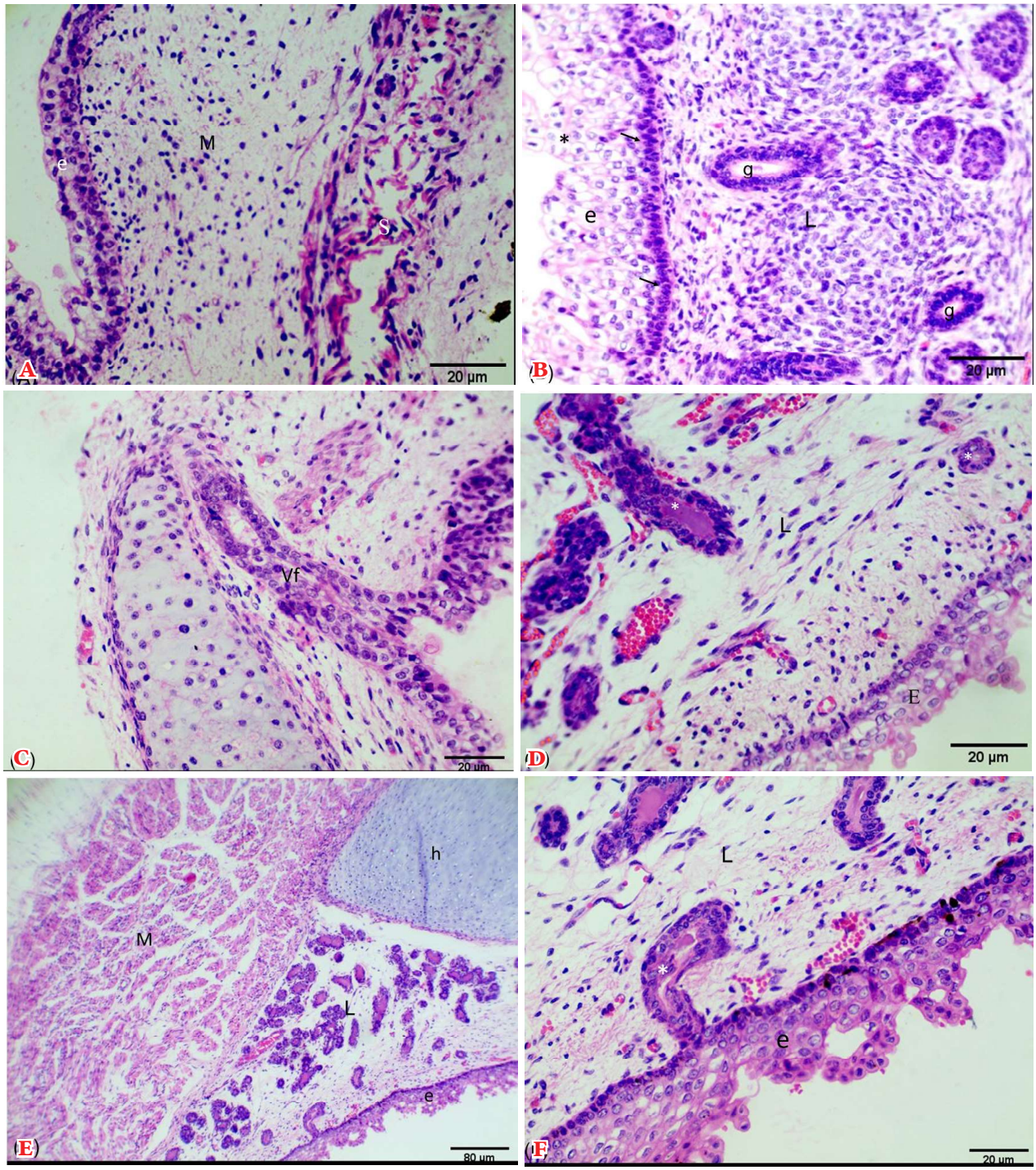

Figure 1.
1A, the larynx at FD 53 showing forming epithelium (e), mesenchymal tissue area (M), and naïve skeletal muscle area (S), H&E stain, x400. 1B, the larynx at FD 69 shows the forming of the epithelium (e) with basal (arrows) and apical (asterisk) areas and lamina propria mucosae (L) with glands (g). H&E stain, x400. 1C, the larynx at FD 71 showing the vestibular fold (Vf). H&E stain, x400. 1D, the larynx at FD 98 shows stratified squamous epithelium (E) and lamina propria mucosae (L) with glands (asterisks). H&E stain, x400. 1E, the larynx at FD 104 shows stratified squamous epithelium (e), lamina propria mucosa (L) with many glands, bundles of skeletal muscles (M), and hyaline cartilage (h), H&E stain. x100. 1F, the larynx at FD 104 shows stratified squamous epithelium (e), lamina propria mucosae (L), and invaginating gland (asterisk). H&E stain, x400

lar lumina were observed (Figure 2B).

Histology of the trachea

The trachea of the red Sokoto goat was made up of four tunics, namely tunica mucosa, tunica submucosa, tunica muscularis, and tunica adventitia (Figures 2C and 2F). The tunica mucosa consisted of lamina epithelialis mucosae, lamina propria mucosae, and lamina muscularis mucosae, while the tunica submucosa contained C-shaped hyaline cartilages whose ends are bridged by smooth muscles.

At FD 53, the lamina epithelialis mucosae was a

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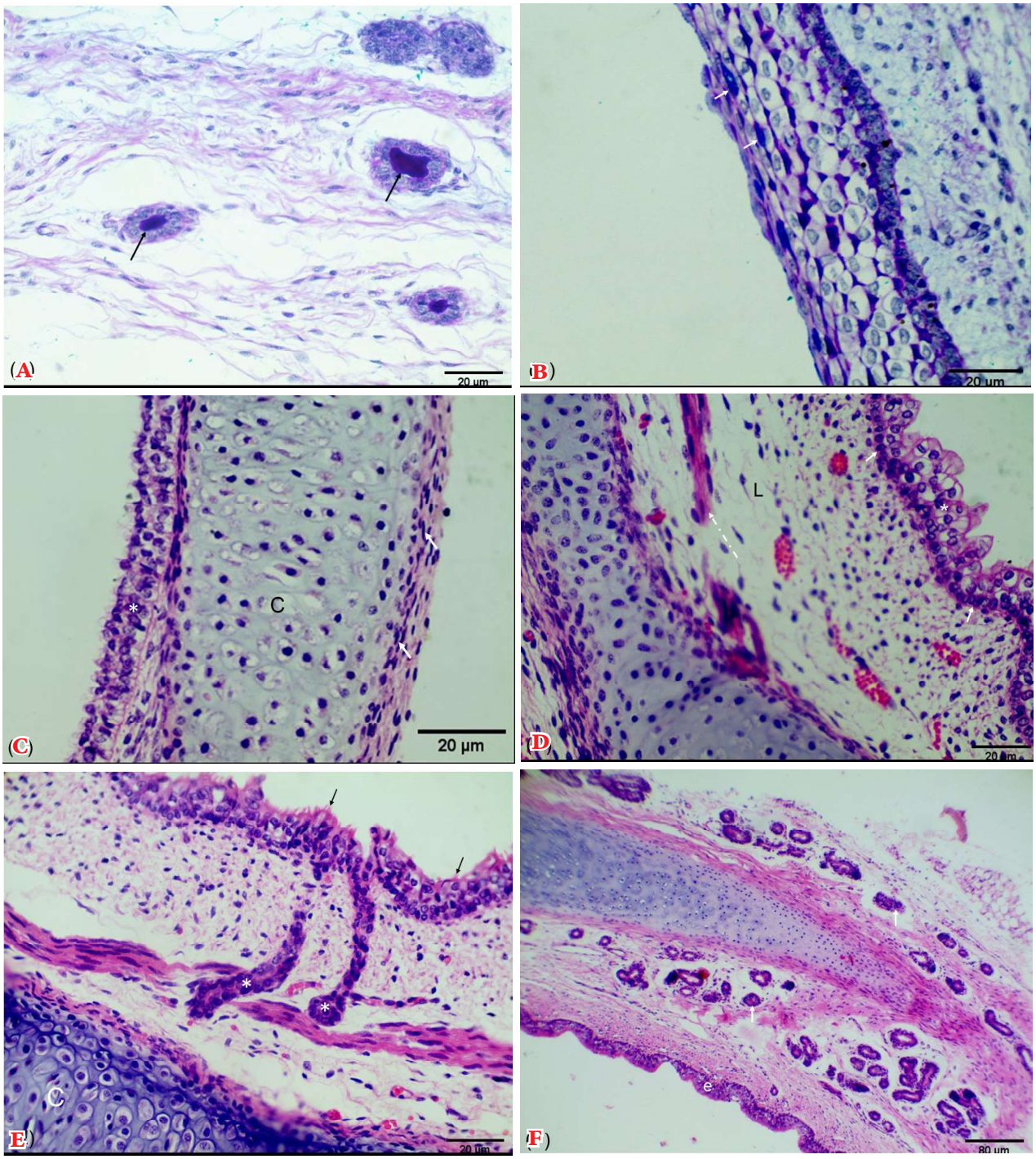

Figure 2.
2A, the larynx at FD 99 showed glandular lumina with magenta-stained secretions (arrows), PAS-Al stain, x400. 2B, at FD 104, the larynx showed bluish-stained areas within the epithelium (arrows), PAS-Al stain, x400. 2C, the trachea at FD 53 showing bi-stratified epithelium (asterisk), hyaline cartilage (c), and smooth muscle cells (arrows), H&E stain, x400. 2D, micrograph of the trachea at FD 80 showing basal (arrows) and apical (asterisk) epithelial areas, lamina propria mucosae (L), lamina muscularis mucosae (dotted-arrow), H&E stain, x400. 2E, the trachea at FD 102 showing tracheal epithelium with apical cells (arrows) and invaginating glands (g), H&E stain, x400. 2F, the trachea at FD 130 shows a substantial population of glands (arrows) in the lamina propria and submucosal areas and ciliated pseudostratified columnar epithelium (e), H&E stain, x100.

bi-stratified epithelium with basal columnar and apical cuboidal to columnar epithelial cell layers. A thin layer of lamina muscularis mucosae closely associated with the cartilaginous rings was observed, while islands of smooth muscle cells existed between the tips of the cartilages (Figure 2C). At FD 71, cells within the bi-stratified epithelium became more staggered, with additional apical dome-shaped binucleated cells forming an extra layer. At FD 80, the lamina muscularis mucosae was more clearly delineated and partly separated from the cartilaginous ring (Figure 2D). At

FD 98, epithelial cells invagination into the wide lamina propria mucosae formed a few glands.

At FD 102, the lamina epithelialis mucosae was a pseudostratified columnar epithelium that showed scanty cilia. Several glands extended from the epithelium throughout the length of the lamina propria mucosae and to the submucosa (Figure 2E). At FD 130, the tracheal epithelium consisted of pseudostratified columnar epithelium with an apical brush border appearance (Figures. 2F, 3A). Each C-shaped cartilage exhibited three lateral, medial, and distal surfaces.

PAS-Alcian blue histochemistry of trachea

Between FDs 53 – 102, staining with PAS-Alcian blue showed no positive reaction within the epithelium. Within the above timings, no goblet cell areas were obvious (Fig. 3B). However, at FD 102, the glandular epithelia contained a bluish-stained area, while the glandular lumina contained bluish-magenta secretions (Figure 3C). At FD 130, distinct bluish-stained goblet cells were found within the tracheal epithelium. The glandular epithelia of the trachea exhibited bluish-magenta stained areas (Figure 3D).

Histology of the lungs

At maturity, the lungs are composed of parenchyma, branches of the bronchial tree (consisting of primary bronchi, secondary (lobar) bronchi, and tertiary (segmental) bronchi), pulmonary arteries, and veins. The segmental bronchi give rise to bronchioles that later forms the terminal bronchioles, respiratory bronchioles, alveolar ducts, alveoli sac, and alveoli (Figure 4)

Early 2nd term

At FD 54, the lung of the red Sokoto goat was at the pseudo-glandular stage (Figure 4A). Here, the developing lungs appear as mesenchymal tissue that

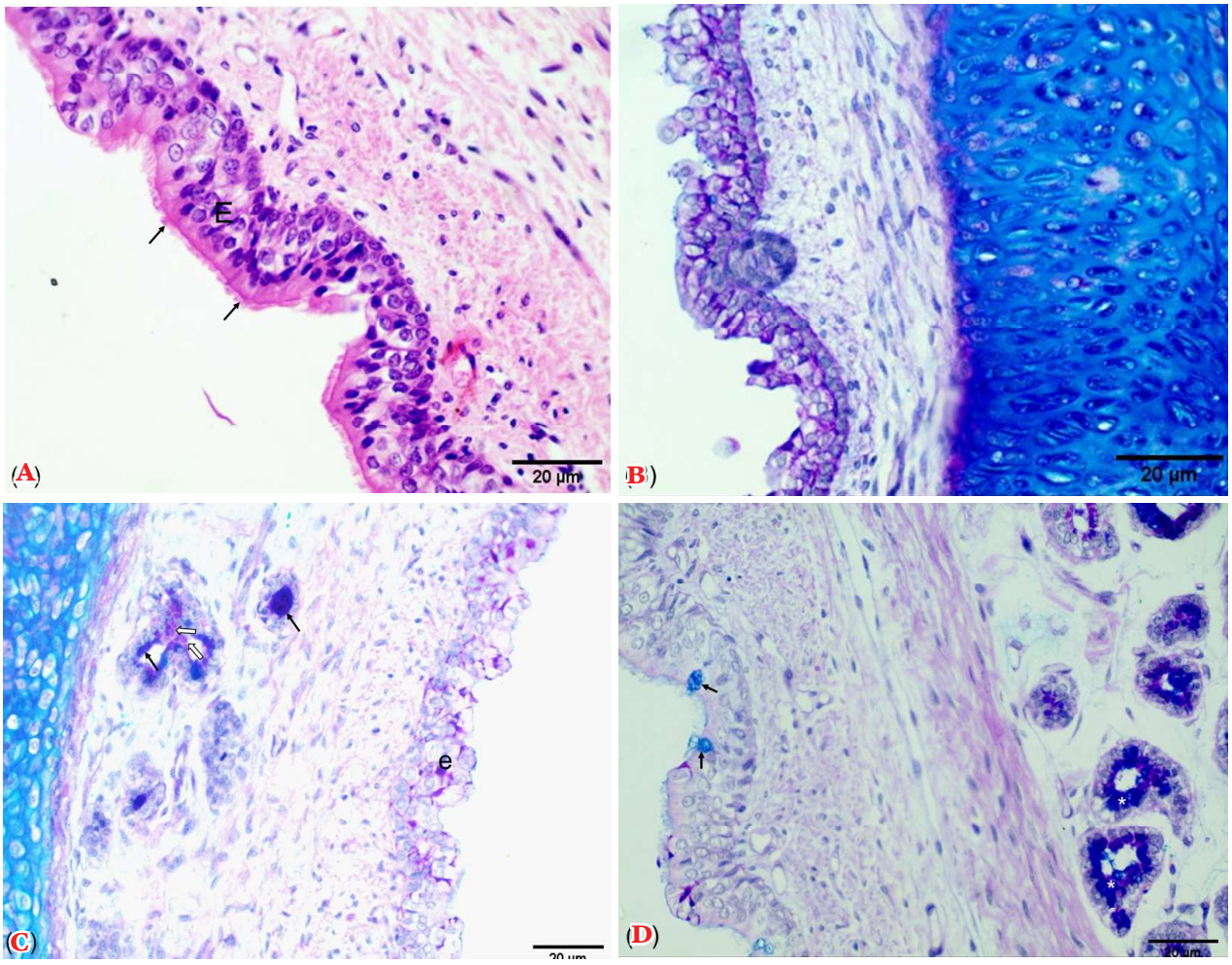

Figure 3.
3A, the trachea at FD 130 showing ciliated (arrows) pseudostratified columnar epithelium (E). H&E stain, x400. 3B, the trachea at FD 80 showing negative PAS-AL blue reactions, PAS-Al stain, x400. 3C, the glandular lumina of the trachea at FD 102 showed bluish (black arrows) and magenta-stained (white arrows) areas, PAS-AL stain, x400. 3D, the trachea at FD 130 shows bluish-stained goblet cells (arrows) and bluish-stained glandular epithelial areas (asterisks). PAS-Al stain, x400.

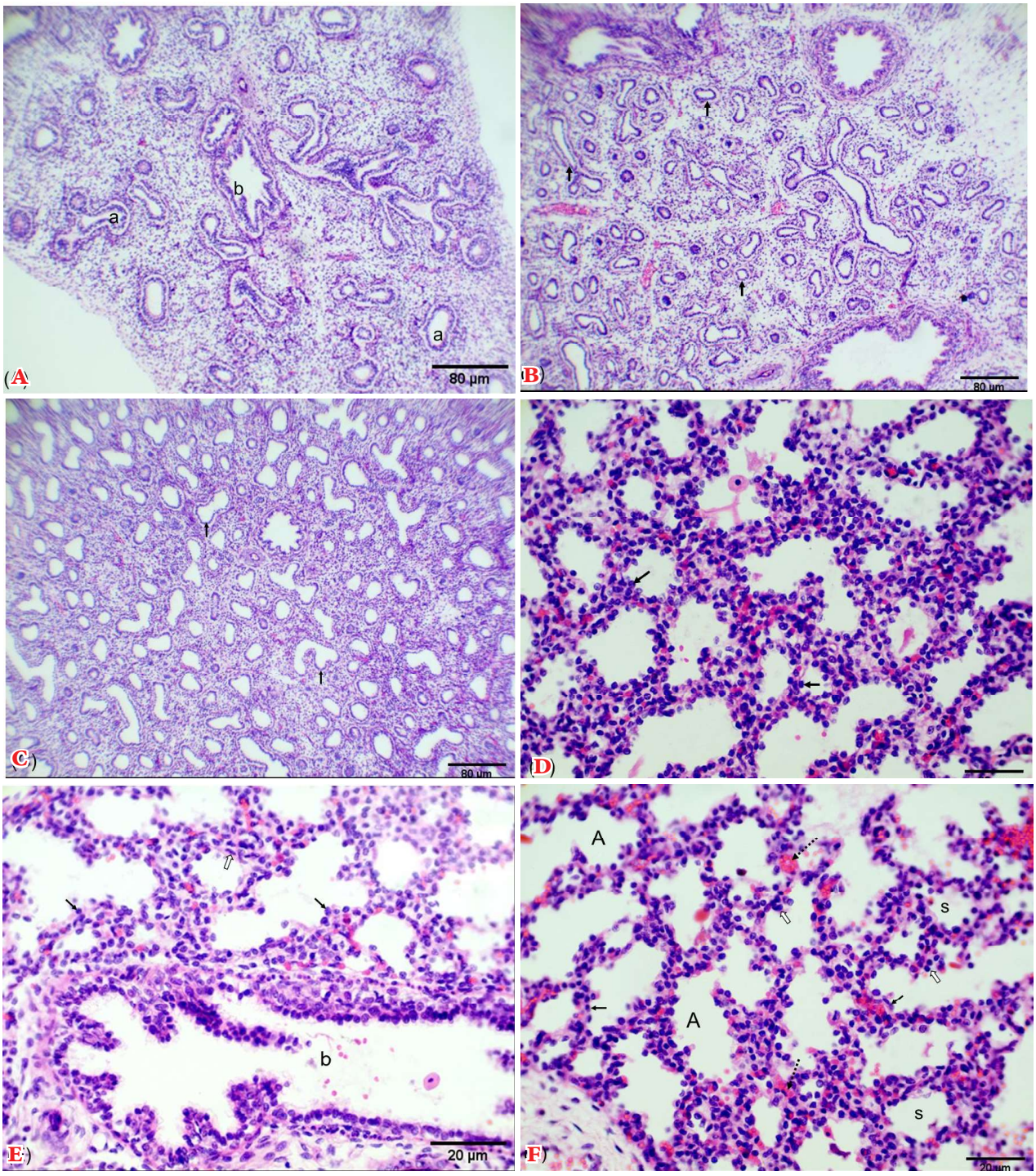

Figure 4.
4A, the lung (FD 54) at pseudo-glandular stage showing mesenchymal tissue with expanding airways (a) and bronchiole (b), H&E stain, x100. 4B, the lung (FD 71) at canalicular stage showing diffusely distributed canaliculi (arrows) within the lung tissue, H&E stain, x100. 4C, the lung (FD 76) at the canalicular stage, transitioning to the terminal sac stage. Note the budding terminal sac (arrows). H&E stain, x100. 4D, the developing lung (FD 99) at terminal sac stage showing sacs lined by simple cuboidal epithelia (arrows), H&E stain, x400. 4E, the lung (FD 104) at terminal sac stage showing bronchiole (b), alveolar sacs with type 1 (white arrows) and type II (black arrows) pneumocytes, H&E stain, x400. 4F, the lung (FD 129) at late terminal sac or alveolar stage showing alveolar sacs (S) and alveoli (A) with type I (black arrows) and type II (white arrows) pneumocytes. Note the blood capillaries (segmented arrows). H&E stain, x400.

contains exocrine glands. All branches of the bronchial tree and bronchioles were present (including terminal bronchioles). At FD 71, the developing lung was at the canalicular stage (Figure 4B). The mesenchymal tissues of the developing lungs contained a large population of mesenchymal cells, with canaliculi widely distributed within the lung tissues.

Late 2nd term

At FD 76, the lung was in the late canalicular stage, transitioning to the terminal sac stage (Figure 4C). Several canaliculi were diffusely distributed, but terminal sacs were beginning to bud. At FD 99, the developing lungs were in the terminal sac stage (Figure 4D). A large population of terminal sacs budded off from the respiratory bronchioles were observed. The terminal sacs were largely lined by simple cuboidal epithelium.

Third term

At FD 104, the developing lungs of red Sokoto goats were in the terminal sac stage (Figure 4E). At this stage, a large number of alveolar sacs were formed from the respiratory bronchioles and were lined by cuboidal epithelial cells. The walls of the sacs had a rich supply of capillaries that were increasingly associated with the epithelium. Areas of type I pneumocytes and type II pneumocytes were observed. At FD 129, the lungs were either in the late terminal sac stage or alveolar stage, meaning that they were transitioning to the alveolar stage (Figure 4F).

Discussion

The framework of the larynx of the red Sokoto goat was fully established in the early 2nd term. Though naïve, a bi-stratified epithelium was clearly seen at FD 53 with basal and upper layers. The laryngeal basal layer may initiate the stratification process of the epithelium and also remain as stem cells for epithelial renewal. In most epithelia, the basal cells serve as multipotent progenitors capable of renewing the epithelia [14, 15]. In mouse embryos the basal layer-initiated stratification at embryonic day 10.5 to form the periderm [16]. At around the 71st day of gestation in this study, the upper layer of the laryngeal epithelium proliferated, largely by apical budding to first form large, hollow cells with small flat to oval nuclei. Later in the 2nd term, the foetal larynx was composed of stratified squamous epithelium. In humans, squamous epithelium develops in the 2nd trimester of fetal life and has been associated with programmed activation of certain genetic signals within the endodermal cells [17]. It is believed that a primitive swallowing ability develops in foetuses [18], thus, the stratified squamous epithelium developed in this study may serve mechanical purposes.

The structure of the larynx in the red Sokoto goat included vocal folds that were similarly lined by an epithelium that varied in structure with age. The vocal cord lamina propria contained laryngeal glands which were first observed in the early 2nd term (around FD 69) in this study. Earlier, the glands formed by epithelial invaginations and proliferated to populate the lamina propria. The observation of the magenta-stained secretions (after PAS-Alcian blue staining) in the glandular lumina at FD 99 shows the onset of neutral mucin secretions which may lubricate the foetal larynx. Bluish-stained areas were seen within the epithelium in the 3rd term. It is unclear if these are cells or orifices of developing glands with a secretory content of acidic mucin. Naïve vocalis muscle areas were observed in the early second term as islands of forming muscles. The vocalis muscle is a key constituent of the true vocal fold which aids phonation [19]. We observed that the naïve vocalis muscle area became bundles of skeletal muscles in 104th day old red Sokoto goats. These muscles, together with the formed cartilages as well as vestibular folds consolidated a complex laryngeal structure just before birth. Mechanical signals from swallowing and breathing were earlier thought to guarantee a more complex/mature larynx [18, 20]. But in this study, the already established complex laryngeal structure fingers the influence of genetic factors than just mechanical factors. According to Jadcherla et al. [21], the swallow peristaltic activity begins in fetal life, thus, the fetal larynx is required to prevent liquid aspiration.

The tracheal epithelium of the red Sokoto goat was poorly developed in the 1st and 2nd terms, most probably because the respiratory system of foetuses is largely nonfunctional. The pattern of development of the tracheal epithelium followed a similar trend as that of the larynx. However, at FD 53, the bi-stratified epithelial lining of the trachea contained basal columnar, and apical cuboidal to columnar-shaped cells. The trachea forms the conductive part of the respiratory system and consists of the ciliated pseudostratified columnar epithelium (respiratory epithelium) in 104-day-old foetuses of red Sokoto goats. The respiratory epithelium which forms in the 3rd term is preparatory for post-natal protective roles. The constituent goblet cells of the respiratory epithelium, its secretory products, and as well as glandular secretions toward birth will likely drive mucociliary clearance of dust and pathogens after birth. The distinct bluish-stained goblet cells in PAS-Alcian blue preparations at FD 130 showed secretion of acidic mucin, while the bluish, bluish-magenta stained glandular areas are indicative of acidic-neutral mucin secretions. These secretions

Structural development of the respiratory tract

will synchronize to defend the respiratory system. Among other cells like goblet cells, basal cells, and neuroendocrine cells, ciliated cells are the most abundant cells of the tracheal epithelium [8, 23].

Five morphological stages of lung development previously recognized in prenatal mammalian lung development included embryonic, pseudo-glandular, canaliculi terminal sac, and alveolar stages. In the current study, the lungs of the red Sokoto goats were at the pseudo-glandular stage at FD 54, canaliculi stage between FDs 71-76, terminal sac stage between FDs 76 – 104, and alveolar stage from 129 days of gestation. This observation is at variance with the report of pseudo-glandular, canaliculi, terminal sac, and alveolar sac stages in sheep, which were reported between 40-90, 95-120, 120-140, and from 140 foetal days, respectively [7]. In experimentally reared perinatal goats of Assam [23], late canaliculi, terminal sac, and alveolar stages were observed at gestation days 116, 139, and 149, respectively. The reports on the varied timing of lung development may represent breed/species-specific features of lung development and may also reflect environmental influences. According to Greenough [24], intra- or extra-thoracic compression, abnormal fetal breathing movement, and reduction in amniotic fluid volume, which may impair antenatal lung growth will adversely affect normal lung functions postnatally. Thus, in this study, the structural maturation of the lungs of red Sokoto goats is obvious in the 3rd term of development and thus prepares the lungs for normal post-natal life. In the late 3rd term, the onset of the alveoli stage is obvious, but it is believed that the lung has a life-long alveolization ability to facilitate any required lung regeneration [25].

Type 2 pneumocytes are considered the major source of both types II and I pneumocytes [26, 27]. In this study, the terminal sacs and the alveoli observed in the 3rd term were composed mostly of type I pneumocytes than type II. Although type II pneumocytes also occurred, there were fewer in population than type I cells. It is unclear in this study when type II pneumocytes first form. However, popular opinion suggests that they arise from the distal tubules during canalicular and saccular stages [28, 29]. The flat appearance of type I cells in this study is typical of the shape of the cells in the mammalian lung. Later in the 3rd term, type I cells were intimately associated with alveolar capillaries in an arrangement that will facilitate the onset of gaseous exchange. The endothelial cells, basement membrane of the type I pneumocytes, basement membrane of the alveolar capillaries, and the endothelial cells of the capillaries form the gaseous exchange barrier in the alveolar wall [28]. Markers of type I pneumocytes are podoplanin (T1α) and aquaprin5 (Aqup5) [26, 29].

In conclusion, the structural modifications observed within the larynx, trachea, and lung in this study highlight essential changes needed for normal post-natal function of the respiratory system in the red Sokoto goat. While four of the five stages of lung development were obvious between FD 54 and 129, the lungs of the red Sokoto goat were structurally mature at FD 129 in readiness for the first breathing exercise.

Materials & Methods

Animals

The intact gravid uterine and their adjoining tissues were obtained from 40 healthy pregnant red Sokoto goats (Figure 5) slaughtered at the Nsukka municipal slaughterhouse, Ikpa, Nsukka Local Government Area, Enugu State. Nsukka is located at 6.86 degree North Latitude, 7.39 degree East longitude, 7.39o East longitude [30]. The gravid uteri were transported to the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, Nsukka for the collection and processing of samples from their fetuses. The fetuses were grouped into the three terms, namely: days 0-50 (first term), 51-100 (second term), and 101-150 (third term). The second term was subdivided into 2, namely: days 51-75 (early second term), and 76-100 (late second term).

Figure 5.
Photograph of the red Sokoto goat showing phenotypic traits of the breed.

Gross anatomy

After the exteriorization of the fetuses, the crown-rump length (CRL) was measured using a thread and meter rule. The distance from the top of the head, passing through the dorsal aspect of the neck, and the curvatures of the spine to the root of the tail represented the CRL. The gestational age (or fetal age) was estimated using the formulae [31]:

$y = 2.74x + 30.15$

The y denotes gestational age in days, and the x is the CRL (cm).

The sections of the larynx, trachea, cranial, middle, and caudal lobes of the lungs were obtained and processed for histological evaluation.

Histological procedures

The sections of the larynx, trachea, and lung were excised and fixed in 10% neutral-buffered formalin for 48 hours. Thereafter, the fixed tissues were dehydrated in the graded concentration of ethanol and cleared in xylene. The cleared tissues were embedded in molten paraffin wax and mounted for sectioning with a rotary microtome. Five micrometer thick sections were obtained and stained with haematoxylin and eosin (H&E), and periodic acid Schiff-Alcian blue at pH 2.5 (PAS-Alcian blue) stains for light microscopy. The staining protocols were according to the methods described by Sheehan and Hrapchak [32] and Mepham [33]. The Motic binocular light microscope was used to evaluate the histological features of the tissue sections. Photomicrographs were captured using an Amscope® digital camera (United Scope LLC) attached to the Motic binocular light microscope.

Authors' Contributions

A.F.U. conceived and planned the experiments. A.F.U., C.L.O., and E.E.U. carried out the experiments. A.F.U. planned and carried out the simulations. A.F.U., C.L.O., and E.E.U. contributed to sample preparation. A.F.U., C.L.O., and E.E.U. contributed to the interpretation of the results. A.F.U. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Isolation, Characterization, and Antibacterial Activity of Actinomycetes from Sheep Feces

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ABSTRACT

Actinomycetes are a vital group of Gram-positive bacteria known for producing a wide range of bioactive secondary metabolites, including important antibiotics. These microorganisms play an essential role in the degradation of organic matter and nutrient cycling, contributing significantly to soil health and fertility. Their capacity to synthesize diverse compounds and the presence of key biosynthetic pathways involving polyketide synthases and non-ribosomal peptide synthetases highlights their potential in antibiotic discovery, particularly against antibiotic-resistant pathogens. This study aimed to isolate and characterize *Actinomycetes* from fresh sheep feces collected in Ilam Province, Iran, focusing on their antibacterial activity and biosynthetic potential. A total of 86 actinomycete isolates were obtained from fecal samples collected from sheep in 2021. Morphological characterization confirmed all isolates as Gram-positive and filamentous. Molecular identification through PCR amplification of the 16S rRNA gene yielded a product of approximately 640 base pairs for all isolates. Antibacterial screening revealed that 17 isolates exhibited activity against various pathogens, with the highest efficacy observed against *Bacillus cereus* (62.1%). Molecular analysis also indicated the presence of biosynthetic gene clusters, with 31 isolates (36.05%) bearing non-ribosomal peptide synthetase (*NRPS*) gene, 15 isolates (17.44%) containing polyketide synthase I (*PKS-I*), and 16 isolates (18.6%) with polyketide synthase II (*PKS-II*) genes. This study highlights the significant antibacterial properties and biosynthetic capabilities of *actinomycetes* from sheep feces, suggesting their potential use in therapeutic, agriculture, and biotechnological applications.

Keywords

Actinomycetes, Polyketide Synthase, Non-Ribosomal Peptide Synthetase, Antibacterial Activity, Sheep Feces

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Number of Pages: 8

Abbreviations

PKS: Polyketide Synthase
NRPS: Non-Ribosomal Peptide Synthetase
BGC : Biosynthetic Gene Clusters
PCR: Polymerase Chain Reaction
NA: Nutrient Agar
SCA:Starch Casein Agar

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Introduction

Actinomycetes, particularly members of the genus *Streptomyces*, represent a significant group of Gram-positive bacteria renowned for their capacity to produce a diverse array of bioactive secondary metabolites, including many clinically relevant antibiotics. These metabolites are crucial in pharmaceutical applications, especially in addressing the escalating issue of antibiotic resistance by providing new compounds for drug development [1].

Actinomycetes' ecological diversity facilitates their thriving in various environments, contributing to their ability to produce different classes of bioactive compounds such as antibacterials, antifungals, and anticancer agents [2]. Notably, actinomycetes account for approximately 45% of all known bioactive microbial metabolites, with over 10,000 compounds reported [3]. Their unique chemical structures and biological activities render them invaluable in the search for new therapeutic agents [4].

Actinomycetes inhabit various ecological niches, with soil and organic matter serving as key reservoirs. They thrive in diverse environments, including marine ecosystems, freshwater habitats, and extreme conditions such as deserts and high-salinity areas [5]. Furthermore, actinomycetes have been isolated from unique habitats like mangrove forests, caves, and endophytic niches within plants, underscoring their adaptability and ecological significance [6, 7]. These microorganisms engage in complex interactions within their ecosystems, significantly influencing their metabolite production. Understanding the ecological context of actinomycetes is essential for discovering novel bioactive compounds, as their secondary metabolites are often linked to environmental interactions [8]. The ongoing exploration of these diverse habitats continues to unveil new actinomycete species with potential therapeutic applications [9].

Sheep feces, rich in organic nutrients, provide an optimal environment for the growth of actinomycetes, thereby promoting their metabolic diversity. The nutrient composition of sheep feces supports a diverse microbial community, including various actinomycete species that thrive in such organic-rich substrates [10]. *Actinomycetes* in fecal environments play a vital role in decomposing organic matter and cycling nutrients, thereby enhancing their metabolic capabilities [11]. The presence of these microorganisms in feces can lead to the production of bioactive compounds, including antibiotics, which are beneficial for ecological balance and potential pharmaceutical applications [12]. Additionally, the interaction between actinomycetes and other microbial communities in feces can further enrich their metabolic profiles, making fecal matter a valuable resource for discovering novel actinomycete strains with unique properties [13].

The microbiota of sheep feces, including actinomycetes, significantly contributes to organic matter decomposition and nutrient cycling. *Actinomycetes* are key players in breaking down complex organic materials, such as cellulose and lignin, facilitating the conversion of organic waste into simpler compounds that plants and other microorganisms can utilize [14, 15]. During the composting process, actinomycetes, bacteria, and fungi function as chemical decomposers, transforming organic matter into stable products like compost, which enriches soil fertility [16]. Their metabolic activities enhance nutrient availability and contribute to forming soil aggregates, improving soil structure and health [17]. Overall, the presence of actinomycetes in sheep feces highlights their ecological importance in maintaining soil health and promoting sustainable agricultural practices through effective nutrient recycling [18].

Polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are essential enzymes involved in the biosynthesis of secondary metabolites, encompassing a wide range of bioactive compounds. PKS and NRPS are characterized by their modular organization, where each module incorporates specific substrates into the final product. This modularity enables the synthesis of structurally diverse compounds through the assembly of various building blocks [19]. Typically, these enzymes are organized in biosynthetic gene clusters (BGCs), facilitating the coordinated expression of the genes required for synthesizing these complex molecules. The arrangement of genes within these clusters can vary significantly, with some clusters containing hybrid PKS/NRPS systems that combine functionalities of both types of synthases [20]. This organization enhances biosynthesis efficiency and allows for the evolution of new compounds through gene rearrangements and modifications [21].

This study focused on the isolation and characterization of actinomycetes from sheep feces, assessing their antibacterial potential and screening for *PKS* and *NRPS* genes. *Actinomycetes* are known for their

Abbreviations - cont'd

TSB: Tryptic Soy Broth
EDTA: Ethylenediaminetetraacetic Acid
SOM: Soil Organic Matter
MH: Mueller-Hinton
bp: Base Pairs
RNA: Ribosomal RNA
SOM: Soil Organic Matter;
B. cereus: Bacillus cereus
E. coli: Escherichia coli
S. aureus: Staphylococcus aureus
P. aeruginosa : Pseudomonas aeruginosa

ability to produce a variety of bioactive secondary metabolites, making them valuable for biotechnological applications, particularly in antibiotic discovery [22]. Research has demonstrated that *Actinomycetes* isolated from various environments, including fecal matter, can exhibit significant antimicrobial activity. For example, studies have reported that a substantial percentage of isolated Actinomycetes possess PKS and NRPS genes, indicating their potential for producing secondary metabolites with antibacterial properties [23]. The presence of these BGCs is crucial for developing new antibiotics, especially in light of rising antibiotic resistance [24]. Thus, this study aimed to isolate *Actinomycetes* from sheep feces and investigate their potential for producing novel antimicrobial compounds, with a focus on the presence of BGCs such as *PKS* and *NRPS*, which are critical for the development of new antibiotics in response to increasing antibiotic resistance.

Results

Isolation and Identification of Actinomycetes

Eighty-six *Actinomycetes* isolates were obtained from sheep feces. These isolates exhibited diverse colony morphologies, including variations in texture and pigmentation. All isolates were Gram-positive and filamentous, characteristic of *Actinomycetes*. The identity of all isolates was confirmed through PCR amplification of the 16S rRNA gene, resulting in a product of approximately 640 base pairs (Figure 1).

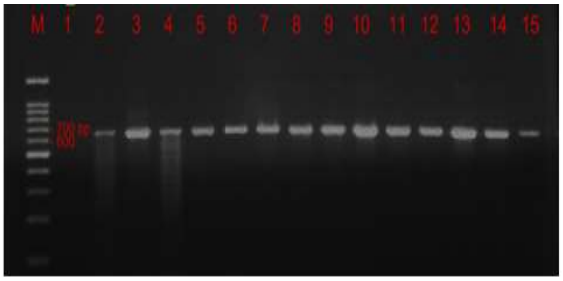

Figure 1.
Agarose gel electrophoresis of 16S rRNA PCR products from bacterial isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from bacterial isolates showing a 640 bp band representing the amplified 16S rRNA gene.

Antibacterial Activity

Out of 86 tested isolates, 17 strains showed antibacterial activity against one or more pathogens. The activity distribution was as follows: against *Staphylococcus aureus:* 16.1% (1 isolate), against *Escherichia coli*: 65.4% (4 isolates), against P*seudomonas aeruginosa*: 32.2% (2 isolates), and against *Bacillus cereus:* 62.1% (10 isolates). The most potent activity was observed against *Bacillus cereus*, underscoring the therapeutic potential of these isolates.

Molecular Identification and Gene Screening

PCR analysis revealed the following distribution of biosynthetic genes: 31 isolates (36.04%) carried *NRPS*, 15 isolates (17.44%) harbored *PKS-I,* and 16 isolates (18.6%) contained *PKS-II* genes (Figures 2-4). These findings indicate substantial biosynthetic potential among the isolates for secondary metabolite production.

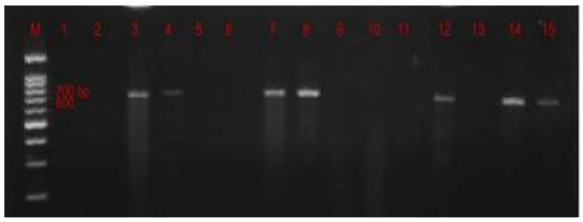

Figure 2.
Agarose gel electrophoresis of *NRPS* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from actinomycete isolates, displaying bands between 700–750 bp, indicative of the amplified *NRPS* gene.

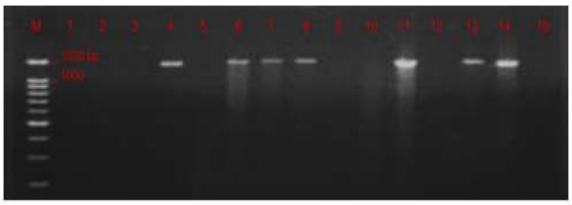

Figure 3.
Agarose gel electrophoresis of *PKS-I* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from actinomycete isolates showing a band at 1200-1400 bp, representing the amplified *PKS-I* gene.

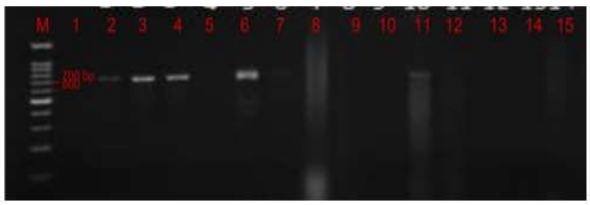

Figure 3.
Agarose gel electrophoresis of *PKS-II* gene PCR products from actinomycete isolates
Lane M: DNA size marker; Lane 1: Negative control; Lanes: 2–15: PCR products from actinomycete isolates exhibiting a band at approximately 600 bp, corresponding to the amplified *PKS-II* gene.

Discussion

The study of *Actinomycetes* isolated from sheep feces has unveiled their potential as a source of antibacterial compounds, which is particularly relevant in the context of rising antibiotic resistance. *Actinomycetes* are renowned for their ability to produce bioactive metabolites that can effectively combat both Gram-positive and Gram-negative bacteria, including notorious pathogens such as *B. cereus* and *E. coli.*

Actinomycetes are recognized for their production of secondary metabolites with antimicrobial properties, making them important candidates in the search for new antibiotics [25, 26]. The successful isolation of 86 *Actinomycetes* strains, with 17 exhibiting notable antibacterial activity, underscores the ecological richness of this niche and the potential for discovering novel antibacterial agents [27]. Previous studies have demonstrated that various *Actinomycetes* strains produce metabolites that inhibit the growth of critical pathogens, suggesting their valuable role in developing new antibiotics, particularly against resistant strains [28].

The microbiota present in sheep feces, particularly enriched with *Actinomycetes*, plays a crucial role in organic matter decomposition and nutrient cycling, which enhances soil fertility [16]. *Actinomycetes* facilitate the breakdown of complex organic materials, contributing to soil organic matter (SOM) and nutrient availability [29, 30]. The diversity of *Actinomycetes* isolated from various habitats, including extreme environments, indicates unique antibacterial activities [31, 32]. This ecological significance supports the notion that environments rich in *Actinomycetes* serve as reservoirs for microorganisms capable of producing bioactive compounds with applications in agriculture and medicine [33].

Molecular characterization of isolates has revealed their potential as producers of bioactive compounds. Research indicates that a significant percentage of isolates possess genes associated with secondary metabolite production, highlighting their capacity for synthesizing antimicrobial compounds [34]. The presence of *PKS* and *NRPS* genes further suggests a robust biosynthetic machinery capable of generating diverse secondary metabolites [20]. Notably, the discovery of hybrid PKS systems has led to the identification of novel aromatic polyketides with therapeutic applications [35].

The antibacterial activity of isolated *Actinomycetes* against *B. cereus* has important implications for food safety and industrial contamination management. For instance, certain strains have shown potential as biocontrol agents in the food industry, effectively inhibiting the growth of *B. cereus* and downregulating its toxin-related genes [36]. Similarly, natural compounds, such as olive oil polyphenol extract, have demonstrated efficacy in reducing *B. cereus* populations in dairy products [37]. The inhibition of *E. coli* also holds significant implications in both veterinary and medical fields, with advancements in monoclonal antibodies and novel small-molecule inhibitors showing promise as alternatives to traditional antibiotics [38, 39].

Future research should prioritize the purification and characterization of bioactive compounds exhibiting antibacterial effects. Techniques such as metabolomic profiling and bioassay-guided fractionation are essential for elucidating the chemical structures and mechanisms of action of these metabolites [40, 41]. Additionally, exploring the regulatory pathways governing the expression of *PKS* and *NRPS* genes is crucial for optimizing metabolite yield and diversity [42, 43]. The study of unconventional habitats like sheep feces for microbial bioprospecting is gaining traction, revealing diverse microbial communities with potential applications in agriculture and biotechnology [44, 45].

In conclusion, combining molecular and microbiological approaches is vital for developing novel antimicrobial agents to combat emerging drug-resistant pathogens. Innovative strategies, including the use of antimicrobial peptides, nanoparticles, and new classes of antibiotics, represent significant advancements in addressing the urgent challenge of antibiotic resistance [46, 47, 48]. The exploration of natural antibacterial agents from sources like *Actinomycetes* not only enhances food safety but also contributes to sustainable practices in veterinary pharmacology and agriculture.

Materials & Methods

Sample Collection

Fresh sheep feces samples were collected from 28 sheep grazing in various geographical regions of Ilam Province, Iran, between March and June 2021.

Sample Preparation and Enrichment

Fresh sheep feces were aseptically collected using sterile forceps. The sample was immediately placed in a sterile zipped bag and transported to the laboratory on ice within two hours of collection.

In the laboratory, each sheep feces sample was thoroughly mixed, and 1 gram of the homogenized sample was serially diluted in sterile distilled water (10-fold dilutions up to *10^-6^*). A volume of 100 µL from each dilution was spread-plated onto two different media: nutrient agar (NA) is a general-purpose medium used for the growth of a wide variety of microorganisms, while starch casein agar (SCA) is a selective medium that enhances the growth of actinobacteria by incorporating starch and casein as sources of carbon and nitrogen.

To inhibit the growth of fungi and other bacteria, both media were supplemented with 50 µg/mL and 50 µg/mL of cycloheximide and

nalidixic acid, respectively.

Isolation and Purification of Actinomycetes

The inoculated plates were incubated at 28°C for 7-14 days. Distinct colony morphologies were observed and selected for further purification. Single colonies were subcultured onto NA and SCA plates until pure isolates were obtained.

Morphological Characterization of Actinomycetes

Isolate identification was based on morphological traits, including colony size, shape, color, margin, elevation; microscopic features such as Gram stain reaction, mycelium formation, and spore morphology; and pigmentation, which may be diffusible or non-diffusible.

DNA Extraction and Molecular Identification of Actinomycetes

Genomic DNA was extracted from pure cultures using a modified method based on Peng et al. (2013) [49]. Briefly, 2 mL of a 48-hour culture grown in TSB at 37°C was centrifuged at 6,000 g for 2 minutes. The supernatant was discarded, and the pellet was resuspended in 300 µL of lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl). This suspension was incubated at 55°C for 60 minutes with gentle shaking, followed by centrifugation at 16,000 g for 5 minutes. The supernatant containing the DNA was transferred to a new tube and stored at -20°C for further analysis. Polymerase chain reaction (PCR) targeting the 16S rRNA gene was performed using Actinomycetes-specific primers, as listed in Table 1 and described previously [50].

Detection of BGCs

Using specific primers listed in Table 1, the presence of *PKS-I*, *PKS-II* and *NRPS* genes was investigated by PCR as described elsewhere [51, 52].

Antibacterial Activity Assay

All actinomycetal isolates were fermented, and their resulting extracts were screened according to previous research without modifications [53].

The antibacterial activity of the actinomycetal strains was evaluated using reference strains of two Gram-negative and two Gram-positive pathogens (Table 2). The bacteria were cultured overnight at 37°C in Mueller-Hinton (MH) broth, and the culture was then adjusted to a turbidity level of 0.5 McFarland standard.

Following the procedure described by Hajizadeh et al. (2023) [53], bacterial lawns were created on MH agar with 6 mm wells, into which 100 µL of crude extracts were added. The plates were left at room temperature for one hour before being incubated at 37°C. After 24 hours, the inhibition zones were assessed in millimeters (mm), utilizing 100

Table 2.
Gram-positive and Gram-negative test pathogens included in the study for evaluating antibacterial activity

Reference strain	Accession number
Bacillus cereus	PTCC 1015
Escherichia coli	PTCC 1330
Pseudomonas aeruginosa	PTCC 1430
Staphylococcus aureus	ATCC 33591

Authors' Contributions

T.M., F.P., and G.H. conceived and planned the experiments. T.M. and F.P. carried out the experiments. T.M, and F.*P.* contributed to sample preparation. T.M., F.*P.*, and K.S. contributed to the interpretation of the results. F.*P.* took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Table 1.
List of oligonucleotide primers used in the study

Primer name	Sequence (5'-3')	Gene	Product size (bp)	Reference
ACT235f	CGCGGCCTATCAGCTTGTTG	16S rRNA	640	50
ACT878r	CCGTACTCCCCAGGCGGGG			
A3F	GCSTACSYSATSTACACSTCSGG	*NRPS*	700-800	52
A7R	SASGTCVCCSGTSCGGTAS			
KIF	TSAAGTCSAACATCGGBCA	*PKS-I*	1200-1400	51
M6R	CGCAGGTTSCSGTACCAGTA			
PKS-II-A	TSGCSTGCTTCGAYGCSATC	*PKS-II*	600	52
PKS-II-B	TGGAANCCGCCGAABCCGCT			

Conflict of interest

The authors declare that there is no conflict of interest.

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بند پایان زهری و سمی درایران، غرب آسیا و خاورمیانه: مروری بر شناسایی، گزیدگی‌ها، نیش زدن‌ها، رفتار، زیست‌شناسی و پراکندگی جغرافیایی آن‌ها

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چکیده

بند پایان به شاخه بندپایان بی‌مهره (Phylum Arthropoda) تعلق دارند که شامل بیشترین تعداد گونه‌ها درزمین است. بندپایان زهرآگین ازجمله مهم‌ترین جانورانی هستند که درمحیط انسانی به فراوانی زندگی می‌کنند. مطالعه حاضر مروری بر اهمیت شناسایی این موجودات، گزش و نیش‌آنها، رفتار، زیست‌شناسی و پراکندگی آن‌ها درمنطقه جغرافیایی ایران، غرب آسیا و خاورمیانه می باشد. پایگاه‌های داده به طور جامع جستجو شده و مقالات و کتاب‌های منتشر شده از سال ۱۹۷۸ تا ۲۰۲۳ با دقت براساس مناسب‌ترین کلید واژه‌های انتخاب شده‌اند. موجودات زهرآگین واجد نیش و توان گزیدگی، درجداول به تفکیک طبقه، راسته و خانواده ارائه شده اند و اهمیت و نقش هر راسته براساس نیش، گزیدگی و وقوع خطرات به طور جداگانه تعیین گردیده است. درنهایت، روش‌های پیشگیری ازنیش و گزیدگی آن‌ها توصیه شده است. شاخه بندپایان درایران شامل دو زیرشاخه قلاب داران Chelicerata با یک کلاس، پنج راسته و ۲۵ خانواده، و زیرشاخهٔ آرواره داران Mandibulata با سه کلاس، نه راسته و ۲۹ خانواده می باشد. آن‌ها در سرتاسر ایران پراکنده‌اند. دستگاه زهرآن‌ها شامل غدد زهر، پدی پالپ یا پاهای انبرکی تغییر یافته، قلابها، نیش در دم (تلسون)، اجزای دهانی مانند هایپوستوم، فنگ (Forcipules) یا دندان نیش، ضمائم (فک‌ها)، خرطوم (proboscis)، اندام تخم‌گذاری (نیش) و موهای سوزنی زهرآگین (موهای تحریک‌کننده) است. دربرنامه‌های آموزشی پزشکان و کادر پزشکی و پرستاری اهمیت نیش و گزیدگی بند پایان زهرآگین بسیار ضعیف یا اصلاً وجود ندارد. برای دستیابی و افزایش کارایی مدیریت گزیدگی و مسمومیت با زهرآنها، اطلاعات دقیق‌تری درباره این موجودات زهرآگین و ترکیب زهرآن‌ها مورد نیاز است.

واژگان کلیدی

بند پایان زهری، سمی، حشره، گزیدگی، نیش، ایران، خاورمیانه

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Abstracts (in Persian)

ارزیابی روش های مختلف استخراج و ویژگی های ضد میکروبی و آنتی اکسیدانی عصاره ریشه *Eremurus spectabilis*

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چکیده

مطالعه حاضر با هدف بررسی فعالیت‌های بالقوه ضد میکروبی و آنتی‌اکسیدانی گیاه Eremurus spectabilis (E.spectabilis) با استفاده از سه روش استخراج انجام شد. سه روش برای استخراج مواد موثره از ریشه گیاه *E. spectabilis* انتخاب شد: استخراج آبی، استخراج الکلی و استخراج هیدروالکلی. برای تعیین بازده استخراج از ۱۰ گرم پودر *E. spectabilis* استفاده شد. آزمون کربوهیدرات با استفاده از روش فنول سولفوریک اسید انجام شد. محتوای پروتئین با روش کجلدال در دو تکرار و بر اساس استاندارد AOAC 2550 اندازه‌گیری شد. غلظت ترکیبات فنولی نیز با استفاده از آزمون فولین-سیوکالتئو تعیین گردید. بر اساس نتایج، ریشه گیاه *E. spectabilis* حاوی ۷۰.۳۳ گرم بر ۱۰۰ گرم کربوهیدرات و ۷.۱ گرم بر ۱۰۰ گرم پروتئین بود. درصد استخراج برای عصاره‌های آبی، الکلی و هیدروالکلی *E. specta-bilis* به ترتیب ۵۰٪، ۱۰٪ و ۲۵٪ بود. نتایج نشان داد که روش استخراج آبی کارآمدترین روش است. مقدار کل ترکیبات فنولی در عصاره آبی *E. spectabilis* برابر با ۱۵۰.۰۴ میلی‌گرم بر گرم بود. خاصیت آنتی‌اکسیدانی عصاره آبی *E. spectabilis* ۵۰.۷۱٪ تعیین شد. تمامی غلظت‌های عصاره آبی فاقد خاصیت ضدمیکروبی در برابر *Staphylococcus aureus, Escherichia coli* و *Pseudomonas aeruginosa* بودند. این یافته‌ها نشان‌دهنده ضرورت بررسی‌های بیشتر با استفاده از غلظت‌های متفاوت و ارزیابی سایر پاتوژن‌هاست.

واژگان کلیدی

ضدمیکروبی، آنتی اکسیدانی، *Eremurus spectabilis*، استخراج هیدروالکلی، استخراج الکلی

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Abstract in Persian

مطالعه آناتومی و رادیوگرافی جمجمه در گورکن بالغ(*Meles meles*)

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۴گروه علوم پایه، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.

چکیده

گورکن اروپایی (*Meles meles*) حیوانی قدرتمند بومی اروپا و بخش هایی از غرب آسیا است. جمجمه به سر گونه‌های حیوانات ظاهر فیلوژنتیکی می‌دهد و به عنوان یک ساختار محافظ برای اندام‌های حیاتی مانند مغز و چشم عمل می‌کند. هدف از مطالعه ما افزایش مجموعه اطلاعات اناتومیکی گوشتخواران وحشی با ارائه تجزیه و تحلیل دقیق از ساختار و اندازه گیری های جمجمه و مندیبل در گورکن بود. این اطلاعات درک پروسه های جراحی و رادیولوژکی نواحی سر این حیوان را فراهم می کند. مطالعه حاضر را بر روی ده جمجمه گورکن و فک پایین انجام شد که شامل پنج نر و پنج ماده بود. این جمجمه ها از لاشه های یافت شده در طبیعت که از مرگ طبیعی مرده بودند، به سالن تشریح دانشکده دامپزشکی انتقال یافت. مطالعات تشریحی روی آنها انجام شد و ویژگی های مورفومتریک اندازه گیری شد. این مطالعه نشان داد که در ویژگی‌های آناتومیکی جمجمه تفاوت قابل‌توجهی بین جمجمه گورکن و سایر گوشتخوران وحشی مانند گرگ و ببر مشاهده نمی شود. وجود دو سوراخ جاگولار در جمجمه گورکن، بعنوان یک ویژگی متمایز که در هیچ حیوان گوشتخوار دیگری یافت نمی شود،قابل توجه بود.هم چنین بررسی های رادیولوژیک وجود دو سینوس مشخص را در حیوان نشان داد که از این نظر با سگ متفاوت بود. نر و ماده در برخی از صفات مورفومتریک تفاوت قابل توجهی دارند که کاملاً با ویژگی های رفتاری و تغذیه ای حیوان سازگار است. تکنیک های تصویربرداری مدرن، مانند سی تی اسکن، برای مطالعات دقیق تر جمجمه گوشتخواران وحشی ضروری است.

واژگان کلیدی

گورکن اروپایی، جمجمه، مورفولوژی، مورفومتری، رادیوگرافی

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جداسازی، شناسایی و فعالیت ضد باکتریایی اکتینومیست ها از مدفوع گوسفند

طلیعه محمدی ، فاضل پوراحمد*، خلیل سالکی

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چکیده

اکتینومیست ها گروه مهمی از باکتری های گرم مثبت هستند که به دلیل تولید طیف وسیعی از متابولیت های ثانویه فعال زیستی، از جمله آنتی بیوتیک های مهم، شناخته شده اند. این میکروارگانیسم ها نقش اساسی در تجزیه مواد آلی و چرخه مواد مغذی ایفا می کنند و به سلامت و حاصلخیزی خاک کمک می کنند. ظرفیت آنها در سنتز ترکیبات متنوع و وجود مسیرهای مهم بیوسنتزی شامل سنتازهای پلی کتیت و سنتازهای پپتید غیر ریبوزومی، پتانسیل آنها را در کشف آنتی بیوتیک، به ویژه علیه پاتوژن های مقاوم به آنتی بیوتیک، برجسته می کند. این مطالعه با هدف جداسازی و شناسایی اکتینومیست ها از مدفوع تازه گوسفند جمع آوری شده در استان ایلام، با تمرکز بر فعالیت ضد باکتریایی و پتانسیل بیوسنتزی آنها انجام شد. در مجموع ۸۶ جدایه اکتینومیست از نمونه های مدفوع جمع آوری شده از گوسفندان به دست آمد. تمام جدایه ها در رنگ آمیزی گرم، گرم مثبت و رشته ای بودند. PCR ژن 16S rRNA هویت جدایه ها را تأیید کرد. غربالگری ضد باکتریایی نشان داد که ۱۷ جدایه فعالیت علیه پاتوژن های مختلف را نشان دادند،که بیشترین اثر بر علیه باسیلوس سرئوس (۶۲.۱٪). بود. درتجزیه و تحلیل مولکولی نیز ۳۱ جدایه (۳۶.۰۵٪) دارای ژن سنتاز پپتید غیر ریبوزومی(*NRPS*)، ۱۵ جدایه (۱۷.۴۴٪) حاوی ژن سنتاز پلی کتیت (*PKS-I*) و ۱۶ جدایه (۱۸.۶٪) واجد ژن سنتاز پلی کتیتII (*PKS-II*) بودند. این مطالعه خواص ضد باکتریایی قابل توجه و قابلیت های بیوسنتزی اکتینومیست های موجود در مدفوع گوسفند را برجسته می کند و استفاده بالقوه آنها را در کاربردهای درمانی، کشاورزی و بیوتکنولوژی نشان می دهد.

واژگان کلیدی

اکتینومیست ها، سنتاز پلی کتیت، سنتاز پپتید غیر ریبوزومی، فعالیت ضد باکتریایی، مدفوع گوسفند

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Author Index

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IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Guide for authors

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Abbreviations: Define abbreviations that are not standard in this field in a list to be placed on the tittle page. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the tittle page. Ensure consistency of abbreviations throughout the article.

Main Text

Introduction: Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

Results: Results indicate the results of an original research in a clear and logical sequence. Do not repeat data that are already covered in tables and illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion: Discussion should include the answer to the question proposed in the introduction and emphasize the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

Materials and Methods: Materials and methods should be described in sufficient details to allow other researchers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by the referees and if necessary, a statistician. Drugs and therapeutic agents, reagents, softwares and equipments should be given in the format: name (trade name, manufacturer name, city, country), e.g. Statview 5 (SAS Institute, Inc., Cary, NC, USA).

Animals: All animal experiments should comply with the ARRIVE guidelines and the authors should clearly indicate in the manuscript the ethical code of the study.

Gene names: The standard gene names, as provided by HGNC should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other

species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

Mouse beta actin gene: Actb

Bovine beta actin gene: ACTB

Chicken beta actin gene: actb

Beta actin protein: ACTB

Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods and Results must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

Protocol for DNA/RNA extraction, including quantification and determination of purity.

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers conce ntration (either random primers or oligonucleotides), reverse transcriptase and master mix components.

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank;

thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves);

validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis confirmed.

References: Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

Example piece of text and reference list :
An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than

half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:
1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.
2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.
3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.
4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.
5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6. Doi: 10.1001/archneur.62.1.112 .
6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.Doi: 10.1001/archneur.62.1.112 .
Use of Italics

Gene symbols, Latin terms (i.e. in vivo, in vitro, ex vivo, in utero, in situ, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. Homo sapiens).

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1. TITLE is clear and adequate
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3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
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