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ON THE COVER

Dye penetration was observed in dead protoscoleces after staining with 0.1% eosin (see page 55).

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Effect of Oral Preanesthesic Sedation with Chlordiazepoxide and Haloperidol Before Anesthesia For Adult Male Bonnet Macaques *(Macaca radiata)*

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ABSTRACT

It is important to capture wild animals with minimal stress to reduce morbidity and mortality. Oral premedicants have the potential to reduce stress during handling and ease the subsequent administration of anaesthetic agents. We evaluated the efficacy of premedication with chlordiazepoxide or haloperidol independently prior to midazolam-ketamine anaesthesia in 12 male Bonnet Macaques. Animals were randomly grouped into two groups of six (n=6). Animals of Group I were administered chlordiazepoxide (10 mg/kg) and animals of Group II were administered haloperidol (1 mg/kg) orally. The temperament of each animal was recorded prior to premedication. Behavioral responses after pre-medication were assessed for 4 h. Glucose and cortisol levels were measured from the venous blood sample collected after anaesthesia induction. Sedation was obtained in both groups, whereas the quality of sedation was comparatively better in Group II. Analgesia was better in Group I compared to Group II. Haloperidol-premedicated animals were easy to handle, but increased cortisol and glucose levels were recorded. According to our findings, pre-medication with chlordiazepoxide and haloperidol produced optimum sedation to handle the Bonnet Macaques for inducing anaesthesia.

Keywords

Preanesthetic Sedative, Chlordiazepoxide, Haloperidol, Non human primate, Stress reduction

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Number of Tables: 4
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Abbreviations

NHP: Non human primate
VTA: Ventral tegmental area
PFC: Cerebral Blood Flow
NAc: Nucleus accumbens
ECLIA: Electrochemiluminescence Immunoassay
CZA: Central Zoo Authority
HPA: Hypothalamic-pituitary-Adrenal axis
CRH: Corticotropin-releasing hormone
ACTH: Adrenocorticotropic hormone

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Introduction

Uncontrolled stress and death due to capture myopathy have been recognized as the most serious concerns during the handling and restraint of wild animals [1]. It is important to capture wild animals with minimal stress to reduce morbidity and mortality. Improvement of the capture technique using appropriate medications to minimize stress is a priority from the welfare point of view in wildlife conservation, zoos, and laboratory-housed NHPs/macaques.

NHPs are routinely used for research in laboratories all over the world [2]. These animals are anaesthetized for several scientific studies in laboratories as well as for routine procedures, such as surgical sterilization and microchipping, in captivity. Capturing and handling NHPs requires experience, thorough knowledge of animal behavior, and technical skills to ensure the safety of animals and handlers. NHPs in captivity may also be darted in their enclosures or hand-injected after physical restraint. Free-ranging macaques are trapped in large cages and anaesthetized by darting or injecting the anaesthetic agents after transfer to squeeze cages or smaller cages. NHPs are not premedicated prior to darting in these conditions. However, darting small-sized unpremedicated monkeys can be dangerous for the animal and is a difficult task because of their frantic fast movements. In addition, NHPs are difficult to handle for drug injection by hand because of their speed, dexterity, intelligence, and their potential to cause serious physical injury to the handler. Moreover, physical and chemical restraint of monkeys is associated with stress as in other wild animals. Premedicating these animals with oral tranquilizers before handling or anaesthesia improves the ease of handling and drug administration as well as reduces their stress response [3].

Tranquilizers, sedatives, and anaesthetic medicines have been established as crucial agents for reducing stress and related issues during wild animal restraint. Chlordiazepoxide [4] and haloperidol [5] have been defined as long-acting tranquilizers. Long-acting neuroleptics have been reported successful in reducing anxiety and producing sedation during treatment and translocation in wild animals [6-9]. Chlordiazepoxide has been observed to produce mild sedation, diminish spontaneous mobility, walking, grooming, and increase the lying period in Rhesus macaques [10]. Haloperidol also has been proven as a long-acting neuroleptic agent in NHPs, dogs, and other wild animals [5, 11, 12].

Both medications can be administered orally as premedicants and have the potential to reduce stress during handling and ease the subsequent administration of anaesthetic agents. The advantage of these long-acting premedicants is that their effect would last for sufficient time to allow gastric emptying before administrating anaesthetic drugs despite being administered orally. Anaesthetic agents may be administered parenterally following the onset of the premedical action and after providing an appropriate period for gastric emptying. Several anaesthetic combinations, such as midazolam and ketamine, have already been proven to produce satisfactory anaesthesia with minimal cardio-respiratory changes in animals and are routinely used for anaesthetising macaques. Therefore, a study was conducted in adult, male, captive Bonnet Macaques undergoing vasectomy at the State Museum and Zoo, Thrissur, Kerala to compare the efficacy of oral premedication with chlordiazepoxide or haloperidol before midazolam-ketamine anaesthesia.

Result

The temperament of each animal is presented in Table 1. Three animals in each group were stoic, two animals in each group were apprehensive, one animal in Group I was calm and relaxed, and one animal in Group II was aggressive. Mean ± SE of estimated body weight and actual measured body weight are presented in Table 2. There was no significant difference between estimated and actual measured body weights in both groups.

Ease of the Acceptance of Premedicants and Duration

The mean ± SE of the corrected oral dose of chlordiazepoxide against the actual measured body weight in the animals of Group I was 10.33 ± 0.20 mg/kg body weight. The mean ± SE of the corrected oral dose of haloperidol against the actual measured body weight in the animals of Group II was 1.12 ± 0.07 mg/kg body weight. The time taken to complete the premedication was 26.16 ± 8.96 and 12.66 ± 7.53 min in Group I and Group II, respectively. Ease of acceptance of fruit juice laced with premedicant, ease of netting or handling, and response to handling or injection were recorded and shown in Table 3. There was no significant difference between Group I and Group II in these parameters.

Behavioural observations

Behavioral responses were observed before premedication and for 4 h at intervals of 30 min after premedication. Various behavioural responses, including anxiety, aggression, and hyperactivity (Fig. 1 and Graph 1); active and playful (Fig. 2 and Graph 2); relaxed, calm, and reduced activity (Fig. 3 and Graph

Table 1.
Temperament of the animal

Parameter	Group I	Group II
Stoic	3 (50 %)	3 (50 %)
Aggressive	-	1 (16.66 %)
Apprehensive	2 (33.33 %)	2 (33.33 %)
Nervous	-	-
Calm and relaxed	1 (16.66 %)	-

Table 2.
Corrected body weight (kg)

Parameter	Mean ± SE		t-value	p-value
	Group I	Group II		
Estimated body weight	7.68 ± 1.07	6.16 ± 0.33	1.345 ns	0.208
Actual measured body weight	7.44 ± 0.96	5.67 ± 0.65	1.515 ns	0.161

Table 3.
Scores for ease of accepting premedicant, netting and resoponse to handling

Parameter	Median		Z-value	p-value
	Group I	Group II		
Ease of acceptance of fruit juice laced with premedicant	1.5	2.0	0.433 ns	0.665
Ease of netting or handling	2.0	2.0	0 ns	1
Response to handling or injection	2.0	2.0	0 ns	1

Ease of Handling after Premedication

The ease of handling and response to handling were recorded during netting and hand injecting of the drug. The median value for the ease of netting or injecting and response to handling or injection was 2 in both groups (Table 3). There was no significant difference in this parameter between the groups. Five (83.3%) of the premedicated animals were observed to be calm with reduced activity 4 h after premedication in both groups. One animal in each group remained active throughout 4 h.

Cortisol and Glucose Levels

Mean ± SE values of cortisol levels were 20.49 ± 3.70 and 40.09 ± 5.96 mg/dl after 4 h of oral premedication in Group I and Group II, respectively. Groups I and II were significantly different in terms of cortisol levels during induction. Mean ± SE of glucose levels was found to be 83.83 ± 7.66 and 98.16 ± 13.11 mg/dL after 4 h of oral premedication in Group I and Group II, respectively. No post-operative complication was observed in either group. All the animals were easily accepted back into their groups without any infighting.

3); stuperous look (Fig. 4 and Graph 4); half-open mouth (Fig. 5 and Graph 5); sitting with relaxed hind limbs (Fig. 6 and Graph 6); self-grooming (Graph 7); interaction with adjacent caged animal (Fig. 7 and Graph 8); ataxia (Fig. 8 and Graph 9); yawning (Fig. 9 and Graph 10); recumbency (Fig. 10 and Graph 11); leaning against wall or grill (Fig. 11 and Graph 12); drowsy (nodding/head down) (Graph 13); and sleeping in sitting posture (Fig. 12 and Graph 14) were recorded. All the observations are presented in Table 4. Graphical observation represents the changes of half an hour in each behavioral response.

Quality of Sedation after Oral Premedication

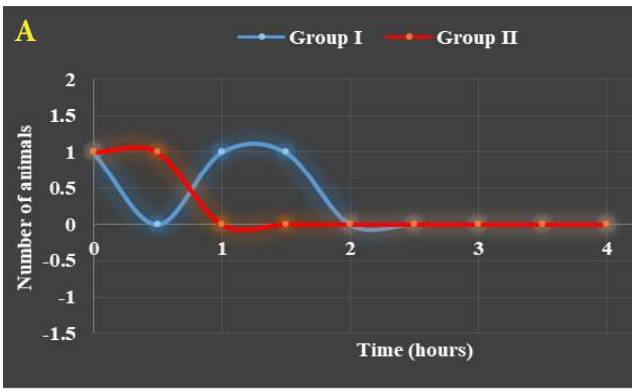
The median value for the quality of sedation after oral premedication was observed to be 1.5 in Group I and 2.5 in Group II. There was no significant difference between Group I and Group II in this parameter.

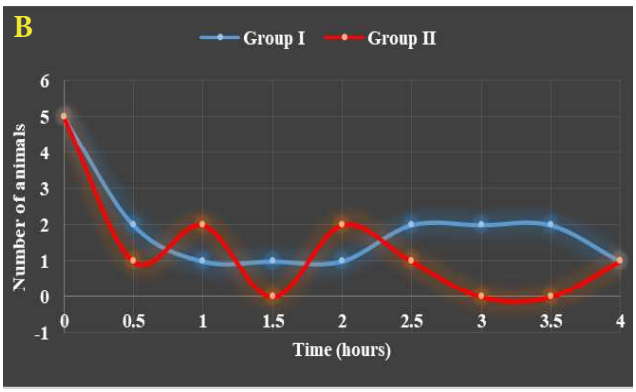
Discussion

The current study aimed to evaluate the sedative effect of two premedicants separately and to assess their practical applications and feasibility for inducing general anesthesia with minimal stress to Bonnet Macacques.

Prior observation of temperament helped assess the change in the behavior of the animals after oral premedication and during post-operative stress evaluation as opined by Fowler (2008) and Murphy (2008) [13, 14]. The corrected doses of drugs used in the present study were observed to be similar to the required dose as reported in many studies [15-19]. All the animals were conditioned to take pineapple juice for 15 days prior to the procedure as opined by CWINP-ILAR

Figure 1.
A-L. Behavioral responses observed after premedication: (A) Anxiety, aggression, and hyperactivity; (B) Active and playful; (C) Relaxed, calm, and reduced activity; (D) Stuperous look; (E) Half-open mouth; (F) Sitting with relaxed limbs; (G) Social behavior; (H) Ataxia; (I) Yawning; (J) Recumbency; (K) Leaning against wall/grill; (L) Sleeping.


A
Group I
Group II
Number of animals
Time (hours)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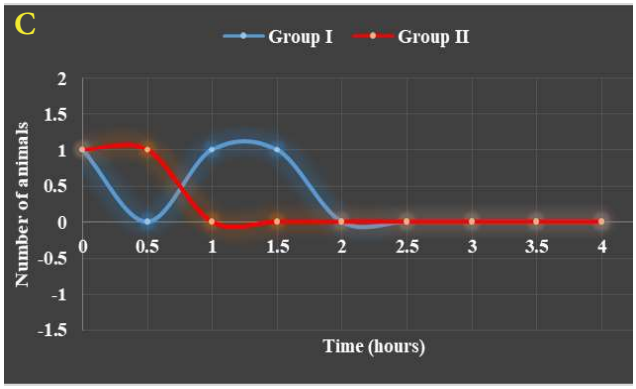
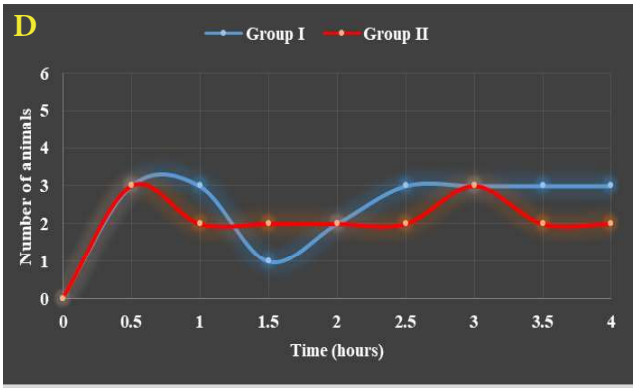

C
Group I
Group II
Number of animals
Time (hours)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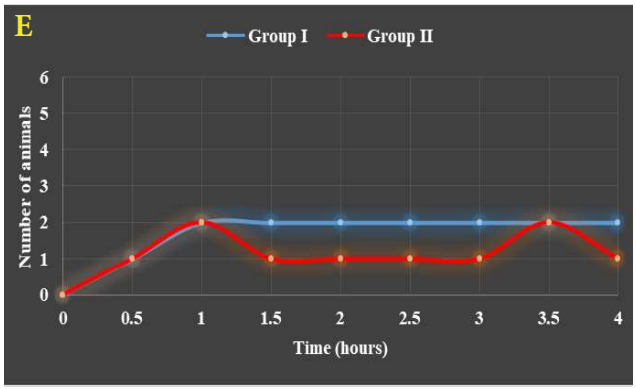
Group I
Group II
Number of animals
Time (hours)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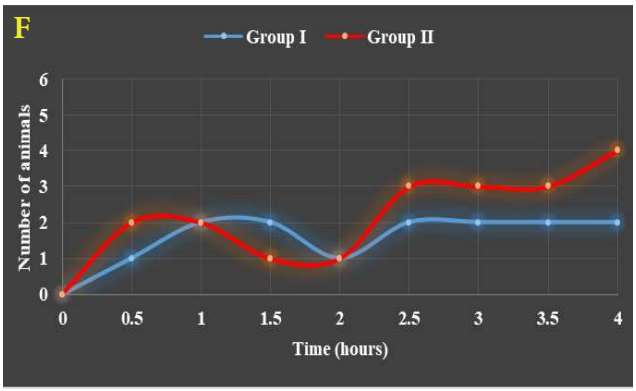
Group I
Group II
Number of animals
Time (hours)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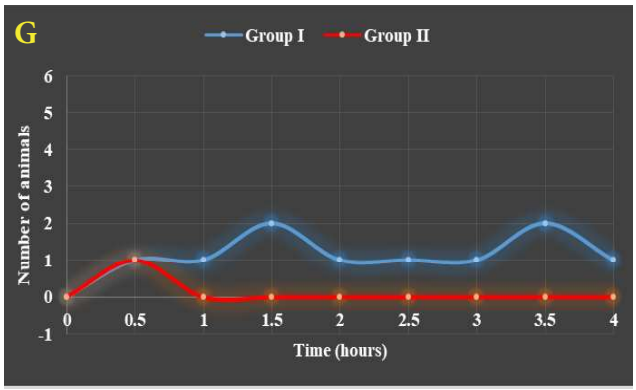
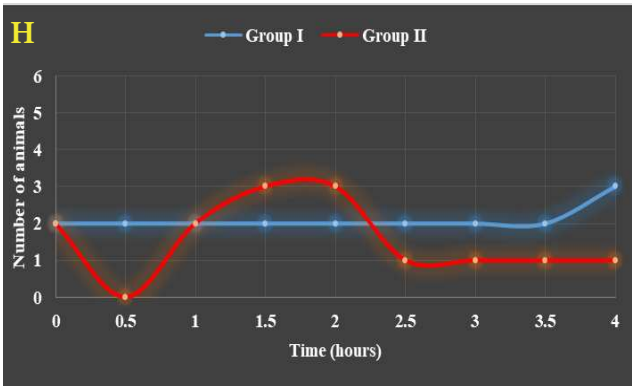
Group I
Group II
Number of animals
Time (hours)


G
Group I
Group II
Number of animals
Time (hours)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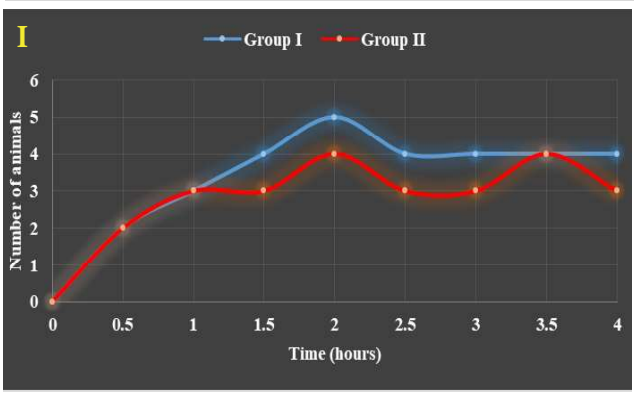
Number of animals
Time (hours)


I
Group I
Group II
Number of animals
Time (hours)

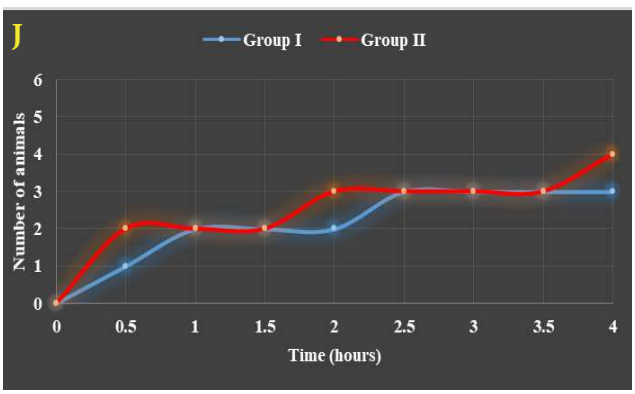

J
Group I
Group II
Number of animals
Time (hours)

Figure 2. Cont.

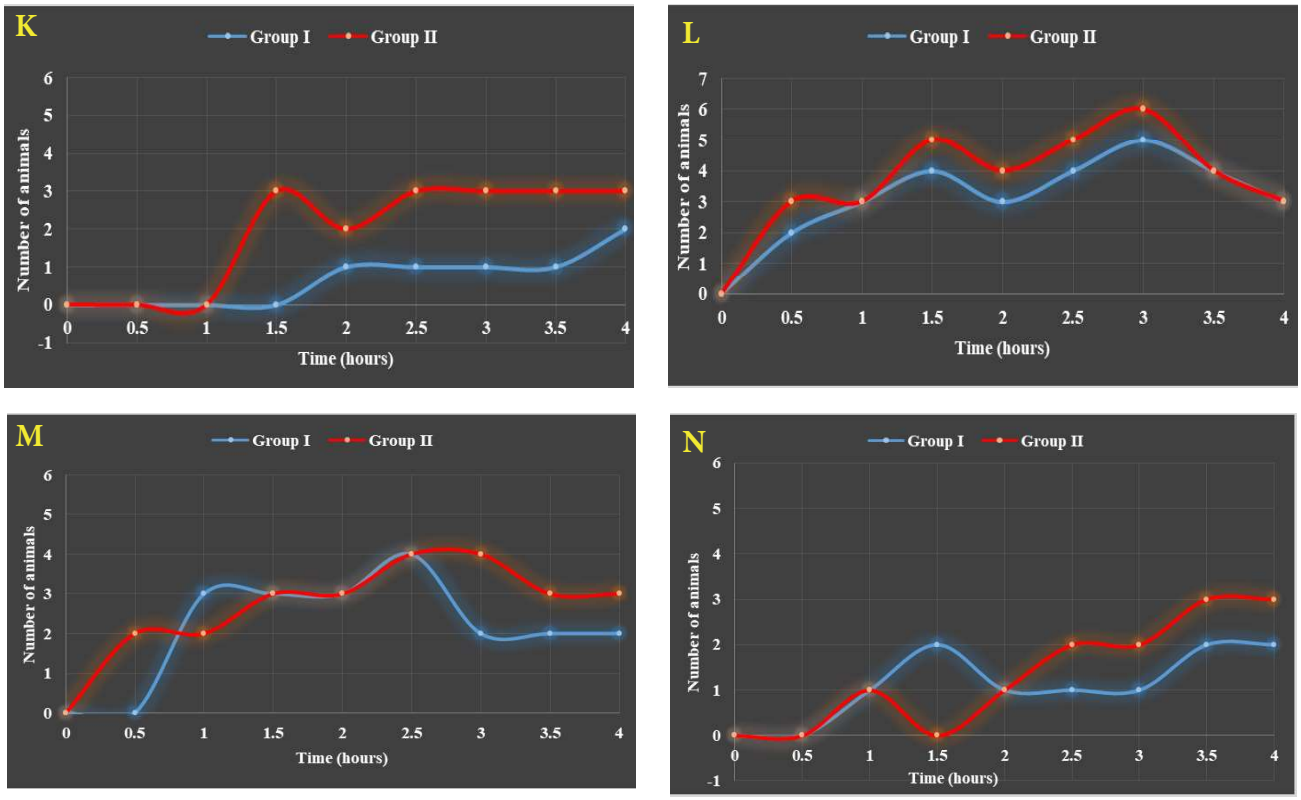


Figure 2.
Graph A-N. Graphical representation of behavioral responses observed after premedication: (A) Anxiety, aggression, and hyperactivity; (B) Active and playful; (C) Relaxed, calm, and reduced activity; (D) Stuperous look; (E) Half-open mouth; (F) Sitting with relaxed limbs; (G) Self-grooming; (H) Social behavior; (I) Ataxia; (J) Yawning; (K) Recumbency; (L) Leaning against wall/grill; (M) Drowsy; (N) Sleeping.

(1998) and Fowler (2008) [13, 20]. The slightly increased time taken to complete premedication and the lesser median value for the ease of administering fruit pulp in Group I could be due to the bitter taste and the larger dose of chlordiazepoxide.

Behavioural Observations

The effectiveness of the use of long-acting neuroleptics in wild animals to reduce stress was described by Fick et al. (2007) [21]. Oral administration of long-acting tranquilizers has been observed to facilitate the handling of NHP [22]. The administered dose of premedication was sufficient to produce the required effect as in earlier studies [10, 12, 23]. The scorecard used by Pulley et al. (2004) [3] in a study involving Rhesus Macaques was effective in monitoring the quality of sedation after oral premedication, which is similar to the present study. The median values for the quality of sedation were observed to be 1.5 (mild-moderate) in Group I and 2.5 (moderate-good) in Group II indicating increased sedation in Group II, but without any significant difference. Haloperidol was more effective in producing sedation than chlordiazepoxide. Increased sedative behavioral responses or tranquillization seen in haloperidol-premedicated animals may be due to its higher sedative effect [12]. The effects of haloperidol are not limited to dopamine D-2 receptor blockade. It also affects other neurotransmitter systems, including serotonin and norepinephrine [24, 25]. Disruptions in these systems have contributed to producing sedation by altering the balance of neurotransmitters causing CNS depression, reduced activity levels, decreased responsiveness to stimuli, and overall sedation.

One animal which had shown calm and relaxed behavior before premedication in Group I had a score of 3 indicating higher sedation. One animal which had shown aggressive behavior before premedication in Group II showed incoordination

Table 4.
Behavioural observations (per cent)

Observations	Prior to premedication		Four hours after premedication	
	Group I	Group II	Group I	Group II
Anxiety, aggression & hyperactive (Grinning and clicking)	16.66	16.66	0	0
Active & playful	83.33	83.33	16.66	16.66
Relaxed, calm & reduced activity	0	0	83.33	83.33
Stuperous look	0	0	50	33.33
Half open mouth	0	0	33.33	16.66
Sitting with relaxed hind limbs	0	0	33.33	66.66
Ataxia	0	0	66.66	50
Interaction with adjacent caged animal	33.33	33.33	50	0
Self-grooming	33.33	33.33	50	16.66
Yawning	0	0	50	66.66
Recumbency	0	0	33.33	50
Leaning against wall/grill	0	0	50	50
Drowsy (nodding/head down)	0	0	33.33	50
Sleeping in sitting posture	0	0	33.33	50

and ataxia (score 2). Two animals that were apprehensive in Group I showed minimal sedation (score 1). All the other animals showed mild to moderate sedation (score 2 - 3). Chlordiazepoxide primarily acts as a positive allosteric modulator of GABA-A receptors that contain alpha-1, alpha-2, alpha-3, or alpha-5 subunits. The alpha-1 subtype, in particular, is associated with sedative effects [24, 25]. By binding to these receptor subtypes, chlordiazepoxide promotes the opening of chloride channels, which further enhances the inhibitory effects of GABA. By enhancing GABAergic activity, chlordiazepoxide can lead to CNS depression. Minimal sedation in Group I could be due to the reduced intake of chlordiazepoxide or reduced dose of the drug leading to mild relaxation and anxiolysis.

None of the animals showed anxiety, aggression, or hyperactivity at the end of 4 h after premedication. Relaxation, calmness, and reduced activity were observed in all animals by the end of 4 h. Reduced aggression, anxiety-relieving, tranquilizing, and muscle relaxant properties of chlordiazepoxide have been also reported in monkeys by Reiser et al. (1962) [5]. Antianxiety, sedative, weak analgesic, and appetite-stimulating effects of the drug were reported in monkeys by Crowel-Davis and Murray (2006) [26]. The effectiveness of chlordiazepoxide in reducing aggressiveness and producing calmness has been already reported by Heuschele (1962) [23]. Hence both medications are proven to reduce anxiety, aggression, and hyperactivity in Bonnet Macaques.

A stuperous look and half-open mouth were noticed more in Group I than in Group II. By binding to GABA-A receptors, chlordiazepoxide increases the frequency of chloride ion channel opening when GABA is present. This hyperpolarizes the neurons, making them less likely to fire [24, 25]. In the context of muscle relaxation, this effect helps reduce the transmission of signals from nerves to muscles. The CNS-depressing effects of chlordiazepoxide affect the transmission of signals along the spinal cord and other pathways involved in muscle contractions. The anxiolytic (anti-anxiety) effects of chlordiazepoxide can indirectly contribute to muscle relaxation. Anxiety and stress can lead to muscle tension, so by reducing anxiety, chlordiazepoxide can help alleviate muscle stiffness and tightness. Muscle relaxation and anxiety-relieving properties of chlordiazepoxide could have increased the occurrence of these behavioral responses.

Long therapy using haloperidol influences dopamine receptors in the basal ganglia of NHP, producing extrapyramidal effects, such as muscle stiffness. The anticholinergic activity of haloperidol causes a reduction in the activity of the neurotransmitter acetylcholine, leading to some de-

gree of muscle relaxation due to the dampening of cholinergic transmission, which is involved in muscle contraction.

Ataxia was noticed in four and three animals of Group I and Group II, respectively. Benzodiazepines have been reported to have benzodiazepine type I (BZ I) receptor predominantly in cerebellum, causing cerebellar ataxia [27, 28]. Haloperidol causes ataxia within 6 h of oral premedication in Spotted Deer [4]. Haloperidol is a potent dopamine receptor antagonist, particularly targeting D2-type dopamine receptors [25]. By blocking these receptors, it decreases the effects of dopamine, a neurotransmitter involved in motor control and coordination. Dopamine pathways in the basal ganglia are important for regulating movement. When these pathways are disrupted due to dopamine receptor blockade, as seen with haloperidol use, it can lead to motor-related side effects, such as ataxia. Dopamine pathways also influence cerebellar function. The disruption of these pathways following dopamine receptor blockade by haloperidol can result in impaired communication between the basal ganglia and the cerebellum, contributing to ataxia. The degree of ataxia varied in each macaque. Factors, such as genetic predisposition, dose of haloperidol administered, and the specific neurochemical makeup of an individual's brain, can influence the severity of ataxic effects. Yawning, recumbency, leaning against wall/grill, drowsiness (nodding/head down), and sleeping in sitting posture could be due to the higher level of sedation shown by animals of both groups. One animal of Group I showed self-grooming which was in agreement with the findings of Kumar et al. (1999) in Rhesus Macaques [29].

Behavioral observations, including sleep-like state; calm and quiet state; and mild hypotension, have been recorded within 5 min of intravenous premedication of haloperidol in dogs [11]. The effectiveness of haloperidol along with zuclopenthixol to produce deep sedation during the transportation of an adult male gorilla from Germany to South Africa has been reported by Redrobe et al. (2008) [30]. Haloperidol produces deep sedation lasting about 2-4 h in Cebus Monkeys and Squirrel Monkeys when administered orally [12]. Haloperidol blocks postsynaptic dopamine (D2) receptors in the mesolimbic system of the brain reducing dopaminergic inputs from the ventral tegmental area (VTA) innervate brain regions involved in executive, affective, and motivational functions, including the prefrontal cortex (PFC), amygdala, and nucleus accumbens (NAc). The CNS-depressant effects of haloperidol can result in sedation and drowsiness. This sedative effect might contribute to improved sleep in NHP.

Five animals of each group could be easily handled after premedication and were observed to be calm with reduced activity by 4 h after premedication. The ease of handling and injecting the drug may have been due to the calming effect of premedication with either chlordiazepoxide or haloperidol. Calming and sedative effects of premedicants have been recognized by Heuschele (1962), Weiss et al. (1977), Crowel-Davis and Murray (2006), and Redrobe et al. (2008) [12, 23, 26, 30]. Administration of anaesthetics after 4 h of premedication helped to achieve peak effect and promoted handling as suggested by Pulley et al. (2004) [3]. Chlordiazepoxide (Rang et al., 2005) and haloperidol (Hofmeyr, 1981) have been defined as long-acting tranquilizers [7, 32]. Prolonged action of premedication agents was observed in Bonnet Macaques in the present study. The ease of handling and injecting drugs may have been due to the calming effect of premedication.

One animal in each group that had an aggressive and apprehensive temperament remained active throughout 4 h. Increased activity of these animals could be due to improper intake of bait or lower doses of premedication agents. The authors opine that pineapple juice could not mask the bitterness of chlordiazepoxide. Better replacement of oral bait can be used to mask the bitterness of the oral premedicants. The authors also suggested increasing the distance between cages to reduce the social behavior and activity of macaques.

Cortisol and Glucose Levels

Higher glucose levels observed in this study in Group II than Group I after 4 h of premedication

might be due to the excessive release of cortisol following psychological and physical stress related to handling. An increase in the cortisol levels may have led to gluconeogenesis in both groups. All the previous reports in Bonnet Macaques had reduced glucose levels in non-anaesthetized, ketamine-anaesthetized, and ketamine-xylazine-anaesthetized animals [32-36].

Circulating cortisol levels have already been reported as an important indicator of stress in wild animals [37, 38]. Normal cortisol levels during tiletamine-zolazepam anaesthesia in trained Rhesus Macaques between 0 and 60 min were between 27.9±1.7 and 21.2±2.0 µg/dL [39]. The significant increase in cortisol level in Group II- may have been due to its sensitivity to physical and psychological stress. HPA axis is a complex neuroendocrine system that controls the body's response to stress and regulates cortisol production. The hypothalamus releases CRH, which stimulates the pituitary gland to release ACTH, which in turn, signals the adrenal glands to produce cortisol [40]. Changes in neurotransmitter activity can influence the release of CRH and ACTH, which could subsequently affect cortisol production. By reducing anxiety and arousal, haloperidol might indirectly influence cortisol levels, especially during situations of acute stress.

Increased cortisol levels due to stress related to cage restraint and ketamine anaesthesia have been reported in Rhesus Monkeys [41]. The injection technique and blood sampling process have been observed to raise cortisol levels in untrained monkeys compared to trained ones [39]. Contradictory results maintaining stable endocrine responses have been reported by Fuller et al. (1984) [42] in Cynomolgus Monkeys. Long-term administration of haloperidol has been observed to produce movement disorders and tardive dyskinesia which resulted in delayed and potentially irreversible motor complications with typically stereotyped abnormal movements (peculiar postures, writhing, stretching, and oral movements) without acute dystonic reactions [43]. None of the animals in the present study showed any adverse effect associated with haloperidol, which may be because the drug was administered only once.

Haloperidol was observed to improve the ease of handling animals when evaluated subjectively by the handler. However, evaluation of parameters associated with stress showed that animals premedicated with haloperidol were more prone to stress than those premedicated with chlordiazepoxide. The quality of recovery in animals premedicated with haloperidol was better than that in animals premedicated with chlordiazepoxide, which showed ataxia during recovery. The present study involved a small group of six to evaluate each premedicant drug. A study involving more animals in the future would be beneficial for the better assessment of the efficacy of chlordiazepoxide and haloperidol as oral premedicants for the anaesthesia of NHP. The authors evaluated the effect of oral premedication using two medications without a control group. To determine the baseline data and to assess whether the premedication was useful in reducing handling stress or not, incorporating a control group would have been superior to the present study. The authors opined to include a control group in future studies.

Chlordiazepoxide and haloperidol at the doses of 10 and 1 mg/kg body weight, respectively, may be used as oral premedicants in Bonnet Macaques 4 h prior to the anaesthetic procedure to reduce stress and to ease the handling of animals during induction. However, bitter taste and a larger dose of chlordiazepoxide may reduce its acceptability. The long-acting nature of chlordiazepoxide and haloperidol ensures sufficient gastric emptying after their oral administration before the induction of anaesthesia and improves the quality of induction and maintenance of anaesthesia with minimal side effects. Pre-medication with chlordiazepoxide and haloperidol during midazolam-ketamine hydrochloride anaesthesia may be recommended for vasectomy and other procedures, such as physical examination, tuberculin testing, and wound dressing.

Materials and Methods

Ethical Considerations

The present study was approved by the Institutional Animal Ethics Committee, Kerala Veterinary and Animal Sciences University, Kerala, India. The study was conducted at the State Mu-

seum and Zoo, Thrissur, Kerala, India. Twelve healthy adult male macaques underwent routine vasectomy procedures to control their population in the zoo as per directions of the CZA of India and were selected for the current study. All the animals were cared for properly and were used humanely during capture, translocation, and study by following the best practices of veterinary care as per the guidelines of the CZA of India.

Animals, Husbandry, and Housing

The twelve animals used for the study were randomly selected from a group of 95 monkeys kept in three enclosures of 30 × 15 × 30 feet (l × b × h). Nutritionally fit animals which weighed more than 4 kg were considered for the study. The selected animals were randomly allotted into two groups of six each (website: random.org). All the twelve animals of the study were separated from their group and were kept in an enclosure sized 30 × 15 × 30 feet (l × b × h). The animals were fed the routine diet of the zoo in the evening. The segregated animals were conditioned in the morning hours to take pineapple fruit juice for 15 days before the procedure. The animals were fasted for 8 h and water was withheld for 5 h prior to the administration of premedication agents.

Assessment of Temperament

The temperaments of the animals were evaluated based on the response to the threatening human in individual cages before the study as suggested by Capitanio (1999) [44]. The temperament of each animal was recorded prior to premedication, which helped to assess the change in behavioral responses later.

Administration of Premedications

The dose of premedication and anaesthetic medications was calculated and administered based on the estimated body weight. These doses were corrected later according to actual measured body weight following anaesthesia. Animals of Group I and Group II were premedicated with chlordiazepoxide (Librium®, Abott Healthcare Pvt Ltd., Himachal Pradesh, India) and haloperidol (Serenace 10®, RPG Life Sciences Ltd., Ankleshwar) at the dose of 10 mg/kg body weight and 1 mg/kg body weight, respectively, orally in pineapple fruit juice. The premedica tablets were powdered, mixed with the fruit juice two minutes before oral administration, and were given in a steel bowl. Fruit juice was administered at a dose not exceeding 3 ml/kg body weight. The time taken to complete the premedication was measured from the moment it was placed in front of the animal until it had completely consumed it. The data relating to the dose of anaesthetics during induction and maintenance of midazolam-ketamine anaesthesia, including the hematological and biochemical parameters during haloperidol premedication have been provided by Kumar et al. (2017) [45].

Efficacy of Oral Premedicants

Ease of administering the premedication agents was scored based on a drug administration index used by Pulley et al. (2004) [3]. The ease of accepting fruit juice laced with premedicant was recorded based on the scorecard prepared (Score Card 1).

Corrected Dose Rate = Estimated Body Weight × Dose Rate of the Agent

Actual Measured Body Weight

Behavioral responses were recorded before and after premedication at intervals of 30 min for a period of 4 h by a blinded observer. All the animals were restrained inside the cages to induce anaesthesia either by netting or physical restraint. Ease of handling was assessed during the intramuscular injection of an anaesthetic mixture into gluteal muscles. Ease of handling before anaesthesia induction was recorded in a scorecard (Score Card 2). Quality of sedation throughout 4 h after premedication was recorded in a graded scorecard modified from one described by Pulley et al. (2004) (Score Card 3) [3].

Cortisol and Glucose Level Assessment

Serum cortisol was estimated from the venous blood collected 4 h after premedication immediately after the induction of anaesthesia by the ECLIA method using a commercially available kit (Cobas ECLIA Kit, Roche Diagnostics, Mannheim, Germany) and an automated analyzer. Plasma glucose was also estimated from the venous blood sample collected after the induction of anaesthesia.

Score card 1:
Ease of acceptance of fruit juice laced with premedicant

Score	Observation
2	Easily taken
1	Taken with apprehension
0	Taken with prolonged time

Score card 2:
Ease of acceptance of fruit juice laced with premedicant

Items of observations	Score	Comments
Ease of netting or handling	2	Easy
	1	Difficult
Response to handling or injection	2	Calm
	1	Excited

Statistical Analysis

The obtained data were analyzed as described by Snedecor and Cochran (1994) using the statistical software SPSS version 16.0. Independent samples t-test was used to compare the means ± SE of dose and time taken for premedication. The Mann-Whitney U-test was used for all the parameters in the study involving scor-

Score card 3:
Quality of sedation after oral premedication

Score	Quality of sedation after oral premedication
0	None: Normal behaviour
1	Mild: Upright, mild incoordination and ataxia (But able to move freely)
2	Moderate: Upright, severe incoordination and ataxia (Sitting with extended hind limbs)
3	Good: Recumbent, easily aroused
4	Heavy/Profound: Recumbent, very little response to stimulus (able to safely hand inject)

ing (ease of administering the premedication agents, ease of the acceptance of fruit juice laced with premedicant, ease of handling prior to the induction of anaesthesia, quality of sedation). Paired samples t-test was used for comparing observations before and after premedication and anaesthesia.

Authors' Contributions

K.S Kamalesh Kumar and George Chandy have planned carried out the experiments. Both of them took the lead in writing the manuscript. S Sooryadas, P T Dinesh have provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare no conflict of interest.

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Comparison of Steinmann Pin and Polymethyl Methacrylate Pin in Experimental Fractures of Humerus in Pigeon Models

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ABSTRACT

Avian orthopedic issues, particularly fractures, pose significant challenges due to birds' unique skeletal anatomy. Their bones, including the humerus (upper wing bone), are often pneumatic (air-filled) and fragile, making fracture management complex. Traditional methods, such as intermedullary pins and plates offer some solutions but have limitations. This study investigated the efficacy of two pin materials for stabilizing humerus fractures in pigeons: Steinman pins (commonly used in veterinary orthopedics) and polymethyl methacrylate (PMMA) pins. We created controlled fractures in the humerus of thirty young adult pigeons. These fractures were then stabilized with either Steinman pins or PMMA pins. Radiographic examinations and histological analysis were performed 2, 4, and 6 weeks post-surgery to assess bone healing progress in both groups. The findings revealed comparable healing outcomes between the two pin types, suggesting that PMMA pins could be a viable alternative for stabilizing fractures in birds, offering the additional benefit of sparing them a second surgery for pin removal.

Keywords

Orthopedic, Bone cement, Bird, Wing

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Abbreviations

PIN: Control group with steinmann pin
PMMA: Polymethyl methacrylate
CT: Callus thickness
CI: Callus Index
CCT: Corrected callus diameter
IM: Intramedullary
ANOVA: Analysis of variance
PDS: Polydiaxonon

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Introduction

Avian fractures, often precipitated by trauma, pose formidable challenges owing to birds' pneumatic and fragile bones. Avian fracture repair necessitates meticulous techniques that ensure the restoration of longitudinal, lateral, and rotational stability of the fractured bone. This facilitates an optimal healing environment while minimizing iatrogenic skeletal and soft tissue damage. Various fixation methods, including IM pins and titanium plates, are deployed to stabilize fractures; however, each method is associated with inherent complexities. The delicate bone cortex in birds, particularly in smaller species, complicates fracture management [1-3].

PMMA, a bone cement, has emerged as a promising adjunct in avian orthopedic surgeries. PMMA offers a versatile and advantageous option for fixing fractures in birds, and has been used alone or in combination with other methods of fracture fixation in birds. The utility of PMMA extends to both pneumatic and marrow-containing bones and facilitates reconstruction in comminuted fractures by aiding fragment reintegration. It is light-weight, relatively inexpensive, fairly easy to apply, rapidly stable, and allows early function restoration without interfering with joint function [3, 4].

Given the imperative for a pragmatic, low-risk fixation modality, this study scrutinizes the efficacy of pin made of PMMA in humerus fracture stabilization in birds.

Result

Radiology

Regarding radiographic assessments, the images provided in Figure 1 illustrate the observed phenomena. Specifically, radiographic examination 2 weeks post-fracture revealed callus formation in the PIN group. Although the fracture line remained visible, the callus exhibited radiodensity and nearly bridged the fracture gap. In addition, no evidence of pin loosening, infection, or refracture was observed. Mirroring the pin group, radiographic examination of the PMMA group revealed callus formation with radiodensity bridging the fracture line. The fracture line remained visible, and similar to the pin group, there were no signs of implant loosening, infection, or refracture.

Four weeks after fracture, radiographic evaluation of the PIN group revealed a significant reduction in callus volume. Notably, the fracture line was no longer fully visible, and satisfactory bony alignment was achieved. The PMMA group demonstrated minimal radiographic evidence of callus, but the fracture line exhibited signs of union. Similar to the pin group, good bony alignment was observed. Importantly, neither group displayed radiographic evidence of pin loosening, infection, refracture, or fragment displacement.

Radiographic examination six weeks post-fracture revealed no discernible callus formation in either group. The fracture lines demonstrated almost complete healing, and satisfactory bony alignment was maintained in both the PIN and PMMA groups. Moreover, no evidence of pin loosening, bone angulation, infection, or implant failure was observed.

The obtained results are shown in Table 1. Statistical analysis of the data revealed no significant difference between the two groups using both the independent-samples t-test and repeated measures ANOVA ($p > 0.05$). However, the significant effect of time was observed within each group, suggesting changes in the measured variable across the sampling period. This finding is consistent with the progress of bone healing in both groups as evidenced by descriptive statistics ($p < 0.05$). Furthermore, the independent-samples t-test, employed to compare the two groups at each sampling point individually, showed no statistically significant difference between groups at any specific time point ($p > 0.05$).

Histopathology

Bone samples of both study groups were evaluated, with results presented separately according to sampling times in each group. Figure 2 shows the histopathology images obtained from the present study . At the second week, in the PIN group, callus tissue forming a bridge across the fracture line was observed, predominantly comprising cartilage and immature bone plates, with minimal fibrotic tissue. Conversely, in the PMMA group, a significant amount of callus tissue, primarily cartilaginous, along with some bone plates and connective tissue, was evident.

By the fourth week, the callus tissue in the PIN group predominantly composed of immature bone, effectively filling the fracture line. Similarly, in the PMMA group, well-formed callus tissue consisting of immature bone plates filled the fracture line. Progressing to the sixth week, a reduction in callus tissue volume was noted in the PIN gromup, with the remaining callus predominantly composed of bone plates. In parallel, the PMMA group exhibited callus tissue composed of bone plates, maintaining continuity of the fracture line.

Statistical analysis was performed by the non-parametric tests due to the qualitative nature of the data. The obtained results are shown in Table 2 and Figure 3. The Friedman test revealed significant changes within each group over the study period ($p < 0.05$). However, according to the Mann-Whitney test, there were no statistically significant differences be-

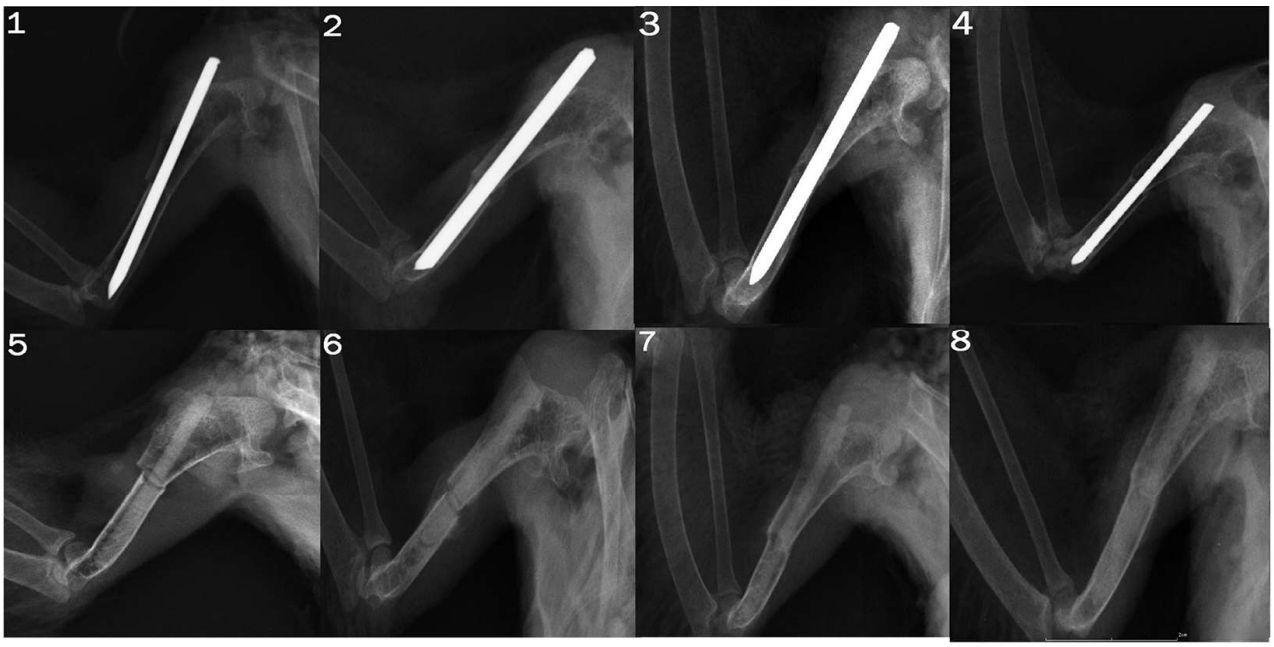

Figure 1.
presents radiographic images obtained during the current study. The upper row depicts the PIN group, with images numbered as follows: 1) post-operative image, 2) image taken two weeks after surgery, 3) image captured four weeks after surgery, and 4) image obtained six weeks after surgery. The lower row represents the PMMA group, with images labeled as follows: 5) post-operative image, 6) image taken two weeks after surgery, 7) image captured four weeks after surgery, and 8) image obtained six weeks after surgery.

Table 1.
Radiologic assessment result

Group	Parameter	Time	Min	Max	Mean ± SD
PIN	CT (Callus thickness)	2w	5.64	7.83	6.67 ± 0.89
		4w	4.25	6.81	5.27 ± 0.97
		6w	4.36	4.98	4.69 ± 0.25
	CI (Callus Index)	2w	1.42	1.64	1.50 ± 0.09
		4w	1.05	1.61	1.22 ± 0.22
		6w	1.02	1.17	1.08 ± 0.06
	CCT (Corrected callus diameter)	2w	1.75	3.07	2.25 ± 0.58
		4w	0.22	2.59	0.97 ± 0.94
		6w	0.09	0.70	0.33 ± 0.26
PMMA	CT (Callus thickness)	2w	5.85	9.62	7.47 ± 1.58
		4w	4.38	5.17	4.73 ± 0.32
		6w	4.17	4.77	4.44 ± 0.22
	CI (Callus Index)	2w	1.45	1.65	1.52 ± 0.08
		4w	1.06	1.18	1.12 ± 0.04
		6w	1.02	1.11	1.08 ± 0.03
	CCT (Corrected callus diameter)	2w	1.91	2.98	2.54 ± 0.45
		4w	0.27	0.77	0.51 ± 0.20
		6w	0.09	0.44	0.32 ± 0.14

CT and CCT parameters are based on size in millimetres.
CI is based on the ratio of the callus thickness to the diameter of the bone cortex in the distal location close to the fracture line immediately after surgery.

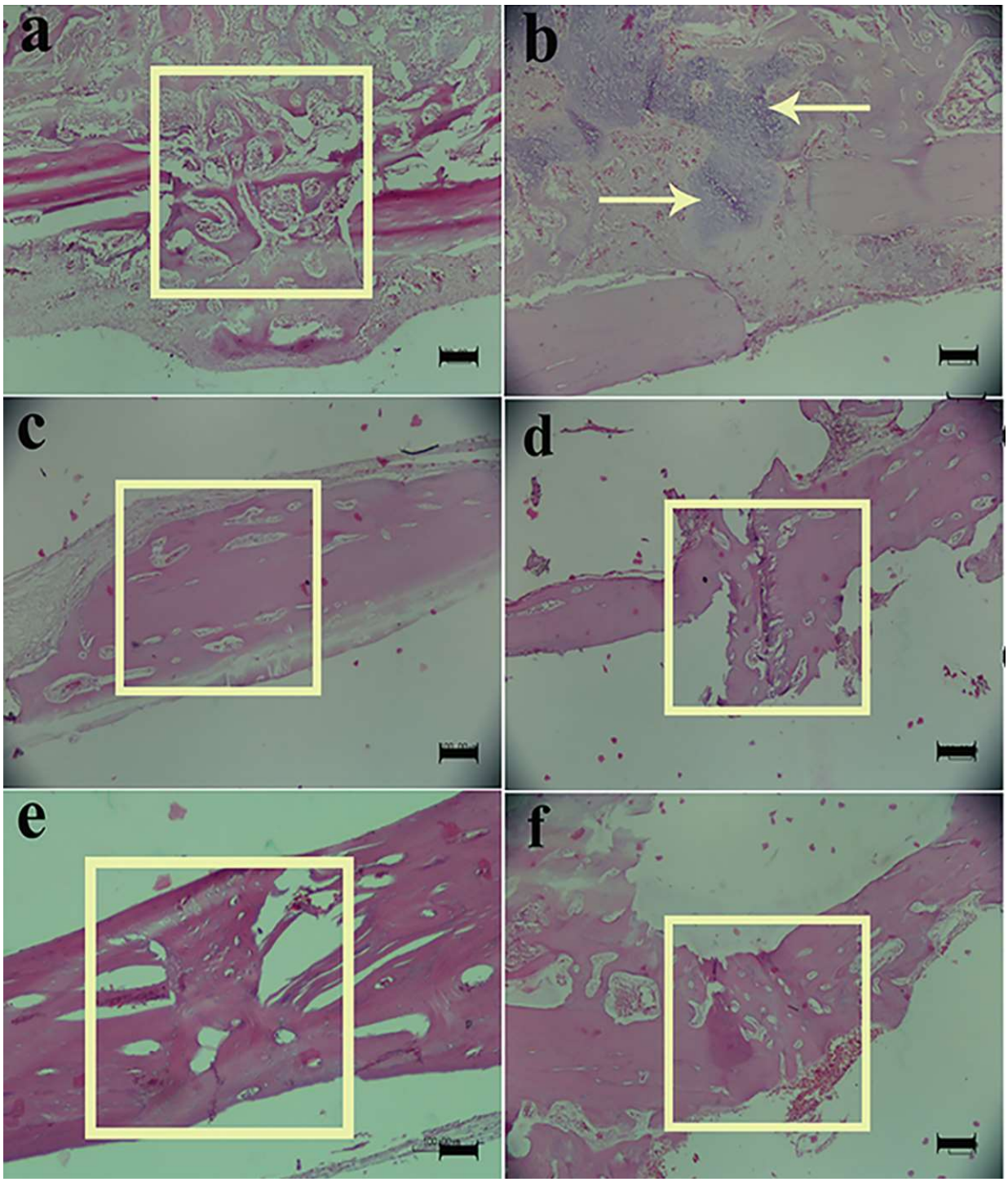

Figure 2.
Illustrates histopathological images obtained during the present study Panel a) depicts the pin2w sample, showcasing the formation of a substantial callus (indicated by the square) comprising fibrous, cartilage, and immature bone within the fracture line. In panel b), representing pmma2w, the callus is primarily composed of cartilage (indicated by the arrow) along with fibrous and immature bone elements. Panel c) exhibits pin4w, revealing complete bone union predominantly comprised of immature bone (indicated by the square). Similarly, panel d) displays pmma4w, where a large protruding callus containing immature bone is evident (indicated by the square). In panel e), representing pin6w, a reduction in the size of the callus is observed, with the composition predominantly consisting of woven bone (indicated by the square). Lastly, panel f) illustrates pmma6w, showcasing a callus containing woven bone exclusively within the fracture line (indicated by the square). These histopathological images were stained with hematoxylin and eosin (H&E), with a scale bar indicating a length of 100µm.

Table 2.
Histological evaluation results in current study

Group	Time	Min	Max	Median	Mean ± SD
PIN	2w	2	3	2	2.4 ± 0.5
	4w	2	3	3	2.8 ± 0.4
	6w	3	4	3	3.4 ± 0.5
PMMA	2w	2	3	2	2.2 ± 0.4
	4w	2	3	3	2.8 ± 0.4
	6w	3	4	3	3.4 ± 0.5

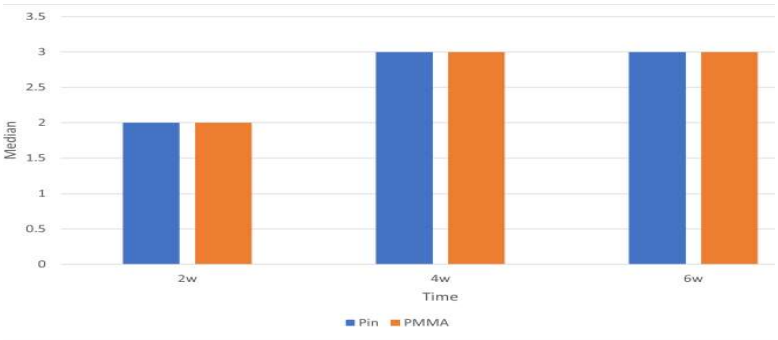

Figure 3.
Diagram of the results obtained from the histopathology data based on the median in the present study

tween the two study groups at any given time point, indicating comparable outcomes.

Discussion

Fracture repair in avian species necessitates techniques that preserve the bone's longitudinal, lateral, and rotational stability to facilitate optimal bone healing while minimizing skeletal and soft tissue damage. Pin fixation represents a widely employed method for stabilizing fractures in birds with the capacity to withstand bending forces and maintain the bone's longitudinal integrity. However, pin fixation may be inadequate in addressing rotational forces, often necessitating supplementary stabilization methods, such as wiring or external skeletal fixation [2].

Two techniques commonly employed for pin placement are Normograde and Retrograde methods. Carrasco et al. advocated for the retrograde method, citing enhanced fracture visualization, albeit with increased manipulation of fracture fragments. Conversely, Ponder et al. recommended the Normograde approach to minimize soft tissue trauma surrounding the fracture site. Consequently, the Normograde method was adopted in the present study to mitigate soft tissue damage during fracture management [8].

Numerous studies in recent years have highlighted significant complications associated with pin usage. Among these, pin migration towards adjacent joints emerges as a primary concern. Such migration can compromise joint integrity, particularly affecting joint cartilage and surrounding structures, potentially leading to arthrosis or joint ankylosis. Furthermore, pin migration may disrupt the bird's rehabilitation process, necessitating secondary surgical intervention for pin removal [3, 8].

Wan et al., in their comparative study between stainless steel and PDS pins, noted that the performance of the PDS pin was comparable to that of the steel pin. In this study, it was stated that at some sampling times, when PDS pin was not absorbed, ethanol was used to dissolve. In addition, they did not observe that a secondary surgery for pin removal was unnecessary with the use of PDS pins [8]. Matthew investigated the use of PGA rod for fracture repair in pigeon. They found that the biodegradable implants elicited a granulomatous foreign body reaction, but this did not impede fracture healing. Moreover, biodegradable repairs resulted in more periosteal callus formation but also a higher incidence of early complications [10]. Although the PMMA pin in the present study was non-absorbable, it achieved comparable performance. The suggested method involving a bone cement pin may offer advantages over conventional pins as it mitigates the risk of migration and eliminates the need for secondary surgical intervention to remove the pin. However, this issue was not observed in the current investigation. Consequently, this approach alleviates the stress associated with anesthesia and additional surgical procedures for the animal.

Fracture healing in avian species is influenced by several factors, such as the degree of bone fragment displacement, adequacy of blood supply, presence of infection, and degree of motion at the fracture site [3]. In avian species, the formation of callus arises from both periosteal and endosteal sources [17]. Research has indicated that in the healing process of the humerus in pigeon models, the formation of callus originating from the periosteum is more prevalent than that originating from the endosteum [18]. Carrasco *et al.* observed in their study that during the healing process of humeral or forearm fractures in pigeons, callus originates from both periosteal and endosteal sources. Initially, within nine days after fracture, the callus comprises cancellous bone, cartilage, and fibrous connective tissue. Subsequently, on the 16th and 21st days, there was an increase in the cancellous bone content accompanied by a decrease in the cartilage and connective tissue amounts. Furthermore, six and twelve weeks post-fracture, well-aligned fractures continued to mature, transitioning towards the development of normal bone components [2].

Yamazoe *et al.* investigated humerus fractures in pigeons and found that periosteal callus formation, comprising spongy new bone, and occurred bilaterally along the bone by the fourth week. By the sixth week, cortical bony union was observed [19]. In their study, Bush *et al.* noted that humeral bone healing without fracture fixation in pigeons progresses through distinct stages. Specifically, by the ninth day post-injury, the formation of fibrous connective tissue was observed. Subsequently, by the sixteenth day, the tissue composition predominantly transitioned to fibrocartilage. By the twenty-first day, callus formation had bridged across both sides of the fracture line [20]. The primary limitation of this study lies in the absence of fracture stabilization. As elucidated by Gandal [3], factors influencing fracture healing suggest that inadequate stabilization may lead to the displacement and movement of fracture fragments, potentially impeding the healing process. Despite this limitation, the current study demonstrated superior outcomes in bone healing compared to the findings reported by Bush. In contrast, Matthew *et al.* employed intramedullary rods to stabilize humeral fractures in their study. Their evaluation of bone healing revealed the formation of callus tissue evident on radiographs 2 and 3 weeks post-fracture [10].

Wander et al. investigated the use of xenografts for fracture healing. Their findings demonstrated the presence of callus formation, which was evident upon radiographic and histopathological evaluation at the 3rd and 6th weeks [21]. However, the specific characteristics of the callus tissue formed in the latter study were not delineated. In contrast, the present study provides comprehensive details regarding the type of callus tissue formed at different time points, which are visually depicted in the histopathology images.

Hatt et al. conducted a study on 28 birds across various species, which sustained fractures in bones, such as the humerus, radius, ulna, and leg bones. Their findings indicated an average duration of 3-5 weeks for bone healing [22] and subsequent removal of the fracture fixation device. Given the clinical relevance of their study, the results align with those obtained in the present investigation, where it was also observed that immature bone callus fills the fracture line by the fourth week.

Similarly, Kayikci *et al.* conducted a clinical study assessing fractures in various avian species, including falcons, owls, and eagles. However, their radiographic evaluation of bone healing was limited to the third week post-surgery [23]. In contrast, the present study utilized evaluation time points consistent with previous research to ensure comprehensive assessment of bone healing progression.

Park *et al.* conducted a case report study wherein they stabilized a humerus fracture in a common kestrel (Falco tinnunculus) using a tie-in fixator and figure-of-eight tension band method. Radiographs were obtained on days 5, 14, and 60 to assess bone healing progression. Their findings revealed bone fusion by day 14, leading to the subsequent removal of the fixation device [24]. In the present study, radiographic evaluation conducted after two weeks in both groups demonstrated complete visibility of callus formation, bridging the fracture lines. Histopathological as-

sessment corroborated the presence of bone and cartilage tissue within the callus.

Conclusion

Our findings suggest that PMMA pins show similar effectiveness to Steinman pins in promoting the healing of avian humerus fractures. PMMA pins offer several advantages over traditional Steinman pins, particularly in terms of post-treatment management. One significant advantage is the elimination of the need for removal surgery, which is often required with Steinman pins due to their permanent nature. This aspect not only reduces the risk of additional surgical procedures and associated complications but also minimizes the stress and discomfort experienced by the bird during the recovery period.

Furthermore, PMMA pins offer versatility in fracture management, as they can be customized to fit the specific anatomical requirements of the bird, ensuring optimal stabilization and alignment of the fracture site. In addition, PMMA pins are lightweight and biocompatible, minimizing the risk of adverse reactions or complications associated with implant materials. This aspect is particularly important in avian patients, where the delicate nature of their anatomy requires careful consideration of implant materials to avoid tissue irritation or rejection.

Overall, the study findings support the use of PMMA pins as a viable alternative for avian humerus fracture stabilization, offering comparable efficacy to traditional Steinman pins while providing additional benefits in terms of post-treatment management and patient comfort. Further research and clinical evaluation may be warranted to explore the long-term outcomes and potential complications associated with PMMA pin fixation in avian patients.

Materials and Methods

Thirty young adult pigeons (Columba livia domestica) were enlisted for this study, adhering to the guidelines set forth by the research Ethics Committee of Shahid Bahonar University of Kerman (ethics code: IR.UK.VETMED.REC.1401.023). The pigeons were kept under standardized conditions, receiving identical rations and having unrestricted access to food, water, light, and darkness on a 12-hour cycle. Moreover, consistent temperature and humidity levels were meticulously maintained throughout the study [5]. The animals were allocated randomly into study groups. A detailed description of each study group and the corresponding procedures conducted within each group are provided in Table 3.

To prepare a bone cement pin (G1A 40™, G21 company, Italy), the cement and its solvent were initially opened under sterile conditions. Following the manufacturer's guidelines, cement powder was mixed with solvent in a ratio of 2:1 in a sterile stainless-steel container. Subsequently, this mixture was injected into a sterile Nelaton catheter (Supa Manufacture of Medical Equipment, Iran) using a sterile syringe. After solidification, the pin was stored in a formalin tablet compartment to maintain sterility until its use. Given the varying diameters of the bone marrow, three sizes of Nelaton catheters (10, 12, and 14) were utilized.

To facilitate the study, thirty minutes prior to surgery, the pigeons were administered tramadol (5 mg/kg IM) and meloxicam (2 mg/kg IM) for analgesia [6]. Subsequently, the pigeons were anesthetized using isoflurane (Piramal, India) via a face mask at a concentration of 4%–5% in oxygen (flow rate of 1–1.5 L/min) for induction. Following induction, the pigeons were intubated using an uncuffed endotracheal tube (with internal diameters ranging from 2.5 to 4 mm) and maintained under isoflurane anesthesia at a concentration of 1.5%–2.5% utilizing a non-rebreathing anesthesia circuit system [7]. Following induction, the pigeons received supplemental fluids in the form of lactated Ringer's solution subcutaneously at a rate of 20 mL/kg. Throughout the surgical procedure, the birds were kept on a heating pad to maintain their body temperature [7].

To access the humerus, the animal was positioned in sternal recumbency, and a dorsal approach was employed. The surgical site was meticulously prepared in an aseptic manner and subsequently draped [8]. In a longitudinal fashion, the skin was incised using a scalpel blade number 10 along the axis of the bone. Next, dissection was performed to gain access to the humerus through the muscular tissue using a medical micromotor (Strong, China). A transverse fracture was created in the midshaft of the humerus. In both study groups, IM pinning was done using the Normograde method [2]. Pin size selection was based on 50%–70% of the diameter in both groups to achieve appropriate coverage of the medullary canal [3, 9]. Following the surgical procedure across all groups, muscle closure was achieved utilizing 0-3 vicryl with a simple continuous pattern, while skin closure was performed using 0-3 nylon sutures. Clindamycin gel was applied to the surgical site, and the wing of the animal was bandaged in a figure-8 configuration using vet wrap [10]. After 14 days, the bandage and suture were removed.

The pigeons received a treatment regimen of enrofloxacin

Table 3.
presents the names of the groups and the respective operations carried out in the current study

Group name	Operations performed on group members (n=5)
control group	Creating a fracture in the middle part of the Humerus and stabilizing it using an intramedullary pin (This group is called PIN for short)
Treatment	Creating a fracture in the middle part of the humerus and fixing it using a pin made of bone cement inside the bone medulla(This group is called PMMA for short).

at a dosage of 10 mg/kg administered subcutaneously for three consecutive days. The enrofloxacin utilized for this treatment was sourced from Baytril Bayer, Germany [11]. Postoperative analgesia was administered in the form of meloxicam at a dosage of 2 mg/kg orally every 12 h for five days [6]. Afterwards, at the intervals of 2, 4, and 6 weeks post-surgery, tissue samples were collected. Initially, the animals were euthanized through the administration of a high dose of isoflurane [12]. Radiographic assessment of the samples was performed using a VD view and Carestream software by a computed radiography system. Callus diameter and corrected callus diameter were determined 2, 4, and 6 weeks post-surgery. The callus diameter is the maximum diameter of the callus at any time. The corrected callus diameter value is the callus diameter at a given time less the diameter of the humerus on the immediate postoperative radiograph [6, 8, 13, 14]. After dissection, bone samples were collected, and the PMMA and Steinmann pins were removed. Next, the bone specimens were fixed in 10% buffered formalin. Upon completion of the fixation process, the samples underwent decalcification utilizing a 5% nitric acid solution [15]. Following the preparation period utilizing the tissue processor, sections with a thickness of 5 µm were generated. These sections were subsequently subjected to hematoxylin-eosin staining, enabling the evaluation of bone healing. The assessment criteria included the following categories: 0 (indicating no sign of union), 1 (representing fibrous union), 2 (reflecting osteochondral union), 3 (indicating bone union), and 4 (denoting complete reorganization of the shaft) [16].

Statistical Analysis

All analyses were conducted using IBM SPSS Statistics version 26 software. Quantitative radiology results were assessed with independent-samples t-tests and repeated measures ANOVA. Non-parametric tests, including the Friedman test and the Mann-Whitney U test, were applied to evaluate the qualitative histopathology data.

Authors' Contributions

Kaveh Aski A, Molaei M, Azari O, Kheirandish R, and Vosough D conceived and planned the experiments. All carried out the experiments. They planned , carried out the simulations, and contributed to the interpretation of the results. They took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare no conflict of interest.

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Study of histopathological effects of electromagnetic field (EMF) on the thyroid gland of rats

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ABSTRACT

The relationship between exposures to Electromagnetic field (EMF) and human health is more in focus. Some studies showed the possible relation between exposure to EMF and cancer. The thyroid gland is one of the most exposed and vital organ and may be a target for any type of electromagnetic radiation; therefore, we studied histopathological effects of electromagnetic field (EMF) on the thyroid gland of rat. In this research, 35 healthy rats were used. The animals were divided to five groups, and then all animals were exposed to 2100MHz (4G) frequency for 0, 15, 60, 120, 180 min every day for a period of 70 days. The animals were euthanized by removing of blood from the heart then tissue samples were prepared from thyroid gland and stained with hematoxylin and eosin (H & E) and Mason trichrome (MT). Stereological studies were done by the Cavalier's principle. Following the EMF exposure, a significant decrease ($p < 0.05$) in the diameter of the thyroid follicles, heights of epithelial follicles and thyroid follicles volume were recorded in 180 min exposure groups.In the EMF exposure groups (120 and 180 min) histopathological effect observed include follicles with decreased colloid, congestion and increase of connective tissue. We also observed formation of apoptotic body that infiltrated inside of follicle and follicular cells with condensed nuclei under the exposure of EMF for120 and 180 min.We concluded that exposure of EMF (4G) for over 120 min for 70 consecutive days has histopathological and stereological effects in thyroid gland in rats.

Keywords

Histopathology, Electromagnetic field, Thyroid gland, Rat

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Number of Tables: 0
Number of References:: 18
Number of Pages: 06

Abbreviations

EMF: Electromagnetic field
TCs: Thyrocyte
T3: Triiodothyronine
T4: Thyroxin
TSH: Thyroid-stimulating hormone

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Introduction

With the development of technologies, human exposure of electromagnetic field (EMF) has increased during the recent years. In 2021, mobile phone subscriptions surpassed eight billion users worldwide, and the number is expected to increase to 8.8 billion by 2026 [1]. Non-ionizing forms of radiation include lower frequencies on the electromagnetic spectrum and are not proven to cause DNA damage directly [2].

Several studies showed the possible relation between exposure to EMF and certain malignancies including lymphomas, leukemia, breast, brain and lung cancers [3-5]. International agency for Research on Cancer (IARC) has classified EMF as possibly carcinogenic to humans [6].

EMF causes morphological and functional changes in endocrine system, central nervous system, immune systems, cardiovascular system, reproductive system, learning and memory [7, 8]. The thyroid gland may be particularly vulnerable to this effect because of its normal anatomical position near the neck.

The finding of another study revealed that exposure of electromagnetic fields with a 940 MHz frequency has increased the permeability of blood brain barrier (BBB) [9]. Previous studies have reported that EMF exposure in rats caused a decreased uptake of iodine by the thyroid gland and reduced levels of plasma TSH [10]. Another research has reported that EMF (50MHZ) exposure increased cyclic adenosine monophosphate (cAMP), T3 and T4 [11]. In another study, exposure of EMF (50MHz) for 2-6 month by using light and transmission electron microscopy revealed the frequent findings of several colloid droplets within the same thyrocyte with the occasional presence of large-diameter droplets and alterations in lysosomes, granular endoplasmic reticulum and cell nuclei compared to the control group [12]. Another research was designed that were epigenetic effect of 50 Hz EMF in vitro, showed alteration of genome-wide methylation and DNMTS expression may play an important role in the biological effect in mouse spermatocyte[13].

Questions have been raised about the safety of prolonged use of EMF in mobile phone and other equipment. Therefore, in the present study, we aimed to investigate the possible harmful effect of EMF (2100MHz) (4G) on thyroid gland. This paper will focus on new generation of EMF (4G) exposure on thyroid gland by using EMF generator.

Result

Stereological findings

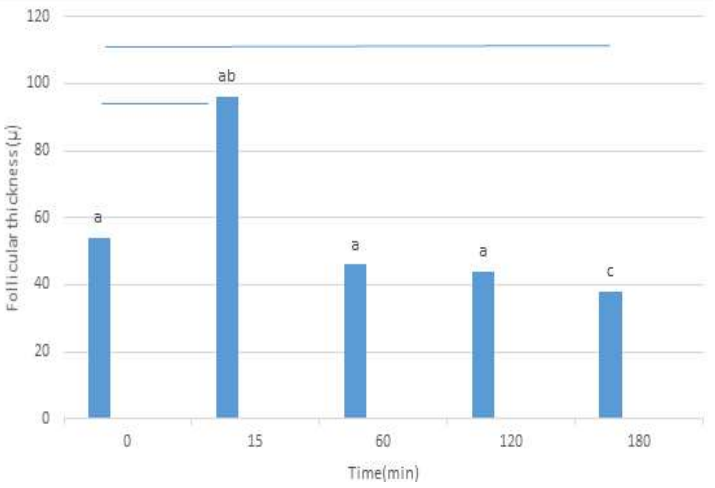
Following the EMF exposure were showed in diameter of the thyroid follicles significant increase in 15 min and decrease in 180 min ($p < 0.005$) (Graph. 1), for heights of thyrocyte were decreased with increased the time of exposure but have not significant change (Graph. 2) and for thyroid follicles volume significant decreased in180 min exposure of EMF (Graph. 3).

Histopathological finding

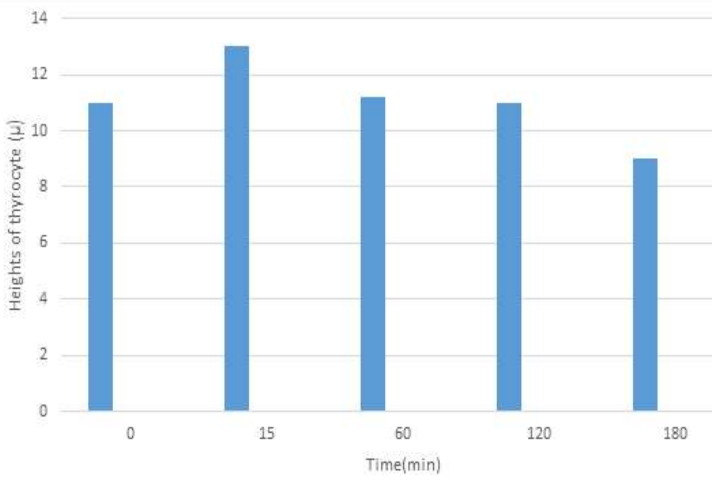
The results of the studies showed that in the control group (0 min) (Fig. 1) and EFM exposure group (15 min.) tissue characteristics including follicles, thymocyte cells, colloidal materials, connective tissue and capsule of the thyroid gland were observed as normal.

Histopathological finding in the EMF exposure groups (60 and 120 min.) include: collapsed follicles with little colloid, congestion and increased apop-

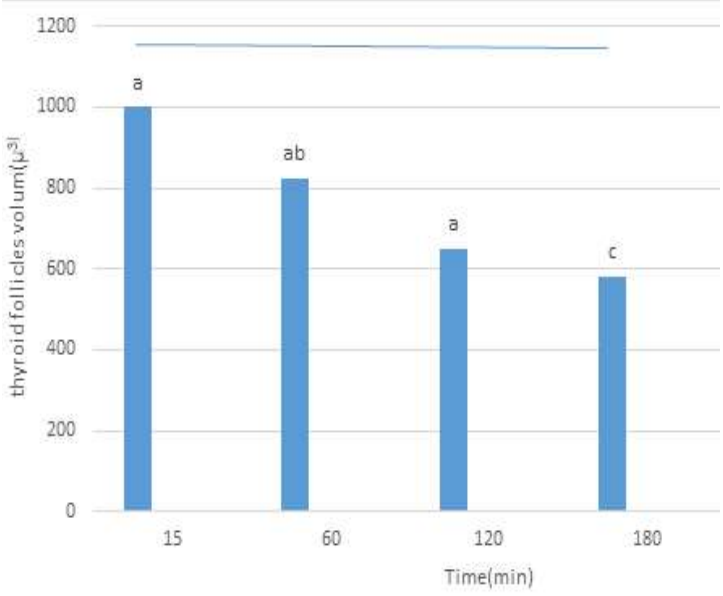
Graph 1.
Measurement of the diameters of the thyroid follicles ($p \leq 0.005$).



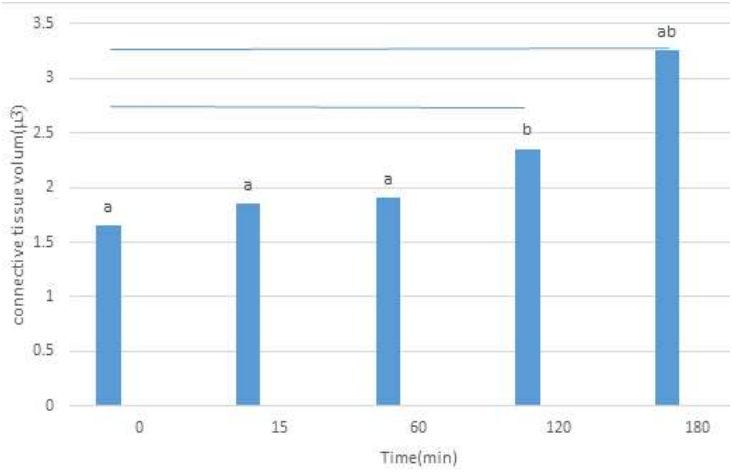
Graph 2.
Measurement of the heights of the epithelial follicles (p≤0.005).



Graph 3.
Measurement of the thyroid follicular volume (p≤0.005).



Graph 4.
Measurement of connective tissue volume ($p \leq 0.005$).



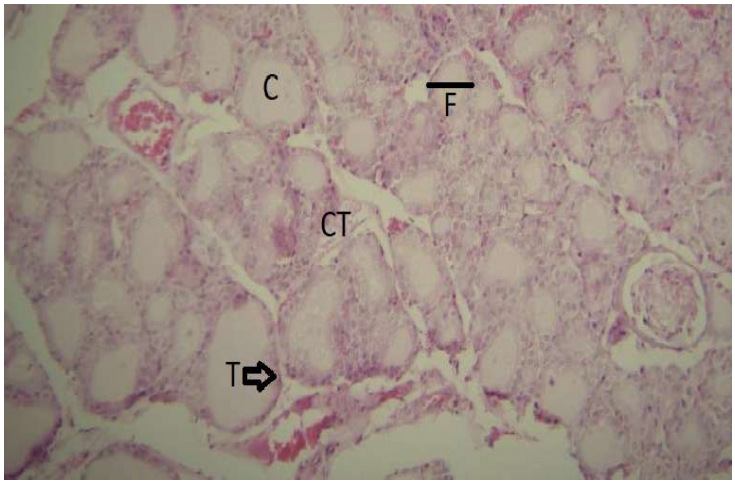


Figure1.
Photomicrograph of thyroid gland from control group (0 min). It shows normal Follicle (F), Connective tissue (CT), Thyrocyte (T) and Colloid(C). H&E staining ×100.

totic bodies. (Fig. 2). We also observed apoptotic bodies (AB) within follicles, rupture of follicles, and TCs with heterochromatin nuclei were infiltrated in interfollicular septa on the thyroid gland that exposure of EMF for 120 and180 min. (Fig. 2, 3).

The results showed that the connective tissue between the thyroid follicles in all of the exposed groups had increased compared to the control group but the only difference between groups of 120 and 180 min were statistically significant ($p < 0.005$) (Fig. 4) (Graph. 3).

Discussion

In 2021, mobile phone subscriptions surpassed eight billion users worldwide, and the number is expected to increase to 8.8 billion by 2026 [1].

The relationship between exposures to EMF and human health is very important. The present study confirms previous finding and contributes additional evidence about histological effects of EMF (4G) exposure on thyroid gland in rats.

The results of this study showed that the diameter of the thyroid follicles increased significantly in the exposure of EMF after 15 min, and with the increase in the time of the influence of the waves, it decreased up to 180 min, and this decrease was significant in 180 min. The reason for this initial increase is not known to us, but with the increase in the time of impact of the waves on the cells, maybe with cell organelles were damaged and with the decrease in the secretion of thyroid hormones, the diameter of the thyroid follicles decreases. We were observed that with increased of time of exposure of EMF the height of thyrocyte decreased but not significantly and we were observed that with increased of time of exposure of EMF the thyroid follicles volume significantly decreased

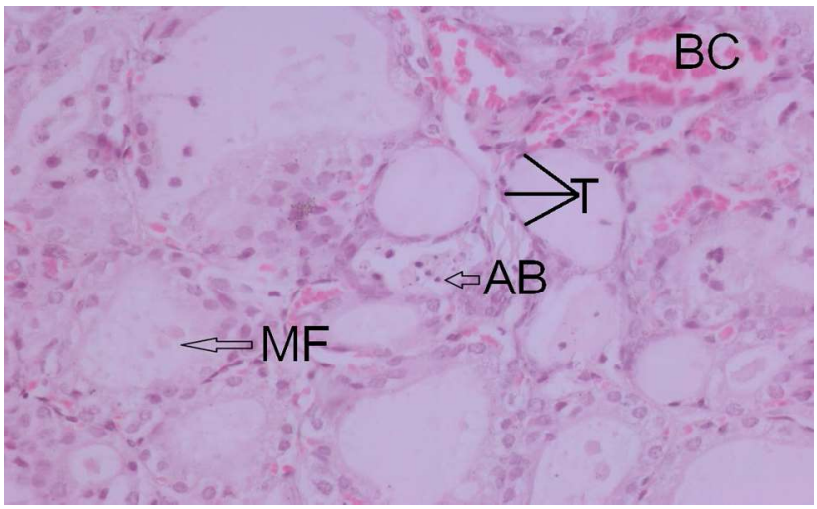


Figure 2.
Photomicrograph of thyroid gland from animals exposed to 2100 MHz (group: 120 min). It shows numerous small follicles (MF) with decreased colloid and low height of TCs (T), congestion (BC) and more apoptotic bodies (AB), H& E staining × 400.

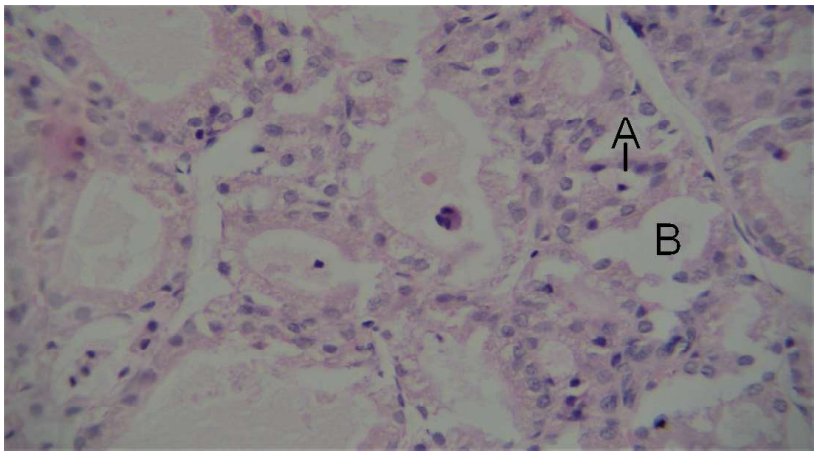


Figure 3.
Photomicrograph of thyroid gland from animal exposed to 2100 MHz (group: 180 min). It shows apoptotic bodies (A), rupture of follicles (B)(RF) ×400.

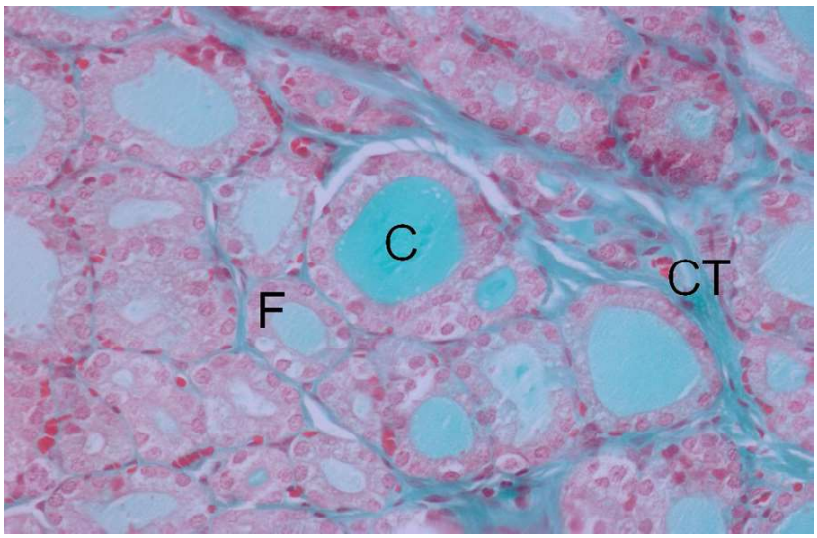


Figure 4.
Photomicrograph of thyroid gland from animal exposed to 2100 MHz (group: 180 min) shows colloid droplets within a TCs (F), coagulated colloid (C) and Connective tissue (CT), Mason trichrome staining ×400.

in 180 minute and connective tissue volume significantly increased in180 minute. We concluded that with increased of time of exposure EMF ,it causes the destruction of cell organelles and DNA of nuclear, and after that, with the decreased in the height of thyrocyte, the secretion of hormones were decreased, and then the volume of follicles were decreased and the volume of connective tissue were increased.

This study showed pathological effects of TCs after exposure of EMF including contracted follicles with decreased colloid, congestion and extension of connective tissue in interstitial space. Also we were observed formation of apoptotic bodies within follicles and follicular cells with condensed nuclei in thyroid gland of the rats under the exposure of EMF for 180 min that it was consistent with other research [14].

Some researcher revealed that mobile phone radiofrequency radiation might be associated with thyroid gland insufficiency and alterations in serum thyroid hormone levels, with a possible disruption in the hypothalamic-pituitary-thyroid axis [15].

Other researcher concluded that EMF exposure at the frequency of 1800 MHz has caused significant changes in the levels of serum TSH, T4, MDA, and MCT8 concentration in the Wistar rats [16].

Our work shows a dilation and an increase in the number of blood capillary in thyroid

gland, which is similar to other researches [12]. Some scientists showed effect of that EMF exposure on mast cells population and degranulation of these cells; therefore, EMF increases released of some mediator from mast cell [17].

According to the obtained results, the long-term use of electromagnetic waves can inhibit the growth and differentiation of cells by affecting the vital mechanisms.

Materials and Methods

To do this research, for the first time an electromagnetic generator, an antenna (output power: 2000 MW, power source lithium-ion battery 127, 2600 MAH) was used with a variable frequency band (2100 to 2600 MHz) (Fig.4).

In this study, 35 healthy adult male Wistar rats aged 2 months old and weighing 300-350 gram were used. The rats purchased from the laboratory animal unit, Mashhad University of Medical sciences, Mashhad, Iran. The Wistar rats were kept in a 12h light: 12h dark cycle, at constant temperature of 25°C, while food and water were accessible on an ad libitum basis. Animals were randomly divided into five groups, each of which consisted of 7 animals. Groups exposed to 2100 MHz frequency continuously for 0, 15, 60, 120, 180 min every day for a period of 70 days. The animals were anesthesiad with CO_2 and after dissection of abdominal cavity, they were euthanized by removing of blood from the heart. Then thyroid glands were removed and the samples collected. Then the samples were fixed in 10% neutral buffered formalin solution. Thyroid samples were transferred from formaldehyde, after dehydration by passing tissue through a series of alcohol solutions, were cleared in xylene and were embedded in paraffin (Merck, Germany).Then the specimen were embedded in large block of paraffin by paraffin dispenser (Didsabz, Iran).Then sample were sectioned at 5 µm thickness using microtome (Leica, Germany).The final step was staining sample with Hematoxylin and eosin (H &E) and Masson trichrome (MT) (Merck, Germany). All the procedures were certified by the relevant Ethical Committee of Ferdowsi University of Mashhad, Mashhad, Iran (Code: IR.UM.REC.1401.089).

Data were analyzed by one-way ANOVA, (statistical package of SPSS version 19). In all tests, $p \leq 0.05$ was considered as statistically significant.

Stereology

Stereological studies were done by the Cavalier's principle in order to estimate the epithelial height, follicular thickness, follicular volume and connective tissue volume in septa [18]. For this purpose, 10 sections were selected from each specimens and photographed by a camera (Olympus/DP25) with light microscope (Olympus CX22). A point grid was used for point counting. Grid was cited on the figures, each parameter was counted, and thyroid gland was blindly estimated by the following formula:

$V (mm3) = d \times \Sigma p \times a (p)$

d= Interval between section and section thickness; Σp = total number of point considered on the area of sections; a (p) = the area represented of each point in the grid.

Authors' Contributions

Investigation, writing the original draft, and statistical analysis: MAM. Conceptualization, supervision,software, draft review, and editing: MAM, AR, MM, MKR. All authors were involved in writing the article and accepted responsibility for its content.

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Competing Interests

The authors declare no conflict of interest.

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Genotypic and Phenotypic Characteristics of the Phylogenetic Groups of *Escherichia Coli* Isolates From Ostriches in Iran

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ABSTRACT

Increased antibiotic use in the ostrich industry could lead to the emergence of virulent antibiotic-resistant bacterial strains transmissible to human. This study investigated the genotypic and phenotypic characteristics of the phylogenetic groups of *Escherichia coli (E. coli)* isolates from ostrich and reveal their health risk potential. One hundred twenty-nine confirmed presumptive commensal (44) and suspected pathogenic (85) *E. coli* isolates from ostrich flocks in Mashhad, Northeast Iran, were phylo-typed by the Clermont quadruplex polymerase chain reaction. The phylogenetic profile of the isolates was comparatively investigated based on antimicrobial susceptibility, resistance, and virulence gene profiles. Results indicated that both groups of presumptive commensal and pathogenic isolates were mostly distributed within phylogroups A (with proportions 31.81% and 32.94%, respectively) and B1 (with proportions 36.36% and 31.76%, respectively). Multi-drug resistance was highest within the phylogroup B2 ($p \geq 0.05$). The phylogroup B1, typically known for commensal strains, unlike B2, showed the most negligible proportions of isolates which were devoid of resistance genes ($p \geq 0.05$) and virulence genes ($p \geq 0.05$). The findings of this study expanded the horizon of the genotypic and phenotypic characteristics of the phylogenetic groups of *E. coli* isolates from ostrich. Moreover, we indicated a complicated inconsistency between both characteristics. Therefore, more comprehensive and comparative studies on *E. coli* isolates from ostrich and human are favoured in future research.

Keywords

Antimicrobial susceptibility, Clermont quadruplex PCR, MDR, Resistance genes, Virulence genes

Number of Figures: 4
Number of Tables: 0
Number of References:: 38
Number of Pages: 09

Abbreviations

E. coli: Escherichia coli
PCR: Polymerase Chain Reaction
MDR: Multi-drug resistance
APEC: Avian pathogenic *E. coli*
ExPEC: Extra-intestinal pathogenic *E. coli*

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Introduction

The presence of antimicrobial residues and the emergence of resistant bacterial pathogens in food and the environment have complicated the strategies for appropriate treatment, raising serious public health concerns. In this sense, the improper or extensive use of antimicrobials in food-producing animals, particularly poultry, for improving growth and health probably plays a significant part. This practice imposes a selection pressure, leading to resistant bacterial strains transmissible to human [1-5]. The emergence and dissemination of diversified phylogenetic groups of antibiotic-resistant *E. coli* strains is a global health concern. Furthermore, *E. coli* strains provide accurate findings on antimicrobial resistance status because of their presence in the environment and as commensal flora in humans and animals. Therefore, monitoring the phylogenetic distribution of *E. coli* strains could benefit the design of preventative and therapeutic strategies with economic significance [6-8]. The phylogenetic background, indicating the ecological distribution, evolutionary history, and virulence of pathogens, could be affected by geographical region, sampling area, site of infection, antibiotic resistance, and host response. Environmental, social, and dietary conditions are also considered to cause phylogenic heterogeneity [9]. Most extra-intestinal pathogenic *E. coli* strains belong to the previously described phylogroups. On the other hand, newly described phylogroups mostly include intestinal pathogenic *E. coli* strains. As a result, the phylotype-related traits necessitate a reliable detection of *E. coli* phylogroups and also the investigation of probable impacts of virulence genes, MDR characteristics, and their cross-talk regarding each phylogroup [7, 10]. Finding the relationship between pathogenic traits and the phylogeny of E. coli is a complicated phenomenon due to distinctive interplays [11]. However, some previous studies indicated that commensal *E. coli* strains are within phylogroups A and B1, while extra-intestinal pathogenic *E. coli* strains belong to phylogroups D and B2. Moreover, E. coli strains within phylogroup B2, which have the highest susceptibility to antimicrobials, have previously exhibited promoted virulence capacity compared to commensal groups [12].

The APEC strain belongs to the ExPEC category [13]. Phylogenic backgrounds and virulence genes within the ExPEC strains from both human and avian sources are identical [5]. Therefore, monitoring the ExPEC strains in poultry is crucial from the perspective of public health [14]. Comprehensive research has demonstrated that APEC and human ExPEC strains have multiple common traits, encompassing serogroups, virulence factors, and sequence types. In addition, the APEC strains might function as a reservoir for the virulence genes of ExPEC in humans. Therefore, for the effective surveillance and control of avian colibacillosis, it is vital to identify the phylogeny, lineage, and virulence of APEC strains that commonly infect poultry flocks. Early identification of these strains using phylogenetic analyses could beneficially provide the needed preventive measures [15, 16].

The ostrich farming industry is rapidly expanding for the human consumption of meat, leather, and plumes. This industry plays an integral part in terms of agriculture, economy, and meat production in Iran. Scarce information on ostrich-originated *E. coli* strains, transmissible to humans, necessitates more attention to the potential zoonosis health threats caused by these strains [17, 18]. To the best of our knowledge, this study is the first that comprehensively compared the phylogenetic profile of *E. coli* isolates from ostrich based on their genotypic and phenotypic traits. This study demonstrated some of the genotypic and phenotypic characteristics of the phylogenetic groups of *E. coli* isolates from ostrich, thereby revealing their potential health threats.

Result

Distribution of Phylogenetic Groups Within Isolates and in Relation to the Source of Isolation

Most isolates were segregated into five phylogenetic groups. However, 11.36% and 4.70% of the presumptive commensal and pathogenic strains were unassignable, respectively. The distribution of both groups of presumptive commensal and pathogenic strains was the highest in the B1 and A phylogroups. Commensal isolates were rarely classified into phylogroups B2 (0%) and C (2.27%). Details on phylogenetic classification results are demonstrated in Figure 1. E. coli strains isolated from each source mostly belonged to the phylogroups A and B1, except that the strains isolated from the lungs were mainly within phylogroups A (42.85%) and E (42.85%). Those isolated from dead-in-shell embryos were mostly within the phylogroups B2 (75%) and E (25%). Moreover, the isolates from the faeces of sick ostriches were abundantly within phylogroups A (53.84%) and Unassignable (23.07%). Isolates from each source, except for the isolates from embryos, yolk sacs, and faeces of sick ostriches, possessed the lowest frequency within the phylogroups B2 and C. The strains within the Unassignable phylogroup were only isolated from faeces (of both healthy and sick birds; $p \geq 0.05$) and yolk sacs ($p \geq 0.05$). The strains within the phylogroup B2 were significantly isolated from dead-in-shell embryos ($p < 0.05$). Details on the phylogenetic groups in relation to the source of isolation are presented in Figure 1.

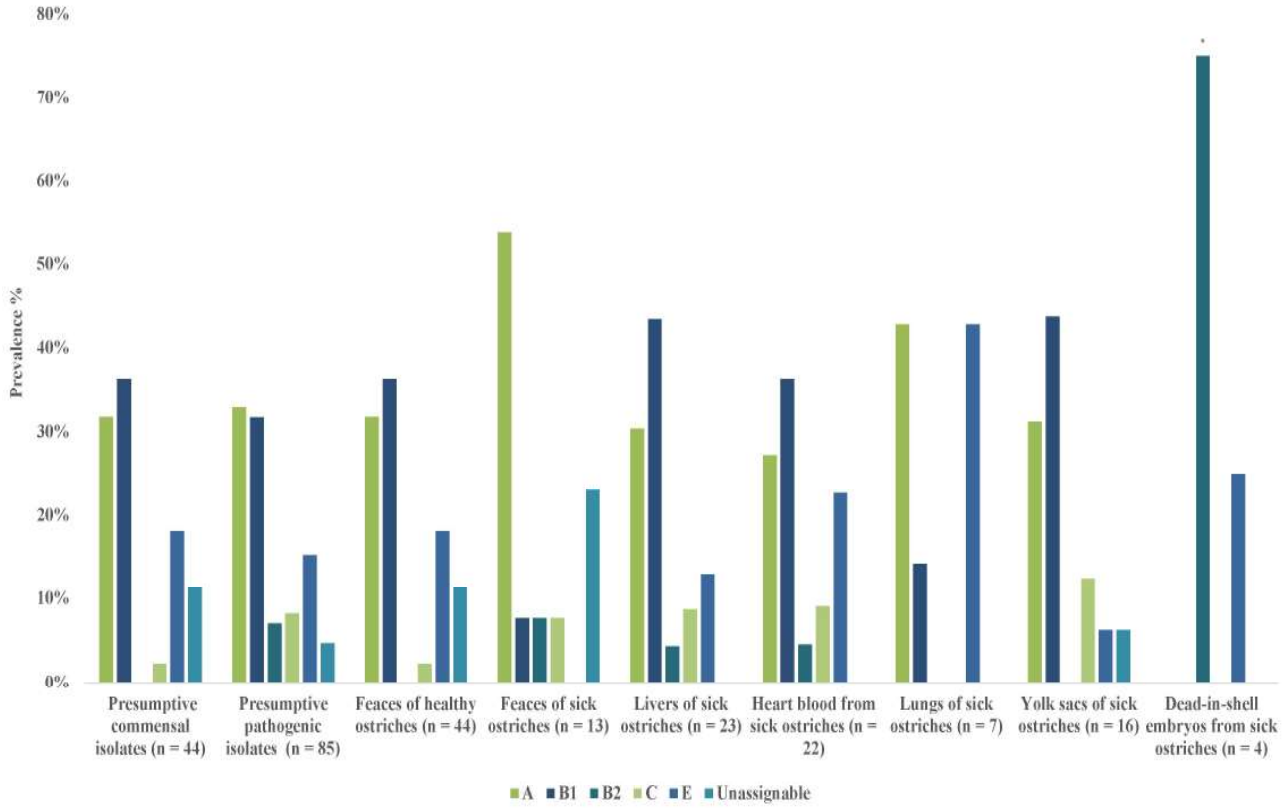

Figure1.
Distribution of phylogroups within the presumptive commensal and pathogenic *E. coli* isolates from ostrich and in relation to their source of isolation. Against categorical variables, *P-value* is ≥ 0.05 for each phylogroup, excluding one indicated by * (*P-value* < 0.05).

Antimicrobial Resistance Profile of Isolates and Phylogenetic Groups

Comparing the resistance of the pathogenic and commensal groups of isolates against four antimicrobials revealed a higher proportion of resistance for the earlier group against tetracycline ($p \geq 0.05$); however, both groups of isolates showed a comparable proportion of resistance against the other two tested antimicrobials, lincomycin ($p \geq 0.05$) and ceftriaxone ($p \geq 0.05$). The distribution of antibiotic resistance within both groups of presumptive commensal and pathogenic isolates was highest against lincomycin and tetracycline, respectively, and lowest against ceftriaxone (Figure 2). Isolates from all phylogenetic groups harboured 100% resistance proportion against lincomycin. The isolates of the phylogroup C were entirely susceptible to streptomycin, trimethoprim + sulfamethoxazole, and doxycycline. Isolates of the phylogroup C also recorded no resistance against enrofloxacin and amoxicillin, similar to unassignable isolates. Isolates of C and B2 phylogroups showed complete susceptibility to florfenicol. While full susceptibility was recorded against ceftriaxone for isolates within most phylogenetic groups, the isolates of C and E phylogroups showed 12.5% and 9.52% resistance to this antimicrobial, respectively ($p \geq 0.05$). Resistance to gentamicin was also low within the isolates of all phylogroups, and only the isolates within phylogroups A, C, and E indicated a negligible resistance (4.76%, 12.5%, and 4.76%, respectively) ($p \geq 0.05$). Details on antimicrobial resistance frequency in relation to phylogeny are demonstrated in Figure 3a. Results also revealed that the highest total MDR proportion belonged to the phylogroup B2 (33%) ($p \geq 0.05$) compared to the MDR observed within the phylogroups A (10%), B1 (21%), C (0%), E (24%), and Unassignable (0%). More details on MDR frequency in relation to phylogeny are demonstrated in Figure 3b.

Antimicrobial Resistance Gene and Virulence Gene Profiles of Phylogenetic Groups

Within phylogroups B2 and C, sul1 and tet(A) genes were undetectable. In addition, sul1 gene was absent within the Unassignable phylogroup ($p \geq 0.05$). Phylogroups B2 and C included the isolates devoid of blaTEM and qnrA gene, respectively. More than half of the isolates within the phylogroup B2 (66.66%) lacked any resistance genes, which was the most abundant but was not significantly different ($p \geq 0.05$) compared to the corresponding rate of the phylogroup B1 (30.23%). Details on antimicrobial resistance gene frequency in relation to phylogeny are demonstrated in Figure 4a. Within phylogroups C and Unassignable, astA and Irp2 genes were absent. Within the Unassignable phylogroup, iucD gene was

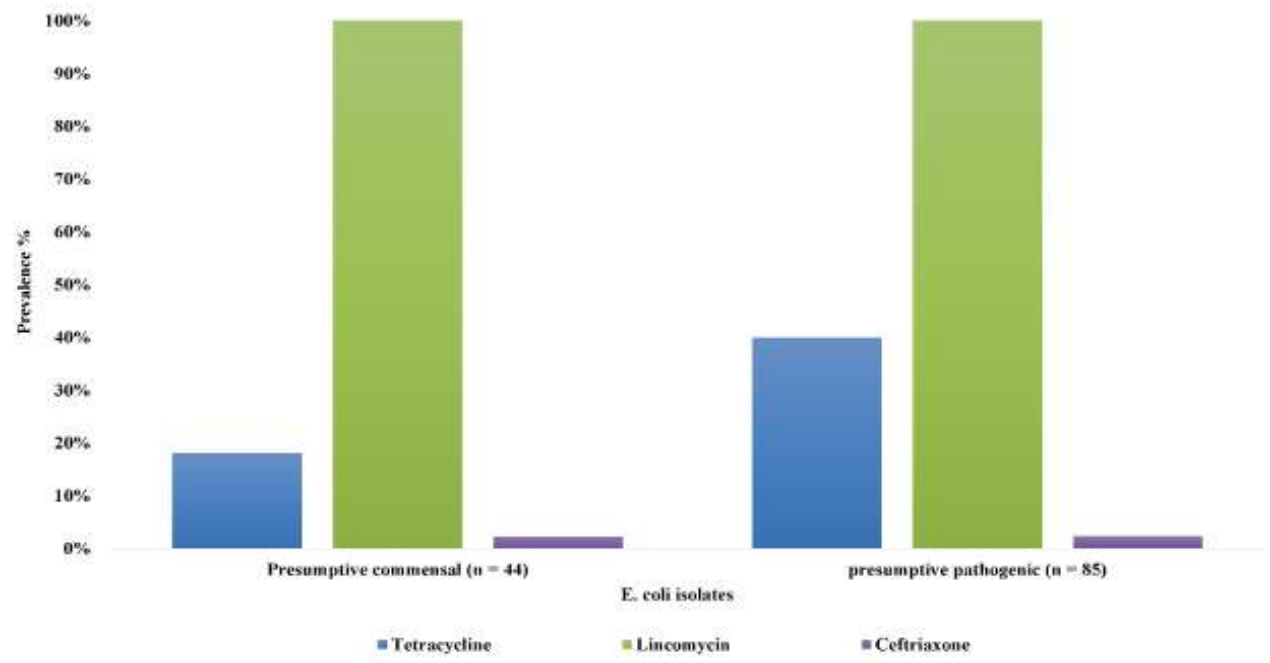

Figure 2.
Distribution of antibiotic resistance against three different antimicrobials within presumptive commensal and pathogenic *E. coli* isolates from ostrich. *P-value* is ≥ 0.05 for each antimicrobial between categorical variables.

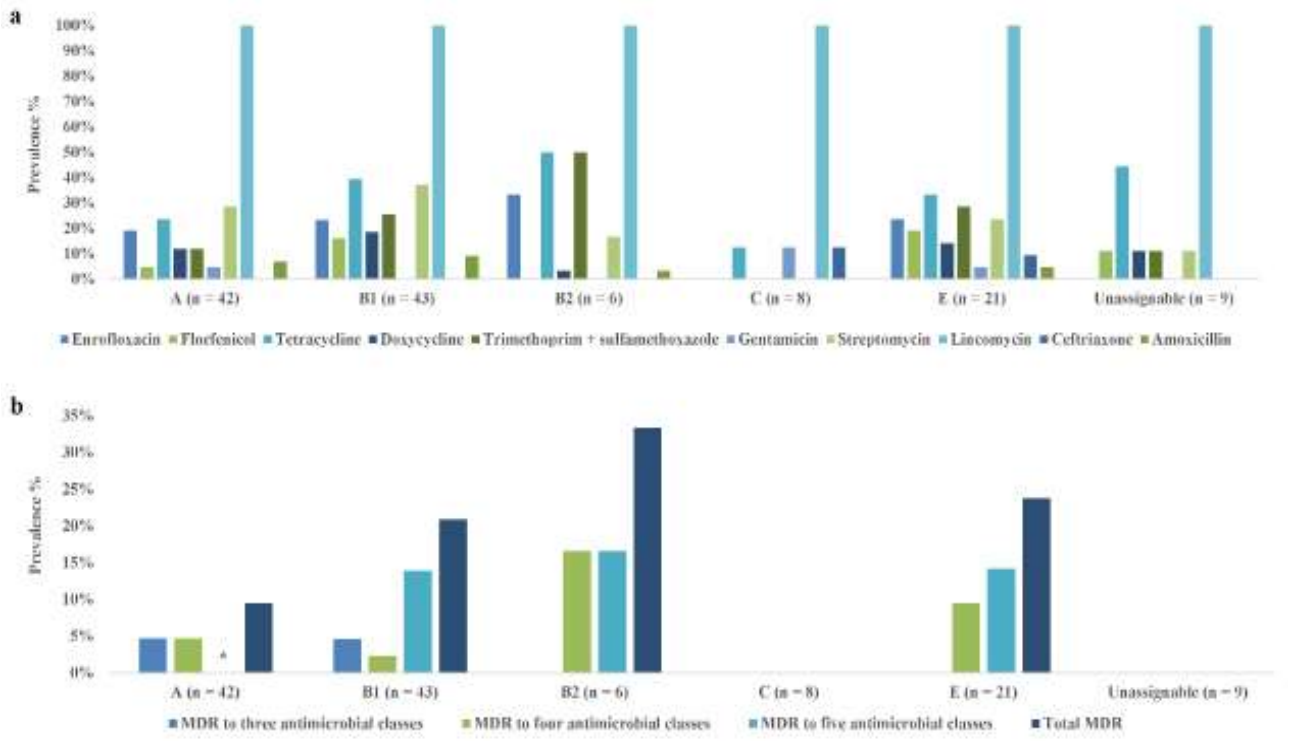

Figure 3.
(a) Distribution of antibiotic resistance against 10 different antimicrobials within phylogroups of E. coli isolates from ostrich. *P-value* is ≥ 0.05 for each antimicrobial against categorical variables. (b) Distribution of MDR within phylogroups of *E. coli* isolates from ostrich. Against categorical variables, *P-value* is ≥ 0.05 for each MDR, excluding one indicated by * (*P-value* < 0.05).

also undetectable. Within all phylogroups, excluding B1 ($p < 0.05$), tsh gene was rare. In addition, B1 was the only phylogroup carrying vat gene (2.32%) ($p \geq 0.05$). Only within the phylogroup B2, cvaA/B gene was undetectable ($p \geq 0.05$), whereas this gene was significantly distributed within the phylogroup C ($p < 0.05$). More than half of the isolates of phylogroup B2 (66.66%) did not have any virulence genes, which was the most noticeable but did not have a significant difference ($p \geq 0.05$) with the corresponding proportion of the phylogroup B1 (37.20%). Details on virulence gene frequency in relation to phylogeny are demonstrated in Figure 4b.

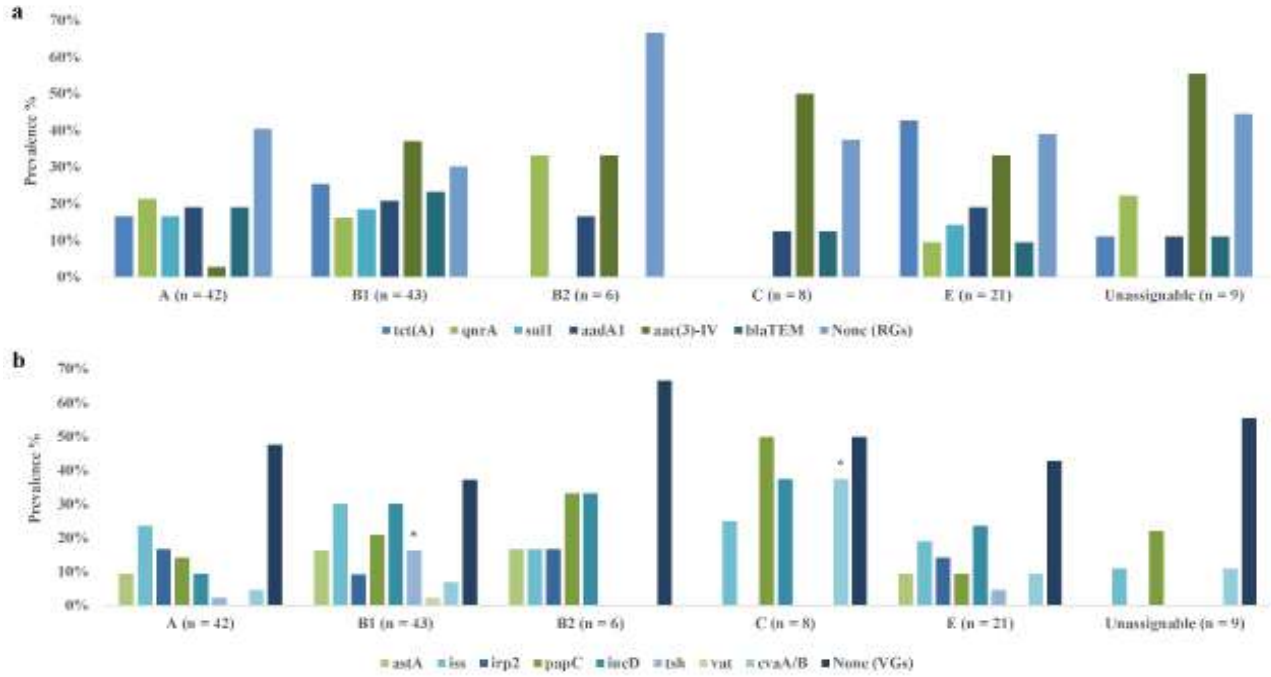

Figure 4.
Distribution of antibiotic resistance genes (a) and virulence genes (b) within phylogroups of E. coli isolates from ostrich. tet(A), tetracycline resistance gene; qnrA, quinolone resistance gene; sul1, sulfonamide resistance gene; aadA1, streptomycin resistance gene; aac(3)-IV, aminoglycoside N(3)-acetyltransferase gene; blaTEM, beta-lactamase resistance gene; None (RGs), None resistance genes. *P-value* is ≥ 0.05 for each resistance gene against categorical variables. astA, enteroaggregative toxin gene; iss, increased serum survival protein gene; irp2, iron repressible protein gene; papC, P-fimbriae gene; iucD, aerobactin gene; tsh, temperature-sensitive hemagglutinin gene; vat, vacuolating autotransporter toxin gene; cvaA/B, colicin V plasmid operon gene; None (VGs), None virulence genes. Against categorical variables, P-value is ≥ 0.05 for each virulence gene, excluding two indicated by * (*P-value* < 0.05).

Discussion

According to the phylotyping results of this study, most isolates were classified into five phylogenetic groups; however, multiple strains were not assignable according to the quadruplex PCR-based method of Clermont et al. [19] (Figure 1). These unassignable strains have also been observed in some previous studies and are probably from extremely rare phylogroups. The loss of specific genes resulting from the plasticity of the genome of *E. coli* or the recombination of isolates from variant phylogroups might also lead to the observation of these unknown strains [19, 23, 24]. Phylogenetic groups B1 and A accounted for most of the phylogenetic profiles of both groups of presumptive commensal and pathogenic isolates with a close distribution proportion (Figure 1). This finding is in line with multiple previous studies on *E. coli* strains originating either from ostrich [25] or other avian species [26-32]. However, some other studies reported different dominant phylogenetic groups within avian-originated *E. coli* strains [7, 13-15, 33-36]. These inconsistencies could be due to the factors causing phylogenic heterogeneity, including geographical region, sampling area, site of infection, antibiotic resistance, host response, as well as environmental, social, and dietary conditions [9]. However, regarding the results of this study, insufficient evidence was obtained to com-

pletely correlate phylogenic heterogeneity to the isolation source of the strains, which justifies the necessity for further studies.

In the present study, the *E. coli* isolates of the phylogroup B2 were all from the suspected pathogenic group (Figure 1) and showed the highest MDR (statistically insignificant; $p \geq 0.05$) (Figure 3b). However, these isolates, unlike typically known commensal strains from the phylogroup B1, harboured the lowest virulence and resistance gene capacity (statistically insignificant; $p \geq 0.05$) (Figure 4). Saha et al. [7] have also reported that all APEC phylogroups, including B2, obtained from poultry farms in Bangladesh, showed MDR. Moreover, enhanced virulence capacity, which probably occurs by acquiring virulence factors through horizontal gene transfer, has also been found previously within the non-B2 phylogroups of *E. coli* strains [13, 31, 34, 35]. Among all resistance and virulence genes examined in this study, only two virulence genes were significantly prevalent ($p < 0.05$) within specific phylogroups, tsh within the phylogroup B1 and cvaA/B within the phylogroup C. This observation provides another evidence for the enhanced virulence capacity obtained through horizontal gene transfer within the non-B2 phylogroups of *E. coli* strains. That is because both genes are located on the Colicin V (ColV) plasmid, which is detectable in most APEC strains and transmissible to the non-APEC strains [35]. Furthermore, a part of the inconsistency observed between phenotypic and genotypic traits within the phylogroups B2 and B1 might result from the expression status of virulence and resistance genes, even those not investigated in the present research. In this sense, Amani et al. [1], examining the virulence and resistance gene panels of the isolates, encountered some strains with a specific antimicrobial resistance phenotype lacking the corresponding resistance gene. The authors also found the investigation of the virulence genes insufficient to discriminate pathogenic from commensal strains [1]. Overall, due to discrepancy between the genotypic and phenotypic characteristics of phylogenetic groups of the examined

E. coli isolates, further comparative studies on the resistance and virulence properties of the phylogenetic groups of *E. coli* isolates from ostrich and human, especially from larger sample sizes and different geographical locations, are suggested.

In conclusion, the findings of this study provided understanding of the resistance and virulence traits of the phylogenetic groups of *E. coli* isolates from ostrich and indicated that further genotypic and phenotypic analyses on these phylogroups are essential.

Materials and Methods

Sample collection

From September 2018 to August 2019, a total of 44 presumptive commensal and 85 suspected pathogenic *E. coli* strains were randomly obtained from apparently healthy and sick ostriches, respectively, from six distinct ostrich flocks in Mashhad, Northeast Iran. The presumptive commensal strains were sampled from the fresh faeces of apparently healthy ostriches, and suspected pathogenic strains were sampled from the fresh faeces of sick diarrheic ostriches (n = 13), dead-in-shell embryos (n = 4) from ostriches suspected to colibacillosis, and dead ostrich chicks (n = 68). These chicks were suspected to be infected with *E. coli* through post-mortem examination and samples from their infected organs, including yolk sac (n = 16), lung (n = 7), liver (n = 23), and heart (n = 22), were aseptically taken.

Isolation and Detection of E. coli Strains

All obtained samples were aseptically streaked on MacConkey agar and aerobically incubated at 37°C for 24 h in the laboratory at the Veterinary Teaching Hospital, Ferdowsi University of Mashhad, Iran. The pure bacterial colonies with morphological and Gram staining characteristics similar to *E. coli* underwent biochemical tests (Indol, MR-VP, Simon Citrate, and TSI) and were confirmed as *E. coli* strains. Pure colonies of each sample were stored at -20°C in microtubes containing 2 ml of BHI medium and 15% sterile glycerol until use [37].

DNA Extraction

Following thawing, *E. coli* isolates were cultured on MacConkey agar and were then incubated at 37°C for 24 h. Subsequently, a pure colony of each cultured isolate was suspended in a microtube containing 150 µl sterile distilled water. Extraction of the whole bacterial genome was performed through the rapid boiling method on the mixture [38]. Following the centrifugation of the suspension at 14,000 rpm for 15 min, the supernatant containing the extracted genome as the DNA template was transferred to a new microtube and stored at -20°C for later PCR.

Phylogenetic Group Assignment

The quadruplex PCR method of Clermont et al. [19] was followed entirely for phylotyping. Based on this method, avian *E. coli* strains are classified into one of the eight phylogroups (A, B1, B2, C, D, E, F, or Escherichia cryptic clade I). All PCR amplifications were conducted on a final volume of 20 µl of the mixture, including distilled water, master mix (Ampliqon®, Denmark), DNA tem-

plate, and appropriate primers [19] based on the following procedure: pre-denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 5 sec, annealing at 59°C (quadruplex and phylogroup C) and 57°C (phylogroup E) for 20 sec, and a final extension at 72 °C for 5 min. The control strain was not included in this practice as arpA gene functions as an internal control, meaning that each *E. coli* strain is anticipated to produce at least one PCR product. Amplified products were analyzed through electrophoresis on 1% (w/v) agarose gel. Following staining with ethidium bromide, DNA bands were photographed under UV light, and those with the size of the target gene were considered to possess that gene. Finally, each isolate was classified into a specific phylogenetic group regarding the obtained amplicons.

Antimicrobial Susceptibility Testing

Those raw unpublished data on antimicrobial resistance of the isolates against antimicrobials commonly used in ostrich farms, including enrofloxacin, florfenicol, doxycycline, trimethoprim + sulfamethoxazole (sultrim®), gentamicin, streptomycin, and amoxicillin, were kindly provided by Amani et al. [1], who previously tested the isolates for the mentioned antimicrobials. In the current study, the isolates were further tested for three different antimicrobials, including lincomycin (2 µg), tetracycline (30 µg), and ceftriaxone (30 µg). The first two antimicrobials are also used in ostrich farms. Ceftriaxone represents a different class of antimicrobials, namely third-generation cephalosporins which are known as critically important antimicrobial in human medicine [6]. Resistance to CIA is a noticeable concern correlated to the poultry industry [15]. The procedure previously used by Amani et al. [1] was followed entirely for antimicrobial susceptibility testing. Briefly, E. coli isolates, adjusted to 1.5 × 108 CFU/ml with 0.5 McFarland standard turbidity, were cultured on plates containing Mueller-Hinton medium. These plates were then aerobically incubated at 37°C for 18 h. All the relevant antimicrobial disks were provided from PadtanTeb®, Iran. The antimicrobial sensitivity testing was based on the modified Kirby-Bauer disk diffusion method and the guidelines of the Clinical Laboratory Standard Institute [20]. Following measuring the diameter of the zone of inhibition (mm) caused by *E. coli* isolates against each antibiotic, resistance was interpreted as susceptible or non-susceptible. E. coli ATCC 25922 was the quality control strain. Subsequently, resistance against the antimicrobials and the total MDR, which is defined as resistance to at least three antimicrobial classes [3], excluding lincosamides herein, was obtained within the phylogenetic groups.

Detection of Antimicrobial Resistance Genes and Virulence Genes

The antimicrobial resistance genes, including tetracycline resistance gene (tet(A)), quinolone resistance gene (qnrA), sulfonamide resistance gene (sul1), streptomycin resistance gene (aadA1), aminoglycoside N(3)-acetyltransferase gene (aac(3)-IV), and beta-lactamase resistance gene (blaTEM) were detected within the isolates using PCR. These genes are associated with resistance to antimicrobials investigated in the present study and have previously been used in this respect [15, 21]. Furthermore, multiplex PCR was employed to detect the virulence genes, comprising enteroaggregative toxin gene (astA), increased serum survival protein gene (iss), iron repressible protein gene (irp2), P-fimbriae gene (papC), aerobactin gene (iucD), temperature-sensitive hemagglutinin gene (tsh), vacuolating autotransporter toxin gene (vat), and colicin V plasmid operon gene (cvaA/B) within the isolates. Employing these genes has proven to provide the capability of differentiation of APEC from non-APEC isolates [22]. Amani et al. [1], who previously performed both PCR detections for each isolate, kindly provided the raw data to be used for the evaluation of genes distribution within phylogenetic groups.

Statistical Analysis

The Fisher's Exact test was used to compare the proportion of each categorical variable with the cumulative proportion of other categorical variables (a specific phylogroup with different phylogroups pooled together; presumptive commensal with presumptive pathogenic *E. coli* isolates) using GraphPad 2 × 2 contingency table analysis (https://www.graphpad.com/quickcalcs/contingency1). The two-tailed p-value was reported whenever the test was performed. The difference between the proportions was considered significant at a two-tailed *p-value* < 0.05.

Authors' Contributions

A.G and M. JM conceived and planned the experiments. MS. ED carried out the experiments. MS.ED contributed to sample preparation. A. G and M. JM contributed to the interpretation of the results. E. T the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare no conflict of interest.

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Effects of trona (sodium sesquicarbonate) on physio-biochemical profiles and cardiovascular risk indices in Wistar rats

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ABSTRACT

Purpose of the study: This investigation aimed to assess the impact of trona on the cardiovascular risk markers and physio-biochemical profiles of rats administered graded dosages over 28 days. Five groups (n = 5) of rats—A, B, C, D, and E—were randomly assigned, with E acting as the control. For 28 days, rats in groups A, B, C, and D were administered trona at doses of 50, 100, 200, and 400 mg/kg, correspondingly. All over the investigation, distilled water was provided to the animals in the control group. We measured body weights, oxidative stress, hematology, hepatorenal profiles, somatic organs, fasting blood glucose (FBG) levels, and cardiovascular risk indices (CVRI). Findings: Rats administered 400 mg/kg had higher FBG levels ($p < 0.05$) than the control group. Group D showed decreases ($p < 0.05$) in erythrocytic indices, total protein level, and heart-body index with concurrent increases ($p < 0.05$) in total leucocyte counts (TLC) and creatinine levels in comparison to the control group. Rats in groups B, C, and D showed higher levels of catalase activity ($p < 0.05$) compared to the control group. Rats given 100 mg/kg or above of trona showed increases ($p < 0.05$) in CVRI compared to controls. Conclusions: There were dose-dependent harmful effects on the erythrocytic indices, high exposure to T2DM (type-2 diabetes mellitus), and increased CVRI levels in rats dosed orally with ≥100 mg/kg trona for 28 days.

Keywords

Cardiovascular indices, hepatorenal profiles, oxidative stress, rats, trona

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Abbreviations

AI: Atherogenic index
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
ANOVA: One-way analysis of variance
BUN: Blood Urea Nitrogen
CAT: Catalase
CHD: Coronary heart disease
CRI: Coronary risk index

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Introduction

In several parts of Nigeria, trona, an earthly mineral, is utilized as an intrinsic food enhancer. [1]. It is traditionally known by different names among various tribes in Nigeria. Kaun (Yoruba in Southwest Nigeria; Igbos in Eastern Nigeria); 'Kanwa' or 'Karu' (Hausa in Northern Nigeria); and 'Okanwa' and 'Ikoro' (Igalas and Egbira, respectively, in the Middle Belt of Nigeria), [2]. There is evidence of trona in the northern regions of Nigeria, especially in Kano and Maiduguri, as well as in neighboring countries like Niger and Chad [2]. Trona is erroneously called 'potash', although, in comparison to sodium, it has very small potassium [2,1]. Calcite makes up the majority of trona's chemical composition; the remaining constituents are hanksite, halite, pirssonite, and borax [3]. According to [4], sodium sesquicarbonate is the primary ingredient in trona. Conventionally, the reddish white (20% sodium carbonate) and whitish (80% sodium carbonate) kinds of trona have different make-ups when it comes to sodium salt. Trona (sesquicarbonate) is said to have sodium carbonate and sodium bicarbonate in an equal molar concentration [2]. As an intrinsically occurring food additive, trona is mostly utilized to soften tough foods including skins, bones, beans, and maize. In the states of Edo and Delta, it is also explored in making a delicacy known as "owo." [2, 1]. It is used broadly in ethno-veterinary practices for the treatment of skin diseases and digestive problems. Trona also serves as a salt lick and decoction for the treatment of reproductive ailments such as retained placenta [5]. The widespread use of trona (sodium sesquicarbonate dihydrate) in Africa, especially Nigeria, as an essential culinary additive and its gross applications in ethno-veterinary practices without minding its impacts on the biological systems of the end users could expose them to high toxicity during prolonged usage. The purpose of the study was to determine a safe dosage for trona to be administered sub-acutely orally for 28 days while also assessing the effects of the drug on the physio-biochemical profiles of rats.

Result

The findings of the metal content analysis of trona are displayed in Table 1. Heavy metals like iron, zinc, arsenic, cadmium, lead, and copper were also present, although sodium had the highest concentration.

Rats' acute toxicity to different trona dosages is displayed in Table 2. The behavioral reactions of the experimental rats did not exhibit any notable negative clinical effects. Rats administered trona orally at a dose of 5000 mg/kg for 24 hours did not exhibit any mortality. As a result, it is anticipated that trona's LD50 is greater than 5000 mg/kg.

The effect of different trona dosages on the fasting blood glucose levels of rats on days 14 and 28 is depicted in Figure 1. When comparing the blood glucose levels of rats exposed to different dosages of trona on day 14 to day 28, there were no observable changes ($p > 0.05$). On both days 14 and 28, the rats in group D had significantly higher fasting blood glucose levels ($p < 0.01$) (67.00 ± 5.24 and 73.25 ± 4.87) than the control group (49.00 ± 4.55 and 55.75 ± 3.90). The effect of weekly graded doses of trona on rats' body weight and the relative organ-body weights of the liver, spleen, heart, and kidneys is depicted in Figures 2 and 3. Rats' mean body weights before trona administration (day zero) were markedly lower ($p < 0.05$) than their mean body weights on day 28 post-trona administration in all groups, and their body weights showed increasing trends overall. Relative weights of the kidney and liver of rats treated with trona did not markedly differ ($p > 0.05$) from those of the rats' control group. Rats given 400 mg/kg (group D) of trona had a discernably lower relative heart weight ($p < 0.05$) than the control group.

Following oral treatment of trona for 28 days, the effects of different dosages on the hematological profiles of rats are displayed in Table 3 and Figure 4. Rat mean PCV values in groups A, B, and C were noticeably higher ($p < 0.05$) than those of the control. When comparing the PCV levels of the rats in group D to the control, there was no discernible difference ($p > 0.05$). Rats in the trona-treated groups showed no appreciable differences ($p > 0.05$) in their HbC when compared to the controls. When compared to the control, the RBC count of the rats in groups A, B, and C increased

Abbreviations-Cont'd

CVD: Cardiovascular disease
CVRI: Cardiovascular risk index
DLC: Differential leucocyte count
EDTA: Ethylenediaminetetraacetic acid
FBG: Fasting blood glucose
HbC: Haemoglobin Concentration
HDL-C: High-density lipoprotein cholesterol
LD50: Median lethal dose
LDL-C: Low-density cholesterol
LPH: Lipid hydroperoxide
LSD: Least significant different
MDA: Malondialdehyde
NOAEL: No observed adverse effect level
OECD: Organization of Economic Cooperation and Development
PCV: Packed cell volume
RBC: Red blood cell
SEM: Standard error of the mean
SPSS: Statistical Package for Social Science
T2DM: Type-2 diabetes mellitus
TAG: Triacylglycerols
TB: Total bilirubin
TC: Total cholesterol
TLC: Total leukocyte count

Table 1.
Metal contents analysis of tronaa

Metal	Concentration[b] (%)
Potassium	0.008
Sodium	0.150
Calcium	0.012
Lead (x 10^{-3})	0.233
Iron (x 10^{-3})	1.104
Copper (x 10^{-3})	0.189
Zinc (x 10^{-3})	1.430
Arsenic (x 10^{-3})	0.015
Cadmium (x 10^{-3})	0.003

a. Sodium sesquicarbonate ore
[b] Dry matter basis

Table 2.
The outcomes of a test on the acute toxicity of trona in rats

Treatment	No of rats	Mortality recorded	Observation
First phase			
50 mg/kg	3	Nil	No visible signs of toxicity
300 mg/kg	3	Nil	No visible signs of toxicity
2000 mg/kg	3	Nil	No visible signs of toxicity
Second phase			
5000 mg/kg	3	Nil	No visible signs of toxicity

Visible signs of toxicity such as changes in gait, drowsiness, hyperexcitability, diarrhea, vomiting, and nose bleeding were watched out for during the acute toxicity test.

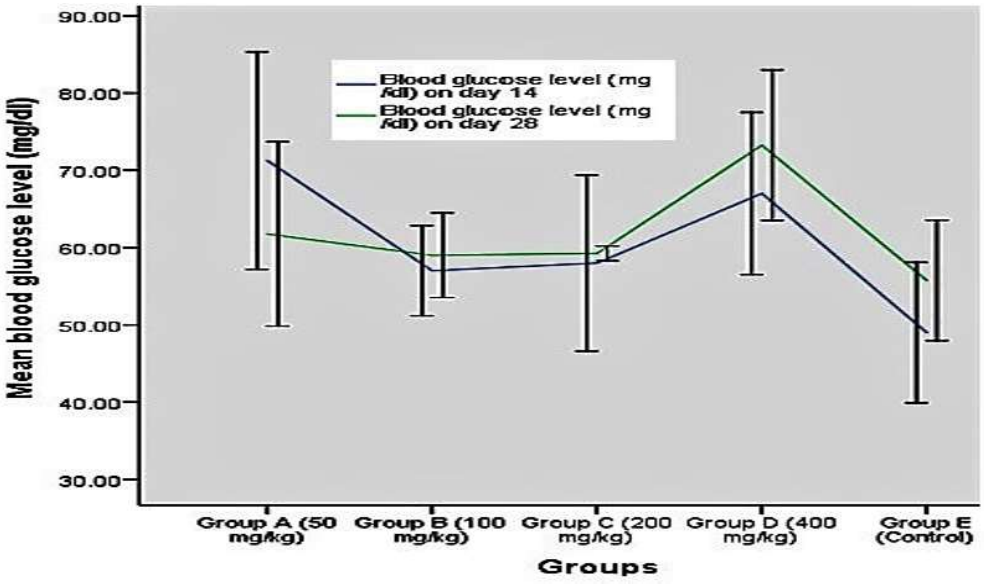

Figure 1.
Fasting blood glucose levels of rats exposed to varying doses of trona on days 14 and 28. Results are shown as mean ± SEM (n = 5)

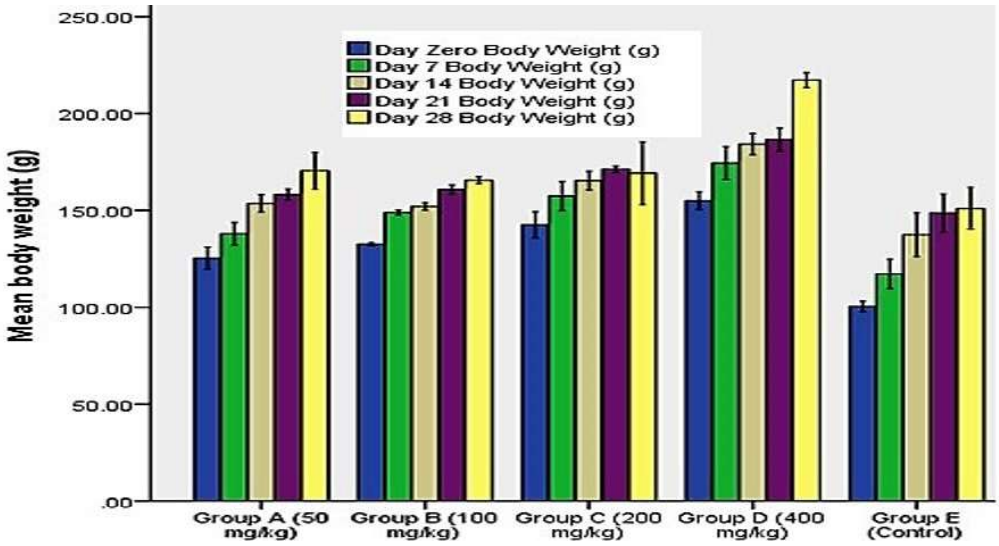

Figure 2.
Mean weekly body weight of rats exposed to varying doses of trona after 28 days oral administration. Results are shown as mean ± SEM (n = 5).

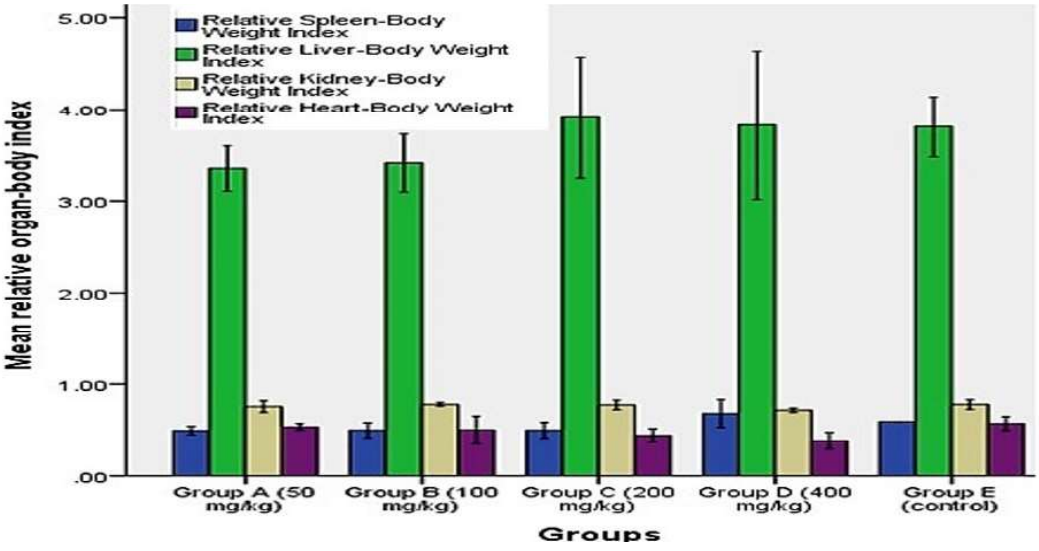

Figure 3.
Mean relative organ-body weight index of rat exposed to varying doses of trona after 28 days oral administration. Results are shown as mean ± SEM (n = 3).

Table 3.
Rats' hematological parameters following 28 days of oral trona treatment at different doses

Group	PCV (%)	HbC (g /dl)	RBC (x 106 / µl)	TLC (x 10^3 / µl)
A (50 mg/kg)	45.50 ± 2.96[a]	18.62 ± 1.34[a]	3.33 ± 0.18[a]	9.97 ±0.32[a]
B (100 mg/kg)	47.50 ± 3.97[a]	16.72 ± 3.40[a]	3.25 ± 0.33[a]	10.27 ± 0.84[a]
C (200 mg/kg)	46.25 ± 3.47[a]	14.69 ± 1.15[a]	2.82 ± 0.20[a]	8.07 ± 0.52[a]
D (400 mg/kg)	36.00 ± 3.11[b]	15.17 ± 1.55[a]	1.98 ± 0.50[b]	13.07 ± 1.10[c]
E (Control)	38.25 ± 1.15[b]	15.00 ± 1.25[a]	0.83 ± 0.2 8[c]	6.80 ± 0.81[b]

The means ± SEM of the four groups is displayed. A one-way ANOVA was used, and post hoc LSD was applied. LSD stands for least significant difference. A different superscript letter (s) in the same column indicates a significant difference of $p < 0.05$ when comparing all the groups. PCV = Packed cell volume; HbC = Hemoglobin concentration; RBC = Red blood cell; TLC = Total leucocyte count.

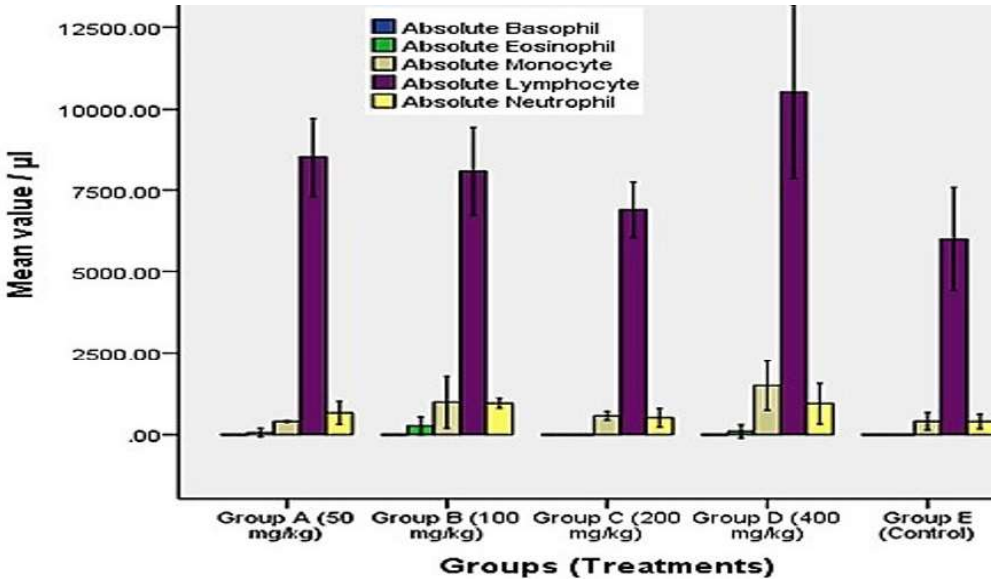

Figure 4.
Mean absolute differential leukocyte counts per of rat exposed to varying doses of trona after 28 days oral administration. Results are shown as mean ± SEM (n = 5).

significantly ($p = 0.000$). Rats in group D had a substantially lower ($p < 0.05$) RBC count compared to rats in groups A, B, and C, but a markedly higher ($p = 0.001$) RBC count when compared to the control. Rats in group D had a considerably higher TLC ($p = 0.000$) than rats in the control group.

Table 4.
Rats given different dosages of trona orally for 28 days and their hepatorenal profiles

Groups	ALT (IU/L)	ALP (IU/L)	TP (g /dl)	TBIL(mg/dl)	BUN (mg/dl)	Creatinine (mg/dl)
A (50 mg/kg)	8.00 ± 0.41[a]	29.25 ± 0.25[a]	4.65 ± 0.16a	0.56 ± 0.16[a]	60.50 ± 5.56[a]	2.18 ± 0.19[ab]
B (100 mg/kg)	8.75 ± 0.48[a]	28.50 ± 0.87[a]	5.13 ± 0.10[b]	0.73 ± 0.10[a]	52.52 ± 4.56[a]	1.88 ± 0.16[a]
C (200 mg/kg)	8.50 ± 0.29[a]	28.50 ± 0.50[a]	5.25 ± 0.20[bc]	0.71 ± 0.12[a]	59.75 ± 4.64[a]	2.13 ± 0.17[ab]
D (400 mg/kg)	8.50 ± 0.29[a]	28.75 ± 0.48[a]	4.58 ± 0.27[a]	1.14 ± 0.23[b]	68.50 ± 5.70[a]	2.45 ± 0.19[bc]
E (Control)	8.50 ± 0.29[a]	29.85 ± 0.48[a]	5.75 ± 0.21[c]	1.06 ± 0.17[b]	59.75 ± 6.14[a]	2.13 ± 0.22[ab]

The means ± SEM of the four groups is displayed. A one-way ANOVA was used, and post hoc LSD was applied. LSD stands for least significant difference. A different superscript letter (s) in the same column indicates a significant difference of $p < 0.05$ when comparing all the groups. ALT= Alanine aminotransferase; ALP= Alkaline phosphatase; TP= Total protein; TBIL= Total bilirubin; BUN = Blood urea nitrogen.

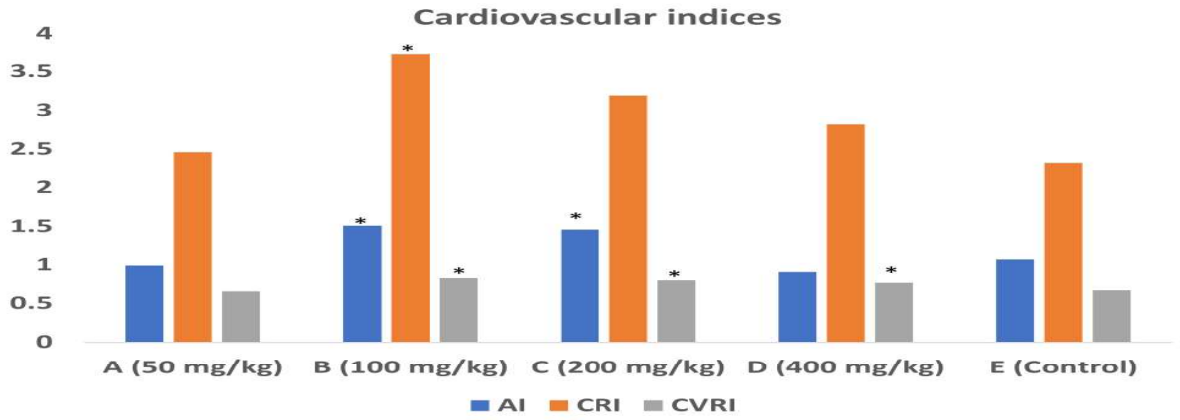

Figure 5.
Effects of varying doses of trona on cardiovascular risk indices of rats after 28 days sub-acute administration. Results are shown as mean ± SEM (n = 4). *AI significant when compared with A, D, and control ($p < 0.05$). *CRI significant when compared with A and control ($p < 0.05$). *CVRI significant when compared with A and control ($p < 0.05$). AI = Atherogenic index; CRI = Coronary risk index; CVRI = Cardiovascular risk index.

When compared to rats in groups C and control, the mean absolute lymphocyte count of rats in group D increased significantly ($p < 0.05$). Rats in group D had significantly higher mean absolute monocyte counts (p =0.000) than rats in groups A, C, and control. When comparing the mean eosinophil and neutrophil counts of rats in the trona-treated groups to the controls, there were no observable differences (p> 0.05).

Following 28 days of oral dosing, Table 4 displays the effect of different dosages of trona on biomarkers of hepatorenal function in rats. Comparing the ALT and ALP activities of the trona-treated groups to those of the control group revealed no marked changes ($p > 0.05$). When compared to the control, the rats in group A (50 mg/kg trona) had a significant ($p < 0.05$) drop in their total bilirubin level. Rats treated with trona showed no appreciable differences ($p > 0.05$) in their blood urea nitrogen (BUN) and creatinine levels when compared to controls. When compared to the control, the total protein of the rats in group D was substantially ($p < 0.05$) lower.

Table 5 displays the results of antioxidant enzyme activity and serum lipid peroxidation in rats given different dosages of trona orally for 28 days.

While the CAT activity of rats in groups B, C, and D increased significantly ($p < 0.05$) when compared with control and group A, there were no discernible changes ($p > 0.05$) in the activity of SOD in trona-treated groups compared with control. When compared to control and group D, the serum lipid peroxidation levels of rats in groups A, B, and C showed a marked decrease ($p < 0.05$).

Following 28 days of oral dosing, Table 6 displays the effects of different dosages of trona on the serum lipid profiles of rats. No significant changes ($p > 0.05$) were observed in triacylglycerols (TAG) or high-density lipoprotein cholesterol (HDL-C) when the trona-treated rat group was compared to the control group. Rats in groups B, C, and D had substantially higher total cholesterol (TC) levels ($p < 0.05$) than the controls. Rats in groups B and C showed a marked (p

Table 5.
Serum lipid peroxidation and antioxidant enzyme activity of rats subjected to different doses of trona orally for 28 days

Group	MDA (mg/ml)	SOD (U/ml)	CAT (U/ml)
A (50 mg/kg)	2.73 ± 0.41[a]	10.60 ± 0.28[a]	3.92 ± 0.35[a]
B (100 mg/kg)	2.84 ± 0.34[a]	10.09 ± 0.51[a]	5.94 ± 0.50[b]
C (200 mg/kg)	3.64 ± 0.49[a]	10.83 ± 0.11[a]	5.71 ± 0.78[b]
D (400 mg/kg)	4.09 ± 0.49[b]	10.70 ± 0.22[a]	5.17 ± 0.19[b]
E (Control)	4.96 ± 0.23[b]	10.64 ± 0.28[a]	4.48 ± 0.33[a]

The means ± SEM of the four groups is displayed. A one-way ANOVA was used, and post hoc LSD was applied. LSD stands for least significant difference. A different superscript letter (s) in the same column indicates a significant difference of $p < 0.05$ when comparing all the groups. MDA= Malodialdehyde; SOD= Superoxide dismutase; CAT= Catalase.

< 0.05) rise in their levels of low-density lipoprotein cholesterol (LDL-C) when compared to the control group. Following oral administration of trona for 28 days, Figure 5 illustrates the effect of different dosages on the atherogenic, coronary risk, and cardiovascular risk indices in rats. In comparison to the controls, the atherogenic index level was substantially ($p < 0.05$) elevated in rats in groups B (100 mg/kg) and C (200 mg/kg). Rats in group B had a higher coronary risk index level ($p < 0.05$) than the control group. On the other hand, when compared to controls, rats administered trona at doses more than 100 mg/kg showed a substantial ($p < 0.05$) elevation in their cardiovascular risk index (CVRI).

Discussion

Only following acute, subacute, and chronic toxicity testing is a food additive safe for human consumption [1]. Therefore, the purpose of this study was to assess how trona affected the physio-biochemical profiles of rats when it was administered orally over a short period.

High amounts of sodium and potassium are found in the metal analysis of trona (Table 1), but calcium and iron values are comparatively low. There was also zinc, cadmium, lead, copper, and arsenic. It has been determined that toxic metals pose a serious risk to human health, mostly due to their capacity to harm DNA and membranes as well as to interfere with the activity of enzymes and proteins [6]. The current study's findings demonstrated that, although there is a possibility of bioaccumulation, the levels of metal pollution, as measured against the FAO/WHO [7] standard, were below the upper limit that was permitted.

The acute toxicity investigation (Table 2) revealed no deaths and observable harm. Since there was no mortality at 5000 mg/kg, suggesting that it is reasonably safe for short-term exposure, the LD50 was not determined.

When comparing the FBG levels of rats in group D (400 mg/kg) on days 14 and 28 to those of the control group, the results (Figure 1) show a substantial rise ($p < 0.01$). T2DM is a possibility for the rats given a 400 mg/kg dosage, based on their hyperglycemia. Following a carbohydrate-rich meal, T2DM is known for a persistent rise in blood glucose [8]. Figure 2 illustrates that the body weights of the rats in each group significantly increased when compared to their baseline weights. This suggests that trona has no negative effect on the rats' body weight. This is consistent with findings from [9], who reported that trona had no negative effects on body weight or testicular shape. As seen in Figure 3, the heart-body weight index of rats in group D (400 mg/kg) decreased significantly ($p < 0.05$) in comparison to the control group. This may be a sign of inflammation in the heart caused by elevated salt levels from a high trona dosage. High sodium concentrations are associated with increased production of reactive oxygen species in cardiac muscles, which is a significant factor in the development of cardiovascular disease (CVD) [10].

The results of this investigation clearly showed that trona damages erythrocytic parameters in a dose-dependent manner (Table 3). This could be because of a high rate of erythrocyte destruction that lowers PCV and RBC levels because of a rise in lead bioaccumulation in bone marrow with increasing doses. Lead disrupts the proper maturation of erythroid components in the bone marrow and is primarily found in bones in both humans and animals [11]. In the present investigation, we found higher TLC in rats dosed with 400mg/kg of trona which was linked to both lymphocytosis and monocytosis in comparison to the control and other groups as shown in Table 3. The monocytosis observed in our study agrees with the previous reports that monocyte recruitment is crucial in the host response to metabolic, atherogenic, and neoplastic stimuli, attributing to wound repair and fibrosis [12]. The concurrent lymphocytosis and monocytosis observed could be attributed to potentiation of B and T lymphocytes by interleukin secreted by monocytes as was demonstrated in previous reports [13].

Analysis of serum liver function enzymes in our present study (Table 4) showed that the dosage of trona could not have any significant effect on the activities of ALT and ALP suggestive of no hepatic tissue injury. However, the marked reduction in the total bil-

irubin level of rats dosed with trona below 400 mg/kg suggests an enhanced glucuronidation process. This is in line with previous studies that increased total bilirubin level above normal (0.3 to 0.8 mg/dl) could be a pointer to liver damage mostly in hepatocytes that affect the glucuronidation process and the presence of erythrocyte hemolysis [14]. Our study's findings (Table 4) demonstrated that, in comparison to controls, rats given 400 mg/kg trona had a significantly lower level of total protein and an increased amount of creatinine. This implies that rats given 400 mg/kg of trona may be susceptible to decreased renal function after extended treatment. This supports a previous study that found a higher blood creatinine level to be a diagnostic marker for a decline in glomerular filtration rate [15].

One important enzyme called catalase uses hydrogen peroxide, a nonradical ROS, as a substrate. It oversees neutralization through the breakdown of hydrogen peroxide, preserving the molecule at the optimal amount in the cell, which is essential for signaling events within the cell [16]. The current study (Table 5) showed elevated catalase activity in dose dose-dependent manner with concurrent elevation of MDA level. This suggests that an increase in the dosage of trona could lead to the suppression of the antioxidant systems with the consequential effect of an increase in lipid peroxidation. This finding is consistent with previous reports that antioxidant enzyme activities are overwhelmed when the levels of MDA and LPH become enhanced [2].

Due to its correlation with the quantity of cholesterol contained in lipoprotein, total serum cholesterol plays a crucial role in the development of cardiovascular disease (CVD) [17]. There is a dearth of knowledge regarding trona's effects on rats' lipid profiles, hence this study is necessitated. This current study (Table 6) showed elevated total cholesterol in trona-treated rats with associated increased low-density lipoprotein cholesterol compared with control. There were no significant effects on triglycerides and high-density lipoprotein cholesterol in trona-treated rats compared with control. This implies that trona administration promotes LDL cholesterol oxidation by increasing total serum cholesterol, a hallmark in the development of atherosclerotic plaque.

The current investigation assessed the impact of trona administration on the experimental rats' lipid ratios (TC/HDL cholesterol, TG/HDL cholesterol, and LDL/HDL cholesterol). An effort is currently being made to maximize the predictive power of lipid profiles against the risk of atherogenicity, coronary heart disease (CHD), and cardiovascular disease (CVD) by evaluating lipid ratios, or cardiovascular risk indices [18]. Rats given 100 mg/kg trona showed significantly higher levels of both the coronary risk index (CRI) and the atherogenic index (AI) when compared to the control group. This suggests that trona (100 mg/kg) treatment in rats may increase the risk of coronary heart disease and atherosclerosis. The LDL/HDL cholesterol ratio takes AI into account [18]. Compared to separately used total cholesterol, LDL cholesterol, and HDL cholesterol of coronary heart disease, CRI is a more powerful coronary risk predictor [19]. In a similar vein, rats administered trona at doses greater than 100 mg/kg showed a significant rise in their cardiovascular risk index (CVRI) values relative to the control group, hence raising the risk of cardiovascular illness. This implies that trona administration at doses greater than 100 mg/kg is associated with increasing levels of sd LDL cholesterol, a tiny and dense subclass of LDL cholesterol linked to cardiovascular risk. A significant risk of cardiovascular disease is associated with elevated levels of sd LDL, a small and dense subclass of LDL, which is indicated by an elevated TG/HDL cholesterol ratio [20].

It is also hypothesized that an increase in CVRI level is associated with hyperglycemia [21] which is in tandem with our finding as shown in Figure 1. The present study reveals that T2DM observed in rats dosed 400 mg/kg trona serves as a predisposing factor to cardiovascular disease which agrees with previous reports that T2DM correlates positively with cardiovascular disease risk factors [22]. In summary, the present study has revealed that animals given high doses of trona 400 mg/kg might be at risk of T2DM, and a dose-dependent deleterious effect on the erythrocytic indices. Our study also reveals that trona at 100mg/kg and above when given to animals could raise their chances of developing myocardial infarction. Thus, for this present study, the no observed adverse effect level (NOAEL) dose for oral administration of trona in rats is estimated to be 50 mg/kg per day in 28-day sub-acute toxicity studies. Hence, caution has to be taken when consuming trona continuously and indiscriminately due to its potential toxicity risk that is associated with cardiovascular disease.

Accordingly, further investigations are recommended for a better understanding of the dose-response correlation of trona, the bioavailability of heavy metals content of trona in the body, and the toxicological effect of trona on other organs such as the brain and lungs over a long period based on the NOAEL dose (50 mg/kg).

Materials and Methods

Ethical approval

The EU Directive 2010/63/EU and the rules of the University of Nigeria, Nsukka's Animal Ethics Committee (UNEC/21/190304) were followed in the conduct of this study. The Faculty of Veterinary Medicine, University of Nigeria, Nsukka's Institutional Animal Care and Use Committee finally approved this study (FVM-UNN-IACUC-202306108).

Materials

The trona was bought at Ogige market in Nsukka LGA, Enugu State, Nigeria. The trona was properly recognized by a geologist at the Department of Geology, University of Nigeria, Nsukka. It was then pulverized into powder form and properly stored in an airtight container for the study.

Metal content analyses

Calcium, magnesium, sodium, potassium, zinc, iron, lead, cadmium, arsenic, and copper were analyzed using standard procedures (23, 24, 25).

Chemicals

The assay kits for ALP, ALT, AST, ALP, total bilirubin, total protein, urea, and creatinine were provided by Randox Laboratories Ltd. (Antrim, United Kingdom). Additional kits for total cholesterol, triacylglycerols, high-density lipoprotein choles-terol (HDL-Chol), and low-density lipoprotein cholesterol (LDL-Chol) were also acquired from Randox Laboratories (Antrim, United Kingdom) as well as catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) were also determined All chemicals utilized were of analytical grade.

Animals

The Department of Zoology's Animal House provided male Wistar strain albino rats that were 5–6 weeks old. They were kept at the University of Nigeria, Nsukka's Laboratory Animal Unit of Veterinary Physiology and Pharmacology. During the two weeks of acclimatization, the animals were given commercially prepared rat food and unlimited water. Because female rats' cycles are characterized by hormonal fluctuations, this study used male rats instead of females to avoid inconsistent responses to the same stimuli. Adopted were the guidelines for the management of laboratory animals [26]. The Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, examined and ultimately accepted this study protocol with scientific reason (FVM-UNN-IACUC-202306108).

Acute toxicity study

Organization for Economic Cooperation and Development (OECD) guideline 423 [27] was used to determine the oral acute toxicity and median lethal dosage (LD50) of trona. The investigation involved the use of twelve male Wistar rats, three of which were employed for testing at 50, 300, 2000, and the 5000 mg/kg limit, respectively. Clinical and behavioral signs of toxicity such as changes in gait, drowsiness, hyperexcitability, diarrhea, vomiting, and nose bleeding were checked out for during the acute toxicity test. With the LD50 shown to be greater than 5000 mg/kg, the oral dosages of 50 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg were selected for the trona-treated groups.

Sub-acute toxicity studies

For the investigation, rats weighing between 110 and 135 g were employed. Five groups (n = 5) consisting of A, B, C, D, and E, were randomly assigned to them, with E acting as the control.

For 28 days, the rats in groups A, B, C, and D received daily doses of trona of 50 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively. Rats in group E were given distilled water throughout the study. Body weights of the rats in the treatment groups and control group are taken at day one (zero) and 7-day intervals for 28 days using a Metler weighing balance.

Blood sample collection and animal sacrifice

The animals were allowed to fast the night before the experiment concluded, and in the morning, blood samples were taken from the retrobulbar plexus of the median canthus of the eye and placed into sterile containers either with or without ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The serum utilized for the examination of lipid and hepatorenal function indicators was separated from the blood in the sterile bottles by centrifuging the mixture at 3000 g for 15 minutes. Blood was utilized for hematological analyses in EDTA sample vials. Following their euthanasia, three (3) rats from each group were slain by intraperitoneal injection of five milligrams of xylazine (Kepro Holland) and ninety mg of ketamine hydrochloride (Laborate Pharmaceutical, India) [28]. Following the animals' dissection, relevant organs including the liver, spleen, heart, and kidney were removed and weighed for organosomatic research using a Metler weighing scale.

Fasting blood glucose level

On days 14 and 28 of the trial, after an overnight fast, blood samples were taken through the tail. The digital blood glucose monitor with microprocessor was filled with blood after drops of blood were placed onto the dextrostix reagent pad. Data were then collected. On days 14 and 28 of the trial, all groups' FBG levels were measured.

Hematological analyses

The hemocytometer method was used to determine the TLC and RBC whereas the microhematocrit method was used to calculate PCV [29]. The stained blood film was used to perform differential leucocyte counts (DLC) [29], and Drabkin's reagent assay method was used to measure hemoglobin (Hb) content [30].

Serum biochemical analyses

Following the manufacturer's instructions, commercial kits from Randox® were used to analyze serum stored at 4 °C for liver and kidney function biomarkers such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), creatinine, blood urea nitrogen (BUN), and total proteins (TP). Utilizing Randox® kits, lipid profiles including total cholesterol (TC), triacylglycerols (TAG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were also assessed. Superoxide dismutase (SOD) and catalase (CAT) activities were measured using conventional techniques [31]. Using the spectrophotometric approach, the lipid peroxidation biomarker malondialdehyde (MDA) was identified following Ohkwa [32].

Organosomatic indices

Vital organs of interest, such as the spleen, heart, liver, and kidneys, were harvested, dissected, and weighed.

Organosomatic index = Weight of organ ÷ weight of animal X 100%

Cardiovascular risk indices

Using the following formulas, cardiovascular risk indices, including atherogenic index (AI), coronary risk index (CRI), and cardiovascular risk index (CVRI), were calculated: [33, 34].

AI (atherosclerotic index) = LDL/HDL cholesterol

TC divided by HDL cholesterol is the coronary risk index (CRI).

TAG divided by HDL cholesterol is the cardiovascular risk indicator (CVRI).

Data analysis

With the use of SPSS version 23, the acquired data were examined using One-Way Analysis of Variance (ANOVA). To distinguish between the variant means, a least significant difference (LSD) post hoc test was employed. It was determined that the probability level ($p < 0.05$) was significant. The means of the SEM are displayed in tabular and graphical style for the results.

Data availability statement

The raw data were produced by the Department of Veterinary Physiology and Pharmacology at the University of Nigeria, Nsukka's Faculty of Veterinary Medicine. On request, the corresponding author (H. E. U.) will provide derived data supporting the study's findings.

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Authors' Contributions

H.E., and O.B. were involved in research conceptualization; supervision; project administration; validation; visualization; drafting, reviewing, and editing the original manuscript while H.E., and M.Z. were involved in data curation; formal analysis; investigation; methodology; resources, and software. All authors read and approved the final manuscript.

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Competing Interests

The authors declare no conflict of interest.

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A Green Way to Combat *Echinococcus granulosus*: Exploring the Scolicidal Effects of *Lycopus europaeus* and *Lythrum salicaria* Extracts

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ABSTRACT

This study assessed the scolicidal activity of Lythrum *salicaria* and *Lycopus europaeus* methanolic extracts on the protoscoleces of *Echinococcus granulosus* using ultrasound-assisted extraction. Protoscoleces were obtained from sheep livers and lungs and were exposed to extracts at concentrations of 125, 250, and 500 mg/mL for 1, 10, 20, and 30 minutes. Over the exposure period, both plant extracts demonstrated progressively stronger scolicidal activity at all tested doses. At the lower concentrations of 125 and 250 mg/mL, *L. salicaria* generally exhibited a higher protoscolicidal effect compared to *L. europaeus.* However, the difference in efficacy was more pronounced at 125 mg/mL. On the other hand, at the 500 mg/mL concentration, the *L. europaeus* extract showed considerably greater scolicidal activity than the *L. salicaria* extract. Statistical analysis revealed that concentration had the biggest impact on mortality, followed by plant species and exposure time. The interaction between concentration and plant type impacted mortality the most, indicating that both factors influenced the overall effectiveness. In conclusion, both *L. salicaria* and *L. europaeus* methanolic extracts showed promise as potential candidates for future studies aimed at developing natural agents to control *E. granulosus.*

Keywords

Echinococcus granulosus, Hydatidosis, Methanolic extract, Medicinal plants, Protoscolex

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Abbreviations

E. granulosus: Echinococcus granulosus
L. salicaria: Lythrum salicaria
L. europaeus: Lycopus europaeus
PAIR: Puncture, Aspiration, Injection, Reaspiration

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Introduction

Hydatidosis, or echinococcosis, is a zoonotic parasitic infection caused by the metacestode stage of the tapeworm *E. granulosus.* In the life cycle of the parasite, adult cestodes reside in the intestines of carnivores from the Canidae family, releasing eggs through the feces of these definitive hosts. Intermediate hosts, including humans and herbivorous mammals, may inadvertently ingest these eggs through contact with contaminated food, water, or soil, leading to the development of the larval stage (metacestode) within their tissues. The larval stage results in hydatid cyst formation, which can have severe health consequences and may be fatal if left untreated [1-5].

Despite the range of treatment options available, surgery remains the preferred approach for managing human hydatidosis, especially in cases involving large, infected cysts or those located in critical organs. Surgical intervention provides a direct method for cyst removal, reducing the risk of complications and recurrence associated with untreated cysts. However, alternative therapeutic methods, such as PAIR and chemotherapy, are recommended for patients with multiple cysts affecting several organs or in cases where cysts are deemed inoperable. The PAIR technique, a minimally invasive approach, is effective in reducing cyst size and rupture risk, while chemotherapy with agents, such as albendazole and mebendazole, targets parasite viability, complementing other interventions for non-surgical cases or to prevent recurrence [6]. The use of effective scolicidal agents during surgical intervention is crucial to mitigate the risk of secondary infection caused by the accidental release of protoscoleces from hydatid cysts. An ideal scolicidal compound should possess high parasiticidal efficacy while minimizing potential adverse effects on the host tissue. A variety of compounds have been explored for their scolicidal properties, including formalin, 10% polyvinylpyrrolidone-iodine, 3% hydrogen peroxide, mannitol, 20% silver nitrate, 95% ethyl alcohol, hypertonic glucose, a combination of 1.5% cetrimide with 0.15% chlorhexidine, and 20% saline solution. Each of these agents offers varying degrees of effectiveness and safety, providing options tailored to specific surgical contexts in hydatidosis treatment [7-14]. While many scolicidal agents have demonstrated effectiveness, several compounds, including ethanol (70%–90%), cetrimide (0.5%), silver nitrate, and hypertonic saline (15%–20%), have been discontinued in clinical practice due to toxicity concerns and undesirable side effects [7-9, 12, 14].

The limitations and safety issues associated with these conventional agents highlight the urgent need for alternative scolicidal solutions. Herbal extracts have emerged as promising candidates, offering potential advantages, such as cost-effectiveness, accessibility, and a lower incidence of adverse effects. Therefore, expanding research into plant-based scolicidal agents represents a crucial step toward developing safer and more effective treatments, ultimately reducing the disease burden on both human and animal health.

L. salicaria, commonly referred to as purple loosestrife, is a perennial herbaceous plant of the Lythraceae family, native to regions across Europe, North Africa, and Asia. In Iran, multiple Lythrum species grow abundantly, particularly in wetland areas adjacent to streams. L. salicaria has a long history of use in traditional medicine, primarily for managing gastrointestinal disorders, such as diarrhea and dysentery. Its extract is also utilized topically to treat inflammatory conditions, such as eye inflammation, sinusitis, varicose veins, hemorrhoids, menorrhagia, hemorrhages, leucorrhoea, and ulcers. Beyond these applications, L. salicaria has been recognized for its effectiveness in urogenital inflammation, rheumatism, rabies, fever, benign prostatic hyperplasia, pruritus, dermatitis, and eczema. The phytochemical profile of *L. salicaria* reveals a rich array of bioactive compounds, predominantly polyphenolic constituents, such as tannins, flavonoids, anthocyanins, catechins, phenolic acids, and coumarins. In addition, various secondary metabolites have been identified, including steroids, triterpenes, phthalates, and alkaloids. This diverse phytochemical composition supports the plant's wide-ranging therapeutic applications, highlighting its potential as a valuable source of natural compounds for medicinal use [15-17].

L. europaeus, commonly known as bugleweed, gypsywort, bitter bugle, or water horehound, is an herbaceous perennial plant in the Lamiaceae family, native to Europe and Western Asia, including Iran. Traditionally, *L. europaeus* has been employed in herbal medicine for mild hypothyroidism and alleviating minor nervous disorders. Recent studies have further substantiated its medicinal potential, highlighting analgesic, antitussive, and anti-inflammatory properties, alongside its anti-thyrotropic and anti-gonadotropic effects. Additional pharmacological benefits include cardiotonic, antioxidant, and antimicrobial activities, which underscore its therapeutic versatility. Although the health benefits

of *L. europaeus* are attributed to its complex phytochemical profile, not all bioactive constituents have been fully elucidated. Phytochemical investigations have identified a range of active compounds, including terpenoids, various phenolic compounds (such as phenolic acids, flavonoids, coumarins, and tannins), as well as alkaloids, glycosides, saponins, and sterols. These constituents collectively contribute to the plant's pharmacological effects, supporting its traditional and contemporary medicinal applications [18-25].

To date, the protoscolicidal potential of extracts from *L. salicaria* and *L. europaeus* has not been investigated. This study aimed to evaluate whether extracts from these plants exhibit protoscolicidal effects against *E. granulosus* protoscoleces in an in vitro setting, with the objective of identifying novel natural protoscolicidal agents. If effective protoscolicidal activity be demonstrated, subsequent studies would focus on isolating and characterizing active constituents, elucidating the mechanisms of action and exploring structure-activity relationships to inform further development. Promising in vitro findings would also support the need for in vivo efficacy and safety assessments, essential for future consideration in clinical applications.

Result

The protoscolicidal activity of *L. salicaria* and *L. europaeus* extracts was evaluated against *E. granulosus* protoscoleces at the concentrations of 125, 250, and 500 mg/mL, with exposure times ranging from 1 to 30 minutes (Table 1). Statistical analysis demonstrated significant scolicidal activity for both plant extracts ($p < 0.01$), with mortality rates exhibiting dose- and time-dependent characteristics.

At 125 mg/mL, both extracts exhibited protoscolicidal activity, with *L. salicaria* consistently outperforming *L. europaeus* at all the time points. After 1 min, *L. salicaria* demonstrated 83.87% activity compared to 55.82% for *L. europaeus*. At 5 min, the activity of *L. salicaria* increased slightly to 84.49%, while *L. europaeus* improved to 59.84%. By 10 min, the activity of *L. salicaria* rose to 85.5%, significantly higher than the 63.47% observed for *L. europaeus.* This trend persisted at 20 min, with *L. salicaria* reaching 88.73% activity versus 64.43% for *L. europaeus.* After 30 min, *L. salicaria* retained superior activity at 89.68%, compared to 69.14% for *L. europaeus.*

Both *L. salicaria* and *L. europaeus* displayed protoscolicidal activity at the concentration of 250 mg/mL, but *L. salicaria* was more effective in killing protoscolices compared to *L. europaeus*. Following a 1-min exposure, *L. salicaria* exhibited an 85.87% protoscolicidal activity, while *L. europaeus* showed a lower efficacy of 69.1%. The difference in efficacy between the two plants became less prominent with increasing the exposure time. After 5 min of exposure, *L. salicaria had* a protoscolicidal activity of 87.24%, whereas *L. europaeus* had a lower efficacy of 83.27%. After 10 min of exposure, *L. salicaria* showed a protoscolicidal activity of 90.44%, which was significantly higher than *L. europaeus* with 87.41% protoscolicidal activity. Similarly, after 20 min of exposure, *L. salicaria* had a protoscolicidal activity of 93.68%, while *L. europaeus* showed 88.95% protoscolicidal activity. After a 30-min exposure period, *L. europaeus* demonstrated a protoscolicidal activity of 95.98%, while *L. salicaria* showed 95.16% activity. Despite the absence of statistical significance in the protoscolicidal activity difference between *L. salicaria* and *L. europaeus* at this concentration and exposure time, the result contradicts previous findings at lower concentrations and exposure times where *L. salicaria* exhibited greater effectiveness.

Table 1.
Protoscolicidal effect of the methanolic extract of *Lythrum salicaria* and *Lycopus europaeus* at the different concentrations following various exposure times

		Protoscolicidal activity (%)				
Plant	Concentration (mg/ml)	1 min	5 min	10 min	20 min	30 min
L. salicaria	125	83.87jk	84.49j	85.5i	88.73g	89.68fg
	250	85.87i	87.24h	90.44f	93.68e	95.16d
	500	90.35f	93.5e	96.86bc	100a	100a
L. europaeus	125	55.82o	59.84n	63.47m	64.43m	69.14l
	250	69.1l	83.27k	87.41h	88.95g	95.98cd
	500	95.35d	97.45b	100a	100a	100a

Values with the same letter have no significant difference.

At 250 mg/mL, the efficacy of both extracts improved, though *L. salicaria* continued to show higher protoscolicidal activity at shorter exposure times. After 1 min, *L. salicaria* demonstrated 85.87% activity, while *L. europaeus* exhibited 69.1%. The efficacy gap narrowed with longer exposure, with the activities of 87.24% for *L. salicaria* and 83.27% for *L. europaeus* at 5 min. By 10 min, *L. salicaria* reached 90.44% activity, slightly exceeding the 87.41% observed for *L. europaeus*. After 20 min, the activities increased to 93.68% for *L. salicaria* and 88.95% for *L. europaeus*. At 30 min, *L. europaeus* surpassed *L. salicaria*, with the activities of 95.98% and 95.16%, respectively. This reversal suggests that relative efficacies may shift at higher concentrations and longer exposure times.

At 500 mg/mL, *L. europaeus* displayed greater protoscolicidal activity, reversing the trend seen at lower concentrations. After 1 min, *L. europaeus* achieved 95.35% activity compared to 90.35% for *L. salicaria*. By 5 min, *L. europaeus* improved to 97.45%, while *L. salicaria* reached 93.5%. At 10 min, *L. europaeus* achieved 100% activity, whereas *L. salicaria* lagged at 96.86%, only reaching 100% activity after 20 min of exposure.

Table 2 highlights that extract concentration, plant type, and exposure time significantly influenced protoscoleces mortality, both individually and through interactions. Among these, concentration had the largest mean square value, making it the most critical factor, followed by plant type and exposure time. The two-way interaction between concentration and plant type produced the highest mean square value among the interactions, indicating that the efficacy of the extracts was strongly influenced by their concentration and botanical source. Interactions between concentration and time, and plant type and time, were also significant, albeit with smaller mean square values. The three-way interaction had the smallest mean square value but was still highly significant, reflecting a combined effect of all three factors.

Table 2.
Analysis of variance for the factors influencing protoscoleces mortality of Echinococcus granulosus, including the concentration of the extract, plant type, and exposure time individually and through interactions

Effect	DF	MS	Sign. F
Plant	1	1826.82256	**
Concentration	2	3949.18783	**
Time	4	366.226815	**
Plant × Concentration	2	1367.98903	**
Plant × Time	4	31.587735	**
Concentration × Time	8	27.90843	**
Plant × Concentration × Time	8	27.320355	**
Residual	60	0.208333333	
Total	89	162.9973569	

**: $P < 0.01$

Discussion

Over the past few decades, there has been significant interest in researching natural scolicidal compounds with favorable safety profiles and without adverse effects. This interest stems from the necessity for effective and safer alternatives to conventional scolicidal agents in treating parasitic infections. For instance, Moazeni and Nazer (2010) evaluated the protoscolicidal efficacy of methanolic extract derived from *Allium sativum*. Their results showed that at the concentrations of 25 and 50 mg/mL, the extract completely killed protoscoleces after 60 and 10 min of application, respectively. Similarly, Moazeni *et al.* (2012) investigated the scolicidal effects of *Rhus coriaria* and *Zataria multiflora* methanolic extracts at varying concentrations and exposure times. Another study by Zibaei *et al.* (2012) reported that the hydroalcoholic extract of *Satureja khuzestanica* leaves demonstrated greater scolicidal activity than the aqueous extract of *Olea europaea* leaves. Furthermore, Taran *et al.* (2013) examined the scolicidal activity of *Hymenocarter longiflorus* methanolic extract against the metacestode of *E. granulosus* and found it to be a potent scolicidal agent. Baqer *et al.* (2014) investigated the scolicidal effect of *Zingiber officinale* ethanolic extract and observed that the concentrations of 50, 100, and 150 mg/mL resulted in the complete killing of protoscoleces after 120, 90, and 60 min, respectively. Moreover, Mahmoudvand *et al.* (2014) demonstrated that the methanolic root extract of *Berberis vulgaris* at the concentrations of 2 and 5 mg/mL killed all protoscoleces after 10 min of exposure. However, the scolicidal effect of the methanolic extracts of *Ocimum bacilicum* and *Allium cepa* was found to be insufficient in a study by Haghani (2014). Abdel-Baki *et al.* (2016) investi-

gated the scolicidal activity of *Salvadora persica* root extract and reported the highest scolicidal effect at the concentrations of 30 mg/mL after 30 min, and 50 mg/mL after 20 and 30 min of exposure, respectively [26-34].

Screening the medicinal plants with established antimicrobial properties represents a logical approach to discovering novel natural scolicidal agents. Previous research has validated the antimicrobial efficacy of *L. salicaria* and *L. europaeus* extracts [16, 21, 22, 25], though their potential scolicidal activity had yet to be investigated. Therefore, this study aimed to assess the in vitro protoscolicidal potential of methanolic extracts from *L. salicaria* and *L. europaeus* against *E. granulosus* protoscoleces.

It has been shown that the scolicidal effect of herbal extracts is both dose- and time-dependent. Increasing the concentration of the extracts while maintaining a constant incubation time, has been reported to increase the mortality rate of protoscoleces. Similarly, prolonged exposure to each concentration has resulted in a significant increase in the scolicidal activity of the extracts. As a result, it can be deduced that raising the extract concentration along with prolonging the application time will result in more potent scolicidal effects.

The difference in efficacy between the two plant extracts was particularly pronounced during shorter exposure durations, specifically at 1 and 5 min. However, this distinction became less significant as the exposure time increased to 20 and 30 min. Furthermore, when tested at a concentration of 125 mg/mL, both plant extracts demonstrated reduced scolicidal activity. Conversely, at higher concentrations of 250 and 500 mg/mL, both extracts exhibited more potent scolicidal activity.

According to the findings, compounds in the *L. salicaria* extract likely exhibit more rapid onset of action against the parasite as well as a more potent overall effect, achieving markedly higher protoscolicidal activity than *L. europaeus* at a concentration of 125 mg/mL and all exposure times tested. In contrast, *L. europaeus* showed a faster initial rate of action but consistently lower activity at all time points, indicating weaker protoscolicidal effects at this concentration. At the concentration of 250 mg/mL, the differences in protoscolicidal activity between the two extracts were less pronounced but *L. salicaria* still demonstrated consistently stronger effects at all time points tested except after 30 min, where *L. europaeus* exhibited 95.98% activity compared to 95.16% for *L. salicaria*. These findings indicated that at a concentration of 250 mg/mL and longer exposure times, *L. europaeus* can achieve comparable or slightly higher protoscolicidal activity compared to *L. salicaria*, as the differences in activity between the two extracts were diminished at 250 mg/mL after 30 min. Interestingly, the relative efficacies of the two extracts reversed at 500 mg/mL, and *L. europaeus* demonstrated significantly higher protoscolicidal activity than *L. salicaria*, achieving near-complete inhibition more rapidly. This result suggests that compounds in *L. europaeus* may act faster against the parasite and/or exhibit greater overall potency at a concentration of 500 mg/mL.

In this experiment, the concentration of the extract emerged as the most influential factor in determining mortality rates, with plant type and exposure time following in importance. The dose-response profiles of the two plant extracts were significantly different, with *L. salicaria* demonstrating more potent effects at lower concentrations and *L. europaeus* displaying greater activity at higher concentrations. Notably, *L. salicaria* exhibited time-independent kinetics, reaching maximum activity within similar timeframes across all tested concentrations, in contrast to *L. europaeus*. These findings highlight the importance of optimizing the dosage and duration of exposure to the herbal extracts when considering their potential use as scolicidal agents. The underlying reasons for these varying concentration-effect relationships remain incompletely understood and necessitate further investigation into the specific active components present in each extract.

In traditional medicine, it is a common practice to use the mixtures or extracts of plants as herbal medicines instead of isolated compounds. This approach is based on the understanding that plant extracts contain a complex mixture of bioactive molecules that may act synergistically to produce more potent therapeutic effects than those obtained using isolated compounds alone. Furthermore, using plant extracts rather than isolates can mitigate the risk of drug resistance because multiple bioactive agents act simultaneously and target different pathogenic mechanisms. Therefore, it is not surprising that the methanolic extracts of *L. salicaria* and *L. europaeus* demonstrated strong scolicidal activity as the extract contains a diverse mixture of active components that may collectively disrupt the viability and integrity of the parasite (Lila, 2014; Rasoanaivo et al., 2011).

The active components found in *L. salicaria* and

L. europaeus extracts, particularly phenolics, and terpenes, have been extensively studied and shown to possess antibacterial, antifungal, and antiparasitic properties. For example, luteolin-7-O-glucuronide and rosmarinic acid have all demonstrated antibacterial activity in several investigations [25, 35-37]. On the other hand, certain terpenes, such as eugenol, α-terpinene, terpinolene, caryophyllene, and α-pinene have all exhibited potent protoscolicidal activity [33, 38, 39]. These bioactive molecules can interfere with the growth, replication, or metabolic activity of microorganisms or parasites, leading to their destruction or inhibition. Therefore, it is likely that the presence of these compounds in *L. salicaria* and *L. europaeus* extracts contributes to their observed protoscolicidal effects [40, 41].

While further research is necessary to identify and isolate the bioactive components responsible for the observed scolicidal activity, the results of this study suggest that these two plants possess potent scolicidal properties. Consequently, they have the potential to be utilized in the development of new scolicidal agents for application in hydatid cyst surgery or the PAIR technique. Further research is required to elucidate the mechanisms underlying this activity and identify the specific active components responsible.

Conclusion

This study demonstrated that the methanolic extracts of *L. salicaria* and *L. europaeus* exhibited significant protoscolicidal activity against *E. granulosus* protoscoleces in a dose- and time-dependent manner. At lower concentrations, *L. salicaria* consistently showed higher efficacy, while at higher concentrations, *L. europaeus* surpassed *L. salicaria* in activity. The interaction between concentration and plant type significantly influenced the mortality rate, with concentration emerging as the most critical determinant.

These findings highlight the potential of both plant extracts as natural scolicidal agents, offering promising alternatives to conventional chemical treatments for hydatid disease. Further investigation into their active components and in vivo applications is essential for their potential clinical use.

Materials and Methods

Collection of protoscoleces

Sheep livers and lungs containing hydatid cysts were collected from a slaughterhouse (Amol abattoir) and were brought to the Parasitology Lab at the Veterinary School of Amol University of Special Modern Technologies. The surface of the hydatid cysts was washed with sterile 0.9% NaCl solution (normal saline), and the protoscoleces-containing hydatid fluid was aspirated using a 50 mL syringe and was placed into a sterile conical urine glass. After settling for 30 min, the protoscoleces sank to the bottom. The supernatant was carefully discarded, and the protoscoleces were twice washed with normal saline. The vitality of the harvested protoscoleces was checked under an ordinary light microscope, and the number of protoscoleces was adjusted to 3×10^3 in 1 mL of normal saline with a minimum viability rate of 95%. The protoscoleces were subsequently transferred to a 50 mL Falcon centrifuge tube containing sterile 0.9% NaCl solution and stored at 4°C for future use [32].

Viability test

To measure the percentage of viable protoscoleces, the eosin exclusion test was employed. To measure the percentage of viable protoscoleces, the eosin exclusion test was employed. Briefly, the eosin exclusion test was conducted by mixing equal parts of 0.1% (W/V) eosin stain solution and protoscolex. Dead protoscoleces absorbed the stain and turned red after approximately 10 min, while live protoscoleces remained colorless (Figures 1 and 2). Viability of the protoscoleces was additionally confirmed through observing the flame cell activity and body movement [42, 43].

Preparation of plant extracts

In 2022, the aerial parts of *L. salicaria* and *L. europaeus* were collected from Amol and transported to the Faculty of Medicinal Plants at Amol University of Special Modern Technologies. The plant materials were washed, air-dried, and homogenized into a uniform particle size at room temperature before extraction. The samples were then suspended in 80% methanol (v/v) and vortexed for 5 min. Next, the mixtures were placed in an ultrasonic bath (Elmasonic S40H, 340 W, 37 kHz) at 30°C and sonicated for 1 h. Finally, the extracts were dried using a vacuum rotary evaporator in a water bath set at 40°C. The resulting dried samples were weighed, transferred into microtubes, and kept at 4°C until use [42, 43].

Scolicidal activity

To determine the protoscolicidal activity of the methanolic extracts of *L. salicaria* and *L. europaeus*, three concentrations (125, 250, and 500 mg/mL) were tested. In each experiment, 1 mL of each concentration was placed in test tubes, and 1 ml of the protoscolex mixture was added to the tubes and gently mixed. The tubes were then incubated at 37°C for 1, 5, 10, 20, and 30 min. Following incubation, the upper part of the solution was removed with a pipette, and the sediment was washed twice with 2 mL of normal saline. Next, the supernatant was discarded, and the sedi-

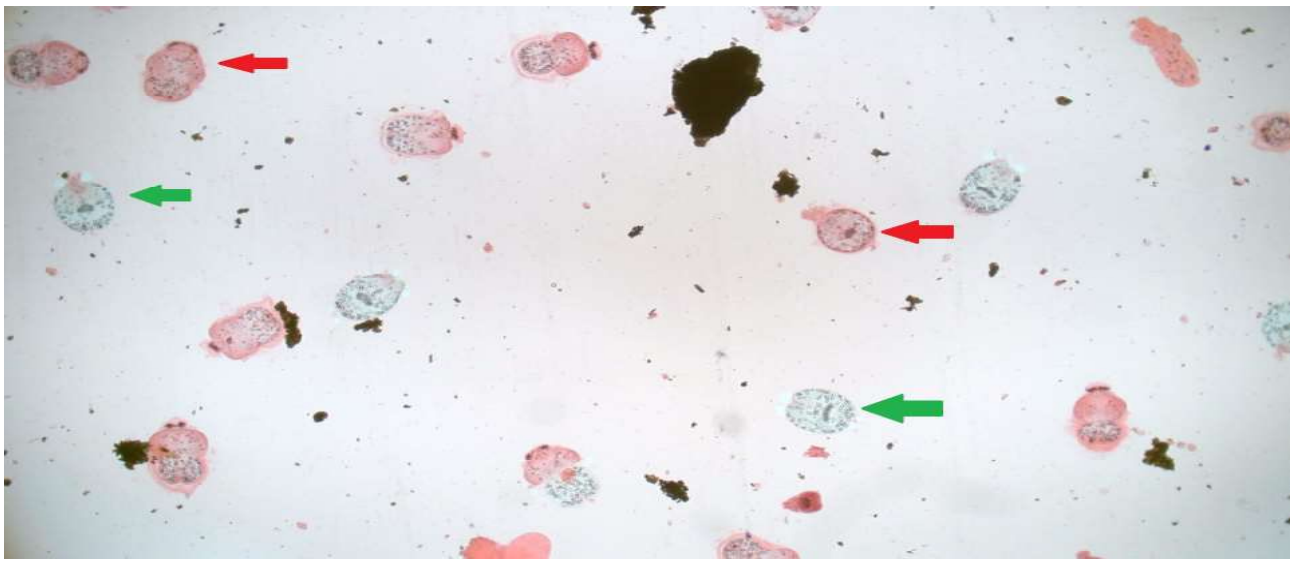

Figure 1.
A comparison of live and dead protoscolex stained with 0.1% eosin. The green arrow points to a live protoscolex, while the red arrow indicates a dead one.

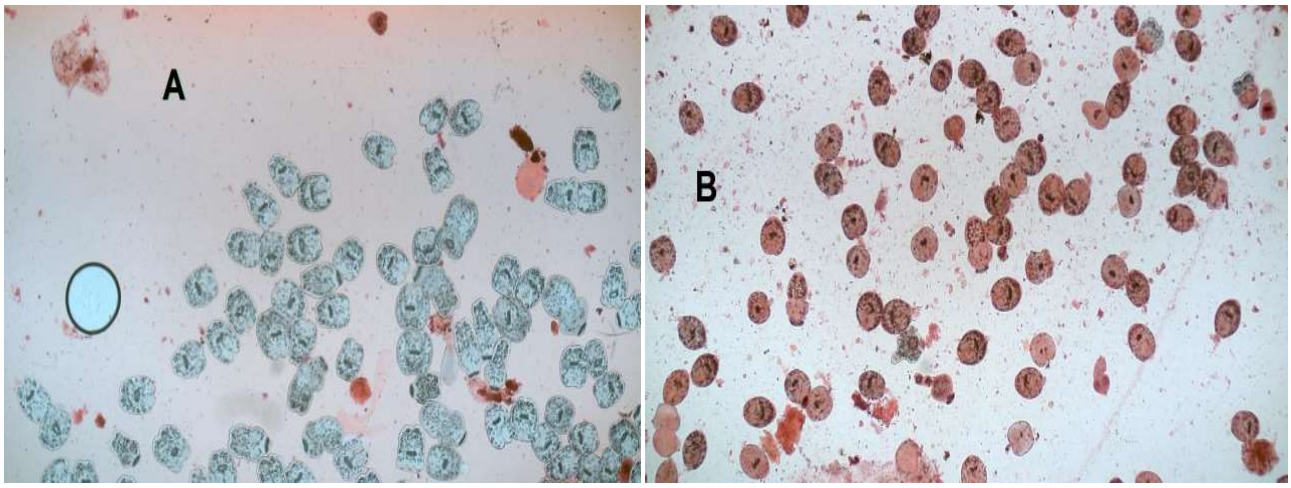

Figure 2.
In the negative control group, untreated living protoscoleces were stained with 0.1% eosin (A). In the positive control group, dye penetration was observed in dead protoscoleces after staining with 0.1% eosin (B).

ment was treated with 2 mL of 0.1% eosin stain solution. After 10 min, one drop of the sedimented protoscoleces was smeared on a glass slide, covered with a cover glass, and examined under a light microscope. The protoscolicidal effect of each treatment was determined by counting 500 protoscoleces. In addition, hypertonic and normal saline solutions were used as positive and negative controls, respectively (Figure 2).

Statistical analysis

All experiments were performed in triplicates, and the data were analyzed using the analysis of variance (ANOVA). Significant differences between the means at $p < 0.01$ were determined by the Duncan's test using SPSS software version 16 (SPSS Inc., USA).

Authors' Contributions

A.N. and M.R. conceived and planned the experiments. A.N. carried out the experiments. M.K. contributed to sample preparation. M.R. contributed to the interpretation of the results. M.K. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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The Fabrication of a Biomedical Scaffold from Human Placenta

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ABSTRACT

The powder derived from human placenta (HP) was successfully used in regenerative medicine. The purpose of this study was to fabricate powder from the human placenta and evaluate it by histological analysis, scanning electron microscopy, and X-ray diffraction. The placenta was decellularized chemically and then lyophilized by a lyophilizer (FTS Systems Bulk Freeze Dryer Model 8–54) for 24 hours at -56 °C and 5 mm Hg until they were totally dried. The assessment used histological analysis, Scanning Electron Microscopy, and x-ray diffraction. The hematoxylin and eosin stain demonstrated that cellular populations and nuclear residues were totally absent from HP tissue. The freeze-drying process of preparing acellular human placenta powder resulted in structures that are made up of highly interconnected, open networks of pores. The particle size mean diameter was approximately ranging from a minimum of 89.44 µm to a maximum of 172.82 µm, and the pore sizes ranged between 44.28 µm and 81.40 µm. Using conventional diffraction database cards, the X-ray diffraction analysis of acellular human placenta powder demonstrated the existence of the constituent organic and inorganic components. It was discovered that the presence of semi-crystalline or amorphous organic components, such as chondroitin sulfate, collagen, and hyaluronic acid. The study concluded from the structural powder that it can be used in regeneration treatments such as treating the spinal cord in animals.

Keywords

powder, X-Ray Diffraction, Extracellular matrix, Scanning Electron Microscopy, histological analysis

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Abbreviations

HP: Human placenta
ECM: Extracellular matrix
SEM: Scanning Electron Microscopy
XRD: X-Ray Diffraction

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The human placenta is an easily accessible, affordable, and morally acceptable source of raw material because it is a transient important organ that is often disposed of as medical waste. Apart from its accessibility, the placenta has other advantageous biological characteristics that are intrinsic to the healing process, such as angiogenic capabilities [1,27], anti-inflammatory properties [2], antibiotic use [3], antifibrotic [4,29], and immunomodulatory [5] with low immunogenicity [6,29]. Placental tissues feature a special extracellular matrix (ECM) with remarkable mechanical and structural characteristics, including tensile strength, stiffness, and elasticity, which complement the desired biological traits [7,17]. As mentioned in the preceding section, the extracellular matrix's (ECM) composition differs depending on the source. Placental-derived biomaterials with living cells, in addition to the ability to function through paracrine pathways, have the above-described qualities[8,33].

The cells found in placental-derived biomaterials facilitate the release of trophic factors, which aids in tissue repair [9,34] and immunomodulation [10]. Furthermore, placental-derived biomaterials' growth factors and cytokines promote anti-inflammatory and antibacterial activities [11,35]. There are several ways to make placental-derived biomaterials, such as using the umbilical cord. (including umbilical cord blood, umbilical cord tissue, and Wharton's jelly), the amniotic sac (including amnion and chorion), amniotic fluid, or a combination of these sources [12]. This produces a great deal of biomaterials but also adds a great deal of variety. The compositions of different sources vary. For example, the extracellular matrix (ECM) of the amniotic membrane is rich in collagen and includes a range of bioactive ECM components, including glycosaminoglycans, fibronectin, laminin, and elastin [13]. While Wharton's Jelly is a mucoid connective tissue composed of a network of glycoprotein microfibrils and collagen fibrils [14,30]. Furthermore, studies have demonstrated that the immunomodulatory qualities vary on the source [15,31]. Even when comparing biomaterials from the same source, there is variation both between and within the donors. [16,32]. The objective of the present study was to prepare a powder from the human placenta and evaluate it using X-ray diffraction and scanning electron microscopy. X-ray diffraction (XRD) was used to study the powdered acellular human placenta in more detail. The Joint Committee on Powder Diffraction Standards (JCPDS) maintained the Powder Diffraction File (PDF-2) from the International Center for Diffraction Data, where standard diffraction data were compared to the XRD patterns.

The acellular human placenta powder contained a variety of organic and inorganic elements, as shown by the analysis of the detected peaks. The following elements were specifically identified as the causes of the peaks:

1. PDF#83-1494 (Chondroitin Sulfate)
2. PDF#50-2241 (Collagen)
3. JCPDS#09-0432 (Hyaluronic Acid)

Abrupt and powerful peaks were seen at 2θ = 7.97°, 11.57°, and 19.13° when certain semi-crystalline or amorphous organic components, such as hyaluronic acid (HA), collagen (Col), and chondroitin sulfate (CS), were present.

The results of the XRD analysis confirmed that the main extracellular matrix (ECM) components, including collagen, hyaluronic acid, and chondroitin sulfate, were present in the decellularized human placenta powder. Understanding the structural and compositional properties of the acellular placental scaffolds is important since they may be used in a wide range of tissue engineering and regenerative medicine applications.

Histological examination was performed in order to evaluate the impact of employing chemical agents in decellularization human placenta (HP). Specifically, hematoxylin and eosin staining was used for confirmation of the decellularization process is illustrated in (Fig. 1 A & B). The staining demonstrated that cellular populations and nuclear residues was totally absent from HP tissue.

One important parameter that can have a big influence on how well biological implants work is the pore width of tissue engineering scaffolds. The porous design of these scaffolds is often the result of water evaporating during the lyophilization (freeze-drying) process, which can lead to the fragmentation of the scaffold's interior particles.

Examination of prepared scaffolds was done by Scanning Electron Microscopy (SEM) revealed differences in particle size and pore size in the bioscaffolds, acellular human placenta powder that was prepared by freeze dry method gave rise to structures that are consisted of open networks of pores with a high degree of interconnection, the particle size mean diameter was approximately ranging from minimum of 89.44 µm to maximum of 172.82 µm (Fig. 2 A), and pore sizes that ranged between 44.28 µm and 81.40 µm (Fig. 2 B).

The mean pore size has a crucial role in the dis-

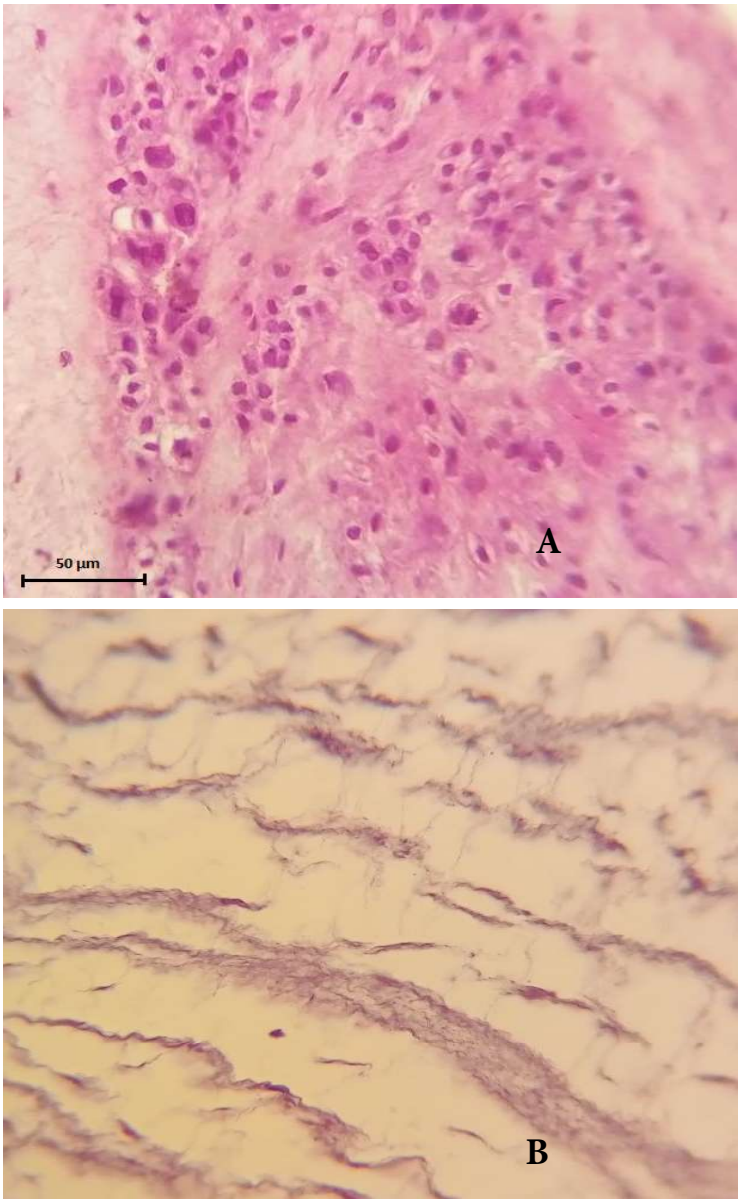

Figure 1.
(A) In the native human placenta shows cell nuclei often arranged in isogenic groups, and evenly distributed in the ECM. (black arrows) (B) Decellularized HP complete loss of cellularity (H&E X200).

tribution of nutrients and removal of waste materials from scaffolds used in tissue engineering. The ability of cells to migrate toward the center of the construct may be restricted by too-small holes, which may obstruct these essential processes. However, the precise relationship between scaffold pore size and cell activity remains unclear. [20]

Similar results on the porosity structure of scaffolds made by freeze-drying have been reported in several prior studies. [21] The morphological structure of urinary bladder powder obtained by freeze-drying is characterized by large interconnected pores in the range of 20 to 100 µm. This pore size range seems to be suitable for certain applications, such as long-size peripheral axon regeneration, which requires pores ranging from 200 to 750 µm, or even up to the millimeter scale. In contrast, smaller pores (20-70 µm) may be more appropriate for the ingrowth and extension of long peripheral axons.

Interestingly, scaffolds with pore diameters around 100 µm have been proposed to be more suitable for neuronal regeneration, depending on the source of the biomaterial [22]. However, according to a study by Hausner et al. 2006, Schwann cells transplanted on fibrin-coated polyurethane scaffolds with a uniaxially-oriented pore structure that is, with 2 µm hole walls and 75 × 750 µm elongated pores showed a significant amount of peripheral axon regeneration [23].

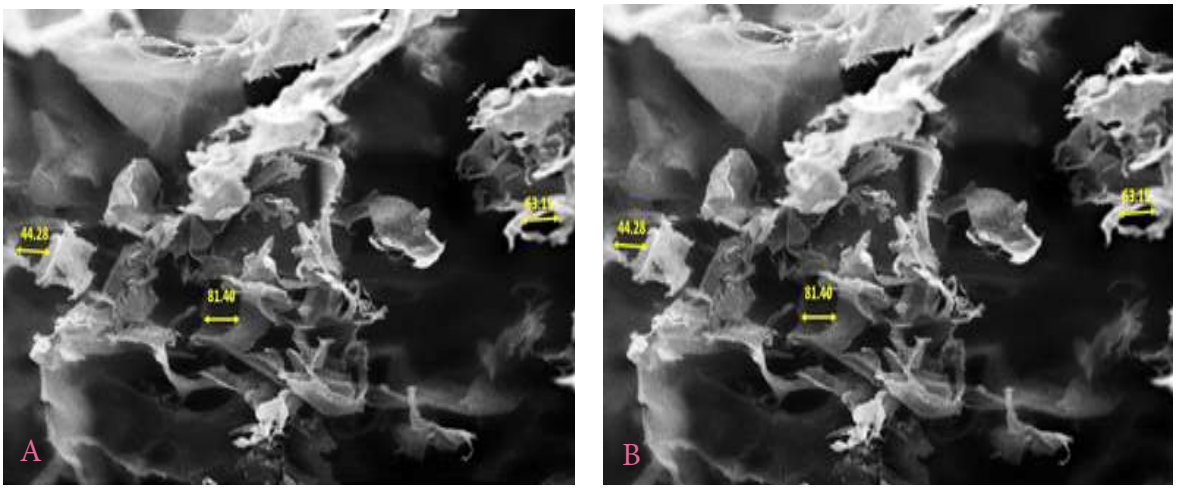

Figure 2.
SEM micrograph the structural morphology of the prepared bioscaffolds shows (A&B) acellular human placenta powder produced by lyophilization.

Additionally, parallel studies [24, 28] have shown that increasing the groove width from 50 to 200 µm in poly(dimethyl siloxane) scaffolds coated with essential proteins like poly-L-lysine and laminin can improve axon regeneration.

In summary, the pore size and architecture of tissue engineering scaffolds play a critical role in facilitating cell infiltration, nutrient/waste exchange, and ultimately, the desired tissue regeneration outcomes. The optimal pore size can vary depending on the specific application and the source of the biomaterial, highlighting the importance of carefully designing and characterizing scaffold properties to achieve the desired biological performance.

The standard diffraction database cards (PDF-2) from the International Center for Diffraction Data were compared to the X-ray diffraction (XRD) examination of the acellular human placenta powder. Diffraction peak analysis revealed that the placenta scaffold includes several significant organic and inorganic components. Specifically, the abrupt and exceptionally potent peaks at 2θ = 7.97°, 11.57°, and 19.13° were accounted for by the presence of semi-crystalline or amorphous organic materials, namely chondroitin sulfate (CS), hyaluronic acid (HA), and collagen (Col). Several previous studies have strongly supported these results. [25].

Scanning techniques such as X-ray diffraction (XRD) provide valuable insights into the crystalline phase and unit cell dimensions of the scaffold materials, making them ideal for the characterization of tissue engineering scaffolds. In the case of the acellular human placenta powder, the XRD analysis confirmed the presence of significant extracellular matrix (ECM) components, including collagen, laminin, fibronectin, proteoglycans, and elastin.

The process of removing cells from scaffold tissue while preserving the extracellular matrix (ECM) structure is known as decellularization. It may be transported physically or chemically. This extracellular matrix (ECM) provides a three-dimensional framework for nerve regeneration and other tissue engineering applications [26].

ECM components such as collagen, chondroitin sulfate, and hyaluronic acid need to be identified by XRD analysis. Within the scaffold, these chemicals are crucial for maintaining cell adhesion, encouraging structural support, and enabling cellular communication and differentiation. To keep the scaffold bioactive and suitable for use in tissue engineering applications, some crucial extracellular matrix elements must be preserved throughout the decellularization process.

In summary, the XRD analysis of the powdered, acellular human placenta provided crucial information on the presence and composition of the scaffold's primary organic and inorganic constituents. Understanding a scaffold's structural and functional properties is essential for determining its potential applications in tissue engineering and regenerative medicine. This understanding is derived from the scaffold's composition.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

HA conceived and planned the experiments. JMK contributed to sample preparation. In addition to leading the paper writing effort and offering insightful criticism, HA and JMK helped analyze the data and shaped the study, analysis, and manuscript.

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Case Report

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Taeniasis, a neglected tropical disease, from Sistan and Baluchestan, Iran

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ABSTRACT

The World Health Organization (WHO) reports that human taeniasis is a neglected tropical disease. It has a worldwide distribution, even in developed countries. Three species of Taenia (*Tania saginata, Taenia solium,* and *Taenia asiatica*) can infect humans. The definitive hosts are humans, while intermediate hosts are cattle or pigs. Consuming raw or undercooked beef can lead to *Taenia saginata* taeniasis, while the primary source of infection for *T. asiatica* and *T. solium* is raw or undercooked pork. *Taenia saginata* taeniasis is the most prevalent in Islamic countries such as Iran, in which pork consumption is very low. It has been reported that human taeniasis has a prevalence between 0.0028% to 3% in Iran. Little is known about the molecular characterization of *T. saginata* in Iran. In this study, *T. saginata* was diagnosed based on its morphological and molecular characteristics. This is the first report on the molecular definition of *Taenia saginata* from Sistan and Baluchestan, Iran.

Keywords

Taenia, Taenia saginata, Taeniasis, neglected tropical disease

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Abbreviations

WHO: World Health Organization

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Case Description

According to the World Health Organization (WHO), human taeniasis is a neglected tropical disease. The disease is globally distributed, even in developed countries [1]. *Taenia saginata* is the most prevalent species of tapeworm worldwide [1].

The epidemiology of taeniasis is associated with cultural practices, such as consuming undercooked meat or eating organs of intermediate hosts infected with viable metacestodes[1].

Humans can be affected by three species of Taenia: (*Taenia solium, Taenia asiatica,* and *Tania saginata*). The consumption of raw or undercooked beef can lead to *Tania saginata* taeniasis in humans, while the primary source of infection for *T. asiatica* and *T. solium* is raw or undercooked pork. The only definitive hosts are humans, while intermediate hosts are cattle or pigs[1].

Although *T. saginata* cannot use humans as intermediate hosts, *T. solium* can cause neurocysticercosis in humans. Human taeniasis caused by T. saginata has been reported in different parts of Iran, including Mazandaran, Guilan, Golestan, Tehran, Alborz, Khorasan-e-Razavi, Ardabil, Esfahan, Fars, and Ilam provinces in the form of case reports or original studies[2-5].

According to previous research, human taeniasis has a prevalence between 0.0028% and 3% in Iran [6, 7].

This study employed morphological and molecular techniques to identify *Taenia spp*. in a human specimen from Sistan-and-Baluchestan, Zabol.

A man aged 46 visited the doctor with complaints of chronic abdominal pain, flatulence, and dyschezia. He reported passing *Taenia proglottids* in his feces. He worked as a government employee and sometimes slaughtered a calf for personal use. However, he stated that he had never eaten raw beef. It is possible that he ingested contaminated material while handling the carcass and touching his mouth. The patient was treated with praziquantel and, after three months, showed no symptoms, with no evidence of eggs/proglottids in the stool sample.

Morphological examination

The proglottids were sent to the parasitology laboratory for morphological analysis. To do this, the specimens were pressed between two microscope slides and stained with the acid carmine method [8]. Under the microscope, morphological characteristics such as the number of uterine branches were carefully examined[9].

Molecular Analysis

Cytochrome c oxidase subunit I (coxI genes) of mitochondrial DNA has been used to study genetic structures in taeniid cestodes. The coxI gene shows genetic diversity among taeniid cestodes, including *E.granulosus, T.taeniaeformis,* and *T.saginata* [10].

The DNA was extracted using the DNA Blood & Tissue kit (MBST, Tehran, Iran) according to the manufacturer's protocol.

The DNA sample was then subjected to PCR amplification of the mitochondrial cytochrome c oxidase (cox1) genes using specific primers : (cox1 F=5`-CATGGAATAATAATGATTTTC-3`) and (cox1 R =5`-ACAGTACACACAATTTTAAC-3`) as mentioned by Anantaphruti [11]. The total reaction volume for PCR was 25 µl, which contained 12.5 µl of 2X Master mix, 1 µl of each primer (10µM), 2 µl of template DNA (approximately 100 ng), and 8.5 µl dH2O.

The PCR procedure consisted of 38 cycles with the following steps: initial denaturation for 5 minutes at 94°C, followed by denaturation for 30 seconds at 94°C, annealing for 45 seconds at 50°C, and extension for 45 seconds at 72°C. Finally, there was a step of final extension for 10 minutes. Each PCR reaction included distilled water as a negative control and Taenia DNA as a positive control. After the procedure, the PCR products were electrophoresed on a 1.5% agarose gel in 0.5× Tris–borate–EDTA buffer and stained with CyberSafe. The PCR product was purified and sequenced using the Sanger method (Pishgam).

A phylogenetic tree was created using the CoxI gene and a representative selection of available sequences from GenBank. The maximum-likelihood method in MEGA was used for this, along with the Tamura-Nei model of nucleotide substitution and 1,000 bootstrap replications. The mitochondrial coxI gene sequence of Echinococcus multilocularis (NC000928) was used as an outgroup for phylogenetic analysis.

Morphological characteristics, such as the number of uterine branches, were examined microscopically. The specimen was identified as T. saginata, because it had over 16 uterine branches (Fig 1). The PCR analysis revealed a PCR with a length above 1200 bp (Fig.1). The amplicon was sequenced and then registered under the accession number OR889487 in GenBank. Upon sequence analysis, it was found that the amplicon had 100% identity to *T. saginata* strains registered in GenBank under the following accession numbers: MT074050 (Cambodia), MW750280 (South Korea), AB533173, AB465239,

AB465235 (Thailand).

The nucleotide sequence obtained in this study exhibited 99.9% similarity to the *T.saginata* sequence data registered under accession numbers: MN452862 (China) and AB107245 (Thailand) and 99.8% identity to the *T. saginata* sequence data registered under accession numbers: MK644930-MK644934 (South Korea), AB984351, AB533172 (China), AB465244(Japan), AB107244 (Thailand), AB107242 (Belgium), AB107240 (Indonesia: Bali), AB107238 (Ecuador). Despite the difference in the nucleotide sequence, the translated amino acid sequence had 100% similarity with the abovementioned records.

Based on phylogenic analysis, the CoxI sequence retrieved from this study clustered with the *T. saginata* cox I gene, distinct from *T. solium* and *T. sciatica* (Fig2).

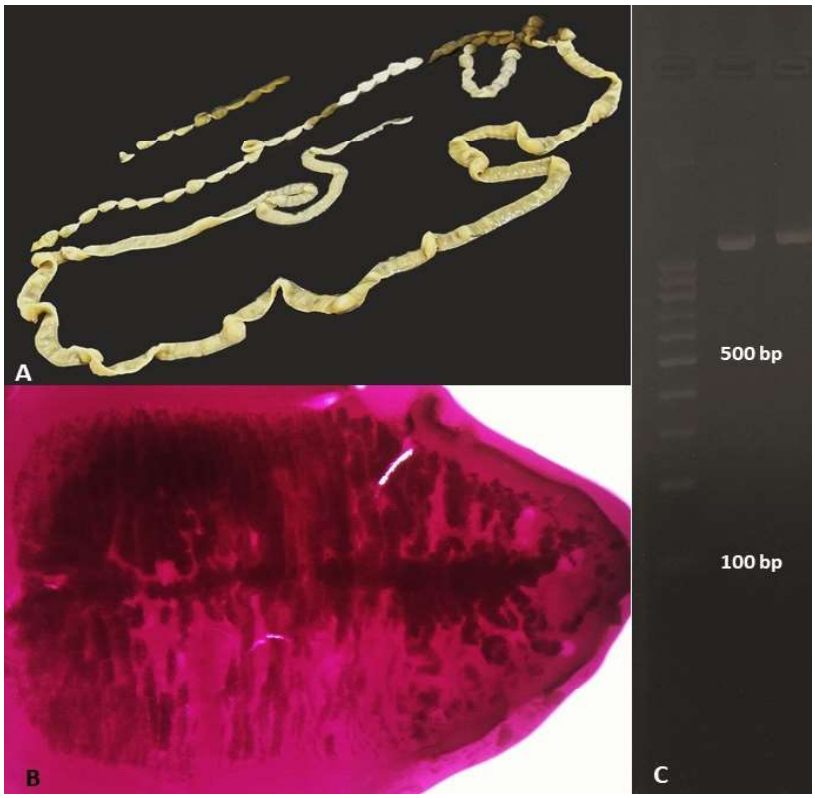

Figure 1.
A) Unstained Taenia B) Carmine acid stained proglottid of Taenia. C: Gel electrophoresis of 924 bp targeting the cox1 fragment of Taenia saginata using PCR.

The diagnosis was *T. saginata* despite the similarity of the lateral branches of the uterus in *T. asiatica* and *T. saginata*. This is because T. asiatica infection is primarily caused by consuming pork and boar meat or internal organs, which are not consumed in Muslim countries like Iran, where this study was conducted[12].

Humans with *T.saginata* taeniasis may be asymptomatic or experience symptoms such as digestive issues, itching of the anus, bloating, abdominal pain and discomfort, mild diarrhea, and weight loss.

In developing countries, human taeniasis is a significant public health problem that is usually diagnosed by observing eggs or gravid proglottids in the stool.

Taenia eggs cannot be used to distinguish between Taenia species. However, stained gravid proglottids can be used to differentiate between *T. saginata* and *T. solium* taeniasis. However, since pork consumption is very low in Iran, it can be assumed that most cases reported based on Taenia eggs are *T. saginata*[12].

Identifying Taenia species by morphological characteristics, such as the number of uterine branches in gravid proglottids, is challenging and requires considerable experience [13, 14]. In a fresh sample of gravid proglottid, proper staining should reveal a clear and sharp uterine structure.

Southeast Asian countries have reported *T. asiatica*, which has a similar morphology to *T.saginata.* However, pork consumption is very low in Islamic countries; its presence is also unlikely.

The final host, intermediate host, and environment influence human taeniasis transmission and spread. The spread of taeniasis in humans caused by *T. saginata* is linked to certain factors, such as consuming raw or undercooked beef, the health status of people, and proper management of municipal wastewater.

Various full articles and case reports [2] have documented cases of human taeniasis caused by *T. saginata* in Iran. Additionally, there have been reports of appendicitis caused by this parasite [5]. However, the genotype of *T. saginata* detected in Iranian patients is poorly understood.

Despite the morphological similarity between *T.saginata* and *T.asiatica*, the nucleotide sequence of the two showed a significant difference in comparison. Anantaphruti et al. conducted a study in Thailand that sequenced a partial region of the cox1 gene (924 bp) and identified 14 haplotypes among *T. saginata* isolates. The most common haplotype was A, followed by B. The study concluded that the sequence obtained from *T. saginata* in this research is similar to haplotype A[11].

Managing *T.saginata* human taeniasis requires a comprehensive approach that includes better health education, improved sanitation, enhanced beef inspection, accurate diagnosis, effective treatment, and

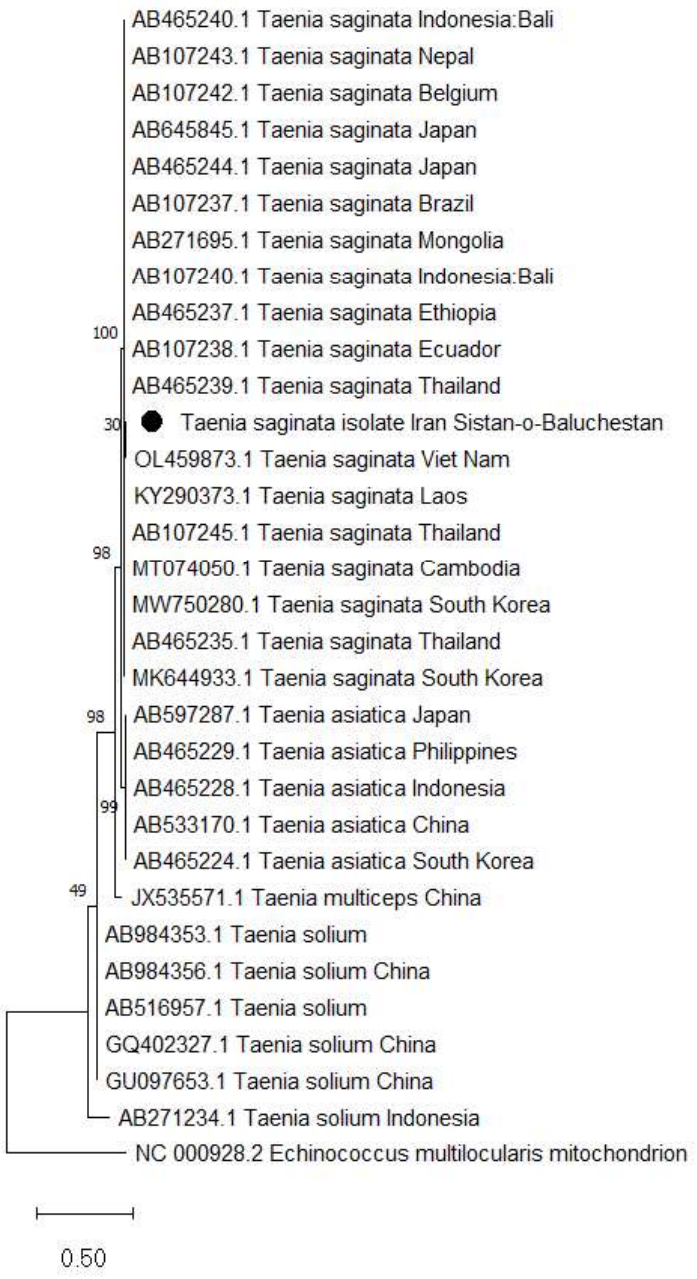

Figure 2.
The phylogenetic tree was drawn based on cox1 sequences of Taenia sequence in this study and retrieved representative sequences from GenBank. The phylogenic tree was inferred using the Maximum Likelihood method and the Tamura-Nei model. Evolutionary analyses were conducted in MEGA11.

close monitoring of taeniasis cases. It is important to remember that greater consumption of illegally slaughtered beef could result in a rise in human taeniasis cases.

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Conflicts of Interest

We have no conflicts of interest related to this work.

Authors' Contributions

M. K. S. collected the sample, E.E. supervised the laboratory tests and wrote the original draft, M.A.D., and J.K. performed laboratory tests, and S.N. edited the manuscript.

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Abstract in Persian

ارزیابی مقایسه ای استفاده از پین اشتایمن و پین ساخته شده از پلی متیل متاکریلات در شکستگی تجربی در استخوان بازو در مدل کبوتر

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چکیده

مشکلات ارتوپدی پرندگان، به ویژه شکستگی ها، به دلیل آناتومی اسکلتی منحصر به فرد پرندگان، چالش های مهمی را ایجاد می کند. استخوان های آنها، از جمله استخوان بازو ، اغلب نوماتیک (پر از هوا) و شکننده هستند و مدیریت شکستگی را پیچیده می کند. روش های سنتی مانند پین های بین مدولاری (پین های IM) و پلیت ها راه حل هایی را ارائه می دهند اما محدودیت هایی دارند. این مطالعه کارایی دو نوع پین را برای تثبیت شکستگی استخوان بازو در کبوتر بررسی کرد: پین های Steinman (که معمولاً در ارتوپدی دامپزشکی استفاده می شود) و پین های ساخته شده از پلی متیل متاکریلات (PMMA). این مطالعه شامل ایجاد شکستگی های کنترل شده در استخوان بازو تعداد سی کبوتر بالغ جوان بود. سپس این شکستگی ها با پین های Steinman یا پین های PMMA تثبیت شدند. معاینات رادیوگرافی و تجزیه و تحلیل هیستوپاتولوژی در هفته های ۲، ۴ و ۶ پس از جراحی برای ارزیابی پیشرفت ترمیم استخوان در هر دو گروه انجام شد. یافته ها نتایج درمانی قابل مقایسه ای را بین دو نوع پین نشان داد. این نشان می‌دهد که پین‌های PMMA می‌توانند جایگزین مناسبی برای تثبیت شکستگی‌ها در پرندگان باشند، و مزیت بیشتری را ارائه می‌دهند که آنها را از جراحی دوم برای برداشتن پین صرف‌نظر می‌کند.

واژگان کلیدی

ارتوپدی، سیمان استخوان، پرنده، بال

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مطالعه اثرات هیستوپاتولوژی امواج الکترومغناطیس (EMF) بر روی غده تیروئید در رت

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چکیده

رابطه بین قرار گرفتن در معرض میدان الکترومغناطیسی (EMF) و سلامتی انسان بسیار مهم می باشد. برخی از منابع رایج EMF عبارتند از تلفن همراه، کامپیوتر، روترهای بی سیم (Wi-Fi)، بلوتوث، مایکروویو، دستگاه MRI، اشعه ایکس، اشعه گاما و اشعه ماوراء بنفش. غده تیروئید یکی از در معرض ترین و حیاتی ترین اندام هاست و ممکن است هدف هر نوع تابش الکترومغناطیسی باشد. بنابراین، ما اثرات هیستوپاتولوژیک میدان الکترومغناطیسی (EMF) بر غده تیروئید رت را بررسی کردیم. در این تحقیق از ۳۵ رت نژاد ویستارسالم استفاده شد. حیوانات به پنج گروه تقسیم شدند و سپس همه حیوانات به مدت ۷۰ روز هر روز به مدت ۰، ۱۵، ۶۰، ۱۲۰، ۱۸۰ دقیقه در معرض فرکانس ۲۱۰۰ مگاهرتز(4G) قرار گرفتند. حیوانات با CO_2 بیهوش شدند. نمونه‌های از غده تیروئید تهیه و با هماتوکسیلین و ائوزین (H&E) و ماسون تری کروم رنگ‌آمیزی شدند. مطالعات استریولوژیکی بر اساس اصل کاوالیر انجام شد. پس از قرار گرفتن در معرض EMF، کاهش معنی‌داری($P < 0.05$) در قطر فولیکول‌های تیروئید، ارتفاع بافت پوششی فولیکول‌ها و حجم فولیکول‌های تیروئید در گروهی که به مدت ۳ ساعت تحت تابش امواج قرار داشتند ثبت شد. مطالعه ما نشان داد که در گروه های ۲ و ۳ ساعت اثرات آسیب بافتی شامل فولیکول های متراکم با کاهش کلوئید، احتقان و گسترش بافت همبند در فضای بینابینی مشاهده شد. علاوه بر آن تشکیل اجسام آپوپتوز در داخل فولیکول ها و همچنین سلول های فولیکولی با هسته‌های متراکم در غده تیروئید رت‌ها ی که به مدت ۳ ساعت تحت تابش امواج EMF(4G) قرار گرفتند مشاهده گردید. در نهایت به این نتیجه رسیدیم که قرار گرفتن در معرض برای بیش از ۲ ساعت به مدت ۷۰ روز متوالی دارای اثرات هیستوپاتولوژیک و استریولوژیک در بافت تیروئید در رت می باشد.

واژگان کلیدی

هیستوپاتولوژی، امواج الکترمغناطیس، غده تیروئید

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Abstract in Persian

خصوصیات ژنوتیپی و فنوتیپی گروه های فیلوژنی جدایه های اشرشیاکلی از شترمرغ ها در ایران

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چکیده

افزایش مصرف آنتی بیوتیک ها در صنعت شترمرغ می تواند منجر به ظهور سویه های حاد باکتریایی مقاوم به آنتی بیوتیک شده که قابل انتقال به انسان باشند. این مطالعه تلاش دارد که خصوصیات ژنوتیپی و فنوتیپی گروه های فیلوژنی جدایه های اشرشیاکلی از شترمرغ را بررسی و پتانسیل خطر بهداشتی آن ها را آشکار سازد. ۱۲۹ جدایه تائید شده ای کولای که همزیست (۴۴) و پاتوژن (۸۵) احتمالی می باشند از گله های شترمرغ مشهد، شمال شرقی ایران، به روش PCR چهارتایی کلرمونت طبقه بندی فیلوژنی شدند. پروفایل فیلوژنی جدایه ها با توجه به حساسیت آنتی میکروبی و پروفایل ژن های مقاومت و حدت ارزیابی مقایسه ای گردید. نتایج نشان داد که بیشتر جدایه های هر دو گروه همزیست و پاتوژن احتمالی متعلق به فیلوگروه A (به ترتیب ۸۱/۳۱ درصد و ۹۴/۳۲ درصد) و B1 (به ترتیب ۳۶/۳۶ درصد و ۷۶/۳۱ درصد) بودند. مقاومت نسبت به چند دارو در فیلوگروه B2 بیشترین بود ($p \geq 0.05$). فیلوگروه B1 که معمولا شامل سویه های همزیست می باشد، برخلاف B2، کمترین درصد جدایه هایی را نشان داد که فاقد ژن های حدت ($p \geq 0.05$) و مقاومت ($p \geq 0.05$) بودند. یافته های این مطالعه افق ادراکی ما را از خصوصیات ژنوتیپی و فنوتیپی گروه های فیلوژنی جدایه های اشرشیاکلی از شترمرغ را گسترش و همچنین یک مغایرت پیچیده بین هر دو خصوصیت را نشان داد. بنابراین مطلوب است مطالعات جامع و مقایسه ای بیشتری بر روی جدایه های اشرشیاکلی از شترمرغ و انسان در مطالعات آینده انجام گیرد.

واژگان کلیدی

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Abstract in Persian

روشی سبز برای مبارزه با اکینوکوکوس گرانولوزوس: بررسی اثرات اسکول کشی عصاره های لیکوپوس یوروپیوس و لیتروم سالیکاریا

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چکیده

این مطالعه فعالیت اسکولکس کشی عصاره متانولی لیتروم سالیکاریا و لیکوپوس یوروپیوس را بر روی پروتواسکولکس های اکینوکوکوس گرانولوزوس با استفاده از استخراج به کمک اولتراسوند ارزیابی کرد. پروتوسکولکس های جدا شده از کبد و ریه گوسفند به مدت ۱، ۱۰، ۲۰ و ۳۰ دقیقه در معرض غلظت های ۱۲۵، ۲۵۰ و ۵۰۰ میلی گرم در میلی لیتر قرار گرفتند. هر دو گیاه فعالیت اسکولکس کشی قابل توجهی را به صورت وابسته به دوز و زمان نشان دادند. در غلظت‌های پایین‌تر (۱۲۵ و ۲۵۰ میلی‌گرم بر میلی‌لیتر)، لیتروم سالیکاریا به طور کلی اثر بیشتری نسبت به لیکوپوس یوروپیوس داشت، اگرچه تفاوت در اثربخشی در غلظت ۱۲۵ میلی‌گرم بر میلی‌لیتر بارزتر بود. در مقابل، عصاره لیکوپوس یوروپیوس در مقایسه با عصاره لیتروم سالیکاریا در غلظت ۵۰۰ میلی‌گرم بر میلی‌لیتر فعالیت قوی‌تری از خود نشان داد. در میان پارامترهای بررسی شده، غلظت بیشترین تأثیر را بر میزان مرگ و میر داشت و پس از آن گونه های گیاهی و زمان در معرض قرار گرفتن در رده های بعدی قرار گرفتند.. اثر متقابل بین غلظت و نوع گیاه بیشترین تأثیر را بر مرگ و میر داشت که نشان دهنده اثربخشی هر دو عامل است. به طور کلی، هر دو گیاه به عنوان نامزدهای بالقوه برای مطالعات آینده با هدف توسعه عوامل طبیعی برای کنترل اکینوکوکوس گرانولوزوس امیدوار کننده هستند.

واژگان کلیدی

اکینوکوکوس گرانولوزوس، هیداتیدوزیس، گیاهان دارویی، عصاره متانولی، پروتواسکولکس

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Abstract in Persian

گزارش یک موردتنیازیس انسانی؛ یک بیماری گرمسیری فراموش شده از سیستان و بلوچستان، ایران

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چکیده

بنا بر گزارش سازمان بهداشت جهانی (WHO) تنیازیس انسانی، یک بیماری گرمسیری فراموش شده است. این بیماری دارای انتشار جهانی، حتی در کشورهای توسعه یافته می باشد. سه گونه تنیا (تنیا ساژیناتا، تنیا سولیوم و تنیا آسیاتیکا) می تواند انسان را آلوده کند. میزبان نهایی انسان است، در حالیکه میزبان های واسط گاو یا خوک می باشد. مصرف گوشت خام یا کم پخته گاو می تواند منجر به تنیازیس ناشی از تنیا ساژیناتا در انسان شود، در حالیکه منبع اصلی عفونت تنیا آسیاتیکا و تنیا سولیوم گوشت خام یا کم پخته خوک است. تنیازیس ناشی از تنیا ساژیناتا در کشورهای اسلامی مانند ایران که مصرف گوشت خوک در آن خیلی کم است شایع تر است. شیوع تنیازیس در انسان بین ۰/۲۸۰۰ تا ۳ درصد در ایران گزارش شده است. اطلاعات کمی درباره مشخصات مولکولی تنیا ساژیناتا در ایران وجود دارد. در این مطالعه تنیا ساژیناتا بر اساس مشخصات مورفولوژی و مولکولی تشخیص داده شده است. این اولین گزارش از مشخصات مولکولی تنیا ساژیناتا از سیستان و بلوچستان، ایران می باشد.

واژگان کلیدی

تنیا، تنیا ساژیناتا، تنیازیس، بیماری گرمسیری فراموش شده

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Guide for authors

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Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) 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 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.

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

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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.

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5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005; 62(1):112-6.

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.

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Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

Figures

Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels

at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than 10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals.

For the use of bar diagrams the following publication should be consulted:

Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015; 13(4):e1002128.

The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.

Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

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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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Iranian Journal of Veterinary Science and Technology is aligned with COPE's (Committee on Publication Ethics) best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

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IJVST requires authors to confirm that they and their co-authors meet all four criteria for authorship based on the guidelines of The International Committee of Medical Journal Editors (ICMJE) (verbatim as follows):

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

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The initial screen will be performed by the editorial office for the structure and format of the manuscript.

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1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
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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.
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10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

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Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

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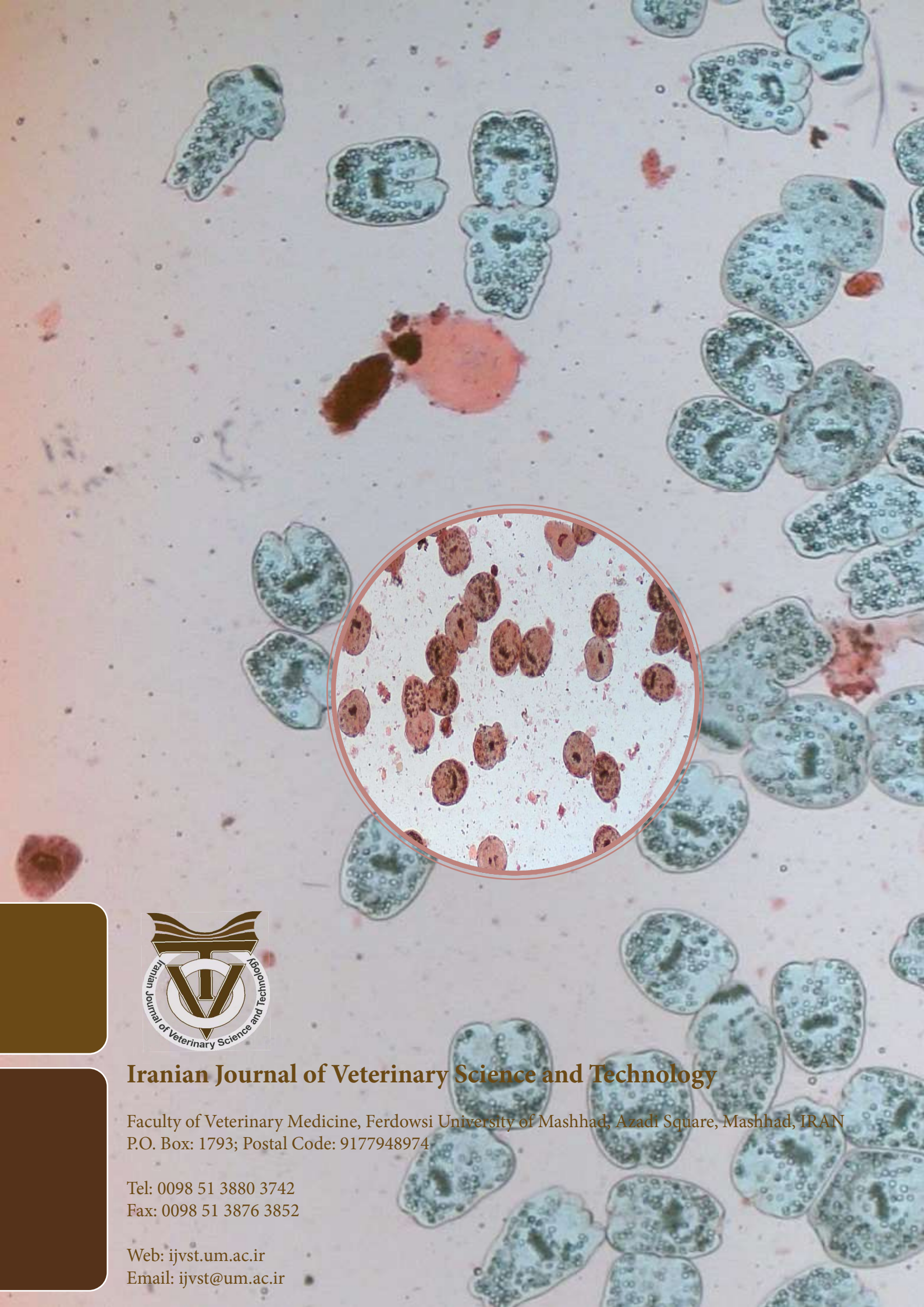

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