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Editorial Office:
Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: 0098 51 3880 3742 ***Fax:*** 0098 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

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ON THE COVER

The growth of *Staphylococcus* colonies with dull haloes on Baird-Parker medium. See page 5.

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The anti-planktonic and anti-biofilm formation activity of Iranian pomegranate peel hydro-extract against *Staphylococcus aureus*

Hadi Ebrahimnejad[a, b], Maryam Ebadi[c], Ladan Mansouri-Najand[b]

[a] Research and Technology Institute of Plant Production (RTIPP), Shahid Bahonar University of Kerman, Kerman, Iran.
[b] Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine; Shahid Bahonar University of Kerman, Kerman, Iran.
[c] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

ABSTRACT

Staphylococcal infections and contaminations have elicited a growing and perennial concern in the medical and food industries. Meanwhile, the manifestation of antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) beside the production of disinfectant-resistant biofilms makes the confrontation with the bacteria more cumbersome and challenging. Pomegranate peel as a waste product of juicing factories is a natural antibacterial agent. The pomegranate peel hydro-extract (PPHE), as a bio-friendly material, was prepared from an Iranian pomegranate cultivar, Rabab, and its phenolic compounds and antioxidant (*via* DPPH and FRAP assays) and anti-staphylococcal (anti-planktonic and anti-biofilm) properties were assessed. The Rabab PPHE inhibited planktonic cells and biofilm formation of three *S. aureus*. The Rabab PPHE produced large and obvious staphylococcal inhibition zones in which their diameters were significantly dose-dependent for the milk isolated *S. aureus* ($p < 0.05$). Despite the resistance of MRSA (ATCC 33591) to beta-lactam antibiotics, the minimum inhibitory concentration (MIC) of PPHE against its planktonic cells was only 3.75mg mL^{-1}. Furthermore, Rabab PPHE inhibited bacterial biofilms formation in a dose-dependent manner. The MIC of Rabab PPHE against planktonic milk-isolated *S. aureus, S. aureus* (ATCC 29737), and MRSA prevented 47, 36, and 26% of their biofilm formation, respectively. This addresses the differences between the anti-planktonic and anti-biofilm activity of Rabab PPHE. The anti-planktonic and to a lesser extent the anti-biofilm forming activity of this water-based extract supports the notion of its effectiveness and salubrious application in food and pharmaceutical industries.

Keywords

Pomegranate peel, Rabab, Staphylococcus aureus

Abbreviations

PPHE: pomegranate peel hydro-extract
MRSA: methicillin-resistant *Staphylococcus aureus*
MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FRAP: ferric reducing ability of plasma
BHT: butylated hydroxytoluene

Corresponding author:
Hadi Ebrahimnejad
Tell/Fax: +98 343 1322940
Email: ebrahimnejad@uk.ac.ir

Introduction

The habitat of *S. aureus*, as a member of the *Micrococcaceae*, is nasopharynx and the hair and skin of more than 50% of healthy people. *Staphylococcus aureus* is the leading cause of staphylococcal food poisoning and extra-intestinal infections. *Staphylococcus aureus* produces many enzymes and toxins that sustain the bacterium and make it resistant to drugs. The enterotoxin of the bacterium is heat resistant, and therefore ordinary cooking, pasteurization, and drying do not easily destroy it (1). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a beta-lactam antibiotics resistant bacterium (2). This bacterium was reported in humans and livestock (mastitic cattle milk) since 1961 and 1972, respectively (3). Antibiotic resistance among pathogens is a worldwide growing problem (4). In 2004, 59.5% of US health centers reported at least one case of MRSA (5). This bacterium has a methicillin resistance gene (*mec-A*). Strains that have this gene also resist many other antibiotics. The resistance makes it difficult to fight them and eventually leads to their further dissemination (6). Staphylococci can form structures called biofilms that attach different surfaces (7). The antibiotic-resistant sessile biofilm forms can further resist the host immune system or be a reason for food contamination and spoilage in the food industry (8-11).

Pomegranate (*Punica granatum* L.) is a native plant of Iran and its neighboring countries which its cultivars have various characteristics (12-14). Rabab pomegranate cultivar which has a thick peel is one of the largest commercial products in the Persian fruit industry (15-17). Pomegranate is classified as a medicinal plant because of its valuable functional compounds (12). Many of its phenolic compounds have drastic antibacterial and antioxidant properties (18-20). The pertinent application of water as a solvent for bioactive compounds extraction from pomegranate peel may provide a safe and relevant extract for the food and pharmaceutical industry which is missed in many evaluations. Until now, studies did not show cytotoxicity towards the by-products of the pomegranate juice industry at arbitrary concentrations (20). Presumably, the staphylococcal complications usually come from both the biofilm and planktonic forms. Lots of the antibacterial agent studies lack a co-assessment of the anti-planktonic and anti-biofilm activity of compounds (20-23). This study aims to assess the antioxidant, total phenolic, and antibacterial effects of an Iranian (Rabab) pomegranate hydro-extract against the planktonic and sessile life of *S. aureus*.

Results

The Rabab PPHE had pale pink to red color. The total phenolic evaluation of the extract revealed that Rabab PPHE has considerable phenolic compounds. Although the Rabab PPHE showed lower antioxidant activity than butylated hydroxytoluene (BHT; synthetic antioxidant), its antioxidant properties in either DPPH or FRAP assays were quite astonishing (Table 1).

Figure 1 shows the staphylococcal inhibition zones produced by Rabab PPHE. The illustrated inhibition zones were quite distinctive in which there was not any tiny colony within their radius. The formation of opaque-milky aura around the dug-wells was probably due to the effect of tannins and astringent compounds of the PPHE on the proteins of Mueller-Hinton agar medium (24).

By increasing the concentration of extract in agar-dug wells the staphylococcal inhibition zones enlarged (Figure 1 and Figure 2). The lowest Rabab PPHE concentration (6mg/well) showed a significantly lower antibacterial activity than other concentrations towards the milk-isolated *S. aureus* ($p < 0.05$). Intriguingly, the higher concentrations (12 and 24mg/well) did not ex-

Table 1
The Antioxidant activity (determined by FRAP and DPPH assays) and total phenolic content of Rabab PPHE.

Extract type	DPPH (IC50, mg mL^{-1})	FRAP (mmol Fe(II) g^{-1})	Total phenols (mg GAE g^{-1})
Rabab PPHE	1.13	0.84	143
BHT (positive control)	0.019	1.38	ND

ND: not determined
PPHE: pomegranate peel hydro-extract
BHT: butylated hydroxytoluene
DPPH: 2,2-diphenyl-1-picrylhydrazyl assay
FRAP: Ferric reducing ability of plasma assay

hibit a significant difference in their antibacterial activity ($p > 0.05$). Statistically, the inhibition zones produced by Rabab PPHE were 6mg/well < 12mg/well = 24mg/well. Notably, the inhibition zones of these three concentrations were not significantly different neither on MRSA nor *S. aureus* (ATCC 29737) ($p > 0.05$).

The minimum bactericidal concentration (MBC) of PPHE was always higher than the MIC (Table 2).

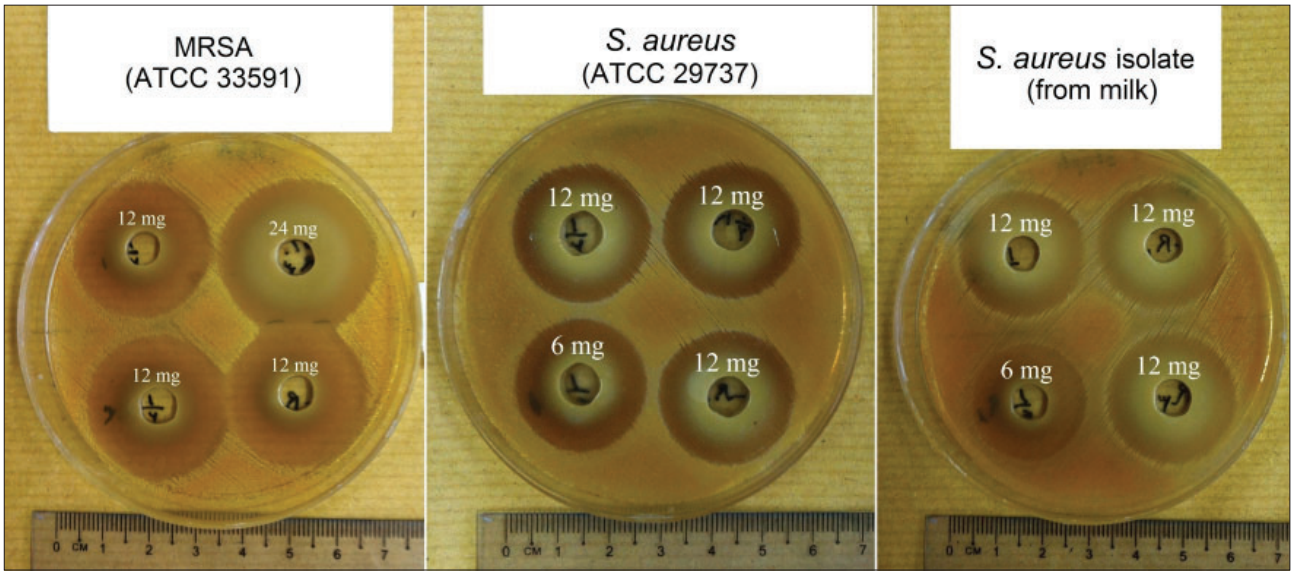

Figure 1.
Antibacterial activity of various concentrations of Rabab PPHE against *S. aureus* by agar well diffusion technique.

Table 2
The MIC and MBC (mg mL^{-1}) of Rabab PPHE against different *S. aureus*.

Antibacterial agent	*S. aureus* (ATCC 29737)		MRSA (ATCC 33591)		*S. aureus* isolate (from milk)	
	MIC	MBC	MIC	MBC	MIC	MBC
Rabab PPHE	15	30	3.75	15	15	60 <
Cefixime (positive control)	8	12	265 <	265 <	8	24

MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
PPHE: pomegranate peel hydro extract
MRSA: methicillin-resistant *Staphylococcus aureus*

The MIC and MBC of PPHE against MRSA were lower than other staphylococci. The lower MIC value of the extract towards MRSA represents its strong anti-planktonic activity. In contrast, the MRSA sensitivity to cefixime antibiotic (positive control) was lower than that of the rest of the bacteria. This antibiotic had a significant inhibitory effect on the other staphylococci that was illustrated by lower MIC and MBC values.

The Rabab PPHE inhibited MRSA biofilm formation by 2.8% at low concentration (0.5mg mL^{-1}), whereas the *S. aureus* (ATCC 29737) biofilm was more sensitive than the MRSA to this concentration of Rabab PPHE. By increasing the concentration of PPHE, the anti-staphylococcal biofilm formation activity was also increased (Figure 3). The extract inhibited the biofilm formation of milk-isolated *S. aureus* at intermediate concentrations (from 1.9 to 30mg mL^{-1}) more than other *S. aureus* strains. This extract at 30mg mL^{-1} inhibited more than 50% of the milk-isolated *S.*

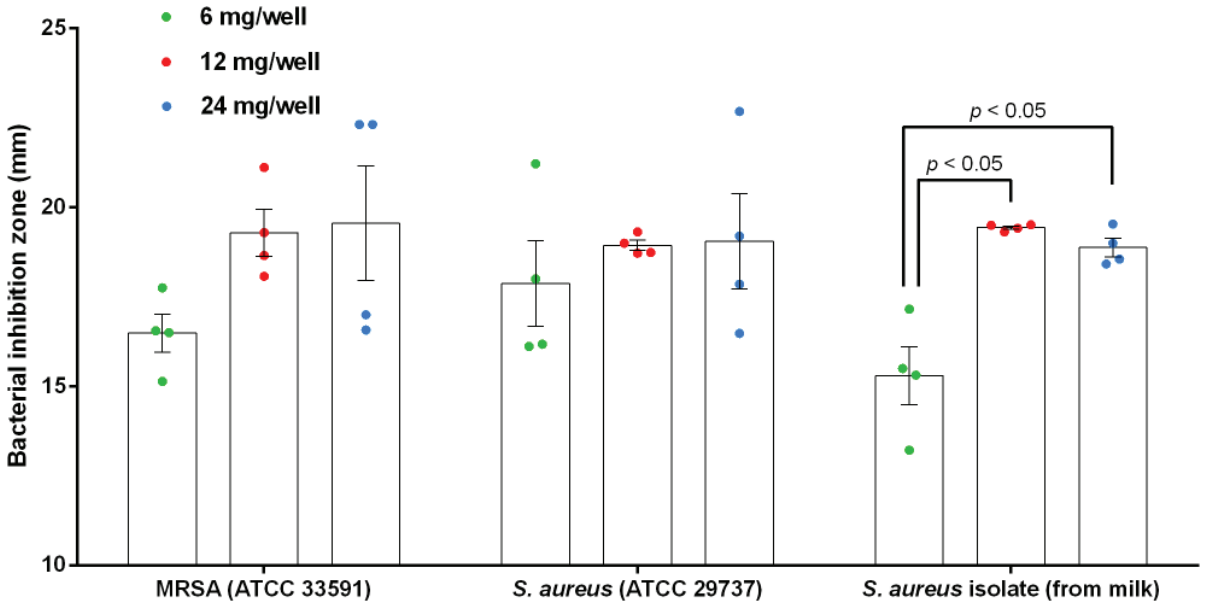

Figure 2.
The effect of three concentrations of Rabab PPHE against *S. aureus* (n=4; mean ± SEM).

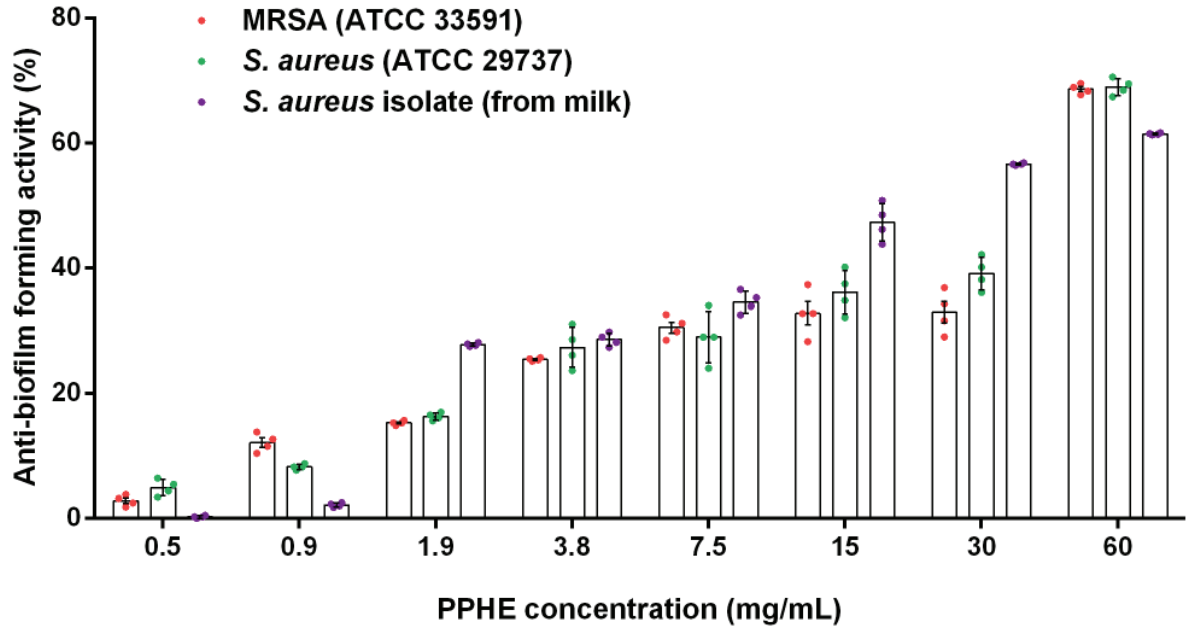

Figure 3.
The anti-biofilm forming activity of different concentrations of Rabab PPHE (n=4).

aureus biofilms.

The anti-biofilm forming activity of the extract was increased profoundly from 30 to 60mg mL^{-1} for MRSA and *S. aureus* (ATCC 29737). Accordingly, Rabab PPHE at 30mg mL^{-1} was not sufficient to inhibit half of the MRSA and *S. aureus* (ATCC 29737) biofilms, while the concentration of 60mg mL^{-1} prevented more than 65% of the biofilm formation by these strains. The milk-isolated *S. aureus* lost 25.7% of its biofilm formation ability while increasing the PPHE exposure from 0.9 to 1.9mg mL^{-1}.

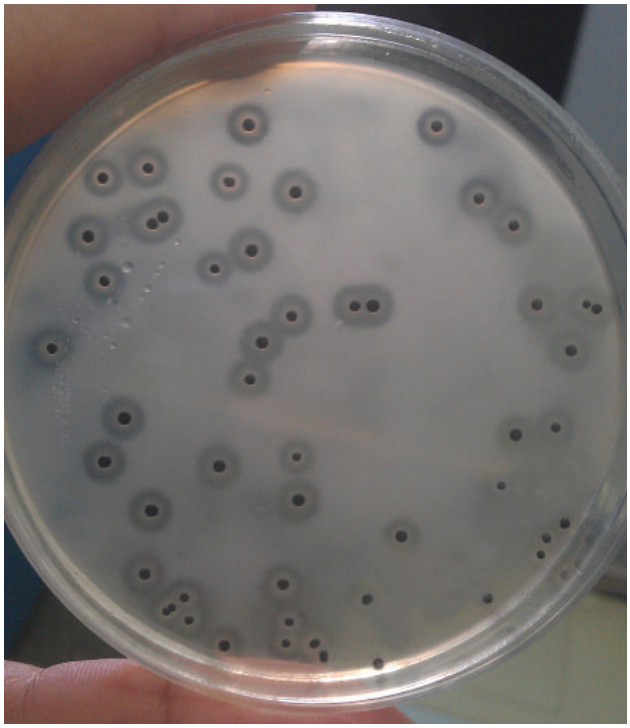

Figure 4.
The growth of *Staphylococcus* colonies with dull haloes on Baird-Parker medium.

Discussion

The extract showed significant anti-staphylococcal and antioxidant activity in this study. It has been shown that the non-edible parts of the pomegranate have higher bioactivity (25). Various pomegranate peel metabolites are very complex (26). Rabab PPHE contains phenolic compounds (Table 1). Many phenolic compounds and organic acids such as gallic acid, chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ellagic acid, malic acid, quinic acid, illogic acid, tannins, punicalin, punicalagin, grantin B, casuarinin, corilagin, methyl gallate, kaempferol, catechol, catechin, epicatechin, epigallocatechin 3-gallate, quercetin, rutin, pelargonidin, naringin, and luteolin, have been detected in pomegranate peel (27-30). Plant polyphenolic compounds have antimicrobial and antioxidant effects. In many studies, pomegranate peel alcoholic extracts demonstrated antimicrobial activity (21, 26, 31). Bioactive effects of pomegranate are due to various and abundant bioactive compounds such as tannins (especially ellagitannin, as a hydrolyzable tannin or prodelphinidin, as a condensed tannin). Two members of ellagitannins (namely ellagic acid and punicalagin) play a significant role in the antimicrobial and antioxidant effects (32, 33). The Precipitation of cell membrane proteins by pomegranate peel phenolic compounds causes bacterial cell membrane leakage and ultimately results in cell lysis and death (25, 26). The toxicity of phenolic compounds against bacteria can also occur when they react with thiol groups of proteins that finally prevents the growth of the microorganism (25). Pomegranate peel extracts (esp. alcoholic) exhibited other deleterious effects on bacteria such as inactivating their enzymes or preventing their protein e.g., staphylococcal enterotoxin A (SEA) production (18, 32).

The concentration of the bioactive compounds in the pomegranate depends on the pomegranate cultivar and the different stages of plant growth (25, 26). Furthermore, the antibacterial and antioxidant activity of pomegranate extract depends on the plant cultivar and geographical origin, harvesting season, and extraction method (34). For example, the hydro-extracts of South Africa or Yemen pomegranate cultivars in previous studies, contrary to the current study, did not show antibacterial activity against *S. aureus* (26, 35). The total phenolics and antioxidant capacity (evaluated by DPPH assay) of Rabab PPHE in this study were less than the methanolic extracts of other pomegranate cultivars in the Fawole et al. (2012) study (26). These differences are probably related to the characteristics of the pomegranate cultivars and the type of fruit peel extract. The extraction method plays an important role in the quality of the extract. It has been stated that the extraction of polyphenolic compounds depends on the type of solvent, plant particle size, solvent to plant solid ratio, and extraction temperature and time. Extract preparation with high temperatures for long times may reduce the level of polyphenols, including ellagitannin (36). Application of hot water or soxhlet extractor to obtain the extract and the autoclave-sterilization probably reduce its antibacterial activity (22, 23).

Pomegranate polyphenols are considerably extracted with hydrophilic solvents. The hydro-extracts lack the toxicity of solvent remnants and therefore can be stored wet while retaining high antioxidant activity (25). Furthermore, hydro-extracts are more compatible with the hydro-nature of the body cells and probably make better systemic effects. The nature of PPHE makes it a highly soluble and diffusible extract in the Muller-Hinton Agar medium. This extract has potent anti-staphylococcal effects, and the combination of this effect along with its facile diffusion generated significant bacterial inhibition zones even at low concentrations (Figure 1). The bacterial inhibition zones did not show any significant difference between the sensitivity of the three staphylococci exposing PPHE ($p > 0.05$).

The planktonic MRSA cells showed a significant sensitivity to the extract in MIC assay. Even the MBC of PPHE against this bacterial strain was lower than other staphylococci (Table 2). The MRSA infections are mainly divided into two hospitals acquired (HA-MRSA) and community-acquired (CA-MRSA) groups (4). Despite hospital infections, the prevalence of MRSA is higher in patients with open ulcers and immune deficiency (37). The sensitivity of MRSA to PPHE is very important due to the resistance of this bacterium to β-lactam antibiotics such as cefixime as a third-generation broad spectrum cephem (Table 2). Methicillin and beta-lactam interfere with bacterial cell wall peptidoglycan by binding the penicillin-binding proteins (PBPs). Nevertheless, MRSA resists β-lactam antibiotics by producing PBP2a instead of PBPs due to the acquisition of the *mec-A* gene (4).

The staphylococcal biofilm formation was also strongly influenced by different concentrations of PPHE. The Rabab PPHE at a concentration of 60mg mL^{-1} prevented nearly 70% of the staphylococcal biofilm formation. The anti-biofilm formation activity of PPHE was dose-dependent and elevated by increasing the extract concentrations. The extract at 1.9mg mL^{-1} made a sharp slope in inhibiting the milk-isolated *S. aureus* biofilm formation. However, this sudden increase in the inhibition of MRSA and *S. aureus* (ATCC 29737) biofilm formation occurred at 60mg mL^{-1} (Figure 3).

The extract at its MIC (15mg mL^{-1}) for the milk-isolated *S. aureus* and *S. aureus* (ATCC 29737) inhibited 47% and 36% of their biofilm formation, respectively. Moreover, 26% of the MRSA biofilm formation was inhibited by the MIC of the extract against this strain (i.e. 3.75mg mL^{-1}). Therefore, the MIC of PPHE against planktonic MRSA shows lower anti-MRSA biofilm formation activity regarding other staphylococci. Notably, the PPHE at the lowest concentration (0.5mg mL^{-1}) inhibited albeit a low percentage (2.8%) but substantial anti-MRSA biofilm formation activity (Figure 3). Hence, concentrations below the MIC also show anti-biofilm forming activity. Plausibly, inhibition of biofilm formation by the extract is achieved not only through bacterial growth inhibition but also through other mechanisms. The exact mechanism for the biofilm formation inhibitory activity of PPHE is still shrouded in mystery. There are some conjectures about especially the alcoholic extracts (32, 38-40). The effect of these extracts on biofilms is probably due to their ellagic acid. Pomegranate extract can precipitate proteins, such as adhesins, which are crucial in biofilm formation. Moreover, tannins such as ellagic acid alter the surface charge of bacteria and subsequently may interfere with the cell-substratum attachment. The pomegranate extract can also disrupt the pre-formed biofilms of various bacteria (32). It has been established that the hydro-alcoholic extract of pomegranate rind shows a good anti-quorum sensing activity. Quorum sensing is a kind of bacterial communication that biofilm formation interconnects with it (38).

In conclusion, Rabab PPHE shows remarkable anti-staphylococcal effects. The extract has high levels of phenolic compounds. Antioxidant and antibacterial effects of PPHE are probably due to its bioactive compounds such as tannins (e.g., ellagitannin or prodelphinidin). The application of this extract will be useful in inhibiting and eliminating staphylococcal food contaminations or body infections. These antibacterial effects against MRSA are of great importance. The PPHE showed lower anti-biofilm formation activity than the anti-planktonic activity against staphylococci. This was more vivid in the case of MRSA. Although inhibition of MRSA biofilm by PPHE begins at low concentrations, the PPHE level equivalent to the MIC has less inhibitory activity against MRSA biofilm formation than other staphylococci. Conversely, the effect of PPHE against some staphylococcal biofilms provokes the use of this water-soluble extract in food and pharmaceutical industries and even milking machines disinfection. However, the efficacy and stability of PPHE and its active ingredients under various industrial processing and simulated body conditions need to be profoundly explored.

Material and methods

Preparation of pomegranate peel hydro-extract (PPHE)

Commercially ripe and fresh pomegranates were harvested during October 2017 from mature trees. The pomegranates were from Rabab-e-Neyriz (from Fars Province) cultivar as a known Persian pomegranate cultivar. The pomegranate was authenticated by the Department of Plant Productions, Agricultural Faculty of Bardsir, Shahid Bahonar University of Kerman. Fifty pomegranates were collected and flushed by tap water and then washed

three times with distilled water. After drying, the pomegranates were peeled while the peel and pulp compartments were carefully separated from each other. The pomegranate peels were shadow dried for 7 days and then grounded with a grinder. The Rabab peel powder mixed with distilled water (0.2g mL^{-1}) and the homogenate was agitated gently at 25 °C for 24h in a shaking incubator (JSSI-100C Compact shaking incubator, JSR). The suspension was centrifugated at 3000 rpm for 30 min at 4 °C using Universal 320R centrifuge (Hettich, Tuttlingen, Germany). The supernatant filtered through a filter paper (Whatman No. 1) and after that filter-sterilized with 0.22µm filters (Millipore Sigma, Millex®-GV). The filtrates were lyophilized and stored at 4 °C as the hydro-extract (41). The anti-staphylococcal activity of the extract was assessed in less than seven days while being filter-sterilized before application.

Total phenolic of PPHE

The total phenolic content of PPHE was determined spectrophotometrically by the Folin-Ciocalteu method (42, 43). A 0.5ml aliquot of diluted PPHE was mixed with 0.5ml of 10-fold-diluted Folin-Ciocalteu's reagent. After 5 minutes of shaking, 0.5ml of sodium carbonate solution (20%) was added. Ultimately, the solution was brought up to 5ml by distilled water and incubated at 25 °C for 90 min in the dark. The absorbance of the mixture was measured at 765 nm against a blank (Shimatzo, Japan, UV-1201). The total phenol content obtained using gallic acid as a standard phenolic acid. The PPHE total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram PPHE.

Ferric reducing/antioxidant power (FRAP) of PPHE

Aliquots of iron (III) chloride solution (20 mM), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ; 10 mM in 40 mM HCl) and acetate buffer (pH 3.6; 300 mM) mixed in proportions of 1:1:10 (v/v), respectively to produce the FRAP reagent. Thence, 100 µL of filtered PPHE were added to 3.0 ml of the 37 °C warmed up FRAP reagent. The Absorbance (593 nm) was recorded after 5 min. Similarly, the standard curve was prepared using iron (II) sulfate solution and butylated hydroxytoluene (BHT) used as a positive control. The antioxidant capacity was expressed as mmol of Fe (II) per g extract (44).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay of PPHE

This assay is based on the ability of antioxidants to decolorize DPPH, a stable free radical. Briefly, the PPHE was diluted in methanol, and 50 µl of each dilution was mixed with 2.5 mL of a fresh DPPH radical methanol solution (0.004%; w/v). The purple mixture allowed to stand for 30 min in the dark at room temperature. The Absorbance (517 nm) were recorded on a spectrophotometer (Shimatzo, Japan, UV-1201) using methanol as a blank. The radical inhibitory activity of PPHE was calculated as follows:

% inhibition = [(A blank – A sample)/A blank] × 100

Where; "A blank" is the absorbance of the control and "A sample" is an absorbance produced by the extract. Extract concentration providing 50% inhibition (IC50) was calculated from the radical inhibition vs PPHE concentration graph. BHT was used as a positive control (45).

Isolation and identification of S. aureus from milk

During the summer season, cow milk samples were collected aseptically from local farms and transferred on ice to the laboratory. Samples were serially diluted in sterilized normal-peptone (0.85% and 0.1%) and 100 µL of each diluted sample were surface plated onto Baird-Parker agar (M043, HiMedia, India) supplemented with egg yolk and potassium tellurite and incubated aerobically for 48h at 37 °C. The black colonies surrounded by an opaque and also clear haloes were considered as staphylococci (Figure 4). The milk isolates were assessed by Gram-staining, catalase, and coagulase tests until finding the intended bacteria. The gram-positive cocci with positive coagulase and catalase results were further identified as *S. aureus* by API Staph system (BioMe´rieux, 20500, France) (46).

The anti-staphylococcal activity of PPHE

The antibacterial activities of PPHE against *S. aureus* (ATCC 29737), Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591) and the milk-isolated *S. aureus* were assessed by agar-well diffusion method. The MIC of PPHE against the planktonic bacteria was assessed via a micro-broth dilution technique. The MBC of PPHE was further assessed. Finally, the ability of PPHE in the prevention of staphylococcal biofilm formation was measured by a microtiter plate test.

Agar well diffusion assay

The anti-staphylococcal activity of the Rabab PPHE was determined by the agar well diffusion method with some modifications (47, 48). The aforementioned *S. aureus* bacteria were inoculated on the cation-adjusted Mueller-Hinton II broth (90922, Fluka) at 37 °C for 18h. The bacterial suspension density was adjusted equal to that of the 0.5 McFarland standard. The density standardized bacterial culture was swabbed on the solidified Mueller-Hinton agar (70191, Merck) and allowed to dry for 10 min. Thence, 6 mm-diameter wells were made with a sterilized cork-borer in the inoculated Mueller-Hinton agar plates. The lower part of wells was first sealed with a few drops of molten agar medium (49). 100 µL of the PPHE (6, 12, and 24mg per well) were added into the wells and allowed to diffuse at room temperature for 15 min. Negative (sterilized distilled water) and positive controls (Gentamicin sulfate salt, G1264 sigma; 10 µg $well^{-1}$) were also placed in wells. The plates incubated at 37° C for 16-18h. The anti-staphylococcal activity of the extract revealed by the formation of bacterial inhibition zones around the wells and the diameter of the halos were measured by a caliper.

Determination of MIC and MBC of PPHE

The MIC of PPHE against the planktonic bacteria was assessed using a 96-well sterile microtiter plate as described before via a micro-broth dilution technique (50). Briefly, the MICs were evaluated after providing 2-fold dilutions of the extract (60 to 0.47mg mL^{-1}) with cation-adjusted Mueller-Hinton II broth. The overnight bacterial suspensions were diluted and added into the wells to provide the final inoculum of 5×10^5 CFU mL^{-1}. After incubation (37 °C, 24h), optical densities (OD620) of the extract exposed bacteria were studied relative to the negative control. The bacterial growth inhibition was calculated as follows:

% inhibition = 100 – [(OD620E – OD620B) / (OD620G – OD620B)] × 100

Where; "OD620E", "OD620B", and "OD620G" are the optical densities at 620 nm for extract containing wells, background control wells, and growth control wells, respectively. The MIC was the lowest concentration of PPHE that completely (100%) inhibited bacterial growth. After the MICs were read and recorded, the 96-well MIC plates were shaken and re-incubated for an extra 4h at 37°C. Subsequently, the bacteria of wells with no visible bacterial growth were enumerated using Trypticase soy agar (22091, Merck) at 37 °C/24h. The minimum bactericidal concentration was defined as the lowest concentration of PPHE that causes ≥99.9%

staphylococcal kill relative to the first inoculum. Cefixime trihydrate (18588, Fluka) was used as a positive control.

The anti-biofilm forming activity of PPHE

To evaluate the effect of PPHE against staphylococcal biofilm formation, a microtiter plate adhesion assay was applied (51). In a 96-well plate, the PPHE was serially diluted with cation-adjusted Mueller-Hinton II broth from 60 to 0.47 mg mL^{-1} in a 2-fold manner. Thence, 100 µL of diluted overnight staphylococcal suspension (1:100) was added to each well. The microtiter plate was incubated at 37 °C for 24h to let the bacteria form different levels of biofilm. After incubation, 200 µL of crystal violet (0.06%, w/v) was added to each well and the plate was shaken three times to help the biofilms stain. After 15 minutes at 25 °C, each well was washed at least three times with sterile normal saline (200 µL) to remove planktonic cells and the unfixed stain. The biofilm-bound crystal violet was further extracted with 200 µL of ethyl alcohol (95%) and transferred to a 96-well plate. The absorbance (595 nm) was recorded by a microplate reader to determine the level of biofilm formation. Culture medium and also different concentrations of the extract without the bacteria were used as the control. The inhibitory activity of PPHE on the staphylococcal biofilm formation was evaluated by comparing the ODs of the treatments with negative controls.

Statistical analysis

Anti-staphylococcal inhibition zones and anti-biofilm forming activities were represented as mean ± SEM of the results in quadruplicates. Data analysis was carried out using SPSS software (SPSS, Chicago, Ill., USA). One-Way ANOVA followed by Duncan's post hoc test (alpha = 0.05) was used to analyze the differences of inhibition zones between staphylococci and also the levels of PPHE.

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Author Contributions

H.E. conceived, designed, and analyzed the data. H.E. and M.E. performed the experiments and wrote the paper. L.M. provided some of the materials.

Conflict of Interest

The authors declare that there is no conflict of interest.s.

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Isolation of broad-host-range bacteriophages against food- and patient-derived Shiga toxin-producing *Escherichia coli*

Golshan Shakeri[a], Abdollah Jamshidi[a], Kiarash Ghazvini[b]

[a] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Microbiology and Virology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

ABSTRACT

This study aimed to isolate bacteriophages specific to Shiga toxin-producing *Escherichia coli* (*E. coli*) strains, particularly EHEC O157:H7, in order to develop a collection of phages against different *E. coli* pathotypes isolated from northeast of Iran. Eighteen samples were screened without any preliminary enrichment and also with small scale enrichment using *E. coli* 12900, which did not result in the phage recovery. Seven samples were prepared with an extensive enrichment. Of them, 5 samples produced plaques. Eventually, seven phages out of thirteen isolated phages were selected for phage host range investigation. Results of the spotting host range assay demonstrated that 22 pathogenic *E. coli* strains and isolates (54%) were susceptible to at least one of the phages. Phage Ecol-MHD1 was polyvalent against *E. coli* and *Salmonella* isolates. The other phages were specific to *E. coli* pathotypes. In conclusion, the phages isolated in this study can be suggested as preventive or therapeutic candidates against foodborne *E. coli* infections in humans.

Keywords

Shiga toxin-producing E. coli (STEC), Bacteriophage, Host range, Enrichment

Abbreviations

STEC: Shiga toxin-producing *Escherichia coli*
EHEC: Enterohemorrhagic *Escherichia coli*
ABR: Antibiotic-resistant

Corresponding author:
Abdollah Jamshidi
Tell/Fax: +98 5138805607

Email: ajamshid@um.ac.ir

Introduction

Escherichia coli (*E. coli*) is the most frequently reported cause of foodborne illnesses (~111 million) worldwide [1]. In 2017, Shiga toxin (Stx)-producing *E. coli* (STEC; also known as verotoxin-producing *E. coli*) caused 2050 cases of infections in the United States [2] and 6,073 cases of infections and 20 deaths in Europe [3].

Pathogenic *E. coli* strains are categorized into six diarrheagenic pathotypes [4]. Of those, STEC can lead to severe gastrointestinal infections, including hemorrhagic colitis or even life-threatening complication of hemolytic uremic syndrome (HUS) [5,6]. The subset of STEC, which is highly pathogenic in humans and has the potential to cause HUS, is named enterohemorrhagic *E. coli* (EHEC) [6]. EHEC outbreaks are linked to the consumption of raw or undercooked contaminated foods such as ground meat products, milk and cheeses, and vegetables and sprouts [7].

Although, there are numerous natural and chemical preservatives or treatments for combating foodborne pathogens, the important thing to watch for is that foodborne illness outbreaks are still occurring and even causing human death [8]. On the other hand, broad-spectrum antibiotics and chemical interventions that are commonly used for treating or preventing infections could lead to antibiotic resistance or side effects on human health [9–11]. These factors intensify a need for an alternative antimicrobial technique for controlling foodborne pathogens or treating their associated diseases. Bacteriophages are biological tools that specifically target pathogens. They are safe and non-toxic to human cells and do not influence the quality and organoleptic properties of foods [12,13]. Therefore, phages can be possible alternatives to antibiotics [14,15] and chemical food preservatives [13,16]. Recently, many studies have been conducted on phage-mediated biocontrol of pathogens and phage therapy. These reports emphasize the significance of isolating novel phages, as well as the determination of phage organismal properties, especially phage host range [17]. Specifying the range of targeted bacteria is a primary requirement for phage biocontrol and therapy, and also for developing efficient phage cocktails that could infect a desirable spectrum of bacteria [15,18]. Although commercial phage preparations against *E. coli* have been approved for food safety applications in the United States and Europe [19,20] and registered as therapy in Russia [21], Georgia, and Poland [22], further phage preparations for reduction of foodborne zoonoses are urgently needed in the developing countries. In the developing countries, not only the prevalence of infectious diseases is higher [23], but also some of the common infections such as those caused by diarrheagenic *E. coli* in children demonstrate high or absolute resistance to current antibiotics [24,25].

This study aimed to isolate bacteriophages specific to STEC strains, in particular, EHEC O157:H7 and to determine their host range for developing a collection of phages against pathogenic *E. coli* strains isolated from northeast of Iran. Additionally, in terms of phage isolation, the influence of methodology on the yield of phage recovery has been reported. The current study could eventually lead to the creation of a bacteriophage cocktail that would be a potential antibacterial against pathogenic *E. coli* isolates for safeguard in food chain or therapy.

Results

Preparation without enrichment and with small scale enrichment

A screening for STEC-infecting phages was performed on filtrates of 18 samples (out of 25; group A in Table 3) directly and following enriching 100 µl of them in combination with *E. coli* O157:H7 ATCC 12900. Neither of the methods led to positive results, and none of 18 samples lysed the lawn of *E. coli* 12900, and field- and patient-derived isolates by either spotting assay or double agar overlay plaque assay.

Preparation with extensive enrichment

Screening of *E. coli*-infecting phages in the remaining samples (7 out of 25 samples; group B in Table 3) was conducted following enriching 30 ml of each sample in combination with *E. coli* O157:H7 ATCC 12900, and three pathogenic food- and patient-derived isolates. According to the results of spot assay in preliminary screening, five samples (septic wastewater of a veterinary clinic, city aqueduct, mixed minced meat, cow cartilage, and vegetables) out of seven (71%) lysed at least one of the tested bacteria. Negative samples were beef and fat that were collected from a cattle slaughterhouse.

Isolation and purification of bacteriophages

Filtrates taken from complete clear spots in preliminary screening were applied for isolating STEC-infecting phages by using the most susceptible hosts based on the spot assay results.

By applying different hosts in double agar overlay plaque assay, 13 STEC-infecting phages recovered from five positive samples. Considering the stability, titer, and clarity of plaques, seven phages (Ecol-MHD1 to Ecol-MHD7) were selected for propagation and further phage host range investigation. Septic wastewater of a veterinary clinic was the richest source that

yielded six phages, including Ecol-MHD4 to MHD7, followed by city aqueduct that resulted in recovering four phages, including Ecol-MHD1 and Ecol-MHD2. Ecol-MHD3 was isolated from minced meat. Plaque sizes ranged from pinpoint to 3.5 mm, as shown in Figure 1. After phage propagation for expanding the phage titer, the final titer of phage suspensions reached 10^8 to 10^9 PFU/ml.

Determination of phage host range

Spotting host range assay was applied for the exploration of the host spectrum of phages. The results (Figure 2) demonstrated that 22 pathogenic *E. coli* strains and isolates (54%) were susceptible to at least one of the phages. As shown in Figure 2, phage *Ecol*-MHD1 was polyvalent and caused lysis on the lawn of 15 *E. coli*, including different pathotypes and also 3 *Salmonella* isolates. The other six phages were specific to *E. coli* pathotypes. Phage Ecol-MHD4 showed a broad spectrum of lytic activity against 15 isolates, followed by Ecol-MHD7 (13 hosts), Ecol-MHD2 (8 hosts), and Ecol-MHD6 (6 hosts), respectively. The narrowest host range belonged to Ecol-MHD3 and Ecol-MHD5, which were only specific to their original host (STEC m145). Whereas, both latter phages in comparison with other isolated phages, produced the largest plaques (~3.5 mm) (Figure 1-A). The phage cocktail could lyse the lawn of 20 *E. coli* strains and one *Salmonella* isolates. Among the phages, only Ecol-MHD2 and Ecol-MHD7 were effective on *E. coli* O157:H7 NCTC12900. The heat-map of host ranges is shown in Figure 2.

Discussion

Developing antibiotic resistance is one of the global concerns in recent years. Bacteriophages could be promising alternatives to antibiotics and food antibacterial agents. Therefore, investigation of novel bacteriophages that are efficient on antibiotic-resistant pathogens is an urgent need, particularly in the developing countries [23]. In our study, we could not isolate any STEC-specific phages from various samples (Table 3, group A) by plating them without enrichment and with small scale enrichment. Similar to our results, Oot et al. [26] could not isolate any *E. coli* O157-infecting phages out of 60 samples by using direct plating without enrichment. These results demonstrated that the levels of O157:H7-infecting phages in environmental samples could be extremely low [26,27]. However, there are some reports to approve the isolation of *E. coli*-infecting phages by applying direct plating protocol, from sewage effluent, wastewater [28,29], and stool samples [30] using other *E. coli* strains instead of 12900 as the indicator host.

It has been suggested that for recovering broad-spectrum phages, which are particularly virulent against desirable bacteria, a volume of sample greater than 1 ml, a variety of host bacteria, and multiple cycles of selection [31], as well as an extensive enrichment, is necessary [27]. Therefore, an extensive enrichment by using different *E. coli* strains and isolates was applied to some of the untested raw samples (Table 3, group B). The results demonstrated that 5 samples out of 7 contained *E. coli*-infecting phages.

Samples taken from cattle fecal slurries are the richest sources for isolating *E. coli*-specific phages [32,33]. Phages in our study were recovered from wastewater, minced meat, cattle cartilage, and vegetables, with the highest concentration and variety in the septic wastewater of a veterinary clinic. In contrast with our data, in a study, no *E. coli* O157:H7-infecting phages were detected in meat and vegetables [29].

According to the results from host range assay, as shown in Figure 2, five phages were efficient on a broad spectrum of *E. coli* isolates including EHEC, STEC, EAEC, EPEC and ETEC pathotypes and were

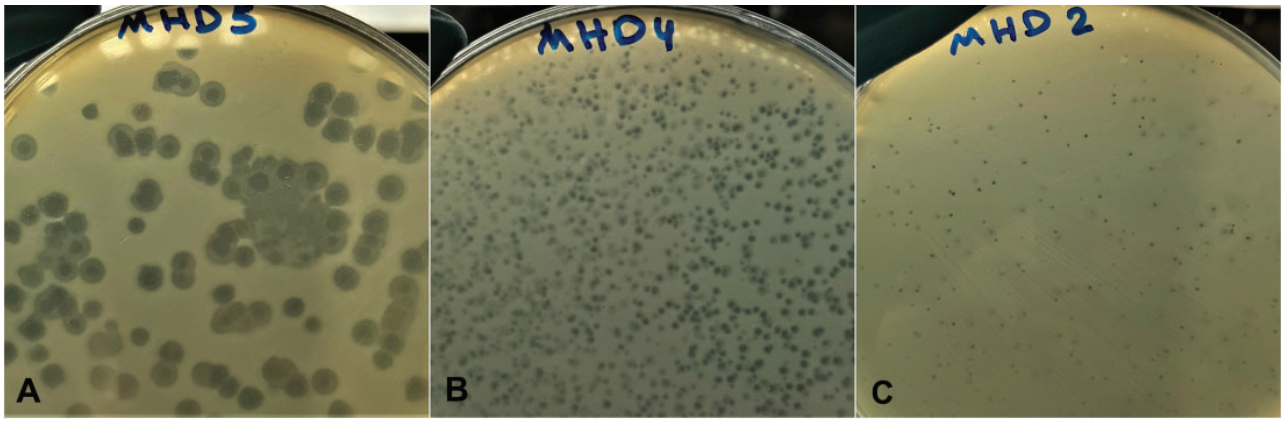

Figure 1.
Different sizes of plaques produced by some of isolated phages such as Ecol-MHD5 (A), *Ecol*-MHD4 (B) and Ecol-MHD2 (C).

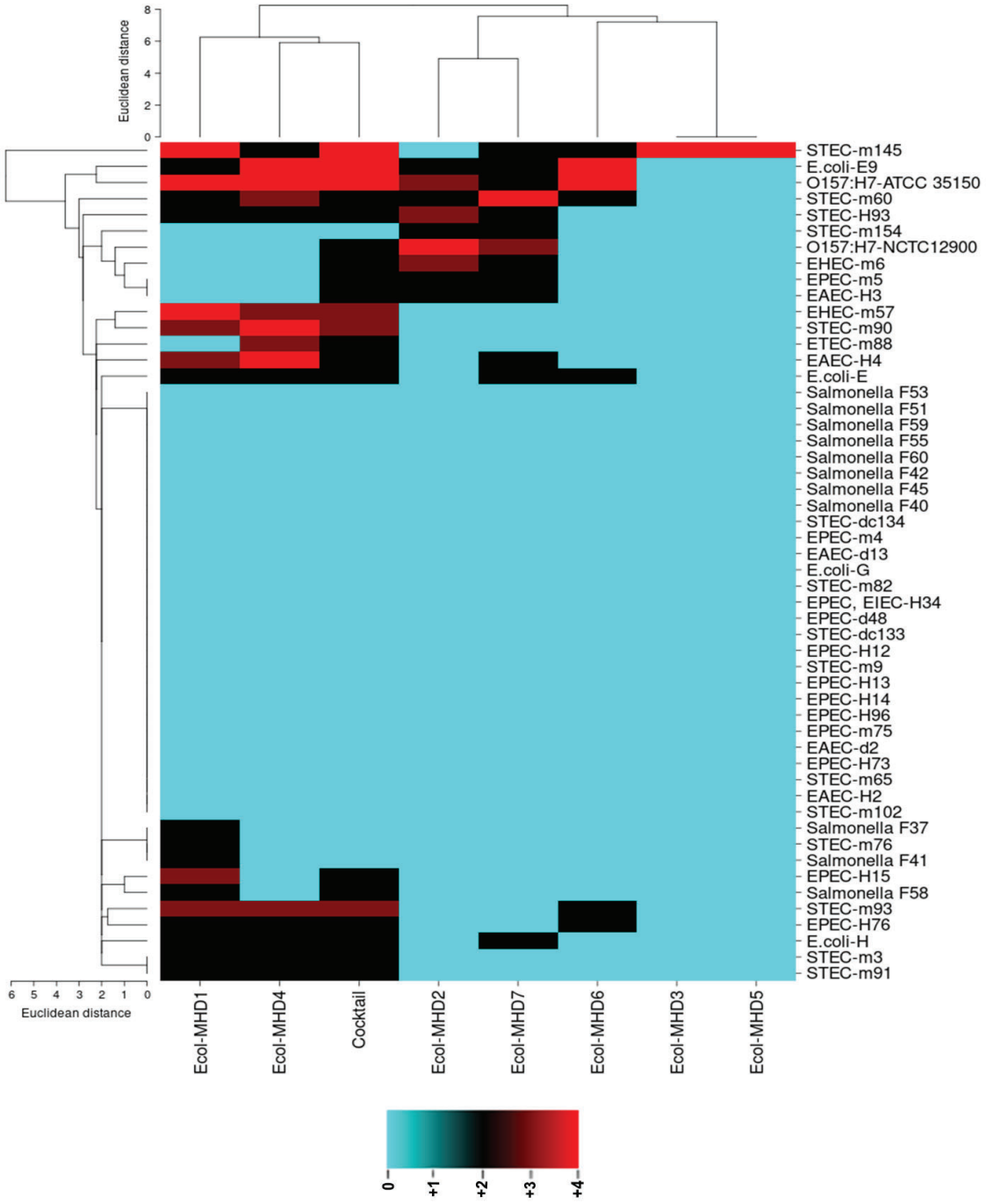

Figure 2.
Heat-map of host range of isolated phages; phages are displayed on the x-axis and bacterial isolates and strains on the y-axis. The degree of lysed lawn scored from +4 to 0. +4: complete clearing, +3: clearing throughout but with faintly hazy background, +2: substantial turbidity throughout the cleared zone, +1: a few individual plaques, 0: no clearing – but a spot may be seen where the pipette tip touched the agar.

highly infective for *E. coli* O157:H7 35150 (presence of eaeA, stx1, and stx2 genes), while only two could infect *E. coli* O157:H7 12900 (stx negative). This implies that the prevalence of *E. coli* O157:H7-specific phages could be higher than if *E. coli* 12900 was the only strain using in phage isolation procedures. In agreement with our findings, in a study conducted by Oot et al. [26] , samples that were negative by using *E. coli* 12900 for phage isolation, yielded 93% phage recovery by changing the indicator host.

Phage Ecol-MHD1 was polyvalent against a variety of field- and patient-derived pathogenic *E. coli* (33%) and three *Salmonella* isolates (out of 11), as shown in Figure 2.

Many phages specific to *E. coli* [34–40], and polyvalent phages against *E. coli* and *Salmonella* strains

[33,41,42] have been previously reported. However, the risk of developing EHEC complications such as HUS depends on virulence factors of EHEC strains as well as host and environmental factors [6]. In view of this fact, the regional prevalence of pathogens and the emergence of antibiotic resistance should be taken into account for evolving the phage biocontrol approach. To our knowledge, this study was the first attempt in Iran to investigate effective phages against pathogenic food- and patient-derived *E. coli* and *Salmonella* isolates from northeast of Iran.

In conclusion, the phages isolated in this study, in particular Ecol-MHD1, MHD4, MHD7, MHD2, and MHD6, can be suggested as potential candidates for phage biocontrol approach and therapy. They could be used in phage cocktails or combination with other techniques as preventive or therapeutic agents against foodborne E. *coli* infections in humans.

Material and methods

Bacterial strains

A total of 52 bacterial strains and isolates, including two standard EHEC, 39 pathogenic *E. coli*, and 11 *Salmonella* isolates used in this study were provided by a simultaneous study (unpublished study). Strains and isolates, their pathotypes, origins, and antibiotic resistance profiles are shown in Tables 1 and 2.

Sampling

25 samples with a variety of sources from wastewater and cow feces to meat and vegetables (Table 3) were collected to be screened for STEC-infecting phages. Of them, 18 samples were prepared both without enrichment and with enriching a small volume of filtrates and categorized as group A in Table 3. While the remaining samples (n=7) were prepared with an extensive enrichment and categorized as group B (Table 3). Sampling occurred during spring and summer in Mashhad and its suburbs.

Sample preparation for bacteriophage screening

Sample preparation procedures are briefly explained as follow:

1. **Without enrichment.** In order to isolate diverse phages with no biases, samples in group A were prepared without enrichment for direct plating. Initial preparation was accomplished, as was described by Gill and Hyman's [43] with some modifications. Briefly, liquid samples (30 ml) were centrifugated directly, while solid samples (5 ml) centrifugated following soaking in 30 ml sodium chloride-magnesium sulfate (SM) buffer (5.8 g NaCl, 2.0 g $MgSO_4$ x 7 H_2O, 50 ml 1 M Tris (Merck, MSD, Darmstadt, Germany) pH 7.5, filled up with distilled water to 1000 ml) and overnight incubation in the refrigerator. The centrifugation was conducted initially for 20 min at 3500 rpm in order to sediment large particles and debris, and then 2 ml of supernatants were centrifuged (Sigma 1-14, GmbH, Germany) for 10 min at 13000 rpm. Supernatants were then filtered through 0.22-micrometer membrane filters (MS® PES, Membrane Solutions, LLC, USA), and filtrates were stored in the refrigerator until further use.

2. **Small scale enrichment.** 100 µl of filtrates from group A samples was added to 5 ml LB broth (Merck, MSD, Darmstadt, Germany) containing 1mM $CaCl_2$ (Merck, MSD, Darmstadt, Germany) and 100 µl overnight culture of *E. coli* O157:H7 NCTC 12900. The suspensions were incubated in an incubator (GFL 30131, mbH, Burgwedel, Germany) shaking at 50 rpm for 24 h at 37 °C. Then they were centrifuged for 10 min at 13000 rpm and afterward filtered through 0.22-micrometer membrane filters. Filtrates stored at 4 °C and used for further phage screening.

3. **Extensive enrichment.** In group B, samples were enriched following the methodology of Van Twest and Kropinski [44] with some modifications. In brief, liquid samples (50 ml) were centrifugated for 30 min at 3500 rpm, and supernatants (30 ml) were mixed with double strength LB broth (30 ml) supplemented with 1mM $CaCl_2$. The mixture was then inoculated with 100 µl of overnight culture of desired bacterial strains including *E. coli* O157:H7 12900, E9 (Hospital isolate), m145 (antibiotic-resistant (ABR) STEC), and m6 (ABR EHEC) and incubated for 48 – 72 h at 37 °C while shaking at 50 rpm. Solid samples (10 ml) were added to SM buffer (40 ml) and kept overnight in the refrigerator. They were then centrifuged for one hour at 3500 rpm, and the supernatants were enriched in the same manner. After the incubation period, all samples were individually filtered through 0.22-micrometer membrane filters, and filtrates were stored at 4 °C until further use.

Sample screening for STEC-infecting bacteriophages

All filtrates were initially screened for the presence of phage by applying spotting assay, as described by Akhtar et al. [45] with some modifications. For this purpose, several bacterial strains and isolates including *E. coli* O157:H7 NCTC 12900 and ACTC 35150, m3 (ABR STEC), m6 (ABR STEC), m57 (ABR EHEC), m60 (STEC), m90 (ABR STEC), m91 (ABR STEC), m145 (ABR STEC), and E9 (Hospital isolate) (presented in Table 1) were cultivated in LB broth supplemented with 1mM $CaCl_2$. 100 µl of their overnight cultures was inoculated into 5 ml molten LB overlay agar (0.4% agar; Quelab, Inc, Montréal, Canada), and the mixture was overlaid on 1.5% LB underlay agar supplemented with 1mM $CaCl_2$. The overlay agar was allowed to solidify at room temperature. Subsequently, 10 µl of each sample was spotted on the top agar and left at room temperature until the drop dried and then incubated for 24 h at 37 °C. Samples that produced clear zones or single plaques on any of the bacterial lawns were considered as positive and selected for phage isolation. Complete clear spots were picked and kept for 24 h in SM buffer in the refrigerator, and after centrifugation and microfiltration, filtrates used for further procedure of phage isolation. All negative samples were rescreened for plaque formation on the lawn of *E. coli* 12900 by using double agar overlay plaque assay.

Isolation, purification, and propagation of bacteriophages

For phage isolation and purification, positive samples were investigated by using double agar overlay plaque assay, as it was described by Kropinski et al. [46]. Briefly, 100 µl of the overnight culture of host *E. coli* isolates and 100 µl of filtrate from each positive sample was inoculated into 5 ml 0.4% Luria-Bertani (LB) overlay agar, then plated on 1.5% LB underlay agar supplemented with 2mM $CaCl_2$ and incubated at 37 °C overnight. Individual plaques were picked by a sterile needle, soaked in 1ml SM buffer, and stored in the refrigerator for 24 h. Phages were purified by centrifugation of the suspensions for 10 min at 13000 rpm followed by sterile filtration. The filtrates were plated a minimum of three times to obtain purified phages.

Phage propagation was accomplished following the methodology of Viazis et al. [33] with some modifications. In brief, 100 µl of each phage at titers of 10^4 to 10^6 PFU/ml along with 100 µl of the

Table 1.
Characteristics of *E. coli* strains.

E. coli	Origin	Pathotype	Antibiotic resistance profile		
			Resistant	Intermediate	Susceptible
d2	raw milk	EAEC	CTX	CAZ, FEP, GEN, AMP, CHL, IPM, AZM	FOX, CIP, SXT, TET
d13	raw milk	EAEC	AMP	CTX, FEP	CAZ, FOX, CIP, GEN, SXT, TET, CHL, IPM, AZM
d48	raw milk	EPEC	CTX, CAZ, AMP	FOX, AZM	FEP, CIP, GEN, SXT, TET, CHL, IPM
dc133	cheese	STEC	CTX, AMP, IPM	CAZ, GEN, CHL, AZM	FEP, FOX, CIP, SXT, TET
dc134	cheese	STEC	CTX, CAZ, FEP, FOX, TET	IPM	CIP, GEN, SXT, AMP, CHL, AZM
m3	minced meat	STEC	FEP, FOX, SXT, AMP, TET, CHL, IPM	CTX, CAZ, GEN	CIP, AZM
m4	lamb	EPEC	CTX, CAZ, CIP, SXT, AMP, TET, CHL	FOX, GEN, IPM	FEP, AZM
m5	minced meat	EPEC	CIP, GEN, SXT, AMP, TET	CTX, CAZ	FEP, FOX, CHL, IPM, AZM
m6	lamb	EHEC	CTX, CIP, SXT, AMP, TET	CAZ, GEN	FEP, FOX, CHL, IPM, AZM
m9	minced meat	STEC	CTX, CIP, GEN, SXT, AMP, TET, AZM	CAZ, FEP	FOX, CHL, IPM
m57	lamb	EHEC	FEP, FOX, SXT, AMP, TET, CHL, IPM	CTX, CAZ, GEN	CIP, AZM
m60	lamb	STEC	-	AMP	CTX, CAZ, FEP, FOX, CIP, GEN, SXT, TET, CHL, IPM, AZM
m65	lamb	STEC	CTX, CAZ, FEP, GEN, SXT, AMP, TET, CHL, AZM	-	FOX, CIP, IPM
m75	minced meat	EPEC	CTX, CIP, SXT, AMP, TET	GEN, IPM, AZM	CAZ, FEP, FOX, CHL
m76	minced meat	STEC	CTX, CAZ	GEN, AMP, CHL, AZM	FEP, FOX, CIP, SXT, TET, IPM
m90	lamb	STEC	TET, CHL	CAZ	CTX, FEP, FOX, CIP, GEN, SXT, AMP, IPM, AZM
m91	lamb	STEC	CTX, AMP, TET	CAZ, GEN, CHL	FEP, FOX, CIP, SXT, IPM, AZM
m93	lamb	STEC	TET	CAZ	CTX, FEP, FOX, CIP, GEN, SXT, AMP, CHL, IPM, AZM
m102	lamb	STEC	SXT, AMP, TET	CTX, CAZ, FOX, AZM	FEP, CIP, GEN, CHL, IPM
m145	lamb	STEC	CTX, CIP, SXT, TET, CHL, AZM	CAZ, FEP	FOX, GEN, AMP, IPM
m154	minced meat	STEC	CTX, CAZ	FOX, AMP	FEP, CIP, GEN, SXT, TET, CHL, IPM, AZM
H2	Hospital*	EAEC	CTX, CAZ, FOX, CIP, SXT, AMP, TET, IPM, AZM	-	FEP, GEN, CHL

H3	Hospital	EAEC	CTX, CAZ, FOX, CIP, SXT, TET, IPM	AZM	FEP, GEN, AMP, CHL
H4	Hospital	EAEC	CTX, CAZ	AMP, CHL	FEP, FOX, CIP, GEN, SXT, TET, IPM, AZM
H12	Hospital	EPEC	-	AMP	CTX, CAZ, FEP, FOX, CIP, GEN, SXT, TET, CHL, IPM, AZM
H13	Hospital	EPEC	CTX, CAZ, FEP, GEN, SXT, AMP, TET, AZM	CIP	FOX, CHL
H14	Hospital	EPEC	CTX, SXT, AMP, TET	CAZ, CHL, IPM, AZM	FEP, FOX, CIP, GEN
H15	Hospital	EPEC	-	CTX, CAZ, AMP	FEP, FOX, CIP, GEN, SXT, TET, CHL, IPM, AZM
H34	Hospital	EPEC, EIEC	-	CTX	CAZ, FEP, FOX, CIP, GEN, SXT, AMP, TET, CHL, IPM, AZM
H73	Hospital	EPEC	CTX, TET	CAZ	FEP, FOX, CIP, GEN, SXT, AMP, CHL, IPM, AZM
H76	Hospital	EPEC	CIP, AXT, AMP, TET	CTX, FOX, CHL, IPM, AZM	CAZ, FEP, GEN
H93	Hospital	STEC	-	CTX, CAZ, AMP	FEP, FOX, CIP, GEN, SXT, TET, CHL, IPM, AZM
H96	Hospital	EPEC	CTX, CAZ, FEP, GEN, SXT, AMP, TET, AZM	CHL	FOX, CIP, IPM
E9	Hospital		-	-	GEN, AMK, LVX, IPM, MEM, CIP, CAZ, NIT, CFZ
H	Cattle farm trough				
E	Cattle farm trough				
G	Cattle farm trough				
O157:H7	NCTC 12900				
O157:H7	ATCC 35150 EHEC				

*Hospital isolates taken from hospitalized children.
CTX: Cefotaxime 30, FEP: Cefepime 30, CAZ: Ceftazidime 30, FOX: Cefoxitin 30, CIP: Ciprofloxacin 5, GEN: Gentamicin 10, SXT: Trimethoprim 1.25-Sulfamethoxazole 23.75, AMP: Ampicillin 10, TET: Tetracycline 30, CHL: Chloramphenicol 30, IPM: Imipenem 10, AZM: Aztreonam 30. AMK: Amikacin 30, LVX: Levofloxacin 5, MEM: Meropenem 10, NIT: Nitrofurantoin 300. CFZ: Cefazolin 30.

Table 2.
Salmonella isolates and their origins

Salmonella	Origin
F37	mixed minced meat
F40	mixed minced meat
F41	mixed minced meat
F42	chicken meat
F45	chicken meat
F51	chicken leg
F53	chicken meat
F55	chicken meat
F58	beef
F59	beef

exponential phase of host strain was inoculated in 10 ml LB broth supplemented with 1mM $CaCl_2$. Cultures were incubated for 72 h with shaking at 37 °C, and meanwhile, 10 ml fresh LB broth was added to them. Subsequently, cultures were centrifugated at 3000 rpm for 15 min, and supernatants were filtered through 0.22 pore-size filters and stored at 4 °C.

Determination of phage host range.

Following the methodology of Kutter [27], spot testing exploration of the host range was accomplished. 39 pathogenic *E. coli* and 11 *Salmonella* isolates, which all were isolated from various meat, milk, cheese, cattle farm trough, and human samples obtained from different zones of Mashhad (unpublished data) and two standard strains (Table 1) were tested for phage susceptibility. Briefly, bacterial strains were grown in LB broth supplemented with 1mM $CaCl_2$ for 4 hours at 37 °C to reach an OD600 of 0.4 to 0.6. Cultures were individually plated on LB underlay agar as it was described earlier for screening samples. When overlay agar solidified, 10 µl of purified phages was pipetted on the overlay agar, and plates were incubated at 37 °C overnight. A phage cocktail consists of the same aliquots of seven selected phages was also tested for the spectrum of susceptible hosts. After 24 h, the sensitivity of bacteria to phages was determined considering the degree of lysed lawn and spots were classified according to a standard system for assessing bacterial infection by phages as described by Kutter [27]. This experiment was accomplished in two repetitions. Cluster analysis performed using CIMMiner software.

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Author Contributions

Conceived and designed the experiments: Golshan Shakeri. Performed the experiments: Golshan Shakeri. Analyzed the data: Golshan Shakeri. Research space and equipment: Abdollah Jamshidi. Contributed reagents/materials/analysis tools: Golshan Shakeri, Abdollah Jamshidi, Kiarash Ghazvini. Wrote the paper: Golshan Shakeri, Abdollah Jamshidi.

Conflict of Interest

The authors declare that they have no competing interests.

Table 3.
Samples and the methods of preparation

Samples prepared without enrichment and with small scale enrichment (Group A)	Samples prepared with extensive enrichment (Group B)
Minced meat	Minced meat
- beef (n=2)	- beef (n=1)
- mixed beef and lamb (n=6)	- mixed beef and lamb (n=1)
Cattle slaughterhouse	
- Septic wastewater (n=3)	Veterinary hospital
- Effluent (n=2)	- Septic wastewater (n=1)
	City aqueduct (n=1)
	Cow cartilage (n=1)
Cattle feces (n=5)	Cow fat (n=1)
	Vegetables (n=1)
Total (n=18)	**Total (n=7)**

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RESEARCH ARTICLE

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Detection of Ciprofloxacin resistance genes in *Escherichia coli* isolated from dogs with urinary tract infections

Muhammad Mustapha[a, b], Parveen Goel[a]

[a] Department of Veterinary Medicine, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana India.

[b] Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Maiduguri, Borno State, Nigeria..

ABSTRACT

This research was performed on uropathogenic *Escherichia coli (E. coli)* isolates and established the genes of resistance to ciprofloxacin between the isolates. A total of one hundred and three urine samples were tested for uropathogenic *E. coli* which were obtained from dogs with urinary tract infections (UTIs) using cultural isolation, antimicrobial susceptibility test, and polymerase chain reaction (PCR). The results revealed that genes associated with ciprofloxacin resistance are 24.3% positive for *E. coli.* The *E. coli* isolates were resistant to both ciprofloxacin and ampicillin (100%), highly susceptible to chloramphenicol (84.0%), and less susceptible to gentamycin (44.0%) and amikacin (40.0%). The PCR tests showed the presence of the ParC (in 25 samples; 100%), GyrA (in 25 samples; 100%), and GyrB (in 4 samples; 16.0%) genes. The findings of the present study showed an upsetting rate of ciprofloxacin and ampicillin resistance among the *E. coli* isolates from dogs with UTIs.

Keywords

Dog, Uropathogenic E. coli, Antibiotic-resistant genes, Ciprofloxacin, PCR

Abbreviations

PCR: polymerase chain reaction
UTI: urinary tract infection
QRDR: quinolone-resistance determining region

Corresponding author: Muhammad Mustapha
Email: tanimuzimbos@unimaid.edu.ng

Introduction

Escherichia coli is the most common pathogen associated with urinary tract infections (UTIs) in dogs and cats, and many fluoroquinolones have been accepted for UTIs treatment in dogs and cats [1,2,3]. Resistance to fluoroquinolones is, however, increasing following their widespread clinical use in veterinary medicine [4]. Thus, treatment can be more complicated, particularly for those fluoroquinolones resistant *E. coli* isolates that show multidrug resistance (MDR) phenotypes [3, 5]. These resistances are both a medical problem for failure of treatment in veterinary medicine practice, and a public health worry for resistant *E. coli* transmission from companion animals to humans, and also in reverse order [2,6]. *E. coli* resistance to fluoroquinolones mainly results from the progressive accumulation of different point mutations in the genes that encode the target enzymes of fluoroquinolones, DNA gyrase (encoded by gyrA and gyrB) and topoisomerase IV (encoded by parC and parE). Such mutations are chiefly found in gyrA's quinolone-resistance deciding regions (QRDR) and its homologous parC region, which lead to multidrug resistance [7, 8]. However, decreased intracellular accumulation of fluoroquinolones in association with AcrAB-TolC system increased efflux pump activity [2]. Manipulation of the fluoroquinolone structure is among the approaches taken by pharmaceutical companies that could mitigate emerging resistance. Different molecular architectures may lead to a change in primacy targets for identical bacteria [9, 10]. Primacy is typically calculated using in vitro selection tests which classify the first mutation that confer resistance in the initial target protein, with mutations that subsequently occurred in the secondary target. Nevertheless, fluoroquinolones are commonly used in the therapy of UTIs due to a high concentration in the urinary tract and good concentrations on tissue [11]. However, there is comparatively less knowledge about the molecular mechanisms for resistance to ciprofloxacin in dog isolated uropathogenic *E. coli*.

The current study aims to use a molecular technique to identify the gene of resistance to ciprofloxacin and its distribution in *E. coli* associated with urinary tract infections.

Results

Assessment of a total of 103 urine samples from dogs of different breeds, genders, and age groups afflicted with urinary tract infections (UTI) divulged 25 (24.3%) of *Escherichia coli*.

Antimicrobial susceptibility profile of E. coli

The findings of antimicrobial susceptibility test showed that the isolates were resistant to ciprofloxacin (25 isolates; 100%), ampicillin (25 isolates; 100%), ceftriaxone (20 isolates; 80.0%), ceftizoxime (19 isolates; 76.0%), nitrofurantoin (14 isolates; 56.0%), streptomycin (13 isolates; 52.0%) and amoxyclav (12 isolates; 48.0%) while they were susceptible to chloramphenicol (21 isolates; 84.0%), gentamycin (11 isolates; 44.0%) and amikacin (10 isolates; 40.0%) as depicted in Table 2.

Ciprofloxacin resistance genes

PCR was used to explore the presence of the ParC, GyrA, and GyrB genes (Figures 1, 2, 3, and Table 1). In all 25 (100%) *E coli* isolates, ParC and GyrA were present, while only 4 (16.0 %) isolates harbored GyrB gene.

Discussion

Urinary tract infections in companion animals, particularly dogs, are life threatening. Nonetheless, if detected sooner, the disease is curable with antibiotics. This may happen at any age, but the highest incidence is observed in adult dogs. Infection occurs mainly in a dog's urinary tract as a result of bacterial colonization. *Escherichia coli* is by far the most important cause of urinary tract diseases in dogs and other pets [16,

Table 1.
Distribution of ciprofloxacin resistance genes among 25 *E. coli* isolates from dogs with UTI

Antibiotics	No. of resistant isolates (%)	No. of positive isolates (%)
Ciprofloxacin	ParC	25 (100)
	GyrA	25 (100)
	GyrB	4 (16)

17]. The prevalence of bacterial infections in dogs with urinary tract is not completely known. In the present study out of 103 urine samples of dogs with UTIs symptoms, *Escherichia coli* was isolated from 25. Chang et al. [18] reported that 114 dogs out of 201 dogs were positive for *E. coli.* Moyaert et al. [19] isolated *E. coli* from dogs with UTIs symptoms from 204 out of 437 urine samples. Kuan et al. [20] recorded that 146 out of 200 urinary specimens from dogs diagnosed with UTIs were cultivated with *E. coli*. Liu et al. [21] also confirmed *E. coli* growth detection in 106 out of 174 dogs investigated. Such data suggest that infections of the urinary tract caused by bacteria are mainly diagnosed by urinalysis, clinical tests, and culture. The pattern of antimicrobial susceptibility of the *E. coli* isolates reported in the current studies has identified a very high level of ciprofloxacin and ampicillin resistance. Fluoroquinolone/quinolone class ciprofloxacin exhibits its antimicrobial activity by inhibiting DNA gyrase and topoisomerase IV through alterations in target enzymes. The presence of mutations in the DNA gyrase enzyme's quinolone-resis-

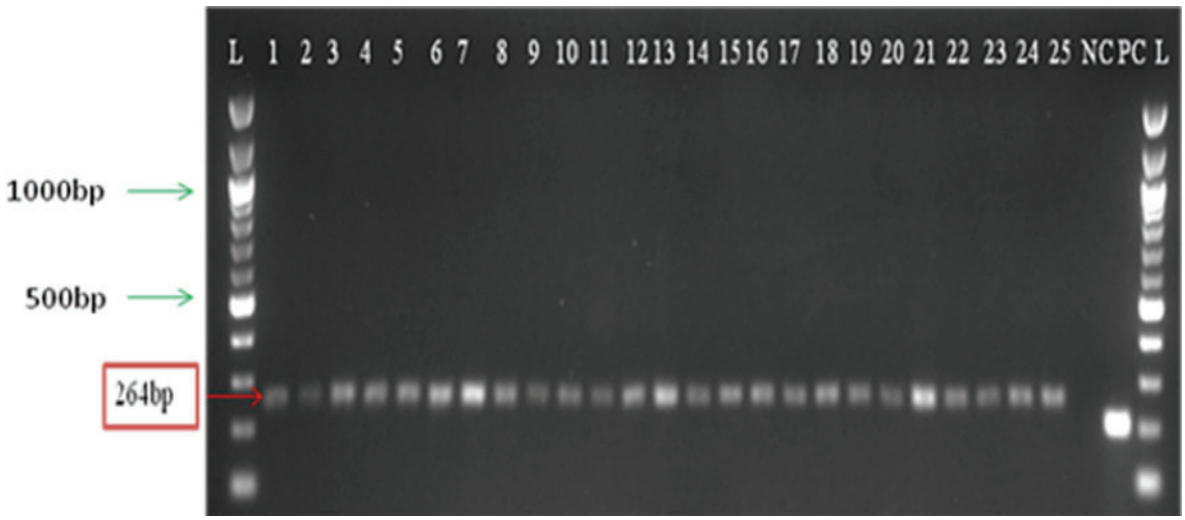

Figure 1.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (ParC) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 1-25 positive samples (264bp)
Lane NC: negative control. Lane PC: positive control

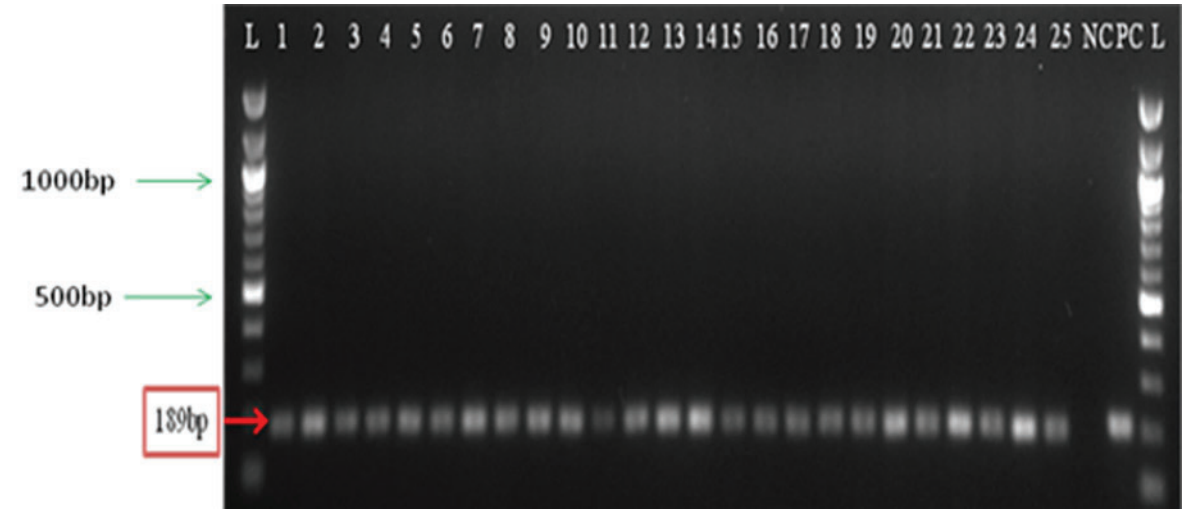

Figure2.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (GyrA) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 1-25 positive samples (189bp)
Lane NC: negative control. Lane PC: positive control

Figure 3.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (GyrB) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 3, 7, 21 & 23 positive samples (203bp)
Lane: 1-2, 4, 5, 6, 8-20, 22, 24 & 25 negative samples
Lane NC: negative control.

Table 2.
Antibiotic susceptibility pattern of *E. coli* isolated from dogs (n = 25)

Antibiotics	No. of resistant isolates (%)	No. of intermediate resistance isolates (%)	No. of susceptible isolates (%)
Amikacin	9 (36.0)	6 (24.0)	10 (40.0)
Amoxyclav	12 (48.0)	3 (12.0)	10 (40.0)
Ampicillin	25 (100)	0 (0.0)	0 (0.0)
Ceftizoxime	19 (76.0)	0 (0.0)	6 (24.0)
Ceftriaxone	20 (80.0)	0 (0.0)	5 (20.0)
Chloramphenicol	3 (12.0)	1 (4.0)	21 (84.0)
Ciprofloxacin	25 (100)	0 (0.0)	0 (0.0)
Gentamicin	9 (36.0)	5 (20.0)	11 (44.0)
Nitrofurantoin	14 (56.0)	5 (20.0)	6 (24.0)
Streptomycin	13 (52.0)	3 (12.0)	9 (36.0)

tance determining region (QRDR) is the main cause of high-fluoroquinolone resistance in gram-negative bacteria such as *E. coli* [22]. This high degree of resistance observed against ciprofloxacin is possibly explained by cross-resistance with the other members of quinolones such as enrofloxacin, nalidixic acid, and norfloxacin [23].This finding is close to that observed in a previous research [21]. However, Oliveira et al. [24], and Thungrat et al. [25] published previously on fluoroquinolone tolerance in companion animals.

Other studies have nevertheless reported a higher degree of canine resistance to ciprofloxacin in *E. coli* [26, 27]. Besides, increased resistance to fluoroquinolone in companion animals might restrict the treatment of uropathogenic *E. coli* infections in humans who might develop *E. coli* infection from their dogs [21]. This also demonstrates that the widespread use of ampicillin and other Beta lactams antibiotics may be related to the selection of antibiotic resistance mechanisms in pathogenic and non-pathogenic *E.*

coli isolates [28]. Beta-lactam antimicrobial resistance in *E. coli* is primarily mediated by B lactamases that mostly hydrolyze the beta-lactam ring and hence deactivate the antibiotics [29]. Ampicillin resistance found in this research was higher than the previously recorded studies of the companion animals. [30], while the results are consistent with those of Gilliver et al. [31], Wedley et al. [32], Wong et al. [33], Nhung et al. [34], Liu et al. [35], Cavalho et al. [36] and Liu et al. [21]. Furthermore, Allen et al. [37] reported high ampicillin resistance. The high degree of antibiotic resistance among the isolates of *E. coli* reported in this study is directly due to the colossal number of cases referred to the Veterinary Clinical Complex (VCC) hospital from primary health care centers and private practitioners with complicated diseases and repeated cases where dogs have already been exposed to various groups of antibiotics without any prior laboratory evaluation as previously stated by Mustapha et al. [38]. The presence of drug-resistant *E. coli* in dogs presents a possible public health threat. The role of livestock as a source of pathogen transmission to humans was well established, mainly through nutritional exposure, but also through direct contact [39, 40]. In Hisar, though, dogs usually share the entire home setting with their owners and are considered as family members [38]. Therefore, close interaction with dogs is typical among humans. It can serve as an essential route of transmission of *E. coli* to humans. Indeed, an increasing body of evidence suggests that resistant bacteria or mobile resistance determinants can be passed between dogs and humans through direct contact. [41, 42]. The isolates of *E. coli* are particularly susceptible to chloramphenicol, gentamicin, and amikacin. This indicated that chloramphenicol has broad-spectrum activity against gram-positive and gram-negative organisms while also providing protection from anaerobic infection. Moreover, gentamicin and amikacin of the same aminoglycoside class have exhibited high activity against uropathogenic *E. coli,* which could be the product of aminoglycoside complexity and possibly due to the route of administration [43].

The genes ParC, GyrA, and GyrB were found to be associated with the resistance of fluoroquinolones to all the *E. coli* urinary tract isolates tested in this study. The genes ParC and GyrA were found in all isolates, while only four isolates had GyrB genes. In addition, the high-level resistance isolates found in this study that contain multiple mutations within the *E. coli* isolates at different stages. This was consistent with the previous research which showed that enrofloxacin-resistant uropathogenic *E. coli* isolates had a mutation of two points, one in ParC and the other in GyrA [18]. In addition, several researchers have documented over-expression of the ACrAB –TolC system causing multiple drug resistance like fluoroquinolone [44].

In conclusion, the study clearly reported a large number of pathogenic *E. coli* strains in the urine of dogs with high levels of resistance to ciprofloxacin and ampicillin. The molecular study shows that the genes responsible for the resistance of ciprofloxacin in clinical isolates of *E. coli* were ParC, GyrA, and GyrB. Monitoring the patterns of antibiotic prescription and resistance in companion animal medicine, serves as an early indicator for the changes in the susceptibility of clinical isolates to antibiotics via cultural isolation, antimicrobial susceptibility, and PCR-based amplification of antibiotic resistance genes that provide useful data to the veterinarians. These are more effective therapeutic protocols in the control of chronic or recurrent UTIs in dogs. Prescription of antibiotics such as chloramphenicol, gentamicin, and amikacin may be more effective for treating UTIs in dogs.

Material and methods

Source of Animals

The present research was performed on dogs of various breeds, different age groups and of both sexes, presented at the Veterinary Clinical Complex (VCC), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar with History / clinical symptoms of anorexia, vaginal discharge, failure to urinate or just dribbling a small amount of urine, dark urine, dysuria, increased water intake, constant licking of urinary opening, soiling in appropriate areas, hematuria, vomiting, lethargy, fever, proteinuria, weight loss, heavy urinal odor (smelly urine), back arching (cystitis), and oliguria.

Sample Collection

A maximum of 103 urine samples was collected aseptically from dogs diagnosed with UTIs in sterile test tubes between February 2017 and January 2018 using cystocentesis. Where each sample was classified with an identifier number and collection date. The samples were transported for evaluation immediately in the central laboratory at the college.

Bacteriological Examination

The aseptically collected fresh urine samples were inoculated and streaked onto a 5% sheep blood agar (BA) (HiMedia, Mumbai, India) and MacConkey's lactose agar (MLA) (HiMedia, Mumbai, India) plates separately with the aid of a 4 mm diameter platinum loop. Over 24 - 48 hours, the plates were aerobically incubated at 37 °C and analyzed for the presence of any standard distinct purple / pink colonies. Positive isolates were streaked onto Eosine Methylene Blue Agar (EMB), (HiMedia, Mumbai, India), a selective medium for *E. coli* and the plates were aerobically incubated for 24 hours at 37 °C. The appearance of blue, green colonies with a metallic luster on EMB was presumptive to indicate the existence of *E. coli*. Biochemical techniques have further confirmed all positive *E. coli* isolates, including Indole, Methyl Red, Voges Proskauer, Glucuronidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose, and Sorbitol) using commercially KB010 HiE.ColiTM identification Kit (HiMedia Mumbai, India) following the guidelines of the manufacturer.

Antimicrobial Susceptibility Testing

The drug sensitivity test of Escherichia coli isolates was assessed using the Bauer-Kirby method [12] by using commercially prepared disc (Himedia, Mumbai, India) with a well-known antibiotic concentration. Using a sterile platinum loop, small amounts of test culture were transferred to a brain heart infusion broth (BHI) tube and incubated at 37 °C for 2-5 hours to achieve turbidity. The broth culture was then uniformly distributed by smearing on the surface of Mueller Hinton agar plates with the aid of a sterile cotton swab. The antibiotic discs were placed on the agar and gently squeezed to provide a smooth, near contact with the medium with a sterile forceps. The inoculated plates were held 3-4 hours at low temperatures to allow the antibiotics to pre-diffuse. For 24 hours, the plates were then incubated at 37 °C. The sensitivity was observed based on the manufacturer's zones size definition map. Sensitive (S), intermediate (I), and resistant (R) results were reported. Amikacin (AK) 30mcg, ampicillin (AMP) 10mcg, amoxyclav (AMC) 30mcg, ceptriaxone (CTR) 10mcg, ceftizoxime (CZX) 30cmg, chloramphenicol (C) 30mcg, ciprofloxacin (CIP) 10mcg, gentamycin (GEN) 30mcg, nitrofurantoin (NIT) 300mcg and streptomycin (S) 25mcg (HiMedia, Mumbai, India) are the 10 antibiotics used in this study.

Bacterial DNA Insolation

Following the manufacturer's instructions, DNA of *E. coli* from all the positive isolates was extracted using commercially available PureLink Genomic DNA mini-kit (Invitrogen, USA). The extracted DNA was stored at -20 °C until further processing.

Detection of Ciprofloxacin resistance gene

The presence of resistance genes to ciprofloxacin in *E. coli* DNA extracts was determined by standard PCR. Table 3 gives the primers sequences, target genes, product size, and references. The standard PCR was conducted in a 25 µl volume reaction in Veriti thermo cycler (ABI, USA) containing 6 µl of template DNA, 1µl of each of the primers (10 pmoles concentration), 12.5 µl Phusion PCR Mastermix (2x) (High Fidelity, USA), 1µl DMSO and 2.5 µl nuclease-free water. The amplification process involved initial denaturation at 98 °C for 30 sec, followed by 35 denaturation cycles at 98 °C for 10 secs, annealing at 60 °C for 30 secs, extension at 72 °C for 30 secs, and a final extension at 72 °C for 5 mins. The PCR products were analyzed with 1.5 % agarose gel electrophoresis and visualized under the Gel Doc UV Trans illuminator XR 170-8171 (BIO-RAD, India).

Descriptive statistics were used to analyze the data obtained with JMP Version 11 (SAS, Cary, NC, USA).

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Author Contributions

The experiment was designed by MM and PG. The experiments were conducted by MM. The data were interpreted by MM and PG. The figures were prepared by MM and the manuscript was written by MM. The manuscript was reviewed by PG. The final manuscript was read and approved by all authors.

Conflict of Interest

The authors have no conflict of interest.

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Table 3.
Oligonucleotide primers used for amplification of ciprofloxacin resistance genes

Target Genes	Primer sequence (5'-3')	Amplified Product (bp)	Reference
GyrA	F:ACGTACTAGGCAATGACTGG R:AGAAGTCGCCGTCGATAGAAC	189	[13]
GyrB	F: CAGACTGCCAGGAACGCGAT R:biotin AGCCAAGTGCGGTGATAAGA	203	[14]
ParC	F: CAGACTGCCAGGAACGCGAT R:biotin-AGCCAAGTGCGGTGATAAGA	264	[15]

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RESEARCH ARTICLE

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The Association between erythropoietin, vitamin D3 and parathormone levels and kidney lesions in buffaloes *(Bubalus bubalis)*

Zana Shahabi[a], Mohammad Razi Jalali[b], Babak Mohamadian[c], Mohammad Rahim Haji Hajikolaei[b], Mohammad Taha Jalali[d]

[a] DVSc student of Clinical Pathology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[c] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[d] Hyperlipidemia Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Renal disorders in livestock may appear subclinically, leading to reduced production performance without any clinical signs, and causeing significant economic losses. In this research, blood and kidney tissue samples were obtained from 102 buffaloes (males and females) slaughtered at Ahvaz industrial slaughterhouse and sent to the laboratory for biochemical and histopathological analyses. Having used the conventional and specific staining methods along with the microscopic examination of the histopathological sections of the kidney tissue samples from buffaloes, 41 samples with no lesion were selected as control and 61 with renal lesions were considered as the lesion group. The samples with renal lesions were then divided into 4 subgroups including acute tubulointerstitial nephritis, chronic tubulointerstitial nephritis, urinary tubular inflammation, and congestion. Serum biochemical tests including parathyroid hormone, erythropoietin, and Vit D3 were measured in blood samples using commercial ELISA kits. After the statistical analysis of the data, the results of Vit D3 in the studied groups did not show any significant differences. However, the levels of erythropoietin and parathormone in buffaloes with renal lesions were significantly lower and higher than those in the control animals, respectively. The results also showed that most of the kidney lesions in the studied buffaloes led to a decrease in the production of erythropoietin hormone following the occurrence of such lesions and their functional effects on the infected kidneys. Besides, the effects of erythropoietin deficiency were reflected in the hemogram, and its resulting anemia was deemed to affect overall body health. As the findings showed, no significant differences were observed between male and female buffaloes in terms of the abundance of renal lesions and laboratory findings.

Keywords

Bubalus bubalis, Kidney lesions, Erythropoietin, Vitamin D3, Parathormone

Abbreviations

EPO: Erythropoietin
Vit D3: Vitamin D3
PTH: Parathormone
ATN: Acute tubulointerstitial nephritis
CTN: Chronic tubulointerstitial nephritis
UTI: Urinary tubular inflammation
CKD: Chronic kidney disease

Corresponding author:
Mohammad Razi Jalali
Tell/Fax: +98 6133336312
Email: jalali_m@scu.ac.ir

Introduction

Water buffalo (*Bubalus bubalis*) is an important livestock resource in many countries of Asia, including Iran, the Mediterranean region, and Latin America. It plays an important role in providing milk, meat, and draught power in agricultural systems, particularly in the developing countries. This productive, adoptive and multipurpose domestic animal species has thus received significant attention in national and international livestock arena in recent years. In addition to supplying the motive power for agriculture allied activities and transport, there is indeed a higher need for raising and successful maintenance of buffalo milk, milk products and meat production in the agriculture-based countries (1).

The breeding of buffalo as a source of milk, meat and labor in tropical regions of Iran and South Asian countries are thus of special economic importance (2). West and East Azarbaijan provinces, Khuzestan, Ardebil, Gilan, Mazandaran, Golestan and Lorestan are considered to be the breeding centers of buffaloes in Iran. According to the statistics provided by the Statistical Centre of Iran in 2017, the number of Iranian buffaloes is estimated to be 127 thousand, showing a decreasing trend in number of buffaloes than in previous years. In effect, due to the lack of proper breeding management, the rise in demand for buffalo meat and the lack of replacement for the slaughtered animals, have resulted in a decline in the population of buffaloes in Iran.

Though its natural habitat consists of hot and humid regions that are very favorable to microorganism and parasite proliferation, Bubalus bubalis is generally a healthy animal (3). However, like many other animal species, Bubalus bubalis is susceptible to most diseases and parasites that afflict cattle, though the effects of such diseases on the buffalo and its productivity are sometimes less evident. Among others, renal diseases are not uncommon in food animals and information from slaughterhouses can be considered a good source of data for the evaluation and monitoring of renal diseases in livestock (4).

Among body organs and tissues, kidney plays an important role in maintaining the health and adjustment of the internal environment of the body, and any damage to it may cause changes in blood biochemical parameters. Infectious agents, toxins, impacts, immune responses, kidney hypoxia, tumors, and the formation of urinary stones are among the causative agents of the kidney. Thus, awareness of such changes in kidney before the advent of clinical symptoms is the first essential step in improving the methods of prevention and management in livestock farms. In addition to economic losses, diseases affecting buffaloes might constitute an epidemiologic and zoonotic threat to humans. As such, problems concerning meat hygiene and possible health risks to consumers should be well documented during both ante-mortem and post-mortem examinations.

Different affections of kidney include developmental anomalies, growth, metabolic and circulatory disturbances, infarction and necrosis, diseases of glomeruli (viral glomerulonephritis, embolic nephritis, immune-mediated glomerulonephritis, immune-complex glomerulonephritis, glomerulosiderosis and glomerulolipidosis), tubulo-interstitial diseases (interstitial nephritis, granulomatous nephritis, and pyelonephritis), chronic renal diseases, urolithiasis and various neoplasms (5). However, there is little information on the renal pathological changes in buffaloes compared to those in other animal species (6, 7).

Erythropoietin (EPO), as a glycoprotein hormone produced primarily by the kidney, is a principal growth factor regulating the red blood cell production (8). Kidney is indeed the major EPO producing organ in adult life, while the liver produces only 10-15% of the total amount of EPO produced in the body (9).

It is also established that vitamin D is not simply a nutrient required for normal skeletal growth and development; rather it serves as a precursor to an intricate endocrine mechanism that maintains calcium and phosphorus concentrations in blood. More recently, vitamin D has been shown to have multiple physiological roles, including the control of cellular differentiation and proliferation as well as the activation of innate immune defenses. The new-found roles of vitamin D, along with its critical role in the calcium and phosphorus homeostasis, highlight the need to examine current practices for vitamin D supplementation in the dairy industry and question whether current recommendations are adequate for dairy cattle (10).

In turn, parathyroid hormone, as a single-chain polypeptide made up of 84 amino acids secreted by the chief cells in the parathyroid gland, augments the absorption of calcium from the intestines and simultaneously conditions its re-absorption from the skeleton. Also, parathormone (PTH) is the principal hormone involved in the fine regulation of the calcium homeostasis. The hypercalcemic biological action of PTH is indeed by its direct influence on the target cells in bones (osteoclasts and osteocytes) and kidney (proximal and distal tubules) along with its indirect action on the duodenum (11, 12).

Kidney lesions, in addition to having a direct impact on regulating the body's homeostasis, disrupt the production and metabolism of some hormones that directly affect the health of the kidney tissue. There-

fore, this research was aimed to study different types of histopathological disorders of kidney, the changes in EPO, PTH, and Vit D3 along with the correlation between the hormonal changes and histopathologic lesions. To those ends, blood and kidney samples were collected from buffaloes slaughtered at Ahvaz industrial slaughterhouse so as to investigate the pathomorphological and hormonal alterations in the samples.

Table 1.
Specimens from studied buffaloes according to gender and renal lesions.

Buffaloes	Male	Female	Total
Normal	32	9	41
Kidney lesion	56	5	61
Total	88	14	102

Table 2.
Changes in the Mean ± SE values of EPO, parathyroid hormone and Vit D3 in buffaloes according to renal lesions.

Group	Number	%	EPO	PTH	Vit D3
Healthy	41	40.20	49.32 ± 4.089 ^{bcdef}	21.34 ± 4.520 ^{c}	69.44 ± 6.399
ATN	18	17.65	31.89 ± 2.747 ^{a}	22.44 ± 3.601 ^{c}	63.06 ± 6.503
CTN	23	22.55	33.50 ± 2.846 ^{a}	31.50 ± 3.417 ^{abdef}	64.25 ± 4.642
UTI	13	12.74	34.69 ± 2.041 ^{a}	22.69 ± 4.385 ^{c}	65.15 ± 5.581
Congestion	7	6.86	36.43 ± 3.036 ^{a}	22.57 ± 3.359 ^{c}	67.86 ± 4.340
Total	102	100	37.28 ± 11.398	22.89 ± 4.693 ^{c}	64.85 ± 6.97

Different lower case letters (a-f) demonstrate significant diffeences between groups ($p < 0.05$)

Results

Histopathological findings

In this study, 102 kidney samples were obtained from apparently healthy buffaloes (88 males and 14 females) slaughtered at Ahvaz industrial slaughterhouse. Among them, 61 kidney samples had lesions and 41 others were healthy. From kidney samples with lesions, fifteen cases (14.7%) had gross lesions such as enlargement of kidney, severe congestion and hemorrhages. All kidney samples were then examined in the laboratory so as to identify the histopathological renal changes in bufalloes. The prevalence of histopathological renal changes including acute tubulointerstitial nephritis (ATN), chronic tubulointerstitial nephritis (CTN), urinary tubular inflammation (UTI) and congestion is presented in Table 2.

Analysis of hormones

The *t*-test analysis showed that the presence or absence of any renal injury was significantly correlated with the serum EPO levels ($p < 0.001$), and it was significantly lower in the groups with renal lesions than in the healthy one. As for vitamin D, there was no significant difference between healthy and kidney lesion groups. However, there was a significant elevation in PTH in CTN group compared to the healthy and other groups with different kidney lesions. This was likely related to the secondary renal hyperparathyroidism.

Statistical analysis

The results of statistical analysis for the groups with different lesions showed that EPO level in healthy group was significantly different from that in all other groups. It was also found that EPO level in the healthy group was significantly higher than that in other groups. Besides, the results showed that there were no significant differences between different groups with lesions in EPO levels. The two-way analysis of variance with two factors of gender and kidney lesions showed that renal injury alone was effective in the EPO levels ($p < 0.001$), while gender had no significant effect on the EPO levels. In the case of Vit D3, neither injury nor gender had a significant effect on the EPO values ($p > 0.05$).

Discussion

Little is known about the pathological kidney lesions in buffaloes in comparison to other animal species. Although some cases of kidney lesions in other animal species slaughtered at Ahvaz slaughterhouse have been investigated in some research studies, no study has been already conducted on buffalo kidney lesions in Khuzestan province. Thus, this research investigated the association between histopathological kidney lesions in slaughtered buffaloes and any possible changes in important indicators such as EPO, parathyroid hormone and Vit D3. As the findings showed, interstitial nephritis was considered as the most common pathological finding in this study. Although interstitial nephritis, either diffuse or focal, is most frequent in post-slaughter inspection in some species, it is rarely detectable in clinical examinations. However, chronic focal interstitial nephritis in kidney, known as a white spot, is a common finding in apparently healthy animals in post-slaughter inspection.

The results showed that from the 102 samples examined, 41 had no renal lesions (40.2%, Fig. 1). Also, most of the lesions were interstitial nephritis, comprising 41 samples (18 acute and 23 chronic samples) or 40.2% of the total cases (Figs. 3, 4). Of these, chronic interstitial nephritis had the highest number of lesions (22.5%), including connective tissue replacement in the interstitial spaces, especially around the glomerular network, infiltrated by mononuclear cells such as macrophages and lymphocytes. Besides, the EPO level was significantly decreased in the groups with chronic interstitial nephritis compared to the healthy group; however, no significant difference was observed in PTH and Vit D3 values between the groups.

Acute interstitial nephritis was, in turn, found to be second in abundance in 18 samples examined (17.65%) and comprised hyperemia and severe infiltration of mononuclear cells, especially lymphocytes and monocytes.

Among others, anemia is one of the main clinical problems in the chronic kidney disease (CKD). Different factors are indeed responsible for the decreased erythrocyte production, especially EPO synthesis. The mechanism by which EPO synthesis deficiency develops in CKD subjects has been attributed to a decrease in the number of kidney EPO-producing cells. In effect, EPO seems to be synthesized by a particular subtype of interstitial fibroblast and the loss of such cells, in the progressive interstitial fibrosis that characterizes CKD, has been supposed to be the cause of EPO deficiency (13).

The EPO levels in the acute interstitial nephritis group were significantly lower than that in the healthy group, but changes in PTH and Vit D3 were not significant in this group compared to those in the healthy group. Besides, it was found that thirteen samples (12.74%) in this group had tubular necrosis and hyaline casts (Fig. 5), and a significant decrease happened in the EPO level compared to that in the healthy group, but no significant change was observed in the PTH and Vit D3 values compared to those in the healthy group. The findings also showed that seven specimens (6.86%) had vascular congestion showing a significant decrease in the EPO values compared to that in the healthy group, while no significant change was observed in the PTH and Vit D3 values in this group compared to those in the healthy group (Fig. 2).

In turn, the active form of Vit D3 plays a very important role in mineral and bone metabolism, although it is synthesized mainly in the kidney. In CKD, there is an unusual accumulation of extracellular ma-

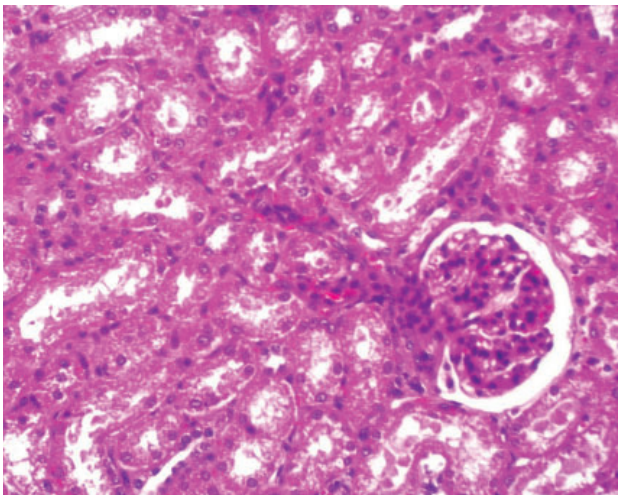

Figure 1.
The structure of the kidney tissue including the glomerular corpuscle and the urinary tubules is normally observed. H & E staining. Magnification: 100x

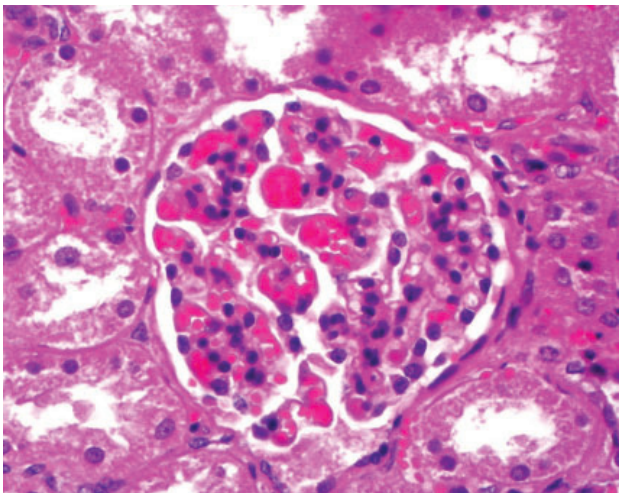

Figure 2.
Glomerular congestion and the decreased glomerular urinary pole space. H & E staining. Magnification: 400x

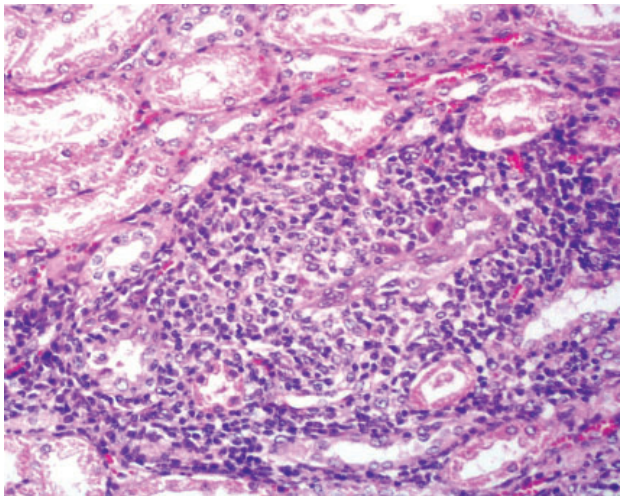

Figure 3.
The presence of mononuclear cells in the interstitial space, necrosis of the urinary tubules and the congestion of the affected areas. H & E staining. Magnification: 100x

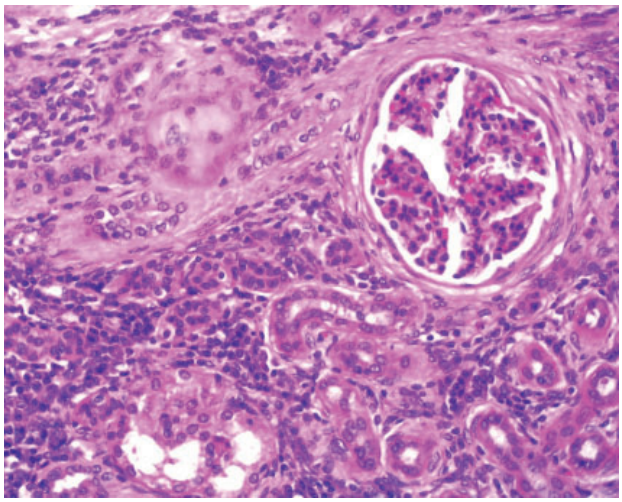

Figure 4.
The aggregation of fibroblasts around glomeruli, infiltration of mononuclear cells into interstitial tissue, the accumulation of connective tissue and the collagen fibers in the interstitial space without the presence of any congestion. H & E staining. Magnification: 200x

trix (ECM) proteins in the renal interstitium, a finding known as interstitial fibrosis (14). The results of Vit D3 concentration showed that all samples examined were within a normal value range, and there was no significant difference between the healthy and lesion groups in this regard. However, a statistically significant negative correlation was found between the levels of EPO and PTH in the CTN group. Indeed, the secondary hyperparathyroidism, as a common complication of CKD, resulted from the interaction of several different factors initiated by the loss of kidney tissue and the inability to excrete the daily load of phosphates, causing an increase in its serum levels. In effect, hyperphosphatemia stimulates the posterior liberation of fibroblastic growth factor 23 (FGF23) by osteocytes, which inhibits proteins NaPiIIa and NaPiIIc in the proximal convoluted tubule generating phosphaturia. It also inhibits the activity of the 1α- hydroxylase renal enzyme, diminishing the synthesis of active vitamin D (1, 25(OH)2D), leading to a reduction in the intestinal absorption of calcium and phosphorus, and serum levels of phosphorus. The final outcome of lowering phosphorus is an increase in the levels of FGF23 and hypocalcaemia, the latter causes an increase in the synthesis and liberation of parathyroid hormone (15, 16).

In Restrepo and Aguirre (2016) study on the level of vitamin D in patients with CKD stages, 21.1% of the patients had normal vitamin D levels, 70.1% were within the insufficient range, while 8.8% were in deficit. Moreover, a negative correlation was found between the levels of vitamin 25 (OH) D and the values of PTH. They also found low levels of vitamin 25(OH) D in patients with CKD, leading to the appearance of secondary hyperparathyroidism. The active vitamin D or calcitriol has indeed important functions in patients with CKD as it promotes the intestinal absorption of calcium and phosphorus, increases the distal tubular absorption of calcium in the kidney and exerts negative feedback on the parathyroid gland, lowering the synthesis and secretion of the parathyroid hormone (PTH). Thus, a sufficient supply of native vitamin D (25(OH) D) in the form of ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) is required for its synthesis in the kidney's proximal convoluted tubule. As such, a progressive reduction in the levels of active vitamin D has been observed in patients with CKD proportional to the decrease of their glomerular filtration rate. It has been assumed that this hap-

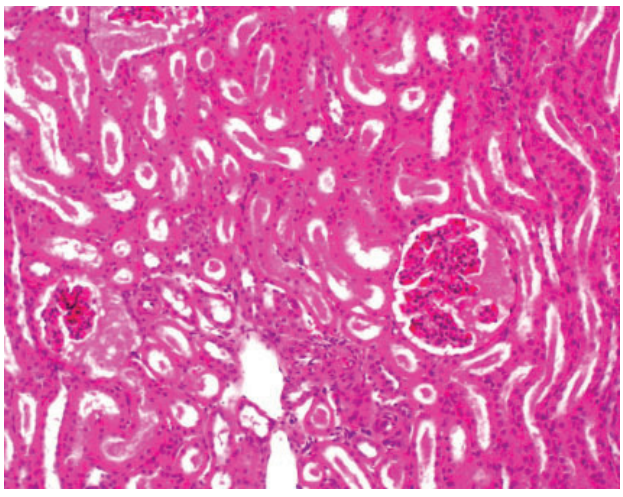

Figure 5.
Protein leakage, hyaline casts, and the necrosis of the urinary tubule cells are observed. H & E staining. Magnification: 100x

pens due to a smaller amount of renal mass and the decrease in the number of proximal tubular cells that absorb the filtered native vitamin D (25(OH) D) to then be hydroxylated to its active form by the 1α-hydroxylase (17).

Nikodimopoulou and Liakos (2011) reported that the secondary hyperparathyroidism (SHPT), as a common disorder in patients with CKD, is characterized by excessive serum parathyroid hormone (PTH) levels, parathyroid hyperplasia and an imbalance in the calcium and phosphorus metabolism. In turn, the SHPT develops early in the course of CKD and becomes more prominent as the kidney function declines (18). Interstitial nephritis is indeed caused by different infective agents including bacterial, viral and protozoal agents such as *Escherichia coli*, sheep pox virus, Adenovirus and *Leptospira* (19).

To determine the prevalence and type of urinary bladder and renal lesions in the slaughtered buffaloes at Ahvaz industrial slaughterhouse, Mohamadian et al. (2016) randomly selected a total of 353 buffaloes for gross histopathological studies. The histopathologic findings showed that 128 (36.3%) of the examined animals had renal lesions including interstitial nephritis (27.2%), acute tubular necrosis (ATN) (3.1%), hydronephrosis (2.5%), glomerular capillary hemorrhage (1.7%), pyelonephritis (1.4%), glomerulonephritis (0.3%). Besides, interstitial nephritis was found to be the major observed lesion (75%) and also the most common histopathological lesions of the kidney identified in buffaloes. As the statistical analysis showed, gender and age were not correlated with the renal lesions (20).

In another research, Nikvand, et al. (2014) showed that the isolation of bacteria from urine was not related to cystitis and pyelonephritis. Indeed, some cases of chronic cystitis were not able to isolate bacteria from urine and for causing pyelonephritis they need predisposing factors which caused urine retention (21).

According to histopathological findings of Baghban and Yaripour (2016), interstitial nephritis was the most abundant condition observed in 69 cases (71.13%) of sheep kidneys and 68 cases (72.34%) of goat kidneys. The other renal lesions observed in sheep included purulent abscess in 15 cases (15.47%), pyelonephritis in 6 cases (6.18%), nephrosis in 5 cases (5.16%) and hydatid cyst in 2 cases (2.06%). In goats, the renal lesions were purulent abscess in 10 cases (10.64%), pyelonephritis in 4 cases (4.25%), nephrosis in 4 cases (4.25%), amyloidosis in 2 cases (2.13%), hydatid cyst in 3 cases (3.19%), infarction in 2 cases (2.25%) and congestion in 1 case (1.07%). The statistical analysis showed a significant difference (p <0.05) in relation to the lesions observed in the condemned kidneys between the two sexes. The results also showed that interstitial nephritis followed by purulent abscesses and pyelonephritis was the most abundant lesion in the condemned kidneys of sheep and goats (22).

Examining sheep from different breeds in Turkey, Hatipoglu et al. (2001) found renal abnormalities macroscopically and microscopically in 316 (3.13%) head of sheep. The most prevalent renal lesions in sheep was interstitial nephritis in 203 (62.24%) head of sheep (23).

In another study, Ansari-Lari (2007) determined gross abnormality changes in the condemned kidneys of sheep and goats to be 1.5-3% in the Shiraz slaughterhouse. Also, Ansari-Lari reported the abundance of kidney condemnation in sheep and goats to be 1.1 and 2.3%, respectively. Moreover, nephritis was identified as the most important reason of kidney condemnation in sheep and goats with 25% and 16% abundance, respectively (4). In another study, Mathur and Dadhich (2005) reported that from 1284 head of sheep examined, 223 head of sheep with apparent macroscopic lesions showed interstitial nephritis in 45 (20.17%) samples (24).

Investigating bovine kidney diseases in Shahrekord district, Kojouri et al. (2008) showed that the prevalence of renal diseases in Shahrekord slaughterhouse was approximately 7.9%. The relative frequency of disorders was reported as interstitial nephritis (34.18%), cyst (27.85%), hydatid cyst (11.4%), glumerulonephritis (3.8%), acute tubular necrosis (5.06%), fibrosis (3.8%), hydronephrosis (3.8%), abscess (2.53%), hemorrhage and congestion (3.8%), renal dysplasia (1.26%), infarction (1.26%) and hyaline cast (1.26%) (25).

Using the slaughterhouse data in Dublin, Monaghan and Hannan (1983) reported that 4.2% of 4166 cattle slaughtered had kidneys that were rejected due to gross abnormalities. The rejection rate was 7.7%, 1.7%, 2.2% and 28% for cows, bullocks, heifers, and bulls, respectively. Besides, the focal interstitial nephritis was found to be the most frequent renal lesion (60.1%) in the slaughtered cattle (26).

Similarly, investigating the prevalence and types of renal lesions, Nourmohammadzadeh et al. (2010) reported that 35 (8.6%) of the examined animals had renal lesions including interstitial nephritis (85.7%), cyst (11.4%), glomerolonephritis (5.7%), ATN (5.75%), pyelonephritis (2.85%), amyloidosis (2.85%), leukosis (2.85%), hydronephrosis (2.85%), and unilateral renal aplasia (2.85%). Moreover, the prevalence of renal lesions in female and male cattle was 8.5% and 9.4%, respectively, which was not statistically significant. Likewise, the prevalence of renal lesions in cows and heifers was found to be 10% and 2.8%, respectively, which was statistically significant (27).

In another study, aiming to assess the prevalence of renal lesions in the slaughtered cattle in the Shiraz slaughterhouse, Taghadosi, et al. (2016) reported the correlation between the rejected kidneys and infection with *Leptospira* using the nested PCR-restriction fragment length polymorphism (RFLP) techniques. The findings showed that out of 1000 inspected animals, 205 (20.5 %) had renal lesions. Among them, chronic nephritis (7.5%), white-spotted kidney (7.3 %), and petechial hemorrhage (3.5 %) were the most prevalent forms of the lesions. Moreover, a direct correlation between increasing the age and a significant increase in the rate of lesions was also observed (29).

Conclusion

As the findings showed renal disorders in livestock may appear subclinically leading to their reduced production performance without any clinical signs. Thus, further studies are required to determine the other aspects of renal subclinical diseases in the cattle slaughtered. Moreover, the changes in the level of kidney function-related hormones such as EPO, PTH, and vitamin D were found likely to affect the metabolism of many organs, causing significant economic losses by reducing the growth and production in the meat and dairy industry. The results of this study could thus help veterinary clinicians and other stakeholders in the field of buffalo breeding improve their knowledge about the role of healthy kidney in the maintenance of buffalo healthy conditions.

Material and methods

Having identified the specifications of buffaloes, 5 ml of blood samples were taken from each animal in clot tubes immediately after the slaughter at Ahvaz industrial slaughterhouse. The blood samples were centrifugated after being transferred to the laboratory at 2500 rpm for 10 minutes. Then, the serum was separated and stored in a freezer at -70 °C until the tests were carried out. The ELISA method was used to measure the levels of EPO, PTH and serum Vit D3. The EPO ELISA kit (Mybiosource, cat no: MBS907898; USA) assay has high sensitivity and excellent specificity for detection of bovine EPO. No significant cross-reactivity or interference between bovine EPO and analogues was observed.

Bovine parathyroid hormone (PTH) was, in turn, measured by the ELISA Kit (Abbkine cat: KTE10155; USA). The PTHELISA Kit employs a two-site sandwich ELISA to quantitate PTH in the samples. Vitamin D ELISA kit (Mybiosource, cat no: MBS744057; USA) assay has high sensitivity and excellent specificity for the detection of Vitamin D. No significant cross-reactivity or interference between Vitamin D and analogues was observed.

After the slaughter was done, kidneys were isolated from the buffalo carcasses to examine their appearance and record any probable macroscopic lesions. In case of any macroscopic lesion or abnormality, a proper sample was taken from the lesion site, while in the absence of any macroscopic lesion, the sample was taken from one of the lobes near the umbilical region of the kidney and transferred to the pathology lab in 10% formalin. The tissue samples were then placed in a 10% formalin buffer container (10 times the sample volume) and 24 hours later, formalin was replaced. After a week, each sample was made up of sections of 2 to 3 mm in diameter and placed in special containers with specified numbers. If the specimen had a macroscopic lesion, it was taken from the same site. Afterwards, the baskets were placed under running water for 4 hours and then transferred to the Autotechnicon (Leica, Germany) tissue processor. The process of tissue passage involving dehydration, clearing and impregnation was carried out on the tissue processor. After 18 hours, the specimens were extracted from the device and paraffin blocks were prepared. The blocks stored in the laboratory temperature and also in the refrigerator for one day were prepared using a 5-micrometer section of the microtome. After placing the tissue sections on the slide, paraffin was melted in a hot air oven at 50-60 degree centigrade depending upon the melting point. Then, the samples stained with the hematoxylin-eosin method were used for histopathological evaluation. If necessary, the Periodic acid–Schiff (PAS) staining method was also used. After careful examination of the prepared slides, healthy kidney specimens were separated and the lesions samples were divided into five groups according to the type and severity of the lesions including ATN, CTN, chronic multifocal tubulointerstitial nephritis, UTI and congestion. Then, different types of histopathological disorders of kidney, the changes in EPO, PTH, and vitamin D values along with the correlation between the hormonal changes and histopathologic lesions were studied statistically using SPSS-22 software and the related tests.

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Author Contributions

Research design: M.R.J and M.R. H.H. Clinical sampling: Z.S. Pathological studies: B.M. Measurement of hormones: M.T.J.

Conflict of Interest

The authors declare that they have no conflict of interest

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RESEARCH ARTICLE

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The Effects of administration of different parts of banana *(Musa cavendish)* fruit extracts and peel powder on the oxidative/antioxidative characteristics and some mineral concentrations in neonatal dairy calves

Nafiseh Keivani Rad[a], Mehrdad Mohri[a,b], Hesam A. Seifi[a,b], Alireza Haghparast[c]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Center of Excellence in Ruminant Abortion and Neonatal Mortality, Ferdowsi University of Mashhad, Mashhad, Iran.
[c] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

The present study examined the effects of overripe banana (*Musa cavendish*) pulp and green banana peel extract and powder on oxidants/antioxidants parameters and some minerals in Holstein dairy calves. Forty newborn calves were randomly divided into four groups of 10 (control, group one, group two and group three). The groups were homogenous for the parity of the dams and the time of birth. Within 12-48 hours of birth, calves were assigned to their treatment groups. In the control group, animals received no banana meal. In group one, calves were supplemented with 2 g (dry matter)/kg body weight/day of overripe banana pulp extract for five days. The calves in group two were supplemented with 1 g (dry matter) of overripe banana pulp extract /kg body weight/day and 1 g (dry matter) of green banana peel extract/kg body weight/day for five days. The animals in group three were supplemented with 2 g/kg body weight/day of green banana peel powder for five days. Blood samples were taken on day 0 (at birth) and on days 7, 15 and 30 through the jugular vein. Age (the time of sampling) had a significant effect on the values of phosphorous, potassium, iron, copper, FRAP and activity of GPx enzyme ($p < 0.05$). Significant group and sampling time interaction was seen for the FRAP concentrations ($p < 0.05$). In conclusion, banana supplementation in the Holstein dairy calf's diet at the concentration and duration that was reported in the present study had beneficial effect on the values of FRAP.

Keywords

Antioxidants, Banana, Calf, Minerals

Abbreviations

MDA: malondialdehyde
SOD: superoxide dismutase
GPx: glutathione peroxidase

Corresponding author:
Mehrdad Mohri
Tell/Fax: +98 513 880 5643
Email: mohri@um.ac.ir

Introduction

Calves are the substitution stocks for cows and bulls in a herd and their initial growth is the most important phase of their life. The nutrition of calves is an important factor in their health. For this reason, the diet of calves has been supplemented with many feed additives. Also, herbs are being used recently. The antioxidants of herbs may reduce the incidence of morbidity and mortality by reducing oxidative damage and improve the pre-weaning calf performance [1].

Banana is one of the most important tropical fruit crops, which belongs to the order of Zingiberales, the family of *Musaceae* and genus *Musa* [2]. Banana can be classified into commercial and non-commercial cultivars. The non-commercial ones are also referred to as indigenous varieties because their cultivation for export or trade is rare [3]. Non-commercial bananas which are cultivated in the south, the east and the southeast of Iran can be utilized as animal food. The use of natural products in the ration of food for animals results in the reduction of the presence of chemical residues in human foods [4]. All different parts of the banana plant including fruits, peels, leaves, roots, bulbs, flowers, gels and barks have medicinal utilizations [5, 6].

Bananas contain high levels of minerals such as potassium and phosphorus. The pulp and the peel possess various antioxidants including phenolic compounds such as catechin, epicatechin, lignin, tannins, anthocyanins, vitamins (A, B, C and E) and β- carotene. Forty percent of the total weight of fresh bananas is peel which is considered as a rich source of protein, crude fat, lipid, dietary fiber, pectin, micronutrients, polyunsaturated fatty acids, and essential amino acids. Different studies have been conducted on the effects of various parts of banana plant supplementation in humans, small and large laboratory animals, chickens and even in prawns. In these studies, the effects of adding banana meal to ration have been evaluated on health, growth performance, feeding behavior, milk production, carcass characteristics, hematological, biochemical and immunological factors, diseases like metabolic disorders, and wound healing [4, 7-31]. *In vitro* studies have also been done for assessing the antimicrobial effects of bananas [5, 6, 32-36].

To the best of our knowledge, there is just one study on calves which has evaluated the effect of dietary supplementation of bananas on immunocyte populations [7]. Hence, this study was conducted to investigate the effects of dietary supplementation of extract of overripe banana pulp, extract of green banana peel with extract of overripe banana pulp together, and powder of green banana peel on oxidants/antioxidants variables and some mineral concentrations of Holstein dairy calves.

Results

The treatment (group) had no significant effect on the measured variables ($p < 0.05$). Age (time of sampling) had a significant effect on the values of phosphorous, potassium, iron, copper, FRAP, and activity of GPx enzyme ($p < 0.05$). No significant effect was seen for calcium, sodium, zinc, MDA, and SOD. The

Table 1
The effects of treatments on measured minerals (LSM and SE) between trial groups.

	LSM							
Parameter	**Control**	**Group 1[1]**	**Group 2[2]**	**Group 3[3]**	**SE[4]**	**Age**	**Group**	**Age × Group**
Ca (mg/dL)	11.33	11.97	12.20	11.37	0.43	NS	NS	NS
P (mg/dL)	7.04	7.15	7.17	6.95	0.36	S	NS	NS
Cu (µg/dL)	71.22	69.44	67.59	67.52	4.80	S	NS	NS
Zn (µg/dl)	152.62	147.3	156.16	140.48	11.28	NS	NS	NS
Fe (µg/dl)	120.84	94.55	86.18	74.98	15.76	S	NS	NS
Na (mEq/L)	148.28	145.98	150.88	146.30	3.44	NS	NS	NS
K (mEq/L)	6.34	6.14	6.12	5.66	0.36	S	NS	NS

[1]: Overripe banana pulp extract supplemented group, [2]: Overripe banana pulp extract + green banana peel extract supplemented group, [3]: Green banana peel powder supplemented group, [4]: Standard error
S: significant effect ($p < 0.05$), NS: non significant effect, Ca: calcium, P: phosphorous, Cu: copper, Zn: zinc, Fe: iron, Na: Sodium, K: potassium

significant treatment and the sampling time interactions (treatment × sampling time) were seen for the quantities of FRAP ($p < 0.05$), while there were no significant interactions for the other variables (Tables 1-3).

Table 2
The effects of treatments on oxidants/antioxidants characteristics (LSM and SE) between trial groups.

	LSM							
Parameter	**Control**	**Group 1[1]**	**Group 2[2]**	**Group 3[3]**	**SE[4]**	**Age**	**Group**	**Age × Group**
FRAP (mmol Fe2+/L)	2.35	2.29	2.36	2.36	0.06	S	NS	S
MDA (nmol/ml)	2.20	3.03	3.31	2.65	0.67	NS	NS	NS
SOD (Units/gr Hb)	13924	13619	13805	13729	233.89	NS	NS	NS
GPx (Units/gr Hb)	2390.66	2386.71	2445.11	2559.58	106.41	S	NS	NS

[1]: Overripe banana pulp extract supplemented group, [2]: Overripe banana pulp extract + green banana peel extract supplemented group, [3]: Green banana peel powder supplemented group, [4]: Standard error
S: significant effect ($p < 0.05$), NS: non significant effect, FRAP: Ferric Reducing Ability of Plasma, MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase

Table 3
The effects of treatments on FRAP amount (LSM ± SE) with a significant age and group interaction at various sampling time (age).

Age	**Control**	**Group 1[1]**	**Group 2[2]**	**Group 3[3]**
	FRAP (mmol Fe2+/L)			
Day 0	2.26 ± 0.08[a]	2.14 ± 0.08[a]	2.30 ± 0.09[a]	2.13 ± 0.08[a]
Day 7	2.43 ± 0.08[a]	2.41 ± 0.08[a]	2.55 ± 0.09[a]	2.44 ± 0.08[a]
Day 15	2.28 ± 0.08[a]	2.33 ± 0.08[a]	2.29 ± 0.09[a]	2.61 ± .08[b]
Day 30	2.42 ± 0.08[a]	2.28 ± 0.08[a]	2.30 ± 0.09[a]	2.27 ± 0.08[a]

[1]: Overripe banana pulp extract supplemented group, [2]: Overripe banana pulp extract + green banana peel extract supplemented group, [3]: Green banana peel powder supplemented group
Means within rows lacking a common superscript, were significantly different ($p < 0.05$ or $0.05 < p < 0.1$)

Discussion

Levels of iron significantly increased from birth to day 30 which is consistent with the results of another study in calves [37], but in contrast with the report from Mohri et al. (2007) [38]. The discrepancy may be resulted from the influence of body iron reserve and the iron content of the diet. In the present study, the concentration of potassium declined on day 30 compared with its amounts at birth except in group 3 because of high potassium content (78.1mg/g) of banana peel [39, 40]. The decreased value of potassium, can be justified by a significantly lower tubular resorption of potassium in calves in postnatal period than sodium and chloride which are similar to the levels in adult animals [41].

In agreement with our results, Mohri et al. (2007) reported that phosphorus levels significantly increased by age [38]. This can be attributed to the enhancement of renal phosphate reabsorption by growth hormone, which is high in growing animals [42]. Copper value

significantly increased from birth to day 30, similarly in the study of Enjalbert et al. (2002) reporting that plasma copper concentration in calves increased after birth and reached its normal values at 3 weeks of age [43]. This can be attributed to the elevation in the concentration of liver copper in the first 2 months of life and the increased absorption of copper in newborn calves in comparison with adults in response to physiological needs for bone and connective tissue growth and development [44].

Age had a significant effect on the activity of GPx enzyme and the values of FRAP. The activity of GPx was significantly declined on day 30 compared with its activity at birth. Previous studies reported similar results and mentioned that age related changes in the antioxidants status can be detected in several species and several tissues, and factors such as nutrition and hormones have important effects on the activity of GPx [45, 46]. Micronutrients such as Zn, Cu, Fe and Mn improve the efficiency of antioxidant system [47], so the results obtained for the values of FRAP may be resulted from the significant increase in the amounts of Cu and Fe.

In the calves supplemented with unripe banana peel powder, the amounts of FRAP were higher than the other three groups on day 15. FRAP assay, is a novel method for evaluating the antioxidant power. The total FRAP is dependent on the individual plasma antioxidants (uric acid, ascorbic acid, vitamin E, bilirubin, albumin, and others) [48]. The reason for the higher value of FRAP in calves supplemented with peel powder compared with those supplemented with pulp extract is the higher antioxidant content of the peel. Although the amounts of ascorbic acid regardless of the ripening stage was constant (10 mg/100 g) in both peel and pulp [49]. The amounts of most other antioxidants are higher in the peel than pulp [50]. In the study of Fatemeh et al. (2012) the amounts of total phenolic and flavonoid contents were higher in the Cavendish variety, the green stage and the peel of banana than pulp [50]. Similarly, Sundaram et al. (2011) revealed that fruit maturation and ripening was accompanied by a decrease in the activities of phenolic compounds and antioxidant enzymes [51]. Devatkal et al. (2014) correlated the antioxidant capacity of bananas to their gallocatechin and dopamine contents [52]. They observed that gallocatechin was more abundant in the peel (158 mg/100 g dry wt.) than in the pulp (29.6 mg/100 g dry wt.). Gallocatechin is a polyphenol and a major catechin in the banana. Catechins are the strongest antioxidants in phytochemicals [50]. In the study of Kanazawa and Sakakibara (2000) on the antioxidants of *Musa cavendish*, large amounts of dopamine in both the pulp and the peel were found. The amounts of dopamine, a strong water-soluble antioxidant in the peel (80-560 mg/100 g) were greater than the amounts in the pulp (2.5-10 mg/100g) and decreased a little with ripening. Flavanone glycoside naringin and flavonol glycoside rutin are two antioxidative phytochemicals in bananas that are mostly in the peel (10 mg) compared with the negligible amounts in the pulp [49]. Also, carotenes and tocopherols were appreciable in the peel and less in the pulp [50]. These reports are in agreement with our results in the group three in which the FRAP values were more than that of calves supplemented with pulp. Furthermore, in calves supplemented with unripe banana peel powder the FRAP values were higher in comparison with those in group two. It seems that the causative agent may be the differences in processing techniques for preparing unripe banana peel supplement (extract or powder) which result in the reduction of the antioxidants in the extract.

Treatment did not have any significant effects on the activity of MDA and the antioxidant enzymes of SOD and GPx. Giri et al. (2016) reported that in Labeo rohita (is a species of fish belonging to Cyprinidae family) supplemented with yellow banana peel flour for 60 days, the activity of antioxidant enzymes SOD and GPx were significantly higher and MDA activity was significantly lower compared with the control group [53]. In prawns supplemented with green banana peel extract for 120 days a significant increase in SOD activity has been observed [30].

Change in antioxidant parameters and lipid peroxidation happens more slowly in RBCs than in the plasma or serum [54]. On the other hand MDA that is generated from lipid peroxidation is known as a marker of oxidative stress in cells [55]. Thus, it can be concluded that after antioxidant supplementation (and increasing FRAP amount in serum), antioxidative defense in cells (GPx and SOD) and consequently the amount of MDA change slowly. In addition, it is important to note that in neonatal calf erythrocyte, the pro-oxidant features predominate over antioxidant features which make it susceptible to oxidative damage. As a result of higher autoxidation, the amounts of peroxide, hydroxyl and superoxide radicals produced from foetal haemoglobin in neonatal erythrocytes are more than these generated from adult haemoglobin. Furthermore, in newborn the activities of GPx and catalase enzymes and the ability to renew fatty acids are reduced and the amounts of vitamin E in young animals' erythrocytes is inadequate which has an important role in preventing membrane lipids autoxidation [56]. As a result of lower antioxidative defense in neonatal calves' RBCs, ROS and lipid peroxidation products including MDA are increased [55, 56]. Moreover, consumption of solid feed in growing calves results in a period of oxidative stress from 2nd

week of life [54].

In addition, in the present study the duration of supplementation with banana (pulp and/or peel, extract or powder) was short in comparison with the previous studies that reported a reduction of MDA activity as a result of banana peel [53] or pulp supplementation [57]. All these reports are in agreement with our results which show no significant differences in MDA level between treatment groups and control and also unchanged GPx and SOD activity.

In conclusion, banana supplementation in the Holstein dairy calf's diet at the concentration and duration that was reported in the present study had beneficial effects on the values of FRAP. Further studies are required to identify the optimal dosage and duration of banana supplementation in dairy calves.

Material and methods

Experimental design

The duration of study was seven months, from 18 August 2017 to 18 March 2018 in a dairy herd with about 210 calves per year at the suburbs of Mashhad (northeast of Iran). This herd consists of pure bred animals of Holstein breed. The herd was totally restricted in open-shed housing with no access to pasture. The ingredients of dry cow ration in both far off dry period and close up dry period are shown in Table 4. The ration was balanced according to NRC recommendations (NRC, 2001).

After parturition, the umbilicus of each calf was treated with povidone iodine and they were allowed to remain with their dams until the umbilicus dried off. The calves were weighed, then their sex was recorded and they were transferred to individual pens bedded with straw. Within the first 6 hours of life, the calves were fed dam's colostrum by nipple bottle in amounts of 10% of their body weight and the colostrum feeding was repeated every 8 hours for 48 hours. Then, herd milk was replaced for feeding twice daily (2 kg every 12 h) until seventy days of life. The calf starter ration was also balanced according to NRC recommendations (NRC, 2001). After transferring them to an individual pen, the animals had free access to clean drinking water. The calves were weaned at seventy days of life.

Forty newborn Holstein dairy calves from both sexes were selected for the study. The animals were randomly divided into four groups of ten. The groups were homogenous for parity of dams and time of birth. In the control group, animals received no banana meals. In group one, calves were supplemented with 2 g (dry matter)/kg body weight/day of overripe banana pulp extract for five days. Calves in group two were supplemented with 1 g (dry matter) of overripe banana pulp extract /kg body weight/day and 1 g (dry matter) of green banana peel extract/kg body weight/day for five days. In group three animals were supplemented with 2 g/kg body weight/day of green banana peel powder for five days. The amounts and duration of supplementation were selected according to a previous report in calves (7). The extract or powder was mixed with milk or warm water and was administered to the calves orally in a milk bottle. All other aspects of their diets were identical for all groups including the control group.

Preparation of aqueous extracts

Ripe and also completely green Bananas (*Musa cavendish*) were purchased locally from a banana local market without any ethylene gas exposure and were stored at 20 °C for 24 h before extraction.

Preparation of green banana peel extract

Green bananas were rinsed thoroughly in tap water, surface sterilized with 70% alcohol and then they were rinsed by distilled water to remove any contaminants. Peels were manually separated from the pulp and they were put into 70 °C water for 20 second to inactivate polyphenol oxidases. The peels were cut into small pieces by using a sharp knife and they were dried in an oven at 60 °C for 38 h. Then, the dried peel was ground into a powder with an industrial grinder. The milled peel was mechanically stirred for 2 h (1 g in 10 ml distilled water) in a vacuum evaporator under reduced pressure at 60 °C. After extraction, the extract was centrifugated for 15 min at 3500 rpm. The supernatant containing the water-soluble extracts was transferred into 50 ml falcon tubes and it was stored at -70 °C until the experiment started.

Preparation of overripe banana pulp extract

Yellow bananas were left at room temperature until peels became yellow brown and the edible portion became leaky (overripe). The peels were separated from the pulps by hand. The flesh was weighed, cut into appropriate sizes, and mixed with a 3-fold weight of deionized water in a vacuum evaporator under reduced pressure at 60 °C for 10 h. The homogenate was centrifuged at 1500 rpm for 15 minutes. The obtained supernatant was transferred into 50 ml falcon tubes and was stored at -70 °C until the experiment started.

Preparation of green banana peel powder

The peels were manually separated from the pulp and were cut into small pieces. Pieces were shade-dried for about two weeks and then they were crushed to make a coarse powder in a pulverizer. The powder was stored in cold, dry and dark place until the experiment started.

Calculating dry matter

The moisture content of the extracts was calculated on the basis of weight loss after the sample had been heated in an oven at 105 °C. The DM content of pulp and peel extracts were 45.86% and 8.5%, respectively.

Blood sampling

The blood samples were taken on day 0 (at birth) and on days 7, 15, and 30 through the jugular vein with the aid of disposable syringes. 2.5 milliliters of blood was transferred into EDTA-3K tubes for hematological analysis and hemolysate preparation and 7.5 ml was transferred to plain tubes for serum separation. As soon as collection done, all tubes were placed on ice and were immediately transferred to the laboratory. The blood in the plain tubes was allowed to clot at room temperature and then it was centrifugated for 15 min at 3000 rpm for serum separation. The serum was aliquoted into 1.5 ml microtubes and the sample code was written on them. The serum was frozen at -20 °C until analysis.

Minerals measurement

Calcium (Ca), phosphorous (P), iron (Fe), sodium (Na), potassium (k), copper (Cu), and zinc (Zn) concentrations in serum were measured by commercial colorimetric kits (for Ca, P and Fe: Pars Azmoon, Tehran, Iran and for Na, K, Cu and Zn: DIALAB, Wiener Neudorf, Austria) using an autoanalyzer (Mindray, BS-200E, Shenzhen, China). The control serum (Centronorm, centronic GmbH, Wartenberg, Germany) was used for controlling measurement accuracy. The inter assay and intra assay of methods were less than 5%.

Estimation of serum oxidant/antioxidant characteristics

MDA in the serum samples was estimated by reaction with thiobarbituric acid (TBA) at a high temperature to generate a MDA-TBA adduct. The pink color adduct was measured spectrophotometrically at 530-540 nm (Nalondi assay kit, Navand Salamat, Orumieh, Iran). Kit sensitivity was 0.1 nmol/well.

Measurement of Ferric Reducing Ability of Plasma (FRAP): Antioxidant potential in the serum samples was measured through reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by single electron-transfer mechanism by antioxidants present in the serum. Change of color of the reaction was estimated colorimetrically at 593 nm (Naxifer assay kit, Navand Salamat, Orumieh, Iran). The antioxidant potential of the samples was determined using a ferrous iron standard curve. Kit sensitivity was 2 µmol Fe^{2+}.

Hemolysate preparation

Blood samples in the anticoagulant containing tubes were centrifugated at 3000 rpm for 5 minutes. The plasma and buffy coat layers were removed immediately and normal saline (0.9% NaCl) with approximately two times the volume of the packed RBC that was added for washing. Then, the tubes were centrifugated at 2000 rpm for 5 minutes. The washing process was repeated four times. After the 4th washing, cold redistilled water was added to the washed RBC in a ratio of 3:1 and vortexed vigorously to make hemolysate. After final centrifugation, the hemolysate was refrigerated at 4 °C for 15 min and then it was aliquoted to 1.5 ml micro tubes on which the sample code was written and they were frozen at -20 °C until analysis.

Estimation of antioxidant enzymes activity

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured colorimetrically in hemolysate samples using Ransod and Ransel commercial kits, respectively (Randox Co., Crumlin, United Kingdom). The inter assay and intra assay of SOD measurement method were 7.07% and 3.58%, respectively and the sensitivity was < 6.13 U/ml. The total assay and intra assay of GPx measurement method were 4.37% and 3.2%, respectively, and the sensitivity was 75 U/L.

Statistical Analysis

The data was subjected to repeated measure analysis of variance (ANOVA) using the PROC MIXED of SAS 9.2 (SAS institute Inc.). Normality of all variables was evaluated by univariate procedure. The outcome variables with Shapiro-Wilk values of ≥ 0.05 were considered as normal (FRAP, phosphorous, sodium) and all the other variables were transformed by using a natural logarithmic transformation to reach a normal distribution. The time of sampling (0, 7, 15 and 30), group (control, group one, two and three), sex and parity of dams were used as independent effects

Table 4
The ingredients of dry cow ration in both far off dry period and close up dry period.

Ingredients	Far off dry period ration (%)	Close up dry period ration (%)
Alfalfa hay	17.79	13.71
Corn silage	25.22	19.45
Wheat straw	31.16	13.73
Barley grain rolled	10.37	16
Corn grain ground dry	5.73	16.68
Wheat bran	7.7	6.14
Fish meal	1.27	-
CaCo3	0.23	-
Salt	0.24	-
Canola meal	-	2.67
Soy meal, expellers	-	6.59
Anionic supplement	-	3.92
TMS	-	1.11
Vitamin/mineral supplement*	0.29	-
Total	**100**	**100**
Calculated dry matter intake	**14 kg**	**17 kg**

*Supplements contain/kg: Vit A 1,000,000 IU, Vit D3 300,000 IU, Vit E 10,000 IU, Ca 6118 mg, P 1500 mg, Mg 5000 mg, Mn 1000 mg, Zn 1000 mg, Cu 500 mg, Se 50 mg, Iodine 50 mg, Fe 1000 mg, Co 5 mg and antioxidant 1000 mg.

and all measured variables were considered as dependent. The results were expressed as Least squares means (LSM) ± standard errors of means (SE) in each group. The effects of independent factors were considered significant at $p < 0.05$, whereas a trend toward significance was noted at $0.05 < p < 0.1$.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received (3/41677). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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Author Contributions

N.K.R contributed to the main design of study, sample collection, laboratory tests, data analysis and drafting the manuscript. M.M contributed to main design of the study, data analysis and reviewed and edited the manuscript. H.A.S and A.H. contributed to the main design of the study. All authors approved the final version of the manuscript for publication.

Conflict of Interest

The authors declare they have no conflict of interest.

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Effect of four local anesthetics (tetracaine, bupivacaine, lidocaine and proparacaine) on intraocular pressure in rabbits- Comparison of an applanation and a rebound tonometer

Arman Emrarian, Ali Asghar Sarchahi

Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

ABSTRACT

The type of device used, the type of local anesthetic agents, and the animal species may affect the intraocular pressure (IOP). Therefore, in order to determine these issues, the effects of four local anesthetics were investigated in 10 adult rabbits by ICare TA01i and Tono-Pen Vet tonometers. In the right eye of half of the rabbits and in the left eye of the other half of the rabbits, one drop of tetracaine was instilled. The IOP in each rabbit was measured using two tonometers, ICare and Tono-Pen Vet, before and each 5 minutes until 40 minutes later. The effects of other drugs were also studied at least with one-week interval. Based on the results of ICare tonometer, tetracaine significantly reduced the IOP immediately and 25 minutes after instillation. IOP changes after instillation of bupivacaine, lidocaine and proparacaine were not significant at any time compared to baseline values ($p > 0.05$). Based on the results of Tono-Pen Vet tonometer, all drugs reduced the IOP immediately after use; however, the effects of bupivacaine and lidocaine on IOP were much lower than that of tetracaine and proparacaine. The average duration of corneal anesthesia were 20, 15.5, 7.5 and 21 minutes for tetracaine, bupivacaine, lidocaine, and proparacaine, respectively. It is concluded that IOP reduction by local anesthetics when Tono-Pen Vet is used is much greater than the ICare tonometer measurements. Also, the reduction of IOP with each of the devices when tetracaine or proparacaine is used is greater than when bupivacaine or lidocaine is used.

Keywords

Bupivacaine, Intraocular pressure, Lidocaine, Proparacaine, Rabbit, Tetracaine

Abbreviations

IOP: intraocular pressure

Corresponding author:
Ali Asghar Sarchahir
Tell/Fax: +98 51 38805596

Email: sarchahi@um.ac.ir

Introduction

Glaucomas are a group of eye diseases that commonly affects the optic nerve head and are caused by various factors, especially the increase of intraocular pressure (IOP). Therefore, in many cases, the intraocular pressure should be measured to diagnose the glaucoma (1). In order to measure the intraocular pressure, it is often necessary to use topical anesthetics (2, 3). The use of topical anesthetics may affect the IOP. Many studies have shown that the use of topical anesthetic agents reduce the IOP (4-8). On the other hand, there are other studies that have reported opposite findings, and have shown that these drugs do not change the IOP (9-11). The causes of these differences can be due to many factors (12, 13). We have previously shown in a study that tetracaine reduced the IOP in healthy and glaucomatous rabbits (8). In that study, the reduction of IOP in glaucomatous rabbits was higher than healthy rabbits. Therefore, one of the causes of differences in reports is the initial amount of IOP. In another study, we investigated the effects of tetracaine, bupivacaine, lidocaine, and proparacaine on IOP in the dogs (14). In that study we used a rebound tonometer (TA01i tonometer, ICare, Finland) that registers the IOP with lower ranges than the other tonometers (15, 16). In that study, tetracaine and proparacaine decreased the IOP, but the effect of lidocaine and bupivacaine on IOP was not significant. Therefore, another cause of the differences in reports is the types of drugs used. We thought that the third major factor is the type of device used to measure the IOP. Therefore, in the present study, we investigated the effect of four local anesthetics (tetracaine, bupivacaine, lidocaine and proparacaine) on intraocular pressure in rabbits using two types of tonometers (rebound ICare and applanation Tono-Pen Vet). With this aim in mind, we asked whether the type of drug, the type of device and animal species are effective in changing the IOP. Also, the duration of anesthesia in rabbits was measured and compared with other studies.

Results

Tetracaine

The results of intraocular pressure measurements with the ICare rebound and Tono-Pen Vet tonometers after the tetracaine instillation are shown in Fig. 1. The ICare tonometer readings showed that, IOP was immediately decreased after the administration of drug so that it was significant at times zero ($p = 0.046$) and 25 after instillation ($p = 0.027$). On the other hand, the Tono-Pen Vet readings showed that, the tetracaine immediately reduced the IOP. This IOP reduction continued up to 15 minutes after drug instillation and then began to increase; however, it was significantly lower than pre-treated values up to 30 minutes after drug administration. When compared to control eyes, the intraocular pressure reduction in treated eyes was

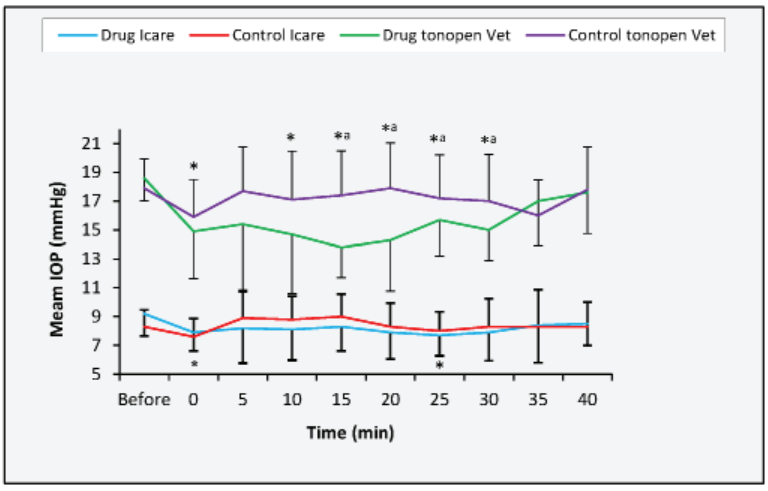

Figure 1.
Mean IOP of treated and control eyes in tetracaine group. The IOP in the treated eyes started to decrease immediately after instillation. The reduction of IOP by Tono-Pen Vet device was much sharper than the rebound Icare device; so that IOP by Tono-Pen Vet device decreased immediately after tetracaine instillation lasting for 30 minutes in treated eyes but by rebound Icare device, IOP reduction was significant only in 0 and 25 minutes after drug instillation. The data are based on the mean ± SD for 10 rabbits.
*: $p < 0.05$ comparing to pretreated baseline values.
a: $p < 0.05$ comparing to control eye values.

significant at times 15 until 30 minutes after drug instillation.

Bupivacaine

The results of intraocular pressure measurements using the ICare and Tono-Pen Vet tonometers after the bupivacaine administration are presented in Fig. 2. The ICare tonometer readings showed that the changes of IOP were not significant after the instillation of bupivacaine at any time compared to before baseline values; however when compared to control eyes, IOP reduction in the treated eyes was significant at times 10 (p = 0.034), 15 (p = 0.040), 20 (p = 0.041), and 25 (p = 0.017) minutes after drug administration. On the other hand, the results of Tono-Pen Vet tonometer indicated that the IOP significantly decreased at times 5 (p = 0.011), 10 (p = 0.011) and 15 (p = 0.027) minutes after instillation and then began to increase afterwards and reached its initial value in 40 minutes. Also, comparison of intraocular pressure in the treated eyes with the control eyes showed a significant decrease at 5 (p = 0.016), 10 (p = 0.008) and 15 (p = 0.013) minutes after drug instillation.

Lidocaine

As shown in Fig. 3, with the use of the ICare tonometer, the changes of IOP was not significant in the treated eyes after drug instillation compared to both baseline and control eyes ($p > 0.05$); but the Tono-Pen Vet tonometer readings showed that, the IOP significantly decreased 5 minutes after drug instillation compared to pretreated baseline values (p = 0.021). When compared to control eyes, the reduction of IOP in the treated eyes was significant at times 0 (p = 0.024) and 5 (p = 0.007).

Proparacaine

As shown in Fig. 4, using ICare tonometer, the IOP changes were minor and not significant. However, the differences of IOP in the treated eyes were significant at times 5 (p = 0.017), 15 (p = 0.016) and 20 (p = 0.027) compared to control eyes. On the other hand, by using Tono-Pen Vet tonometer, the IOP in the treated eyes started to decrease immediately after instillation of proparacaine and reduction of IOP was significant until 20 minutes after the administration of drug. The IOP in the treated eyes was significantly lower than that in control eyes immediately after drug instillation up to 25 minutes later.

Duration of anesthesia and side effects of drugs

All four drugs caused corneal anesthesia immediately after instillation. This effect was evaluated by corneal reflex, touching a piece of cotton with the cornea and seeing the animal's blink. Also, the returning of corneal sense was evaluated with this reflex. The mean duration of anesthesia was 20 minutes for tetracaine, 15.5 minutes for bupivacaine, 7.5 minutes for

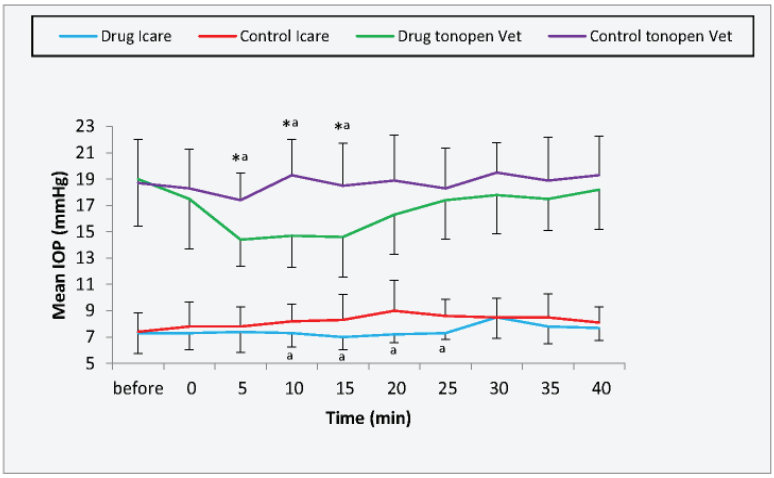

Figure 2.
Mean IOP of treated and control eyes in bupivacaine group. The IOP in the treated eyes started to decrease 5 minutes after instillation and lasting until 15 minutes after instillation by Tono-Pen Vet device. The IOP in the treated eyes by rebound Icare device were not changed comparing to pretreated baseline data. The data are based on the mean ± SD for 10 rabbits.
*: $p < 0.05$ comparing to pretreated baseline values.
a: $p < 0.05$ comparing to control eye values.

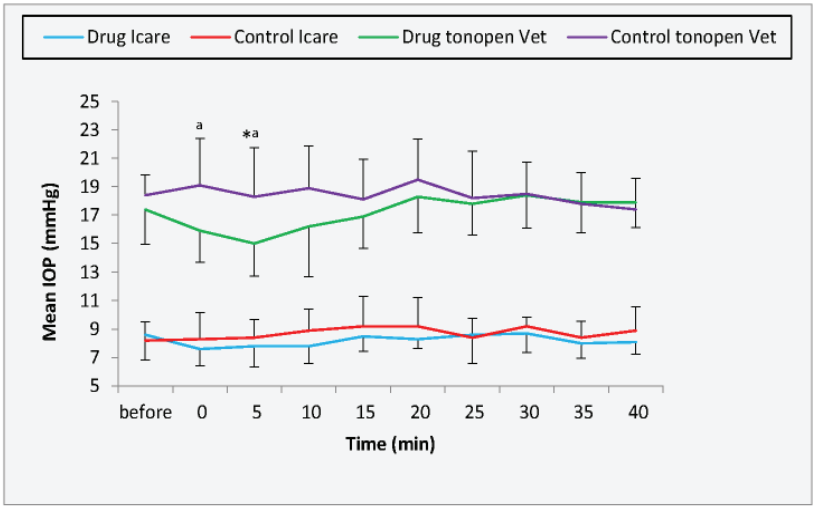

Figure 3.
Mean IOP of treated and control eyes in lidocaine group. The IOP in the treated eyes started to decrease immediately after instillation and was significant after 5 minutes by Tono-Pen Vet device. The IOP in the treated eyes by rebound Icare device were not changed ($p > 0.05$). The data are based on the mean ± SD for 10 rabbits.
*: $p < 0.05$ comparing to pretreated baseline values
a: $p < 0.05$ comparing to control eye values.

lidocaine, and 21 minutes for proparacaine. In this research, no adverse effects of drugs were observed in rabbits.

Discussion

Effects on IOP

Tetracaine

Sarchahi and bozorgi (2012), and Wang et al., (2013) evaluated the diurnal variation of IOP in rabbits, and reported that the intraocular pressure may change over the course of the day (8, 17). Thus, it is necessary to pay attention to this point and to investigate the effects of drugs in a short time. Therefore, in the present study, intraocular pressure in rabbits was

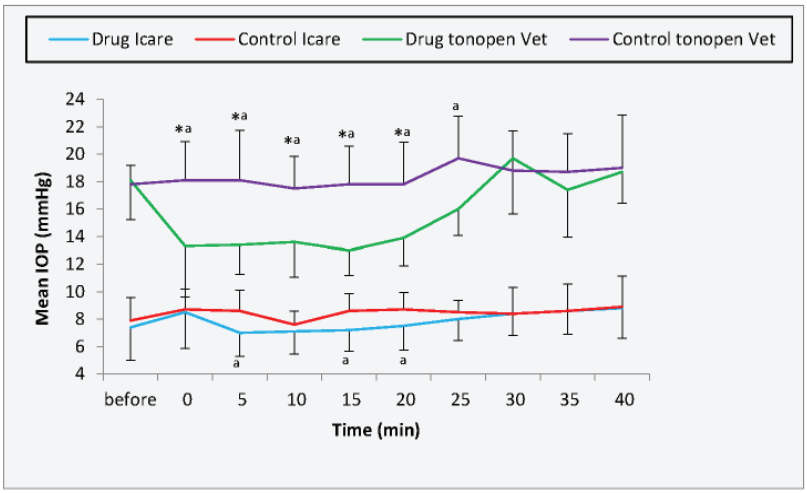

Figure 4.
Mean IOP of treated and control eyes in proparacaine group. The IOP by Tono-Pen Vet device decreased immediately after proparacaine instillation lasting for 20 minutes in treated eyes but by rebound Icare device, IOP reduction were not significant after drug instillation compared to baseline values. The IOP reduction by rebound Icare device was significant in 5 minutes after drug instillation lasting for 20 minutes compared to control eyes. The data are based on the mean ± SD for 10 rabbits.
*: $p < 0.05$ comparing to pretreated baseline values.
a: $p < 0.05$ comparing to control eye values.

taken from 13:00 to 18:00 h.

In the current study, the intraocular pressure, measured with both devices, has been reduced after instillation of tetracaine. The comparison of the IOP measured by two devices, Tono-Pen Vet and ICare, shows that firstly, the IOP measured with the Tono-Pen Vet is higher than that of the ICare device. Knollinger et al. (2005) and Leiva et al. (2006) comparing two Tono-Pen devices (Tono-Pen Vet, and Tonopen XL) and ICare in dogs and horses reported that the IOP shown by ICare was lower than that of Tono-Pen (15, 16). Secondly, because of the high pressure measured by the Tono-Pen Vet device, the effect of tetracaine on the IOP was more pronounced; While the IOP measured with ICare was lower and the reduction of IOP produced by this device was not very clear. The results of this study are almost the same as our two previous studies. In the first study, we compared the effects of tetracaine on healthy and glaucomatous rabbits and concluded that the greater the initial IOP, the more it decreases by tetracaine, so that the reduction of IOP in glaucomatous rabbits was more than healthy rabbits (8). In our previous second study, we investigated the effect of tetracaine on the intraocular pressure in dogs with a rebound ICare tonometer (14). In the previous and current studies, since the IOP measured by the ICare tonometer was low, the reduction in the IOP produced by tetracaine was low.

The results of this study and other researchers indicate that tetracaine reduces the IOP; however, the rate of reduction in IOP in various studies is different. One of the main causes of these differences appears to be the difference in baseline IOP before tetracaine instillation.

Bupivacaine

In a study done by Baudouin and Gastaud in the healthy and glaucomatous subjects, it was found that bupivacaine reduced the intraocular pressure within minutes 1, 5, and 15 after instillation (4). In our previous study, performed in dogs with ICare tonometer, bupivacaine did not significantly affect the IOP (14). In the present study, the results of the ICare device are consistent with our previous study. However, using of Tono-Pen Vet tonometer showed a decrease of IOP in 5-15 minutes. This finding is in agreement with the findings of Baudouin and Gastaud (4). This again emphasizes that the higher the initial IOP, the more it decreases, thus the decrease in IOP becomes significant. Nociti, et al., (2001) also reported a decrease in intraocular pressure 15 minutes after retrobulbar injection of bupivacaine. They have suggested that the reason of IOP decrease is the relaxation of extraocular muscles (18).

Lidocaine

There are some reports that show the using of lidocaine by other methods may affect the IOP; For example, Lerman and Kiskis (1985) and Abdulla and Flaifil (1991) reported that the use of lidocaine as an intravenous injection prevented the increase in IOP after tracheal intubation and laryngoscopy in children, and even 3 minutes after tracheal intubation, the IOP was also lower than that of zero time (19, 20). Hassanein et al. (2016) also reported similar results for lidocaine during the withdrawal of tracheal tubes (21). In the previous study, using the Icare tonometer, we concluded that lidocaine did not have a significant effect on IOP in dogs (14). In the present study, the effects of lidocaine on IOP were similar to those of ICare results and did not affect the IOP in rabbits; however when the IOP was measured with Tono-Pen Vet tonometer, it was found that lidocaine had some effect on the IOP; So that, IOP started to decrease immediately after administration and dropped to its lowest point within 5 minutes. Comparison of the effects of lidocaine with tetracaine and proparacaine in the present study showed that the reduction effect of lidocaine on IOP is similar to that of bupivacaine and is very low.

Proparacaine

The results of ICare tonometer showed that the proparacaine increased the IOP immediately after instillation (time 0), then IOP began to decrease and reached its lowest level in 5 minutes and then gradually increased. All these changes were not significant. On the other hand, results of Tono-Pen Vet tonometer showed that the IOP decreased immediately after instillation so that IOP was significantly lower than pretreated and control values until 20 and 25 minutes later respectively. Dosunmu et al. (2014), using ICare tonometer, evaluated the effects of 0.5% proparacaine on IOP in children (22). They reported that IOP slightly increased compared to before, and then, within 8 minutes after drug administration, a slight decrease in IOP was created, which, of course, was not significant. The results of ICare tonometer in the present study are similar to Dosunmu et al.'s study. Herse and Siu (1992), Ko et al., (2005) and Nam et al. (2006) reported that proparacaine causes a transient increase in the thickness of the cornea, thereby temporarily increases IOP (23-25). Leiva et al. (2006) Compared the IOP values of ICare and Tonopen XL tonometers in the eyes of healthy dogs (16). The results showed that ICare values were significantly lower than those of Tonopen XL ($p < 0.0001$), however, they concluded that the ICare tonometer could be an appropriate measurement method for daily clinical use after calibration for the dogs. As previously mentioned in this

discussion about tetracaine, the results of Leiva et al. and present study show that the IOP values obtained by the ICare tonometer are low. Therefore, the reduction effect of topical anesthetics such as proparacaine on IOP is less likely to be detected. Therefore, by measuring with this device, it seems that the drug has no effect on the IOP, but Tono-Pen Vet tonometer shows higher IOP values; As a result, the reducing effect of proparacaine on IOP is more visible.

Duration of anesthesia

In the present study, the average duration of corneal anesthesia after instillation of a drop tetracaine in rabbits was 20 minutes. This time has been reported 9.4, 16 and 30 minutes in humans, dogs and horses, respectively (14, 26, 27). Therefore, the duration of corneal anesthesia caused by tetracaine also vary in different species. We have already reported that the duration of corneal anesthesia were 20 and 22 minutes in healthy and glaucomatous rabbits, respectively (8). The findings of the present study confirm our previous findings on the duration of anesthesia in rabbits. Since two studies have been conducted in two separate geographical areas, the consistency of the results strongly confirms the effect of the species on the duration of corneal anesthesia.

The mean duration of corneal anesthesia after instillation of a drop bupivacaine in rabbits in the present study was 15.5 minutes. Sun et al. (1999) found that bupivacaine, and especially its buffered solution, had a greater effect than procaine or benzocaine on corneal anesthesia. The anesthetic effect of bupivacaine begins in the first minute after use, and if the acidity is adjusted, the duration of the effect becomes greater (28). In a study conducted by Liu et al. in rats, Bupivacaine had less toxic effects than proparacaine, and the duration of its effect is doubled by increasing the pH of the drug from 5.7 to 6.5 (29). In our previous study, the duration of corneal anesthesia by bupivacaine was 22 minutes in dogs (14). These findings indicate that the duration of corneal anesthesia caused by bupivacaine vary in different species, and in the present study, which is done on rabbits, it is less than the rest.

In the present study, lidocaine immediately after instillation caused corneal anesthesia but the mean duration of corneal anesthesia was very low (7.5 minutes). Assia, et al., (1999) concluded that lidocaine gel in human eye surgery was more effective than lidocaine drop, and had a good lubricating property (30). Shah et al. (2010) found that lidocaine (akten) gel produced longer anesthesia than lidocaine solutions in the eye and, due to containing of hydroxypropylcellulose, protects the corneal epithelium (31).

The duration of corneal anesthesia created by proparacaine varies in different species. Bartfield et al., in a study on humans, have shown that the degree and the duration of anesthesia created by proparacaine is greater than that of tetracaine (26). The results of the present study indicate that the duration of anesthesia created by proparacaine is longer than that of three other drugs. The reported duration of anesthesia induced by proparacaine in cats is 25 minutes (32), in dogs 45 and 34 minutes (14, 33), and in the horse 25 minutes (34).

Bupivacaine and lidocaine are amide anesthetics and they are classified as long-acting local anesthetic drugs. We expected their duration to be greater than tetracaine and proparacaine. But the duration of anesthesia caused by bupivacaine and lidocaine in the present study was in contrary to our expectations. One of the reasons for this can be the type of drug form used. In the present study, due to lack of topical forms of bupivacaine and lidocaine, we used an injection formulation of these drugs. This problem can be resolved in the future by producing and testing the topical form of these drugs. A second and more important reason can be the species in which the drug is used. As mentioned above, the effects of local anesthetic drugs vary in different animals and it seems that the effect of these drugs on rabbits is less than that of other species. Comparing the results of present study in rabbits with our previous study in dogs confirms this idea (14).

One of the limitations of the present study was that the injectable forms of these drugs were used. The second limitation was a small number of samples. However, this was a preliminary study, and these limitations can be overcome in the future by producing the topical form of these drugs and evaluating them in a larger number of samples.

It can be concluded that all drugs used in this study (tetracaine, bupivacaine, lidocaine and proparacaine) reduce the IOP immediately after use; however, the effects of bupivacaine and lidocaine on IOP were much lower than that of tetracaine and proparacaine. Thus, since they do not intensely affect the IOP and since lidocaine has antimicrobial and even positive effect on corneal cells (35), it is recommended that these two drugs be used topically before measuring IOP. Tetracaine and proparacaine reduce the IOP and this should be taken into account in the glaucomatous animals to avoid mistakes.

Material and methods

In the present study, 10 healthy adult white rabbits with a weight of 1.63-3.27 kg (mean±SD: 2.17 ±0.54) were used. Ages of rabbits were 6 months. Five male and five female rabbits were used. The study was approved by the research council of the Facul-

ty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. The rabbits were kept in individual cages and were fed with dry commercial food and water ad libitum. All rabbits were carefully examined and were healthy, and showed no abnormalities in fluorescein staining, direct and panoptic ophthalmoscopy. Intraocular pressure of rabbits was measured at least once a day from a week before the start of the study to habituate the rabbits to this procedure.

To evaluate the effect of the first drug, one drop of 0.5% tetracaine (Anestocaine, Sina Darou, Tehran, Iran) was instilled in the right eye of five rabbits and the left eye of the other five rabbits. One drop of normal saline was instilled in the opposite eyes as controls. IOP was measured before and at 0, 5, 10, 15, 20, 25, 30, 35 and 40 minutes after drug instillation using an electronic rebound tonometer (TA01i tonometer, ICare, Finland) and immediately afterwards by an applanation tonometer (Tono-Pen Vet, Reichert, New York, USA). After an interval of at least one week, the effects of 0.5% bupivacaine (Marcaine® Spinal Heavy, Astrazeneca, Sweden), 2% lidocaine (Lignodig, Caspian Tamin, Iran) and 0.5% proparacaine (Alcaine, Alcon, Canada) were studied in the same way. Because IOP may vary throughout the day, IOPs were measured at 13:00–18:00 h in all rabbits. The sensation of the eyes was also examined every 5 minutes by corneal reflex (touching a piece of cotton with the cornea and observing the animal's blink). The rabbits were placed on a table in a relaxed state and prevented from any stress and minimal restraint was done on the head and neck without the use of systemic anesthetics or tranquilizers (Fig. 5). The eyelids were slowly opened and avoided any pressure on the eyelids and neck to prevent a change in IOP. Restraint was performed by the same assistant at all times. All measurements were also performed by a person who was unaware of the medication or placebo used in individual eyes and experienced with the use of both devices.

Statistical analysis: The normality of the data was analyzed using Shapiro-Wilk's statistical method. Since some of the data were abnormal, nonparametric tests were used for statistical comparisons. To compare the effect of each drug on time Friedman test, and in the case of significance, the Wilcoxon test was used to compare two sets of scores. The Wilcoxon test was also used to compare the IOPs of treated and control eyes. The Pearson correlation coefficient was used to test the relationship between IOP and the weight of rabbits. The Spearman correlation coefficient was used to determine the relationship between IOP and sex. The data are based on the mean ± SD for 10 rabbits. The level of significance was set at $p < 0.05$.

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Author Contributions

Design of Study: A.A.S, IOP measuring: A.A.S, A.E

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure 5.
Restraint of rabbit and tonometry with rebound Icare (a) and Tono-Pen Vet (b) tonometers. Probes are in the center of the cornea.

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The effects of bedding materials on learning and memory performance and texture preference in rats

Mehdi Abbasnejad[a], Razieh Kooshki[b], Saeed Esmaeili-Mahani[a], Abbas Tajabadi[a], Reyhaneh Naderi[a]

[a] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran.
[b] Department of Biology, Faculty of Sciences, Lorestan University, Khorramabad, Iran

ABSTRACT

The present study was designed to investigate the effect of different available bedding materials on learning and memory performance, bedding texture preference as well as intra-cage ammonia concentration in rats. The animals were housed on different bedding types for two weeks. Bedding materials were produced in the same sizes from poplar, walnut, pistachio, apricot, almond woods and alfalfa steam and live. Spatial and passive avoidance learning and memory were assessed by Morris water maze (MWM) and shuttle box tasks. A modifying six-arm radial maze was used to assess bedding texture preference by rats. For each bedding groups, average ammonia level (ppm) over a week was calculated. The data indicated that the rats that had walnut and almond chips show better learning and memory performance in both MWM and shuttle box tests than other groups. The weakest learning and memory performances were observed in rats exposed to alfalfa bedding. In texture preference test, the rats spent more time in walnut and almond arms, and less time in alfalfa. Besides, the total water and food intake as well as the number of visit to alfalfa arm were decreased as compared to other arms. Moreover, in alfalfa bedding cage, average intra-cage ammonia level was utmost. Overall, current bedding materials may contain diverse biochemically effective compounds or individual micro edges which alter learning and memory performances of rats.

Keywords

Bedding materials, Learning and memory, Texture preference, Rats

Abbreviations

MWM: Morris water maze
STL: Step-through latency
TDC: Time spent in the dark compartment
LTP: Long term potentiation

Corresponding author:
Razieh Kooshki
Tell/Fax: +98 9168568557
Email: Kooshki.r@lu.ac.ir

Introduction

The normal physiological functions of laboratory animals were strongly affected by their housing and husbandry conditions. In particular, bedding is one of the most important housing elements, which has influence on various neurobiological functions of laboratory animals [1-3]. Ethogram of mice behaviors including agonistic interaction, feeding, drinking, locomotion, nest-building and resting show bedding material-related changes [4]. On behalf of rodents, various kind of wood shavings such as paper, corncobs and hill rice are known as common bedding substances. There is no definition for ideal bedding, however, ideal bedding must have a low ability of infection, high absorbency, low allergenic activity, minimal chemical toxicity, low cost and high accessibility and compatibility [4-7].

It has been indicated that variations of bedding materials affects the stress and immune system [8], somatosensory signaling [9], neuropathic pain[10], vocalizations [11], body mass [12], temperature regulation, metabolism [13, 14], as well as liver enzyme levels in laboratory rodents [15, 16]. In particular, rodent's neurocognitive development has been affected by bedding materials and housing conditions. It has been indicated that corncob bedding suppresses estrogen-dependent aggressive behavior in rats [17]. In another study, mice housed in cages containing pulp chips, for 8 week, show better water maze performance than wood flakes group [18]. It has been indicated that exposure to an enriched environment induces dendritic branching and synaptogenesis in cortex and hippocampus of rats. Besides, housing enrichment can improve learning, memory, and synaptic plasticity in rodents[19]. Housing rats in cages with limited nesting/bedding materials impaired spatial learning and memory and hippocampal long term potentiation (LTP). Providing enriched environment can overcome the memory impairments through the recovery of LTP [1].

Preference testing has indicated that rodents have different preferences for bedding substances. Additionally, another study on rats and mice indicated that animals had strong preference for large fibrous bedding particles than relatively small particles [20]. It has been also reported that mice prefer cloth bedding type in comparison to wood shavings, paper and polycarbonate bedding substances [21]. Bedding material preferences for animals are determined by various chemical and physical features including color, odor, chemical compounds, and texture properties such as condition of surfaces, edges and coarseness.

In the present study, bedding texture preference was evaluated by housing the rats on six different bedding types including standard hard bed poplar chip, dried alfalfa as typical soft bedding material; walnut, apricot and almond as unusual bedding, and pistachio chip as very exclusive hard bed available in Iran and a few countries. Regarding climate change in Iran many large quantities of pistachio, walnut, apricot, and almond at low cost are available and can be used as bedding surface. We also examined bedding material mediated possible alterations in learning and memory performance of rats. In each bedding cage, ammonia concentration was also assessed.

Results

MWM test

There was a significant difference in the escape latency time among experimental groups during acquisition blocks [$F(3,480) = 44.52$, $p = 0.001$]. In the first day, the latency time to find the hidden platform was significantly decreased in the rats housed on walnut and almond as compared to other groups ($p < 0.05$). In the second day, there was a significant decrease in the escape latency of rats housed on almond as compared to apricot and poplar groups ($p < 0.05$) (Fig. 1, upper graph). In addition, the main escape latency was significantly different among groups [$F(5,165) = 15.93$, $p = 0.001$]. The rats housed on walnut and almond bedding showed the lowest latency time to find the hidden platform (Fig. 1, lower graph).

Significant differences were observed in the travelled distance to find hidden platform among experimental groups during acquisition days [$F(3,480) = 31.47$, $p = 0.001$]. In the first day, the rats housed on walnut and almond travelled lower distance to find the hidden platform as compared to rats that were housed on poplar and pistachio ($p < 0.05$). In the second day, rats kept on almond travelled lower distance to find the hidden platform in comparison with pistachio, apricot and alfalfa group ($p < 0.05$). In the third day, rats kept on almond and poplar travelled lower distance to find the hidden platform as compared to walnut, pistachio, apricot and alfalfa group ($p < 0.05$) (Fig. 2, upper graph). In the fourth day, however, rats subjected to walnut travelled lower distance to reach the hidden platform as compared to alfalfa and pistachio groups ($p < 0.05$). Furthermore, the main travelled distance to find the platform was significantly decreased in rats reserved on almond ($p < 0.05$) and walnut bedding ($p < 0.05$) in comparison to pistachio, alfalfa, and apricot (Fig. 2, lower graph).

The results of probe test indicated that time spent in the target zone was significantly increased in rats kept on walnut and almond as compared to poplar, apricot and alfalfa groups ($p < 0.01$) (Fig. 3).

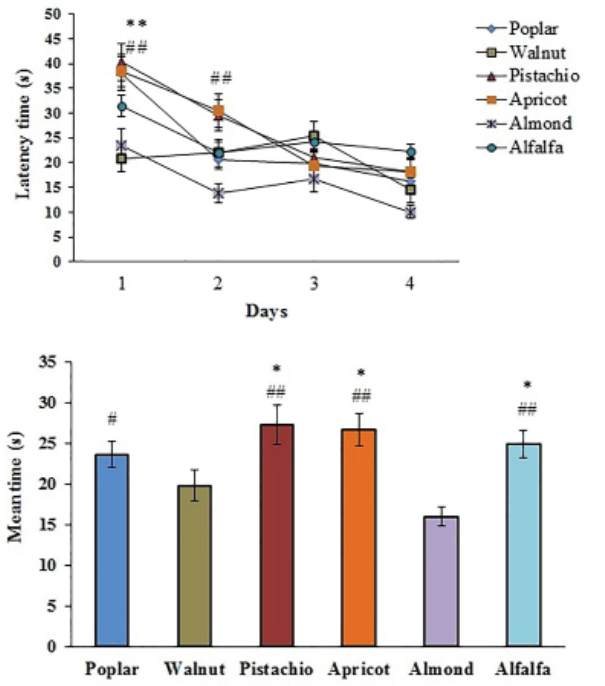

Figure 1.
Evaluation of the escape latency in each acquisition block (upper graph) and the mean scape latency (lower graph) in rats subjected to different bedding surfaces.
*: $p < 0.05$, **: $p < 0.01$ vs walnut group
#: $p < 0.05$, ##: $p < 0.01$ vs almond group

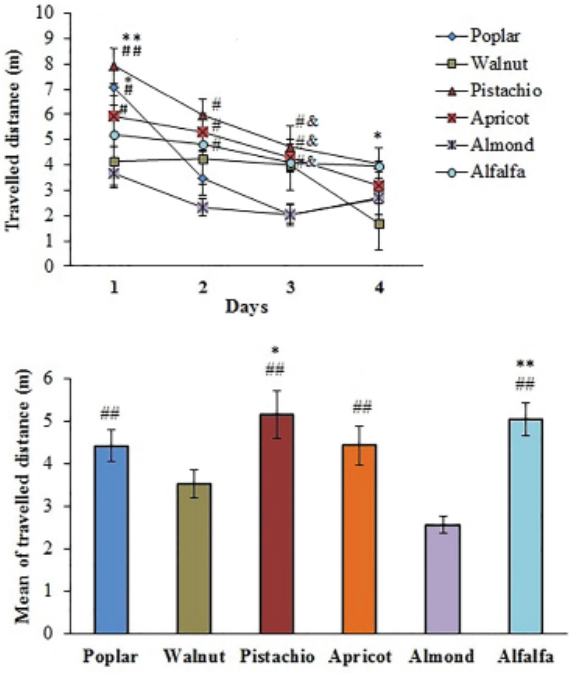

Figure 2.
Evaluation of the travelled distance in each acquisition block (upper graph) and the mean travelled distance (lower graph) in rats subjected to different bedding surfaces. Data are presented as mean ± SEM.
*: $p < 0.05$, **: $p < 0.01$ vs walnut
##: $p < 0.01$, #: $p < 0.05$ vs almond
&: $p < 0.01$ vs poplar.

Shuttle box test

As shown in Fig. 4A, the number of trials required to reach acquisition were significantly decreased in walnut, apricot and almond groups in comparison with poplar group ($p < 0.05$). In the retention test, rats housed in alfalfa bedding substance showed significant decrease in STL ($p < 0.05$) and increase in TDC ($p < 0.001$) as compared to other groups (Fig. 4B and 4C).

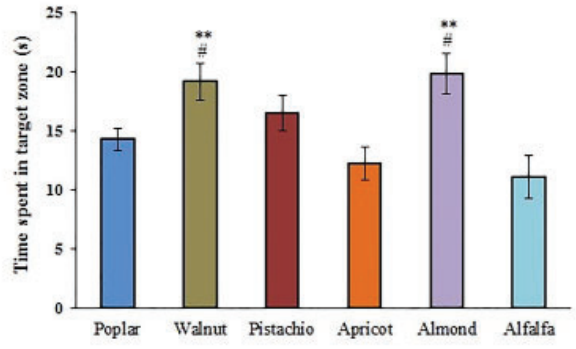

Figure 3.
Evaluation of the time spent in the target zone in probe test between the groups of rat subjected to the different bedding surfaces. Data are presented as mean ± SEM.
**: $p < 0.01$ vs apricot and alfalfa
#: $p < 0.05$ vs poplar

Ammonia concentration

Intra-cage ammonia level was recorded during the first week of experiment. As shown in Fig. 5, the mean ammonia concentration in alfalfa cage was significantly increased in comparison with others groups ($p < 0.001$).

Bedding preference

During the first week, the rats spent highest time in walnut and almond ($p < 0.001$) and lowest time in the alfalfa comprised arms ($p < 0.001$) (Fig. 6A). In addition, the rats had more visit to arms containing almond than pistachio and apricot ($p < 0.05$) and alfalfa ($p < 0.001$). Besides, rats visit into the cage containing alfalfa bedding was lowest (Fig. 6B). Moreover, As shown in Fig. 7A, the total water consumption by rats were significantly increased in walnut, almond as well as poplar cages in comparison with pistachio and alfalfa cages ($p < 0.01$). In addition, the total food consumption was significantly increased in almond cage as compared to pistachio and apricot ($p < 0.05$) as well as alfalfa cage ($p < 0.01$). Moreover, food consumption

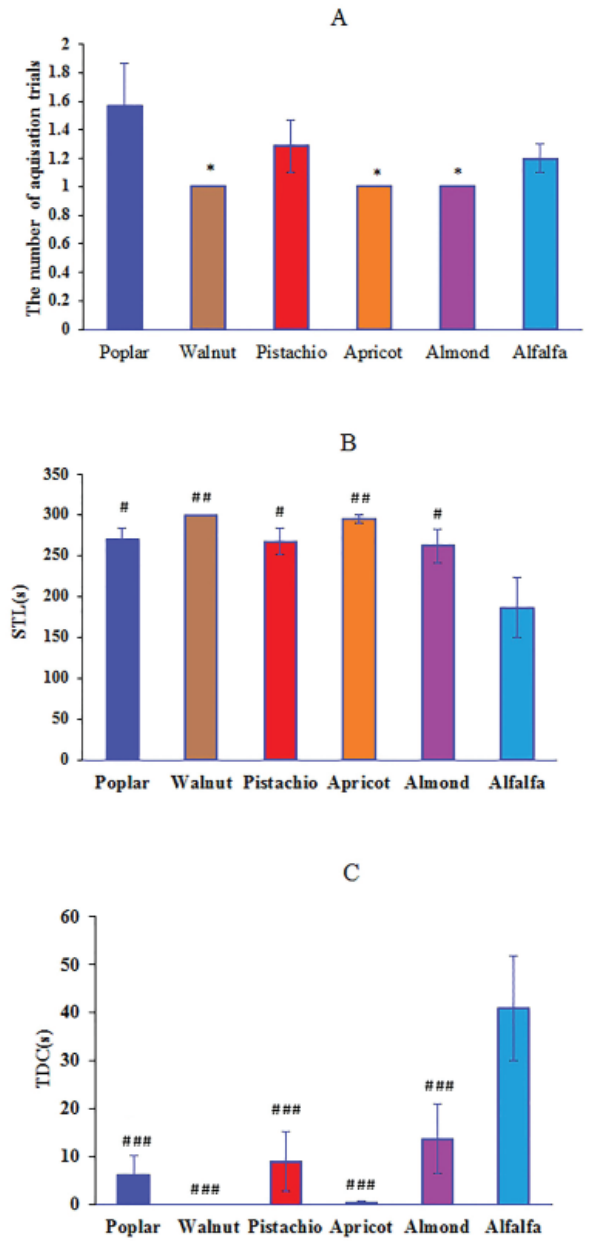

Figure 4.
Evaluation of the number of acquisition trials to reach successful learning (A), the step through latency (STL) (B) and time spent in dark chamber (TDC) (C) in shuttle box test in the groups of rats subjected to different bedding types. Data are presented as mean ± SEM.
*: $p < 0.05$ vs poplar group
#: $p < 0.05$, ###: $p < 0.001$ vs alfalfa

was significantly increased in poplar and almond cages in comparison with alfalfa ($p < 0.05$) (Fig. 6B).

Discussion

The present study investigated the learning and memory performances of rats subjected to different bedding chips including poplar, walnut, pistachio, apricot, almond, and alfalfa. According to the results, in comparison with other groups, rats housed on walnut and almond chips showed better learning and memory performance in MWM and shuttle box tests. The learning and memory performances of rats subjected to alfalfa bedding were lesser than other bedding types. Besides, investigation preferences of rats for types of bedding by a six-arm radial maze showed that rats spent more time in chips of walnut in comparison with pistachio, apricot and alfalfa bedding types. In addition, the rats had highest and lowest visit into almond and alfalfa-containing cage, respectively. Likewise, food and water intake by rats in alfalfa contain cage was lowest. There are only a few studies on the substance quality as bedding material. The present study, tried to fill the gap of data about pistachio, apricot and almond.

It has been well documented that bedding and husbandry have influence on rodent's neurophysiologic responses. However, just a few studies have shown bedding texture property on cognitive-related behaviors. A study by Tanaka and colleagues showed that mice subjected to pulp chips bedding, over eight weeks period, had better water maze performance than those kept on wood flakes [18].

The results of this study also showed that the rats find walnut and almond sawdust more suitable as a resting surface than other four bedding types. It shows direct relationship between comfortable bedding texture and learning and memory performances. The bedding materials used here due to their structure have diverse edge and surface which may result in changed stimulation of the plantar foot area [22]. In other words, edge of the bedding show different mechanical effect on rat planter surface. It has been indicated that the daily behaviors of rats such as nesting and sleeping are affected by kind of bedding materials [10]. Besides, bedding material features may affect sensory processes even down to the molecular and cellular levels [10].

Here, the rats subjected to walnut and almond chips showed better learning and memory performance. Nuts, leaves, woods and hulls from walnut and almond are highly valued for their biological properties. In particular, the compounds have potential health-promoting activities because of their phenolic-enriched contents. It has been indicated that walnut polyphenol improves learning and memory performances of hypercholesterolemia mice [23]. It has been indicated that flavonoid and other polyphenols material improve learning and memory performances mainly via their antioxidant capacity and cholinesterase activities [24-26]. In the present study, it is likely that beddings different influences on learning and memory are partially mediated by differences in their polyphenolic capacity. However, we did not explore polyphenols capacity of different chips and to clarify

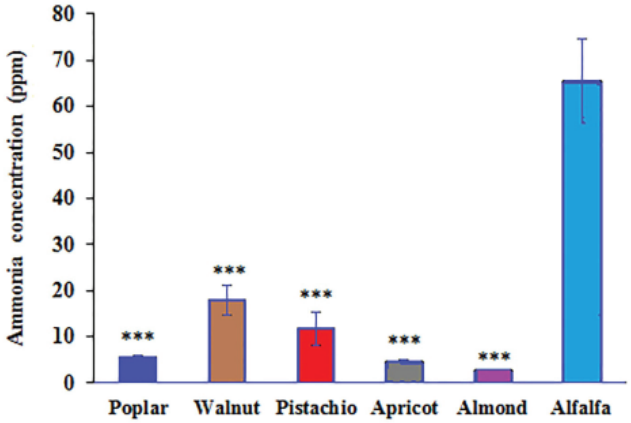

Figure 5.
The average of ammonia concentration in each bedding cage. Data are presented as mean ± SEM.
***: $p < 0.001$, vs alfalfa

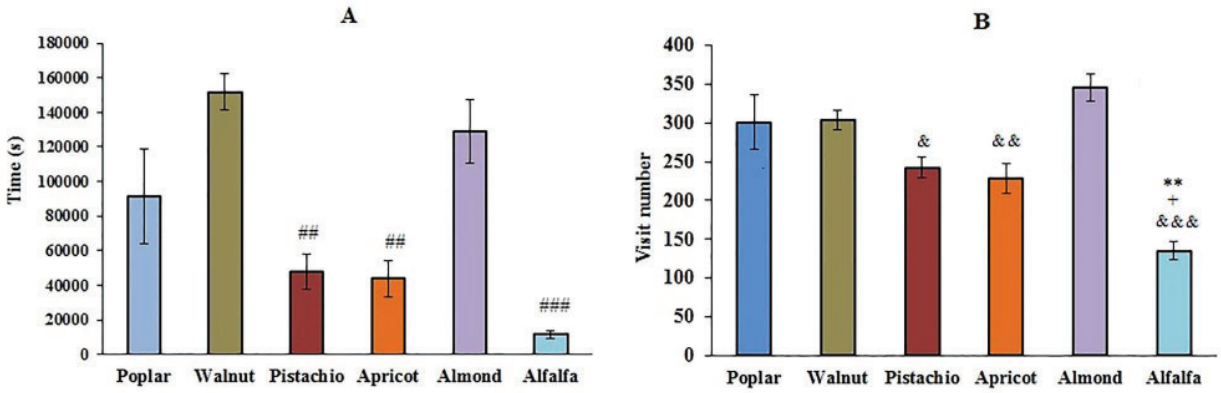

Figure 6.
Assessment time spent and the number of visits by rats into each arm of radial maze containing different bedding substances during a week. Data are presented as mean ± SEM.
**: $p < 0.01$ vs poplar and walnut
##: $p < 0.05$, ###: $p < 0.001$ vs walnut and almond
+: $p < 0.05$ vs pistachio and apricot
&: $p < 0.05$, &&: $p < 0.01$, &&&: $p < 0.001$ vs almond

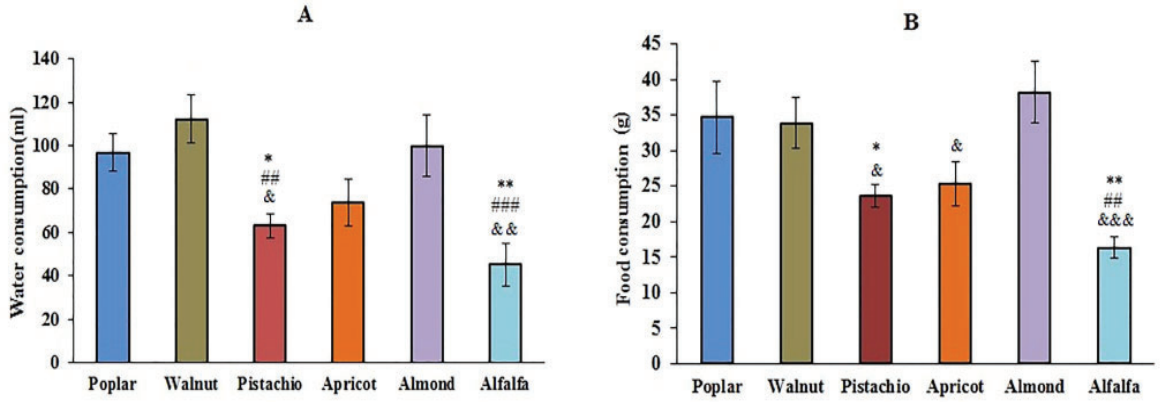

Figure 7.
Comparison of food and water consumption by rats in the maze containing different bedding substance in each arm. Data are presented as mean ± SEM.
*: $p < 0.05$,**: $p < 0.01$, vs poplar
##: $p < 0.05$, ###: $p < 0.001$ vs walnut
&: $p < 0.05$, &&: $p < 0.01$, &&&: $p < 0.001$ vs almond

such relationship further studies are still required.

To the best of our knowledge the study was the first to consider association between bedding preference and learning and memory related behaviors. However, some studies have indicated that improvement of nesting conditions positively modulate learning and memory -related functions. It has been demonstrated that environmental enrichment improves learning and memory in various laboratory tasks [2]. In addition, environmental enrichment can induce morphological changes in the cortex and hippocampus of rat such as enhancement of neuron numbers, synapses and dendritic branches [3, 27]. Increasing bedding volume has been correlated with intracage ammonia decrease [4, 28]. Moreover, there is significant relationship between the depth of bedding and animal preference as well as animal physiology [4].

Welfare of laboratory animals are closely related to optimum intracage features like the level of ammonia, moisture, and absorbency and bacterial growth [29]. In the current study, assessment of ammonia concentration in each cage showed that there was the highest intracage ammonia in the cage containing alfalfa bedding. As shown in the results, the mean ammonia level was lower than adverse level 100 ppm or 130 ppm [22, 30]. Metabolized urea from urine and feces of the animal is responsible for ammonia production [31].

Interestingly, the rats housed on alfalfa bedding surface showed lowest learning and memory performance. Various studies have been reported on the distractive effect of ammonia on brain functions. Increased ammonia concentration in the brain as a result of diseases could induce a range of neurobehavioral dysfunctions like learning and memory deficient, sleep-wake inversions, brain edema and seizures [32, 33]. It has also been reported that inhalation of ammonia was able to reduce cognitive performance for culture fair, digit symbol and vocabulary[34]. Besides, increased intra cage CO_2 levels and fecal cortisol concentrations have crucial role in animal function and behaviors [4], but were not included in this study.

Conclusion

This study provides some primary data supporting the relationship between bedding texture preference and learning and memory function of rats. The effect may be partially mediated by bedding differences in ammonia absorption capacity. It also could be related to biochemical diversity and individual micro edges of distinct bedding materials.

Material and methods

Animal and housing conditions

Adult male Wistar rats (230–270 gr) were used. The animals were housed in conventional animal room (3× 8× 3 m) under a 12 h light/dark cycle in controlled condition with temperature of 22 ± 2 °C. The ventilation rate was 8-15 times per hour. During the experiment the same amount of food and water was available ad libitum. All experimental procedures were approved by the Animal Research Ethics Committee of the Shahid Bahonar University of Kerman, Iran.

Experimental design

Experiment 1: Evaluation of the spatial and passive avoidance learning and memory performances

The rats were divided into six experimental groups and kept on the same amount of different bedding substances for 2 weeks. Bedding materials included were chip of poplar, walnut, pistachio, apricot, almond and dried alfalfa. The wood ship average particle sizes were 15 × 4 × 1 mm with a moisture of 8%, which was sieved (purchased from zist mehvar-pajoheshe Pars Company, Kerman, Iran). The number of animals per group and per cage were seven. The amounts of animal biomass per cage were the same in the beginning and during the study. The rats learning and memory performances were evaluated using Morris water maze (MWM) and shuttle box tests. Besides, during first week of the experiment daily changes in intracage ammonia concentration was measured using multigas transmitter (ModBus, TM-1280). To take a reading, the sampling tube was inserted in the middle of bedding. The monitor took continuous reading for 5 min, for measuring ammonia level according manufacturer's recommendations. The experimenters were not blinded to the bedding types; they had no expectations of any group differences.

Experiment 2: bedding material preference

Bedding texture preference by rats (n=7) was assessed using a modified bedding preference test system introduced by Blom [20]. Briefly, the maze consisted of a central wire mesh circle area surrounded by six enclosed wooden cages (50 × 10 × 40 cm). The central zone was raised 2 cm above the floor than the cages. Equal amounts of six bedding materials, almost the same in size and shape, were situated on different cages of the maze. In each cage food and water was ad libitum. For beginning the experiment, each rat was placed on the central area of maze and during one-week period the rats' behavior including the number of visit and time spent in each arm were monitored by a video camera system. Besides, rats' food and water intakes in different cages of maze were measured. Before this test, rats were housed on pine shavings.

Assessment of learning and memory performance

Shuttle box test

PA learning and memory was assessed by a shuttle box apparatus as previously described. The test protocol was divided into a learning session on the first day and a test trial 24 h later. For the learning trial, each animal was placed in light chamber of shuttle box apparatus. Then, the door was opened and the animal was allowed to enter into the dark sector. The animal received an electrical shock (0.5 mA, 50 Hz, 2 s once) upon entrance to dark sector via the stainless steel floor. The learning trial was terminated when the rat remained in the light chamber for 5 consecutive minutes. After one day, the retention test for assessing memory, each rat was placed in the light compartment of shuttle box device. After 30

seconds, the door was opened and the step-through latency (STL), the time before the first entry of the rat to the dark sector, and total time spent in the dark compartment (TDC) were recorded.

Morris water maze (MWM) test

Spatial learning and memory was assessed by MWM pool. It consisted of a dark circular pool (136 cm in diameter and 60 cm high) filled with water (20 ± 1 °C) to a depth of 25 cm. The extra maze cues were placed in consistent locations on the walls which were visible to the rats. The pool was divided into four quadrants defined by the four cardinal directions. A circular platform was located 2 cm below the water surface in the middle of one of the quadrants. At the beginning of experiment, each rat was lightly placed in the water facing the wall of the pool from one of the directions. The location of each rat was tracked by a digital TV system and analyzed using the Ethovision video tracking system (Noldus Information Technology, the Netherlands). One day prior to the beginning of training; the rats were habituated to the pool by allowing them to swim for 60 s without the platform.

The test included acquisition and probe trials. The acquisition test was performed on 4 consecutive days with four trials per day with a 5 min interval between trials. The rats were allowed to swim within 60 s to find the hidden platform at each trial. Once the platform was found, the animal would have to stay on the platform for 30 s. If unsuccessful within 60 s, it was gently guided to the platform for 30 s. The escape latency, traveled distance and swimming speed for each rat were evaluated. In probe test, one day after acquisition test, the hidden platform was removed from the pool and rats were placed in the quadrant opposite the target quadrant and allowed to swim freely for 60 s. The time spent in the target quadrant was recorded and analyzed as a measure of spatial memory retention.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The acquisition test data related to four acquisition days of MWM test were analyzed using repeated measures ANOVA. The statistical significances for probe and preference tests were determined by one way ANOVA followed by post-hoc Tukey's test. The results of shuttle box test were analyzed by Friedman and Kruskal-Wallis tests followed by Mann-Whitney U test.

Acknowledgments

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Author Contributions

M.A and S.EM designed the study; A.T and R.N collected data; R.K analyzed the data and wrote the paper.

Conflict of Interest

None .

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RESEARCH ARTICLE

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Biological effects of agricultural bio-materials on some blood and tissue factors in Balb/c mice

Manijeh Rostaminia[a], Davood Habibi[b], Samira Shahbazi[c], Behzad Sani[a], Alireza Pazoki[d]

[a] Department of Agronomy, Shahr- e- Qods Branch, Islamic Azad University, Tehran, Iran.
[b] Department of Agronomy, Karaj Branch, Islamic Azad University, Karaj, Iran.
[c] Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Alborz, Iran.
[d] Department of Agronomy, Yadegar-e-Imam Khomeini (RAH) Shahr-e- Rey Branch, Islamic Azad University, Tehran, Iran.

ABSTRACT

Pseudomonas infections are an important cause of morbidity and mortality and saprophytic fungi are now increasingly being recognized as serious pathogens in immunocompromised patients. To investigate the effect of using bio-materials on mammalian tissues, two experiments were designed; the first one was feeding of Balb/c mice with irrigated lettuce with bio-fungicide (mutant and wild) and bio-fertilizers prepared with *Pseudomonas* (p) *fluorescens*, *p. putida*, *p. aeruginosa*, and the second was the usage of drinking water containing (*Trichoderma* (T) spores (mutant and wild) or *P. fluorescens*, *P. putida*, *P. aeruginosa* suspensions). Then, blood factors and inflammation of tissues (liver, kidney, spleen and large intestine) in all mice were analyzed after two months. Blood samples were taken from the mice to examine some of the hematological (RBC, MCV, MCH, MCHC) (data not shown) and biochemical (AST, ALT, ALP) factors, and also observed under a microscope. The study of tumor marker carcinoembryonic antigen (CEA) in all treatments showed that the strains in these bio-fertilizers did not stimulate carcinogenic indices. The results from the other blood factors were normal for all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in alkaline phosphatase (ALP). The results also showed that the effect of bio-fungicide on mammalian tissues (spleen and large intestine) was normal. But a small number of mild liver necrosis was seen in the treatment groups with wild *Trichoderma*, and moderate necrosis in the the liver tissue after treatment with mutant *Trichoderma* isolates. More investigation should be made to determine the impact of these biotic factors on the mammalian tissues before commercialization.

Keywords

Pseudomonas, Fertilizer, Biological Fungicide, Trichoderma

Abbreviations

CEA: Carcinoembryonic antigen
ALP: Alkaline phosphatase
P: *Pseudomonas*
T: *Trichoderma*

Corresponding author:
Samira Shahbazi
Tell/Fax: +989122644209
Email: sshahbazi@aeoi.org.ir

Introduction

According to the statistics published in Iran, per capita consumption of poison in agricultural products for each person is 400 grams, and the use of chemical fertilizers has been increased from 2.5 to 3.5 million tons over the past 10 years. In the traditional agriculture, over 300 types of hazardous chemicals such as pesticides, herbicides, and fertilizers are used to control pests, insects and to increase soil fertility. The residue of these materials will be absorbed into plants by infecting groundwater and air. In addition, it will accumulate in agricultural products such as fruits and vegetables and will be transmitted to the human body. For many years, to tackle these problems, the bio-fungicides such as commercial products made by Trichoderma and bio-fertilizers made with Pseudomonas have been used in different countries (1). Pseudomonas spp. is an aerobic, gram negative, rod shaped, non-spore forming and fast growing bacterium. The most important fluorescent species are Pseudomonas aeruginosa, Pseudomonas putida, and Pseudomonas fluorescens (2). Pseudomonas fluorescens unlike P. aeruginosa has generally been regarded to be of low virulence, and an infrequent cause of human infection (3). However, it has been reported to cause infections such as blood transfusion-related septicemia (4, 5), catheter-related bacteremia (3), and peritonitis in peritoneal dialysis patients (6). Pseudomonas aeruginosa being the most common species isolated from clinical specimens (7). Its pathogenicity has generally been related to its exotoxin. These exotoxins can produce leukopenia, acidosis, circulatory collapse, necrosis of liver, pulmonary edema, hemorrhage, and tubular necrosis of kidneys. The extracellular toxins that cause damage to the tissues of different hosts may differ. Pseudomonas aeruginosa causes several different infections including endocarditis, pneumonia, malignant otitis externa, bacteremia, and also, gastrointestinal tract, skin and soft tissue, skeletal, eye, and burn infections (8, 9). Pseudomonas putida is an uncommon cause of skin and soft tissue infections. It is often associated with trauma or immunocompromised states, and in patients possessing medical devices or catheters (10, 11). Pseudomonas putida is considered a low-virulence pathogen and has been recognized as a rare cause of bacteremia. Despite the fact that this organism causes health care-related infections, clinical data on P. putida infections are lacking, owing to the rarity, relatively lower virulence, and higher antimicrobial susceptibility of P. putida compared with other Pseudomonas species, especially Pseudomonas aeruginosa (10, 12-14).

It is necessary to reduce the consumption of chemical fertilizers and pesticides on greens and vegetables, such as lettuce, which are eaten fresh. However, problems with residual pesticides and pathogenic chemical compounds indicate that the production and use of bio-fertilizers has become more important. The current research was conducted to evaluate the possibility of undesirable effects from these organisms in greenhouse cultivation systems, groundwater and the mammalian food chain.

Trichoderma spp., has been widely investigated in recent years and is the most widely used as a bio-control agent against phytopathogens (15). Over the past decade, infections caused by opportunistic filamentous fungi have become increasingly common among patients after allogeneic stem cell transplantation (allo- HSCT). Trichoderma species are considered plant saprophytes, but have recently been linked to severe cases of invasive infection in immunocompromised human hosts [16-19]. The aim of this study was to investigate the biological effects of Trichoderma rifaii (mutant and wild types) on Balb /c mice (as a model mammal). Obviously, a more comprehensive study should also be carried out on the other isolates that are used as bio-fungicides and pesticides in the production systems. The goal is to draw the attention of other researchers, before the recommendation and application of bacterial microorganisms. The possible adverse effects of inoculation with different species of Pseudomonas spp. and the risks associated with the use of these biological compounds on mammalian health has also been investigated.

Results

The present study examined the biological effects of these bacteria and *Trichoderma* as a bio fungicide on inflammation or necrosis of tissues such as kidney, liver, spleen and large intestine, blood factors (alkaline phosphatase), carcinoembryonic antigen (CEA) and hemoglobin in a mammalian model (mouse). The study of tumor marker CEA in all treatments showed that the strains in these bio-fertilizers did not stimulate carcinogenic indices. The results from the blood factors were normal for all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in alkaline phosphatase (ALP). In this study of the inflammatory processes in the liver, kidney and large intestine, of the 12 mice studied, only minor liver and kidney necrosis and the large intestine necrosis were observed for the different bacterial strains (Figure 1). No pathologies were found in the spleen tissue (Figure 1). The data acquired from 12 mice indicates the need for further studies on the effects of bio-fertilizers on mammals (Table 1). The results of blood factors ALP and CEA in all treatment groups showed

that the bio-fungicide propagule did not stimulate carcinogenicity indices. The study of inflammatory process in the liver and kidney of the twelve studied mouse tissues showed that a small number of mild liver necrosis were seen in the treatment with wild-type *Trichoderma*, and moderate necrosis in the liver tissue after treatment with *Trichoderma* mutant isolates (Figure 1-b2). There were no effects on the spleen and large intestine (Table 2) (Figure 1c, d2).

Discussion

According to the results of this study, tumor marker CEA was not affected by different bacterial strains. Similar results were reported by Kiyama et al. (20). Blood factors were normal in all groups treated with all strains (data not shown). Only *P. putida* showed no adverse effect on the increase in ALP (21, 22).

The pathological changes in the liver, spleen, lung and heart were similar to the changes reported by Al-Muhammadawi (23). Olgerts et al., (24) reported the histopathology and serum enzyme levels of mice inoculated intravenously with *Pseudomonas aeruginosa* exotoxin. The toxin had a significant effect on the liver but did not cause any microscopic changes in other organs. Microscopic changes resulting from an injection of two 50% lethal doses (LD50) of toxins (2.3 g) into the liver are characterized by necrosis, cell swelling, and fat change within 4-8 hours and it was similar to necrosis of cells in the kidney, after 48 hours. Liver necrosis was associated with a parallel increase in serum levels of aspartate and alanine aminotransferases and alkaline phosphatase.

A single injection of 10 LD50 elicited similar but somewhat more rapid degeneration. No progressive lesions were seen after injection of toxoid or of 0.5 LD50 of toxin. Our results were similar to these results. Our microscopic observations are similar with those made by Liu (25). He has briefly reported liver necrosis, edematous and hemorrhagic lungs, and necrotic and hemorrhagic kidneys in mice given intraperitoneal toxin. The enzyme activity in serum (a relatively small increase in levels of alkaline phosphatase) was consistent with the histologic pattern of necrosis.

Based on the Rees (26) report, T*richoderma harzianum* strain T-39 was not infectious, pathogenic or toxic to rats when administered orally at 1.4 to 2.0 x

Table 1
Effect of bio-fertilizer on blood factors and inflammatory processes in extremities of mice treated with bacterial cells and lettuce.

Treatment	Blood		Vital organs			
	CEA[2] Normal (<2.5 ng/ml)	ALP[1] Normal (230-55 U/L)	Kidney	Spleen	liver	gastrointestinal tract
Control (tap water)	0.31	139	0	0	0	0
P. fluorescens + (water)	0.39	485	0	0	1	1
P. putida + (water)	0.21	142	1	0	0	0
P. aeruginosa + (water)	0.3	296	1	0	1	1
Control (lettuce)	0.27	129	0	0	0	0
P. fluorescens + (lettuce)	0.33	330	0	0	0	1
P. putida + (lettuce)	0.29	165	1	0	1	0
P. aeruginosa + (lettuce)	0.25	288	0	0	1	0

[1] alkaline phosphatase
[2] carcinoembryonic antigen
Ranked inflammatory process: negative=0; mild necrosis=1; moderate necrosis=2; severe necrosis=3

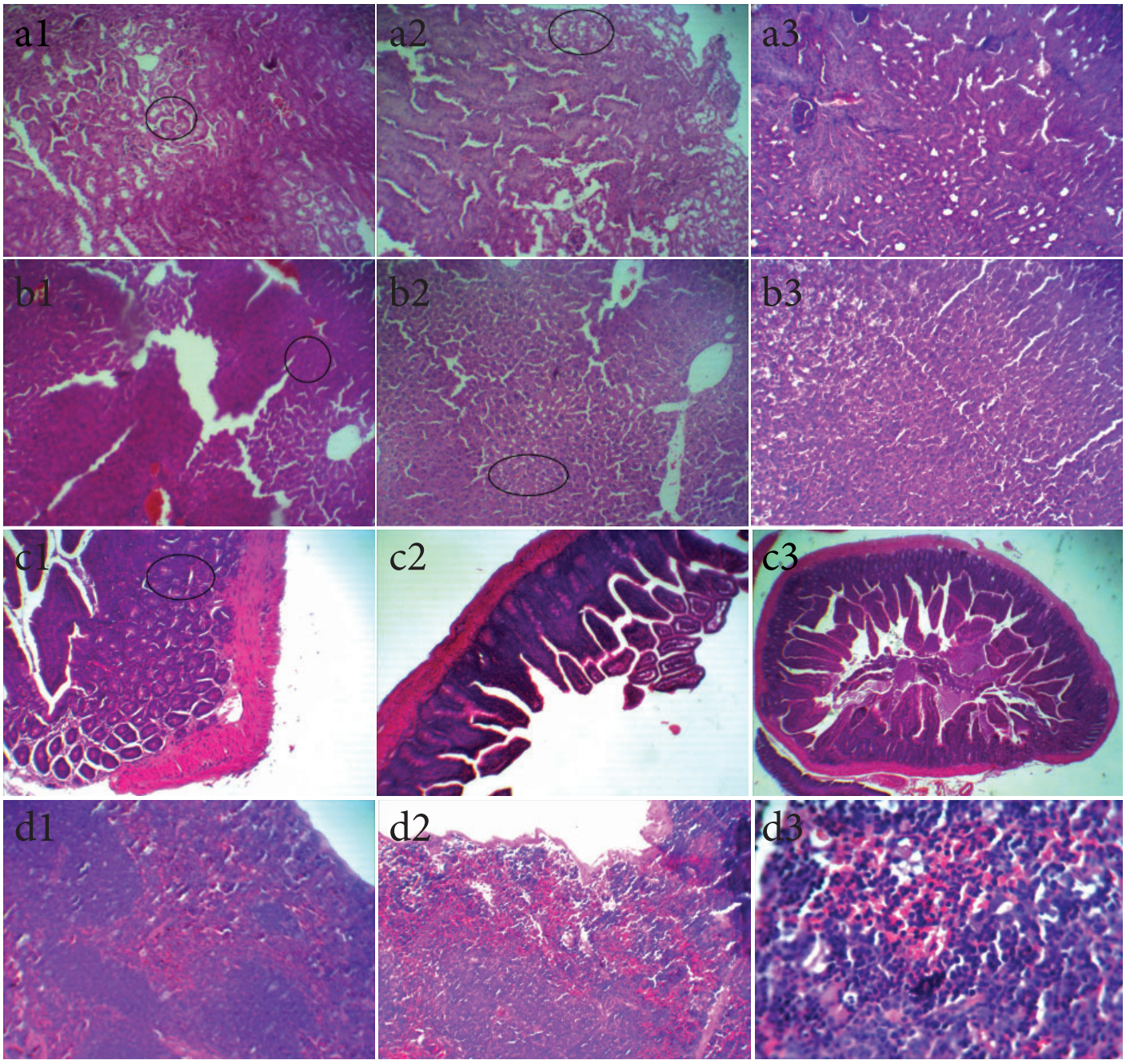

Figure 1.
Comparison of microscopic image of different organ tissues treated with biological materials. a1: The kidney tissue of mouse fed with *P. putida*. a2: The kidney tissue of mouse fed with mutant *Trichoderma.* a3: The kidney tissue of control. b1: The liver tissue of mouse fed by *P. aeroginosa*, b2: The liver tissue of mouse fed with mutant *Trichoderma.* b3: The liver tissue of control. c1: The gastrointestinal tract tissue of mouse fed with *P. fluorescens*, c2: The gastrointestinal tract tissue of mouse fed with mutant *Trichoderma.* c3: The gastrointestinal tract tissue of control. d1: The spleen tissue of mouse fed with *P. putida.* d2: The spleen tissue of mouse fed with mutant *Trichoderma.* d3: The spleen tissue of control. Dark ovals in the shapes indicate the presence of necrosis or cell degeneration. The nucleus becomes swollen and dark and eventually disappears

10^8 CFU/animal. Clearance and infectivity were evaluated in the brain, blood, lymph nodes, kidney, liver, spleen, lungs, caecum and feces. According to Leuschner's (27) findings. Rats were given an oral dose of *trichoderma asperellum* strain ICC 012 (*Trichoderma asperellum conidia* 4.2 x 10^9 CFU/g) at 6-7 weeks of age, then the mice were evaluated over a period of 14 days. The results of this study showed that the *Trichoderma* strain was not toxic at a concentration of 2000 mg / kg body weight. No deaths occurred during the study. *Trichoderma* infection and pathogenesis are also unknown and no clinical signs of treatment or weight change were observed.

According to a report, a suspected case of invasive pulmonary infection with *T. longibrachiatum* in a patient with severe aplastic anemia who received allo-HSCT and was successfully treated with liposomal amphotericin B (L-AmB). There are few reports on the effects of *T. harzianum* on mammalian cells (28) or humans, while the effects of biologically active peptides produced by other *Trichoderma* species have been extensively investigated (29).

According to the Biotechnology Committee of biological products the total cultivated area of crops produced in Iran without the use of pesticides and fertilizers is about 239 thousand and 364 hectares, including 125 thousand and 802 hectares of horticultural products and 113 thousand and 659 hectares of agricultural crops. Generally, the amount of cultivation of agricultural and horticultural that produce them without using fertilizers and poisons is 1% and 2.7% of the total cultivated in Iran, respectively.

In order to establish a bio-agronomic system in Iran, at the same time as the development of this production, the reliable and verified planning in the field of supply these compounds in the distribution network is essential because economization of bio-agriculture is necessary for its development and expansion. Before all this, accurate and sequential reviews on the effect of the use of this fungicide and bio-fertilizers on consumers in long-term should always be included in the agricultural research program in order to prevent the potential harm of such compounds or to make informed choices based on scientific data to the consumer.

Conclusion

The results for the effect of bio-fertilizers on mammals show that they have a minor effect on the liver, kidney and large intestine. The effect of active bio-fertilizers on blood factor such as Carcinoembryonic antigen (CEA) and Alkaline phosphatase(ALP) were normal for all strains. The results also show that the effect of bio-fungicide on mammal's tissues (spleen

Table 2
Effect of active fungicide on blood factors and inflammatory process in vital organs of mice treated with suspension of *Trichoderma* wild type and mutant spores and lettuce treated with fungicide.

Treatment	Blood		Vital organs			
	CEA[2] Normal (<2.5 ng/ml)	ALP[1] Normal (230-55 U/L)	Kidney	Spleen	liver	gastrointestinal tract
Control (water)	0.31	139	0	0	0	0
Wild type *Trichoderma* + water	0.32	170	0	0	1	0
Mutant *Trichoderma* + water	0.33	290	1	0	1	0
Wild type *Trichoderma* + lettuce	0.10	250	0	0	1	0
Mutant *Trichoderma* + lettuce	0.42	198	1	0	2	0
Control (lettuce)	0.27	129	0	0	0	0

[1] alkaline phosphatase
[2] carcinoembryonic antigen
Ranked inflammatory process: negative=0; mild necrosis=1; moderate necrosis=2; severe necrosis=3

and large intestine) was normal. But a small number of mild liver necrosis were seen in the treatment with Trichoderma, and moderate necrosis in the liver tissue after treatment with mutant Trichoderma isolates that it reminds to researchers that the observation of some mild liver necrosis requires a re-examination and double-check on the effects of bio-fungicides.

The protocol of the research entitled Biological effects of Agricultural bio-materials on some blood and tissue factors in Balb/c mice was performed according to Iranian animal ethics society and local university rules.

Material and methods

Bio-materials

Three commercial bio-fertilizers containing *p. aeruginosa*, *P. putida* and *P. fluorescens* were obtained from the Soil and Water Research Institute in Karaj, Iran. *T. rifaii* species (wild and mutant isolates) were collected from fungal collections of the Karaj Nuclear Agriculture Research Center. The tested mice were white, male and female, prepared from the Razi Vaccine and Serology Research Institute.

The effect of Trichoderma spp and Pseudomonas on mammals

The bio-fungicide propagule (suspension of the spore from isolated wild and mutant *Trichoderma*, with a concentration of 10^7 cells per ml) and the bio-fertilizer propagule (suspension of bacterial cells with a concentration of 10^5 cells per ml) were added to drinking water for two months, two times per week to the first group of Balb/c mice (3 males and 9 females). In the second group, mice (3 males and 9 females) were fed with lettuce treated with bio-fungicide and three biological fertilizers three times per week. Blood factors (ALP, AST, CBC), tumor marker carcinoembryonic antigen (CEA), hemoglobin and the inflammatory process of tissues such as kidney, liver, spleen and large intestine in all mice were examined. Only alkaline phosphatase (ALP) and CEA as the main carcinogens are presented here (Tables 1, 2).

Preparation of tissues and blood samples

Mice were 3.5-4 months old and of balb/c strain. The average weight of balb/c mice was from 20 to 25 grams. At the end of the mentioned time (50 days), the mice were anesthetized by peritoneal injection, and after the completion of the procedure, organs such as the spleen, kidney, liver and large intestine were sampled. The tissue samples were fixed in 10% formalin. After the fixation, the digestion and molding stages were done by alcohol and paraffin, respectively. Then, the transverse sections of the anterior, middle, and posterior tissues were prepared by a microtome. The slides were stained with hematoxylin and eosin and then observed by an optical microscope. Blood samples were directly taken from the heart by an insulin syringe, so that the needle was inserted into the area by observing the heartbeat, and when the heartbeat was felt as vibration of the syringe, complete blood sampling was done. After blood sampling, the samples were transferred to two vials with and without anti-coagulant EDTA. The EDTA-free samples were used for analysis of liver enzymes.

Statistical analysis

Data obtained from the experiments was analyzed in SPSS (ver. 13). The groups were compared using ANOVA followed by Duncan's multiple range test at the ($p < 0.05$) level of significance.

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Author Contributions

Conceived and designed the experiments: S.S, H.D. Performed the experiments: S.S. Analyzed the data: S.S. Research space and equipment: R.M, H.D, S.S. Contributed reagents/materials/analysis tools: R.M, H.D, S.S. Wrote the paper: R.M, S.S. Read and edit the paper: S.B, P.A.R..

Conflict of Interest

The authors declare that they have no competing interests.

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SHORT COMMUNICATION

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Anti-quorum sensing and antibacterial activities of *Satureja sahendica* hydroalcoholic extract against avian isolate of *Salmonella* Typhimurium

Razieh Sharchi, Jalal Shayegh, Somayyeh Hosseinzadeh

Department of Veterinary Medicine, Shabestar branch, Islamic Azad University, Shabestar, Iran

ABSTRACT

Quorum sensing (QS) is a cell density dependent mechanism used by many pathogenic bacteria for regulating virulence gene expression. Inhibition or interruption of QS by herbal remedies has been suggested as a new strategy for fighting against antibiotic resistant bacteria. The aim of this study was to evaluate the antibacterial activity of *Satureja sahendica* hydroalcoholic extract (SSHE) against *Salmonella* Typhimurium (*S.* Typhimurium) isolates, and to assess the impact of sub-inhibitory concentrations of this extract on the QS-associated gene (*sdiA*) expression. Using the Soxhlet method, hydroalcoholic extract of *S. sahendica* leaves was prepared and antimicrobial effects of the SSHE were determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) method*S.* The reverse transcription quantitative PCR (RT-qPCR) assay was used to analyze the expression of *sdiA* in 20 *S.* Typhimurium isolates from poultry flocks in response to the treatment of sub-inhibitory concentrations of SSHE at 60-min time point. The MIC values of SSHE against *S.* Typhimurium isolates were ranged from 0.29-4.68 mg/ml^{-1} and MBC values were ranging from 75-150 mg/ml^{-1}. The results also indicated that the expression of *sdiA* gene was reduced in *S.* Typhimurium isolates triggered by the treatment of SSHE comparatively with those from the control culture*S.* Findings suggest that SSHE possess the antibacterial and anti-QS activity and can be used to control the expression of virulence genes in pathogenic bacteria, such as *S.* Typhimurium.

Keywords

Satureja sahendica, Hydroalcoholic extract, sdiA gene, gene expression, Salmonella Typhimurium

Abbreviations

QS: Quorum sensing
SSHE: *Satureja sahendica* hydroalcoholic extract
S. Typhimurium: *Salmonella* Typhimurium
MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration
RT-qPCR: Reverse trasncription quantitative PCR

Corresponding author:
Jalal Shayegh
Tell/Fax: +98 4133341260
Email: Jalalshayegh@iaushab.ac.ir

Salmonella enterica, subspecies *enterica* serotype Typhimurium (*S.* Typhimurium) is one of the most important causes of bacterial food-borne outbreaks and systemic diseases in humans and animals (1). In *S.* Typhimurium, various secreted proteins and regulatory cascades of virulence genes are located within the *Salmonella* pathogenicity islands (SPIs) and the *Salmonella* plasmid virulence (spv) that are regulated by a microbial cell to cell communication process called quorum sensing (QS) (2, 3). It is widely accepted that QS is a form of bacterial communication that involves the production, secretion, detection, and response to extracellular signaling molecules called autoinducers (AIs) (4). There are three different QS autoinducer systems in *Salmonella* that include acyl-homoserine lactone (AHL), autoinducer-2 (AI-2), and autoinducer-3 (AI-3) signals (5). *S.* Typhimurium encodes a LuxR homolog, named SdiA (suppressor of cell division inhibition) that responds to AHLs which is produced by other bacterial species (6). Existence and expression of the *sdiA* and *sdiA*-regulated genes would be beneficial to *Salmonella* within the gastrointestinal tract, and the observed upregulation of this gene in the population brings the relevance of the differentiated state closer to the milieu of the host environment (7). Also, the potential roles of *sdiA* may be construed from the recognized functions of genes known to be regulated by SdiA. SdiA regulates seven genes located in virulence plasmid and chromosomes of *Salmonella* which are involved in *Salmonella*'s colonization of the intestine. These genes include *pefI*/*srgC* operon, *srgE* (SdiA-regulated gene E), and *sirA* (*Salmonella* invasion regulator) (7).

One of the common infection management strategies to treat, prevent, and control common salmonellosis in human and animals include the use of antibiotic therapy (8). However, increased occurrence of antimicrobial-resistant *S.* Typhimurium strains have been reported from different food animals around the world (9-11). For example, emergence of multidrug-resistant (MDR) *S.* Typhimurium definitive phage type 104 (DT104), is a particular concern for animal husbandry, and in human medicine (12).

In recent years, strategies have been taken to combat bacterial resistance by new antimicrobial agents from natural sources (13-15). Among the new therapeutic strategies, anti-virulence strategies have emerged as promising alternatives, since instead of killing the pathogens, these strategies try to deprive the bacteria from their virulence factor*S.* One of the novel therapeutics that are currently being developed is focused on quench pathogen QS systems, because QS is a key regulatory system in the pathogenesis of various bacterial infections (16).

It has been demonstrated that plant-derived natural products contain abundant sources of anti-bacterial or anti-QS compounds (17, 18). The genus *Satureja* is known as "Marze" in Persian, belongs to the *Lamiaceae* family. This genus consists of 16 species in Iran, 10 species are endemic (19). *Satureja sahendica* (*S. sahendica*), one of these species, is a perennial and bushy aromatic herb with small white-viola colored flower*S.* It is a late flowering species, grows in the rock walls and mountains of northwestern and western Iran. The aerial parts of some *Satureja* species have been widely used as a flavoring compound in food and as a traditional plant for the treatment of gastrointestinal disorders (20).

Although, many studies reported that the essential oils and extracts of *Satureja* species have potential antimicrobial activities against different pathogenic bacteria (20-22), the antimicrobial and anti-QS effects of *S. sahendica* hydroalcoholic extracts (SSHE) against the *S.* Typhimurium are still poorly understood. Therefore, the objectives of this study were to observe the antimicrobial and anti-QS effects of SSHE on *S.* Typhimurium isolated from poultry flocks.

The Antimicrobial susceptibility testing

In the assessment of the antimicrobial activities of SSHE, the MIC and MBC values against 20 *S.* Typhimurium isolates were ranged from 0.29-4.68 mg/ml^{-1} and from 75-150 mg/ml^{-1}, respectively.

Confirmation of presence of 16S rRNA and sdiA genes by PCR

Among 20 different *S.* Typhimurium isolates, all stains showed the presence of *16S rRNA* and *sdiA* genes (100%). PCR results confirming the presence of these genes are shown in Figure1.

Growth of S. Typhimurium *in the presence of sub-inhibitory concentrations of SSHE*

In this study, the MIC value of *S.* Typhimurium RITCC1730 versus SSHE was 2.34 mg/ml$^{-1.}$ The growth curve of *S.* Typhimurium RITCC1730 is shown in Figure 2. The test revealed that after 30 min of SSHE treatment, there was no obvious difference in the OD600 value among all culture*S.* A steady increase in the optical density with 0.58, 1.17 mg/ml^{-1} of SSHE treatment and control occurred after 45 min. The optical density increased at a slower rate than the lower concentration*S.* After 300 min, the OD value of the *S.* Typhimurium treated with 0.58, 1.17, 2.34, 4.68, and 9.37 mg/ml^{-1} of SSHE was approximately 95.03%,

89.17%, 66.62%, 41.52% and 33.63% of the control cultures, respectively.

These results show that SSHE concentration of 1MIC, 2 MIC and 4 MIC strongly inhibited the growth of *S.* Typhimurium RITCC1730. To study the effects of a low SSHE concentration on the transcription of the *S.* Typhimurium, we reduced the inhibitory SSHE concentration to 1/2 MIC according to the growth curve. It was claimed that compound concentrations should be at concentrations that are just low enough not to affect the growth of the organism (23). We chose 60 min time point for SSHE treatment in the experiment based on the growth curve mentioned above and our preliminary experiment*S.* In the preliminary experiments, cultures were harvested for RNA preparation following 45, 60, and 120 min of SSHE treatment. Our results demonstrated that time point 45 min delivered the most meaningful results (data not shown).

Influence of subinhibitory concentrations of SSHE

Expression level of *sdiA* gene in 20 treated *S.* Typhimurium strains with SSHE were examined using RT-qPCR and compared to that in non-treated ones of the same strain. The results indicated that *sdiA* gene expression in the SSHE-treated strains was significantly down regulated in comparison to that in the non-treated isolates (p =0.0246) (Figure 3).

The widespread emergence of resistance to a large number of antimicrobial agents in pathogenic bacteria has become a significant global public health threat (24). Most plant extracts studied have various antimicrobial activities and have been used to cure many infections (25). The findings of our study show that SSHE has a good inhibitory effect on *S.* Typhimurium with a minimum inhibitory concentration in the range of 0.29-4.68 mg/ml^{-1} and minimum bactericidal concentration of 75-150 mg/ml^{-1}. These results clearly indicated that SSHE has a strong antimicrobial activity against *S.* Typhimurium isolate*S.* To our knowledge this is the first report on the antimicrobial activity of the SSHE. Previous studies reported that the essential oils of the *S. sahendica* are known to possess antibacterial activity against both Gram positive and Gram negative bacteria (20-22). In a report of Yousefzadeh et al. the minimum inhibition concentration of *S. sa-*

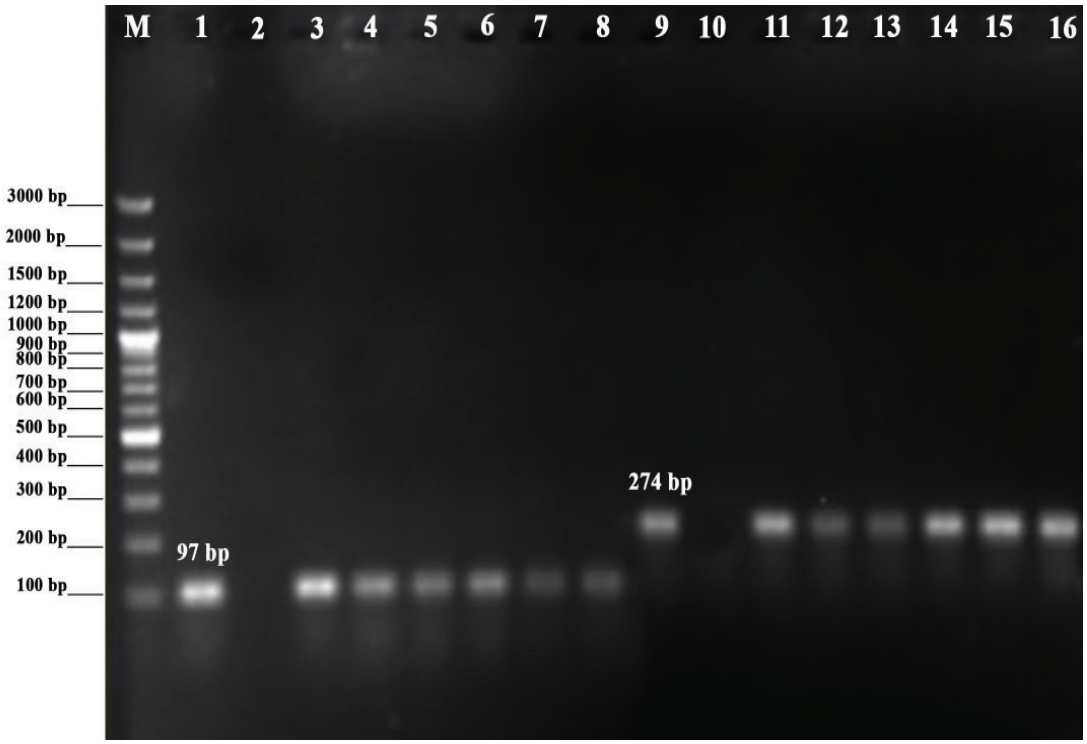

Figure 1.
Agarose gel electrophoresis of the PCR product of *16S rRNA* and *sdiA* genes for DNA extracted from analyzed *S.* Typhimurium isolate*S.* Lane M: GeneRuler™ 100 bp plus DNA ladder; lane 1 and 9: positive control for *16S rRNA* and *sdiA* genes (*S.* Typhimurium RITCC1730), respectively; lane 2 and 10: negative control for *16S rRNA* and *sdiA* genes, respectively; lanes 3-8 and 11-16: amplified fragments of *16S rRNA* and *sdiA* genes in the isolates.

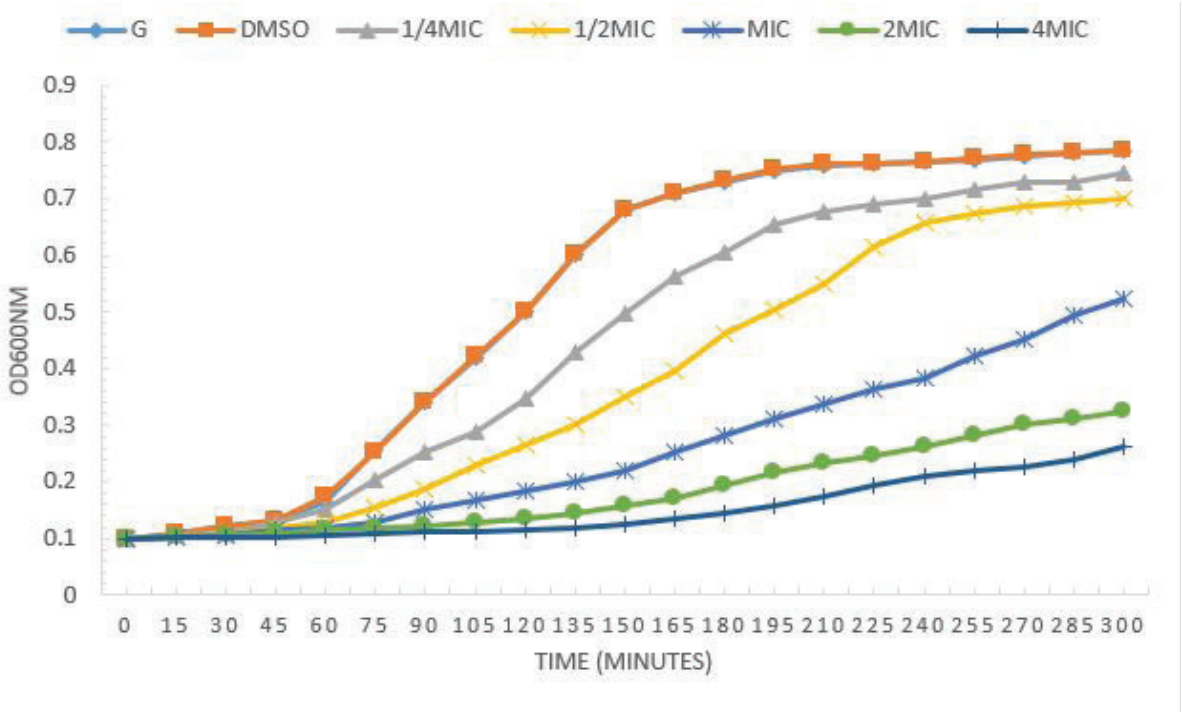

Figure 2.
Growth curve for *S.* Typhimurium RICC1730 in the presence or absence of SSHE. a) untreated *S.* Typhimurium; b) *S.* Typhimurium plus 10% DMSO; c) *S.* Typhimurium plus 0.58 mg ml^{-1} SSHE; d) *S.* Typhimurium plus 1.17 mg ml^{-1} SSHE; e) *S.* Typhimurium plus 2.34 mg ml^{-1} SSHE; and f) *S.* Typhimurium plus 4.68 mg ml^{-1} SSHE; g) *S.* Typhimurium plus 9.37 mg ml^{-1} SSHE.

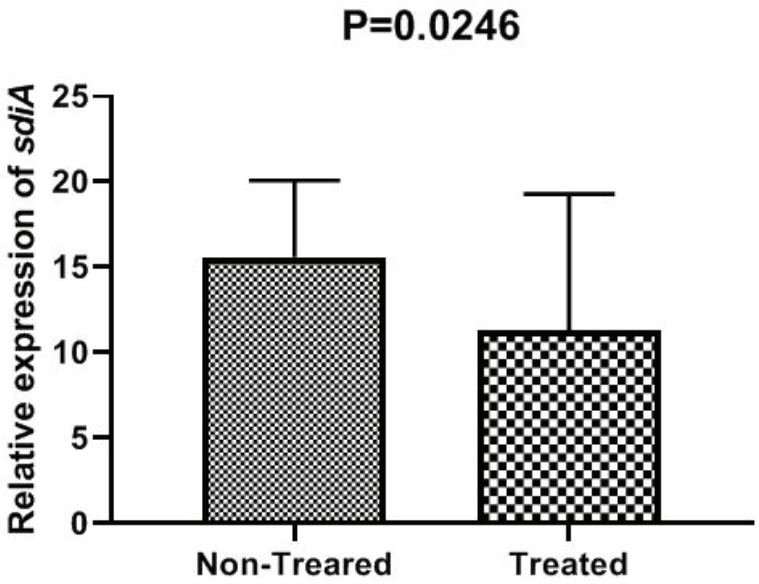

Figure 3.
Relative expression of *sdiA* in response to SSHE. Relative expression of *sdiA* mRNA was detected in 20 SSHE -treated and -nontreated *S.* Typhimurium using RT-qPCR, and it's expression in each sample was normalized to the level of *16S rRNA* mRNA expression in each sample.

hendica essential oil in Gram negative bacteria was in the range of 3.5-15 mg/ml^{-1} (21). Serrano et al. showed that the essential oil and ethanol extracts of *Satureja* contain several compounds with antimicrobial properties (26). The composition of the essential oil isolated from aerial parts of *S. sahendica* has previously been reported. Thirty-three component were identified in *S. sahendica* essential oil. In fact, the major components in *S. sahendica* essential oil were thymol, P-cymene, γ-terpinene and α-terpinene, respectively (27). The high antimicrobial activity of *S. sahendica* essential oil could be explained by the higher percentage of thymol that is well known to has antibacterial activity (28). Most studies on the mechanisms of thymol indicate that it disturbs the structure of cellular membranes, altering their function, and reacts with the active sites of enzymes in Gram positive and Gram negative bacteria (29, 30). The ethanol and methanol extracts of other *Satureja* species were assessed against some Gram-positive and Gram-negative bacteria. The MIC values of *S. bachtiarica* ethanol extract against Gram negative bacteria have been reported in the range of 100-200 mg/ml^{-1} (31) and the corresponding value of methanol extract of *S. khuzestanica* were in the range 4-10 mg/ml^{-1} (32). In the another study, the MIC values of methanol extract from the aerial parts of *S.* hortensis plants against Gram negative bacteria were ranging from 0.03 to 0.25 mg/ml^{-1} (33). Although it has been reported that essential oils of plants contain more antimicrobial substances than their extracts including methanol, ethanol and water extracts, but the results of our study indicate that hydroalcoholic extract of *S. sahendica* can be stronger than essential oil and other type of extract*S.* These differences can be due to ecological factors or species variation*S.* In total, these data indicate the possibility that SSHE may find an application as an antibacterial agent against salmonellosi*S.* However, conducting further studies with more focus on antimicrobial mechanisms of this extract is suggested. For this reason, in this study the attention has been given to it's QS inhibition activity.

A number of studies have shown that extracts from traditional medicinal plants could interfere with bacterial cell-cell communication and can be used for the development of novel anti-infective measures (34-36). Overall, plant extracts or compounds usually target the bacterial QS system via three different ways: stopping the signaling molecules from being synthesized by the luxI encoded AHL synthase, degrading or modifying the signaling molecules, and/or targeting the luxR signal receptor (17, 37). In this study, we have quantitatively assessed the expression level of *sdiA* gene (one of LuxR family member) in SSHE -treated, and non SSHE treated *S.* Typhimurium isolate*S.* According to the results, *sdiA* gene was found to be significantly down-regulated in SSHE treated isolates as compared to the matched non-treated one*S.*

Many Gram-negative bacteria, including *Salmonella*, employ the QS system to control the expression of several virulence genes (38). One of the most significant regulators of *S.* Typhimurium QS system was SdiA which responds to AHLs produced by other bacterial species (39). Thus, downregulation in the expression of this gene was associated with a decrease in expression of the genes involved in QS system. Genes that are controlled by SdiA include: the *pefI*/*srgC* operon, the *srgE* gene, and the *sirA* gene (7). Hence, reducing the expression of the *sdiA* gene can affect the expression of these genes, and subsequently on their virulence factors, including flagella formation (motility), fimbria formation, bacterial invasion, biofilm production, type 3 secretion system, and the phenotypes derived from genes located on the pathogenic islands 1 and 4 (40-43).

Overall, the results of this study showed that SSHE has antimicrobial and anti-QS effects by decreasing the expression of *sdiA* gene. Although there is no documented scientific report on the Quorum Quenching (QQ) potential of SSHE, we found that SSHE also possessed significant anti-QS activity against *sdiA* gene. These results can be in agreement with the results of other studie*S.* The results of Sharifi et al., 2018 revealed that *S. hortensis* essential oil can act as an anti-QS agent against *Staphylococcus aureus* (44). Likewise, in the study of Bacha et al. two of the eighteen plant extracts were found interfering with bacterial QS (18). In another study, Adonizio et al. reported that medicinal plants and their extracts exhibited anti QS activities (45).

To our knowledge, this is the first report which introduces SSHE as an effective antibacterial medicinal plant agent against *S.* Typhimurium isolates and shows noticeable anti-QS effect. Nevertheless, further studies about the safety and toxicity of this extract are needed to evaluate possible clinical applications in the therapy of infectious diseases.

In conclusion, the results of this study revealed that MICs of SSHE can be used to control the expression of virulence genes involved in QS system of *S.* Typhimurium. Also, the present study introduced SSHE as an anti-QS agent with natural origin against *S.* Typhimurium. Nevertheless, more efforts are required to conduct clinical trials of these compounds in the future.

Plant material and extraction procedure

S. sahendica plants were collected from the wild growing plants at the full flowering stage, from East Azerbaijan province of

Iran. The plant was authenticated by Herbal Museum of the Faculty of Pharmacy, Tabriz University of Medical Science, Tabriz, Iran. A voucher specimen (No. 4035) has been deposited at the Herbarium of Medicinal Plants, Faculty of Pharmacy, Tabriz University of Medical Science, Tabriz, Iran.

The collected plant materials were air-dried under shade and the plant leaves were separated from the stem. Then dried leaves were powdered and stored in an airtight container.

Preparation of the ethanol extract

SSHE were prepared with aqueous ethanol (70%; v/v) by using Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent. The ethanol extracts were filtered through Whatman filter paper (No.1) and then concentrated vacuo at 40 °C by means of rotary evaporator. The residues obtained were stored in a freezer at -80 °C until future test*S.*

Bacterial strains

S. Typhimurium RITCC1730 was obtained from Razi Institute Culture Collection Center. A total of 20 local clinical isolates of *S.* Typhimurium, which were isolated from poultry flocks, were provided from Faculty of Veterinary Medicine, Tehran, Iran. These isolates had previously been identified by biochemical and molecular tests to be representative of *S.* Typhimurium (46).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of SSHE against the *S.* Typhimurium isolates described above were determined according to the CLSI (Clinical and Laboratory Standards Institute) procedures (47). The stock solution extract of *S. sahendica* (300 mg/ml) was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich). The MIC was defined as the lowest concentration of extract that prevented resazurin colour change from blue to pink. The minimum bactericidal concentration (MBC) values were determined by removing 100 µL of bacterial suspension from subculture, demonstrating blue color in wells and inoculating on Muller Hinton agar (MHA) plate*S.* MBC was recorded as the lowest concentration of SSHE, which killed 99.9% of bacterial inoculate after a 24-hour incubation at 37 °C (19). The assays were repeated in triplicate.

Confirmation of presence of 16S rRNA and sdiA genes by PCR

PCR amplification was carried out in a 25 µL reaction mixture containing 2 µL of the DNA as the template, 12.5 µL of 2X PCR master mix (3 mM MgCl2, 0.04U/ µL Taq polymerase, reaction buffer, 0.4 mM of each dNTPs), 1 µL (0.4 Mm) of the forward and reverse primers (Sinaclon, Iran) (Table 1). PCR amplification was conducted in a Touchgene Gradient (Model FTGRAD2D, UK). The cycling program consisted of: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. Both positive and negative control reactions were included in each PCR amplification experiment. For negative controls template DNA was replaced with sterile water. *S.* Typhimurium RITCC1730 was used as positive control. PCR products were resolved by electrophoresis in 2% (w/v) agarose gel stained with SYBR Safe DNA gel stain. Visualizations were made using a UV transilluminator (BTS-20, Japan), and the 100 bp plus DNA ladders were used as molecular size markers.

Growth curves

S. Typhimurium RITCC1730 was grown to an optical density of 0.1 at 600 nm in Muller Hinton Broth (MHB), and was distributed as 100ml volumes into six 500ml Erlenmeyer flask*S.* SSHE (dissolved in 10% DMSO) was added to six of the cultures to obtain final concentrations of 0.25 MIC, 0.5 MIC, 1 MIC, 2 MIC, and 4 MIC respectively. The control cultures included the addition of 10% DMSO alone. The cultures were incubated further, and cell growth was monitored spectrophotometrically in the optical density at 600 nm. Three ml samples of each culture were collected immediately at 15 min intervals after the addition of SSHE. In addition, the total number of viable bacteria was estimated by plating dilutions of the culture on MHA without antibiotic and counting the numbers of CFU after 24 h at 37 °C.

Treatment with SSHE

To obtain RNA for investigating the effects of SSHE on *sdiA* gene expression, *S.* Typhimurium RITCC1730 and each of the clinical isolates of *S.* Typhimurium were grown overnight at 37 °C in 10 ml of MHB. Two 250 ml Erlenmeyer flasks, each of which contained 100 ml of MHB, were inoculated with an overnight culture to an initial OD600 of 0.1. Subsequently, the tock solution (SSHE), prepared in 10% DMSO was added to one of the cultures (experimental culture), giving a final concentration of 0.5 MIC. The other culture containing 10% (v/v) DMSO lacking SSHE was used as the control. All bacterial suspensions (both experimental and control suspensions) were further incubated for 60 min at 37 °C. RNA isolation was then performed at this time. Three independent bacterial cultures for SSHE treatment or control condition were prepared as biological replicates for RNA isolation on different days.

RNA Extraction and cDNA Synthesis

Four hundred µL of the bacterial suspension were removed and combined with 800 µL of RNA protect Bacteria Reagent (Qiagen, Valencia, Calif., U.S.A.) to minimize RNA degradation immediately before harvesting for RNA isolation, then cells were collected by centrifugation and kept at -80 °C.

Extraction of the total RNA was carried out from both treated and non-treated bacteria using the Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's instructions, and then each RNA sample was treated with RNase-free DNase I (TaKaRa Bio Inc., Shiga, Japan) to remove contaminating DNA. The RNA quality and quantity was monitored by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Extracted RNAs were stored at -70°C until required for the experiment*S.* The cDNA synthesis of each sample was measured using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran), following the manufacturer's instruction*S.* All the samples were stored at -20 °C until used in the analysis.

Relative Quantitative Real-Time PCR

YTA qPCR Probe Master Mix (Yekta Tajhiz Azma, Iran) was used for amplification and the real-time PCR analysis, according to the manufacturer's instruction*S.* The primer pairs listed in table 1.

The amplification was done with the Applied Biosystems Step One Plus (Thermo Fisher Scientific, ABI, U.S.). Each reaction mixture (20 µL) was prepared as follows: 10 µL of 2X One-Step SYBR RT-PCR Buffer III (Takara), 0.4 µL of TaKaRa Ex Taq HS (5 U

Table 1.
Primers used in the PCR reactions

Primer	Sequence	Size	References
16s rRNA for	AGGCCTTCGGGTTGTAAAGT	97 bp	(Lee et al., 2009)
16s rRNA rev	GTTAGCCGGTGCTTCTTCTG		
sdiA for	AATATCGCTTCGTACCAC	274 bp	(Halatsi et al., 2006)
sdiA rev	GTAGGTAAACGAGGAGCAG		

µL-1), 0.4 µL of PrimeScript RT enzyme Mix II, 0.4 µL of each primer (4 mM), 0.4 µL of ROX reference dye I, 2 µL template RNA and 6 µL of RNase-free dH2O. Amplification and detection were performed with StepOne Real-Time PCR System (Applied Biosystems, Waltham, USA). Cycling condition included 42˚C for 5 min, 95˚C for 10 sec and then 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. To verify the identity of the amplified product, post-amplification melting curve analysis was conducted as follows: 95˚C for 15 sec followed by stepwise elevation of the temperature from 60˚C to 95˚C at a rate of 0.3˚C per sec with continuous fluorescence collection.

All the samples were analyzed in triplicate and in order to confirm that there was no background contamination, a cDNA template-free negative control was included in each run, and the *16S rRNA* gene was used as internal control. To minimize data variation in separate runs, paired treated and non-treated samples from the same isolate were examined on the same run*S*. The expression of the target gene *sdiA* was determined as relative to the expression of the endogenous control gene *16S rRNA* by using the comparative CT (ΔΔCT) method of RT-qPCR.

Statistical analysis

All the experiments were performed in triplicate and repeated for three times and the data are expressed as the mean ± SD. The statistical calculations were performed using GraphPad Prism software (version 8). A paired *t*-test was used to analyze the data. A *p*-value of < 0.05 was considered to be statistically significant.

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Author Contributions

Conceived and designed the experiments: SH, JSH. Performed the experiments: SH, RSH. Analyzed the data: SH. Research space and equipment: SH, JSH, RSH. Contributed reagents/materials/analysis tools: SH, JSH, RSH. Wrote the paper: SH, JSH.

Conflict of Interest

The authors declare that there is no conflict of interest in relation to the publication of this paper.

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Abstracts (in Persian)

فعالیت عصاره آبی پوست انار ایرانی علیه سلولهای پلانکتونیک و تشکیل بیوفیلم استافیلوکوکوس اورئوس

هادی ابراهیم نژاد*[۲،۱]، مریم عبادی[۳]، لادن منصوری نژند[۲]

۱ پژوهشکده فناوری تولیدات گیاهی، دانشگاه شهید باهنر کرمان، کرمان، ایران.
۲ گروه بهداشت مواد غذایی و بهداشت عمومی، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان، کرمان، ایران.
۳ گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان، کرمان، ایران.
* نویسنده مسئول

چکیده

عفونت ها و آلودگی های استافیلوکوکی باعث نگرانی رو به رشد و مکرر در صنایع پزشکی و غذایی شده اند. در ضمن، ظهور سویه های مقاوم به آنتی بیوتیک مانند استافیلوکوکوس اورئوس مقاوم به متی سیلین (MRSA) در کنار تولید بیوفیلم های مقاوم در برابر ضد عفونی کننده ها، مقابله با این باکتری ها را سختتر و چالش برانگیزتر می کند. پوست انار به عنوان یک محصول ضایعات کارخانه های آبمیوه گیری، یک ماده ضد باکتری طبیعی است. عصاره ی آبی پوست انار (PPHE)، به عنوان یک ماده ی دوستدار حیات، از یک رقم انار ایرانی، رباب، تهیه شد و ترکیبات فنلی و ویژگی های آنتی اکسیدانی (توسط آزمونهای DPPH و FRAP) و ضداستافیلوکوکی آن (علیه سلولهای پلانکتونیک و بیوفیلم) ارزیابی گردید. عصاره ی آبی پوست انار رباب سلولهای پلانکتونیک و تشکیل بیوفیلم توسط سه استافیلوکوکوس اورئوس را مهار کرد. عصاره ی آبی پوست انار رباب هاله های واضح و بزرگ مهار استافیلوکوک را تولید کرد بطوریکه قطر آنها در رابطه با استافیلوکوکوس اورئوس جداشده از شیر بطور معنی داری وابسته به دوز بود ($p < ۰/۰۵$). با وجود مقاومت استافیلوکوکوس اورئوس مقاوم به متی سیلین (ATCC 33591) در برابر آنتی بیوتیک های بتا-لاکتام، حداقل غلظت مهارکننده عصاره علیه سلولهای پلانکتونیک آن تنها ۳/۷۵ میلی گرم در میلی لیتر بود. علاوه برآن، عصاره ی آبی پوست انار رباب تشکیل بیوفیلم های باکتریایی را بطور وابسته به دوز مهار کرد. حداقل غلظت مهارکننده عصاره آبی پوست انار رباب علیه سلولهای پلانکتونیک استافیلوکوکوس اورئوس جداشده از شیر، استافیلوکوکوس اورئوس (ATCC 29737) و استافیلوکوکوس اورئوس مقاوم به متی سیلین از تشکیل بیوفیلم های آنها به ترتیب به میزان ۴۷، ۳۶ و ۲۶٪ جلوگیری کرد. این مساله به تفاوتهای بین فعالیت ضدپلانکتونیک و ضدبیوفیلمی عصاره ی آبی پوست انار رباب اشاره می کند. فعالیت ضدپلانکتونیک و به میزان کمتری ضدبیوفیلمی این عصاره ی برپایه ی آب، باعث تصور کاربرد موثر و سودمند آن در صنایع غذایی و دارویی می شود.

واژگان کلیدی

پوست انار، رباب، استافیلوکوکوس اورئوس

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Abstracts (in Persian)

جداسازی باکتریوفاژهای وسیع الطیف علیه ایزوله های باکتری اشریشیاکلای تولید کننده شیگاتوکسین جدا شده از مواد غذایی و بیماران

گلشن شاکری[1]، عبدالله جمشیدی*[1]، کیارش قزوینی[2]

۱گروه بهداشت مواد غذایی و آبزیان، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
۲ گروه میکروب شناسی و ویروس شناسی، دانشکده پزشکی، دانشگاه علوم پزشکی مشهد، مشهد، ایران
* نویسنده مسئول

چکیده

هدف از این مطالعه جداسازی باکتریوفاژهای اختصاصی STEC و جهت تولید یک مجموعه‌ی باکتریوفاژ علیه پاتوتایپ های اشریشیاکلای موجود در شمال شرق ایران بود. روشهای پلیت مستقیم و غنی سازی در حجم کم، در گروه A منجر به تشخیص فاژ نشد. در حالیکه غنی سازی کامل در گروه B، منجر به تشخیص فاژ در ۵ نمونه از مجموع ۷ نمونه شد. در ٪۵۴ باکتری های اشریشیاکلای نسبت به فاژها حساس بودند. فاژ پلی والان Ecol-MHD1، علیه اشریشیاکلای و سالمونلا مؤثر بود. در نتیجه، این فاژها برای کنترل عفونت های ناشی از اشریشیاکلای مناسب هستند.

واژگان کلیدی

اشریشیاکلای تولیدکننده شیگاتوکسین، باکتریوفاژ، دامنه میزبانی، غنی سازی

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Abstracts (in Persian)

مطالعه سطوح اریتروپویتین، ویتامین D3 و پاراتورمون در ارتباط با ضایعات کلیوی در گاومیش های رودخانه ای (*Bubalus bubalis*)

زانا شهابی[۱]، محمد راضی جلالی*[۲]، بابک محمدیان[۳]، محمد رحیم حاجی جاجیکلائی[۲]، محمد طه جلالی[۴]

۱ دانشجوی دکتری تخصصی کلینیکال پاتولوژی، دانشکده دامپزشکی، دانشگاه شهید چمران اهواز، اهواز، ایران.
۲ گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهید چمران اهواز، اهواز، ایران.
۳ گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه شهید چمران اهواز، اهواز، ایران.
۴ مرکز تحقیقات هیپرلیپیدمی، دانشگاه علوم پزشکی جندی شاپور اهواز، اهواز، ایران.
* نویسنده مسئول

چکیده

اختلالات کلیوی در نشخوارکنندگان، ممکن است به صورت تحت بالینی ظاهر شده و منجر به کاهش تولید شوند. در این تحقیق از تعداد ۱۰۲ راس گاومیش نرو ماده به ظاهر سالم نمونه خون و قطعه مشخصی از بافت کلیه از کشتارگاه صنعتی اهواز اخذ گردیده و جهت انجام آزمایش های هورمونی و هیستوپاتولوژیک به آزمایشگاه ارسال گردید. پس از انجام رنگ آمیزی های متداول و اختصاصی نمونه های بافتی مورد ارزیابی میکروسکپی قرار گرفته و از میان ۱۰۲ نمونه تعداد۴۱ نمونه فاقد ضایعه بعنوان گروه شاهد و تعداد ۶۱ نمونه به عنوان گروه ضایعه در نظر گرفته شدند. نمونه‌های ضایعه دار به ۴ زیرگروه نفریت بینابینی حاد، نفریت بینابینی مزمن، التهاب لوله‌ای ادراری و پرخونی تقسیم شدند. مقادیر هورمون های اریتروپویتین، پاراتورمون و ویتامین D3 با استفاده از کیت های اختصاصی به روش الیزا اندازه گیری شدند. آنالیزآماری نشان دادکه مقادیر ویتامین D3 در گروههای ضایعه دار اگر چه کمتر از متوسط گروه شاهد است ولی این تفاوت معنی داری نبوده است. با این حال، مقادیر هورمون اریتروپویتین و پاراتورمون(در گروه نفریت بینابینی مزمن) در گاومیش های با ضایعات کلیوی به طور معنی داری بترتیب پایین تر و بالاتر از گروه شاهد بوده است. نتایج حاکی است که بیشتر ضایعات کلیوی در گاومیش های مورد مطالعه از جمله موارد ذکر شده در بالا منجر به کاهش تولید هورمون اریتروپویتین در پی بروز این ضایعات و اثرات آن برعملکرد کلیه ها می شود. از آنجائیکه ارتباط مستقیمی بین تولید اریتروپویتین و خونسازی وجود دارد رخداد اختلالاتی همچون آنمی با ضایعات کلیوی مرتبط بوده که این امر به نوبه خود عملکرد بسیاری از سیستم های بدن را تحت الشعاع قرار می دهد.

واژگان کلیدی

گاومیش رودخانه ای، ضایعات کلیوی، اریتروپویتین، ویتامین D3، پاراتورمون

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Abstracts (in Persian)

تأثیرات تجویز عصاره‌ی قسمت‌های مختلف میوه موز بر شاخص‌های اکسیداتیو/ آنتی‌اکسیداتیو و غلظت برخی مواد معدنی در گوساله‌های شیری هلشتاین

نفیسه کیوانی راد[1]، مهرداد مهری*[1,2]، حسام الدین سیفی[1,2]، علیرضا حق پرست[3]

۱ گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
۲ قطب علمی مطالعات سقط جنین و مرگ ومیر نوزاد نشخوار کنندگان، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
۳ گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
* نویسنده مسئول

چکیده

در مطالعه حاضر تأثیر عصاره پالپ موز زیاد رسیده، عصاره و پودر پوست موز نارس بر پارامترهای اکسیدان/ آنتی‌اکسیدان و برخی مواد معدنی در گوساله‌های شیری هلشتاین بررسی شد. ۴۰ گوساله تازه متولد شده بصورت تصادفی به ۴ گروه ۱۰ تایی (کنترل، گروه ۱، گروه ۲ و ۳) تقسیم شدند.گروه‌ها از نظر شکم زایش گاوها و زمان زایش مشابه بودند. طی ۴۸-۱۲ روز پس از تولد، گوساله‌ها در گروه درمانشان قرار گرفتند. در گروه کنترل، گوساله‌ها مکمل موز دریافت نکردند. در گروه یک، به گوساله‌ها ۲ گرم (ماده خشک)/ کیلوگرم وزن بدن/ روز از عصاره پالپ موز زیاد رسیده به مدت ۵ روز داده شد. به گوساله‌های گروه دو، ۱گرم (ماده خشک) از عصاره پالپ موز زیاد رسیده/کیلوگرم وزن بدن/ روز و ۱گرم (ماده خشک) از عصاره پوست موز نارس/کیلوگرم وزن بدن/ روز به مدت ۵ روز خورانده شد. در گروه سه به گوساله‌ها پودر پوست موز نارس به میزان ۲ گرم/ کیلوگرم وزن بدن/ روز بمدت ۵ روز تجویز شد. نمونه‌های خون از ورید وداج و در روزهای ۰، ۷، ۱۵ و ۳۰ اخذ شد. سن (زمان نمونه‌گیری) تأثیری معنی‌دار روی میزان فسفر، پتاسیم، آهن و مس ($p < ۰/۰۵$) داشت. واکنش گروه × زمان برای میزان FRAP معنی‌دار بود ($p < ۰/۰۵$). یافته های حاضر نشان می دهد که افزودن مکمل موز به جیره‌ی گوساله شیری هلشتاین تأثیری سودمند بر مقادیر FRAP دارد.

واژگان کلیدی

آنتی اکسیدان ها، موز، گوساله شیری، مواد معدنی

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بررسی اثر چهار داروی بی‌حسی موضعی (تتراکائین، بوپیواکائین، لیدوکائین و پروپاراکائین) بر فشار داخلی چشم در خرگوش– مقایسه دو نوع دستگاه اپلناسیون و ریباند

آرمان امراریان، علی اصغر سرچاهی*

گروه علوم درمانگاهی، بهداشت و پیشگیری بیماریهای دامی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.
* نویسنده مسئول

چکیده

نوع دستگاه اندازه گیری، نوع داروی بی حسی موضعی و گونه حیوانی، ممکن است فشار داخلی چشم (IOP) را تحت تاثیر قرار دهند. لذا جهت بررسی اثر این موارد، اثر چهار داروی بی حسی موضعی روی فشار داخلی چشم در خرگوش بوسیله دو نوع دستگاه اندازه‌گیری (تونومتر آی‌کر و تونوپن‌وت) مورد ارزیابی قرار گرفت. در چشم راست نیمی از خرگوش‌ها و در چشم چپ بقیه خرگوش‌ها، یک قطره تتراکائین چکانده شد. فشار داخلی چشم در هر خرگوش با دو نوع تونومتر، قبل و هر ۵ دقیقه تا ۴۰ دقیقه بعد از چکاندن دارو اندازه گیری شد. حداقل با فاصله یک هفته، اثرات داروهای دیگر نیز مورد مطالعه قرار گرفت. بر اساس نتایج تونومترICare، تتراکائین بطور معنی‌داری IOP را بلافاصله و ۲۵ دقیقه پس از چکاندن دارو کاهش داد. تغییرات IOP پس از چکاندن بوپیواکائین، لیدوکایین و پروپاراکائین در هر زمان نسبت به مقادیر پایه معنی دار نبود ($P<۰/۰۵$). بر اساس نتایج تونوپن‌وت، تمام داروها بلافاصله بعد از استفاده IOP را کاهش دادند؛ با این حال، اثرات بوپیواکائین و لیدوکایین بر IOP بسیار کمتر از تتراکائین و پروپاراکائین بود. میانگین مدت زمان بی حسی قرنیه به ترتیب ۲۰، ۱۵/۵، ۷/۵ و ۲۱ دقیقه برای تتراکائین، بوپیواکائین، لیدوکائین و پروپاراکائین بود. بنابراین نتیجه‌گیری می‌شود که کاهش IOP توسط داروهای بی حسی موضعی هنگام استفاده از تونوپن‌وت بسیار بیشتر از زمانی است که از تونومتر ICare استفاده می‌شود. همچنین کاهش IOP با هر دستگاه زمانی که تتراکائین یا پروپاراکائین استفاده می شود بیشتر از زمانی است که از بوپیواکائین یا لیدوکائین استفاده می‌شود.

واژگان کلیدی

بوپیواکائین، فشار داخلی چشم، لیدوکائین، پروپاراکائین، خرگوش، تتراکائین

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Abstracts (in Persian)

اثر مواد بسترساز بر عملکرد حافظه و یادگیری و ترجیح جنس در موش های صحرایی

مهدی عباس نژاد[1]، راضیه کوشکی*[2]، سعید اسماعیلی ماهانی[1]، عباس تاج آبادی[1]، ریحانه نادری[1]

۱بخش زیست شناسی، دانشکده علوم، دانشگاه شهید با هنر کرمان، کرمان، ایران
۲بخش زیست شناسی، دانشکده علوم، دانشگاه لرستان، خرم آباد، ایران
* نویسنده مسئول

چکیده

امروزه انواع مواد بستر ساز براساس درجه سلامت، در دسترس بودن و هزینه برای دام و حیوانات آزمایشگاهی مورد استفاده است. در مطالعه حاضر، عملکرد حافظه و یادگیری و ترجیح به مواد بستر ساز در موش های صحرایی نگهداری شده تحت مواد بستر ساز مختلف بررسی شد. حیوانات در مواد بستر ساز در اندازه مشابه از چوب خاک اره ، گردو، پسته، سمندر، بادام و ساقه و برگ یونجه برای دو هفته نگهداری شدند. حافظه و یادگیری با استفاده از تست ماز آبی موریس(MWM) و شاتل باکس بررسی شد. یک ماز شعاعی تغییر یافته برای سنجش ترجیح جنس مواد بسترساز استفاده شد. برای هر یک از مواد بسترساز سطح آمونیاک درون قفس طی یک هفته محاسبه و نتایج نشان داد حیواناتی که در چوب بادام و گردو نگهداری شدند توانایی حافظه و یادگیری بهتری در تست MWM و شاتل باکس دارند. ضعیف ترین حافظه و یادگیری در موش های صحرایی قرار گرفته در یونجه به عنوان ماده بستر ساز مشاهده شد. در تست ترجیح جنس، حیوانات زمان بیشتری را در بازوهای حاوی گردو و بادام و زمان کمتری را در ناحیه حاوی یونجه گذراندند. علاوه براین، مصرف آب و غذا و همچنین تعداد ورود به ناحیه حاوی ماده بستر ساز یونجه در مقایسه با دیگر مواد بستر ساز کاهش یافت. همچنین، در قفس حاوی یونجه بیشترین سطح آمونیاک مشاهده شد. در مجموع نتایج نشان داد، مواد بسترساز با جنس متفاوت و خواص شیمیایی معین اثرات متفاوتی بر عملکرد حافظه و یادگیری موش های صحرایی دارند.

واژگان کلیدی

مواد بستر ساز، حافظه و یادگیری، ترجیح جنس، موش صحرایی

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Abstracts (in Persian)

اثرات زیستی مواد بیولوژیکی کشاورزی بر برخی از فاکتورهای خونی و بافت در موش نژاد Balb/c

منیژه رستمی نیا[1]، داود حبیبی[2]، سمیرا شهبازی*[3]، بهزاد ثانی[1]، علیرضا پازکی[4]

۱ گروه زراعت، واحد شهر قدس، دانشگاه آزاد اسلامی، تهران، ایران.
۲ گروه زراعت و اصلاح نباتات، واحد کرج، دانشگاه آزاد اسلامی، کرج، ایران.
۳ پژوهشکده کشاورزی هسته ای، پژوهشگاه علوم و فنون هسته ای، سازمان انرژی اتمی، البرز، ایران.
۴ گروه زراعت و اصلاح نباتات، واحد یادگار امام خمینی(ره) شهرری، دانشگاه آزاد اسلامی، تهران، ایران.
* نویسنده مسئول

چکیده

عفونت های سودوموناس و قارچ های ساپروفیتیک از عوامل مهم مرگ و میر، به طور افزاینده ای به عنوان عوامل بیماری زای جدی در بیماران مبتلا به نقص سیستم ایمنی شناخته می شوند. این مطالعه به بررسی اثرات زیستی (کود بیولوژیک تجاری تهیه شده از سودوموناس فلورسنس، سودوموناس پوتیدا، سودوموناس آئروجینوزا و بیو قارچ کش های مبتنی بر تریکودرما) بربافت و فاکتورهای خونی پستانداران می پردازد. بدین منظور، دو آزمایش طراحی شده است: اولین مورد تغذیه موشهای نژاد بالبسی با کاهو تیمار شده با قارچ کش زیستی (جهش یافته و وحشی) و کود بیولوژیک تهیه شده از(سه گونه سودوموناس)، دوم استفاده از آب آشامیدنی حاوی اسپور تریکودرما (جهش یافته و وحشی) یا سوسپانسیون سه گونه سودوموناس بود. سپس فاکتورهای خون و التهاب بافت های حیاتی (کبد، کلیه ،طحال و دستگاه گوارش) پس از دو ماه اندازه گیری شد. مطالعه تومورمارکر CEA در تمام تیمارها نشان داد که سویه های موجود باعث تحریک شاخص های سرطان زا نمی شوند. نتایج فاکتورهای خونی برای همه گونه ها طبیعی بود (داده ها نشان داده نشده است) فقط سودوموناس پوتیدا اثر منفی در افزایش ALP نشان نداد. همچنین نتایج اثر بیو قارچ کش بر بافت پستانداران (طحال و دستگاه گوارش) طبیعی بوده است. کمی نکروز خفیف کبد درتیمار با تریکودرما وحشی و نکروز متوسط کبد پس از تیمار با جدایه های جهش یافته تریکودرما مشاهده شد. این مطالعه نشان می دهد که برای تعیین تاثیرات عوامل زیستی بر بافت پستانداران قبل از تجارت باید تحقیقات بیشتری انجام شود.

واژگان کلیدی

سودوموناس، کود، قارچ کش بیولوژیکی، تریکودرما

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Abstracts (in Persian)

اثر ضد کوئوروم سنسنیگی و ضد باکتریایی عصاره هیدروالکلی مرزه سهندیکا بر روی جدایه های طیوری سالمونلا تایفی موریوم

راضیه شارچی، جلال شایق*، سمیه حسین زاده

۱گروه دامپزشکی، واحد شبستر، دانشگاه آزاد اسلامی، شبستر، ایران.
* نویسنده مسئول

چکیده

کوئوروم سنسینگ مکانیسم وابسته به تراکم سلولی است که توسط بسیاری از باکتریهای بیماری زا جهت تنظیم بیان ژن های حدت، مورد استفاده قرار می گیرد. مهار یا متوقف کردن این سیستم توسط داروهای گیاهی به عنوان یک استراتژی جدید برای مبارزه با باکتری-های مقاوم به آنتیبیوتیک محسوب می گردد. هدف از این مطالعه، بررسی فعالیت ضد باکتریایی عصاره هیدروالکلی مرزه سهندیکا بر روی نمونه های سالمونلا تایفی موریوم جدا شده از طیور و همچنین ارزیابی اثر این عصاره بر روی بیان ژن مرتبط با کوئوروم سنسینگ (sdiA) در این جدایه ها بود. عصاره هیدروالکلی مرزه سهندیکا با استفاده از روش سوکسله تهیه شد و حداقل غلظت مهارکنندگی (MIC) و حداقل غلظت کشندگی (MBC) آن با استفاده از روش ریز رقیق سازی در محیط مایع مشخص گردید. برای آنالیز بیان ژن sdiA در جدایه های سالمونلا تایفی موریوم تیمار شده با غلظت تحت مهاری عصاره هیدروالکلی مرزه سهندیکا در مدت زمان ۶۰ دقیقه، از روش real-time PCR کمی استفاده شد. مقدار MIC عصاره هیدروالکلی مرزه سهندیکا در برابر جدایه ها در محدوده ۶۸/۴ -۲۹/۰ میلی گرم بر میلی لیتر و میزان MBC در برابر جدایه ها ۷۵ تا ۱۵۰ میلی گرم بر میلی لیتر بود. همچنین نتایج نشان داد که بیان ژن sdiA در جدایه های سالمونلا تایفی موریوم تیمار شده با عصاره هیدروالکلی مرزه سهندیکا نسبت به جدایه های تیمار نشده به طور معنی داری کاهش یافته است. بطور کلی نتایج نشان داد که عصاره هیدروالکلی مرزه سهندیکا دارای فعالیت ضدباکتریایی و ضدکوئوروم سنسینگی است و میتواند برای کنترل بیان ژن های حدت در باکتری های بیماری زا از جمله سالمونلا تایفی موریوم بکار رود.

واژگان کلیدی

مرزه سهندیکا، عصاره هیدروالکلی، ژن sdiA، بیان ژن، سالمونلا تایفی موریوم

Guide for Authors

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Example piece of text and reference list :

An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

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5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;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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Publication ethics

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The manuscripts are evaluated by editor and a member of editorial board (depending to the field of study) for the scope, 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. This stage is performed within 2 weeks in order to not waste authors' time, allowing them to submit the manuscripts to another journal. Those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage. We aim to reach a first decision on all manuscripts within two or three weeks of submission.

External review:

The manuscripts which are found to be appropriate after the initial screen, will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods and results.
3. INTRODUCTION is well-structured and provides rationale for experiments described.
4. MATERIALS AND METHODS is sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and are supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are in good quality and well-designed and clearly illustrate results of the study.
10. References are appropriate.
11. There are no issues relating to author misconduct such as plagiarism and unethical behavior.
12. The article is imprtant and worthy of publication.

Final Decision:

Based on reviewers' recommendations a final decision is made by the editor and if needed the help of a member of editorial board (depending to 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.

Author Index

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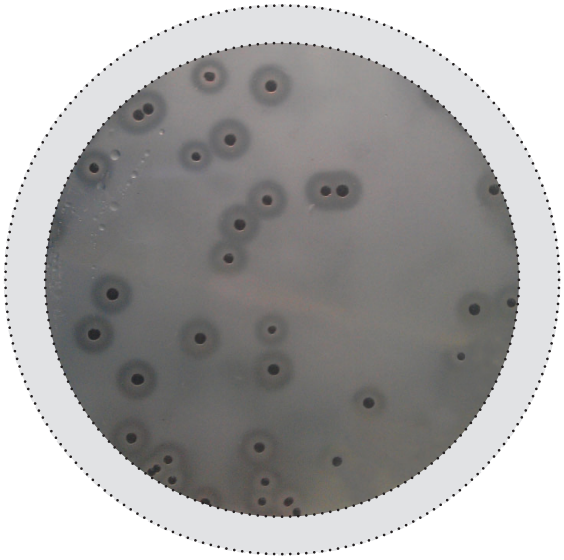

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Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: 0098 51 3880 3742
Fax: 0098 51 3876 3852

Web: ijvst.um.ac.ir
Email: ijvst@um.ac.ir