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ORIGINAL PAPER

The Effect of Environmental Stresses on *lipL32* Gene Expression in Pathogenic *Leptospira* spp. through Real-Time PCR

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Abstract

Leptospirosis is a worldwide infectious and zoonotic disease. The incidence of this disease is high in temperate regions, especially in northern Iran. The aim of this study was to investigate the effects of temperature, pH, and *Phyllanthus amarus* plant extract on the *lipL32* gene expression in pathogenic *Leptospira* spp. Fifty water samples were collected. Culture and PCR technique were used to isolate and identify the bacterium and the presence of the *lipL32* gene. The samples were exposed to different temperatures and pH levels for one day and the *Ph. amarus* plant extract at different concentrations for one and seven days. RNA was extracted, and cDNA synthesis was performed for all the samples. All cDNAs were evaluated by the real-time PCR (SYBR green) technique. Out of the 50 samples, ten samples (20%), using PCR were determined to contain the pathogenic *Leptospira*. Fold change of the expression of the *lipL32* gene associated with stresses was as follows: temperature stress of 40°C, 35°C, and 25°C reduced the *lipL32* gene expression in all three isolates, especially in the isolates type 1. The pH stress, i.e., pH values equal to 8 or 9 reduced the gene expression in three types of isolates, and pH = 6 stress increases the *lipL32* gene expression in the isolates of type 1. *Ph. amarus* plant extract stress reduced the mentioned gene expression only in isolates of type 2. Temperature and pH stresses could lead to differences in the expression level and cause the *lipL32* gene expression decrease in three pathogenic isolates. The MIC results showed anti-leptospiral effect of *Ph. amarus* plant extract.

Key words: *Leptospira*, *Phyllanthus amarus*, *lipL32* gene expression, real-time PCR, stress

Introduction

Leptospirosis is a contagious zoonotic disease spread worldwide and is caused by the bacterium *Leptospira* (Zakeri et al. 2010). The genus *Leptospira* includes pathogenic, intermediate, and non-pathogenic species with the pathogenic species causing infection in humans and animals (Ko et al. 2009). Leptospirosis is widespread throughout the world except in Antarctica, and it is endemic in tropical regions with high rainfall (Costa et al. 2015). The World Health Organization recognizes leptospirosis, also known as Weil's disease, as a neglected tropical disease with a significant global health burden. Globally, it has been estimated to be 1.03 million cases annually with 58,900 deaths (Fann et al. 2020) The highest estimates of disease morbidity and mortality were observed in Global Burden of Disease (GBD) regions of South and Southeast Asia, Oceania, Caribbean, Andean, Central, Tropical Latin America, and East Sub-Saharan Africa) Costa et al. 2015).

The natural reservoirs of *Leptospira* are rodents, but reservoirs include a variety of wild and domestic animals, livestock, and insectivores (De Vries et al. 2014). The exposure may occur through direct contact with an infected animal or indirect contact via soil or water contaminated with urine from an infected animal. Individuals with occupations at risk for direct contact with potentially infected animals include veterinarians, farmworkers (particularly in dairy milking situations), hunters, animal shelter workers, scientists, and technologists handling animals in laboratories or during fieldwork. Agricultural workers at risk for

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leptospirosis include rice field workers, banana farmers, sugar cane, and pineapple field harvesters (Haake and Levett 2015).

Many virulence factors are involved in the pathogenesis and infection of this bacterium, including lipopolysaccharide (LPS), hemolysin, surface proteins, adhesion molecules, and the ability to move and swim in liquid environments (Fraga et al. 2011). The 32-kDa lipL32 lipoprotein, a type of outer membrane protein of *Leptospira*, which is highly conserved in pathogenic species, exists only in pathogenic strains and is expressed during infection (Podgorsek et al. 2020). LipL32 binds to collagens V, VI, and I as well as to lamin. It also binds to fibronectin via the calcium-dependent pathway (Shen-Hsing et al. 2017).

Bacteria rely on the ability to sense and respond to environmental stressors, including changes in temperature, pH, osmolarity, oxygen availability, and nutrient conditions. Environmental signals, such as increased oxidative stress or temperature, can stimulate the response of regulators of virulence genes to pathogenic *Leptospira* during infection (Fraser and Brown 2017). *Phyllanthus amarus* is a plant widely used for the treatment of human diseases. The aqueous and methanolic extracts of this plant possess an excellent and useful inhibitory activity against *Leptospira* (Verma et al. 2014).

The aim of this research was to isolate and identify the leptospires from the farming fields of Tonekabon town situated in northern Iran as well as to study the effect of different environmental stresses, including pH, temperature, and *Ph. amarus* plant extract on the survival capability and the *lipL32* gene expression in the pathogenic leptospires. Study of the influence of *Ph. amarus* plant extract on these bacteria could reveal the important role of this plant in the prevention, control, and treatment of leptospirosis. So far, no research has been performed to investigate the effect of pH on the *lipL32* gene expression in pathogenic *Leptospira* spp. and our study is a part of new investigations.

Experimental

Material and Methods

Samples collection, cultivation, and isolation. 50 samples from the stagnant waters of the rice field were collected under the aseptic conditions, including wearing gloves and the boot in Tonekabon city (Iran, Mazandaran province). Sampling was performed in four seasons of the year from the autumn of 2017 to the end of the summer of 2018 on days with stable weather conditions (sunny weather and a few days after rain). A sampling of creeks and water canals of agricultural fields was done from ten places, including: Valiabad, Mir Shams-ol Din, Bagh-e Nazar, Tilpordehsar, Alkaleh, Rud-e Posht, Nematabad, Shiraj Mahalleh-ye Bozorg, Abkele Sar, and Lashkarak (Fig. 1). The samples were placed in on ice after sampling each time and transferred to Tonekabon University's Microbiology Research Laboratory as soon as possible.

Samples were centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the remaining contents were passed through 0.45 µm and 0.2 µm filters, respectively, and transferred to liquid EMJH medium (Ellinghausen and Mccullough 1965; Johnson and Harris 1967). All the samples were incubated at 30°C for 30 days. Aliquots of the medium were transferred

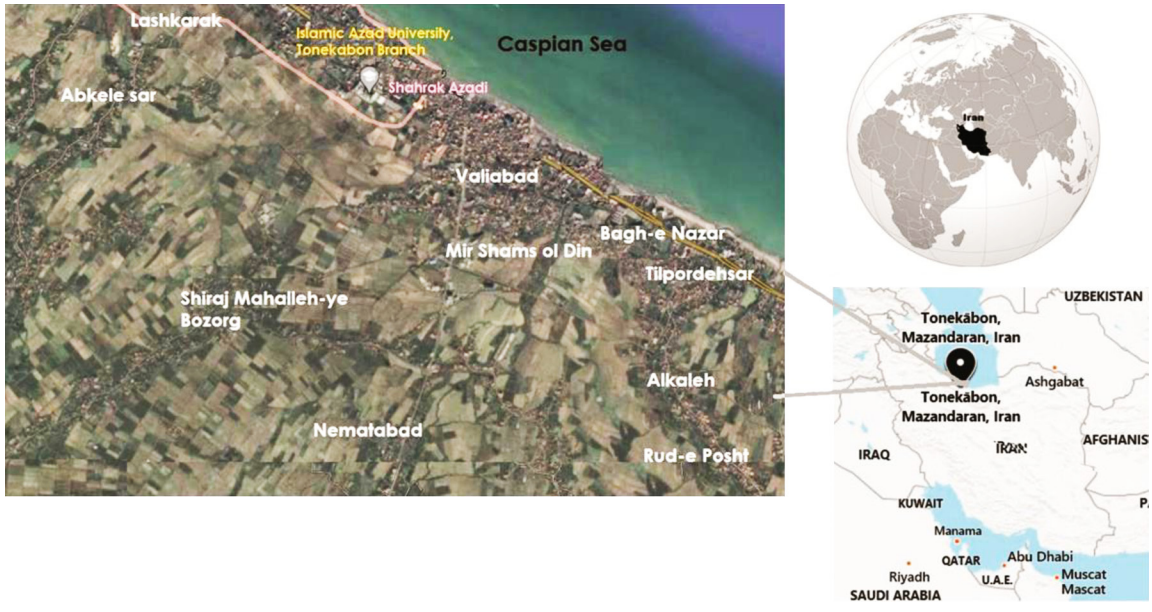

Fig. 1. Localization of ten environmental sites investigated.

Table I
The specific primers of *Leptospira*.

Name of primer	Type	Sequence of primer
16S rRNA	Forward	5'-GAACTGAGACACGGTCCAT-3'
16S rRNA	Reverse	5'-GCCTCAGCGTCAGTTTTAGG-3'
LipL32	Forward	5'-ATCTCCGTTGCACTCTTTGC-3'
LipL32	Reverse	5'-ACCATCATCATCATCGTCCA-3'

to the solid EMJH medium and cultured. Plates were incubated at 30°C for 30 days. *Leptospira interrogans* serovar Icterohaemorrhagiae (RTCC2823) was used as a reference strain. The mentioned strain was from a *Leptospira* Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran.

DNA extraction. DNA was extracted from all *Leptospira* isolates along with the reference strain using the Purification Kia Spin polymerase chain reaction (PCR) Kit (Kiagen, Iran). The purity of the extracted DNA was evaluated by optical absorption at 260 and 280 nm wavelengths using a Biophotometer (Eppendorf, Germany).

Amplification of the *lipL32* and *16S rRNA* genes by PCR method. At this stage, the presence of the *16S rRNA* and *lipL32* genes was evaluated using specific primers (Table I). The primers were produced by TAG Kopenhagen (Denmark) and used to amplify the mentioned genes (Vital-Brazil et al. 2010). Each reaction was performed in a total volume of 25 µl and included 14 µl dd water, 2.5 µl 10 × PCR buffer, 1 µl of 10 pmol of each primer, 0.5 µl of 10 mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 0.25 µl Taq polymerase enzyme (CinnaGen, Iran) and 5 µl of DNA. *Escherichia coli* was used as a negative control sample, and *L. interrogans* serovar Icterohaemorrhagiae (RTCC2823) as a positive control sample. The mentioned control samples were amplified along with the other samples.

PCR amplification conditions using a thermocycler (Sensoquest, Germany) were as follows: one denaturation cycle at 95°C for 5 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

Agarose gel electrophoresis. PCR products were run on 1.5% agarose gel with 100 bp DNA ladder (Fermentas, Russia). Five microliters of the PCR products were electrophoresed at 75 V for 40 min. DNA fragments were visualized using Safe view-II Nucleic Acid stain (Kiagen, Iran) and UVDoc (England) imaging.

The *16S rRNA* gene sequencing. *16S rRNA* PCR products were sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing.

Bioinformatics applications. All sequence data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to definitively identify each respective amplicon of the *16S rRNA* gene.

Thermal and pH stress conditions. Three isolates were selected from *Leptospira* colonies whose PCR confirmed the presence of the *lipL32* gene. A similar number of colonies was cultured in liquid EMJH medium for seven days until the bacteria reached the log phase (Larson et al. 1959). One ml of the above culture was added to 4 ml of liquid EMJH medium and incubated at 25, 30, 35, and 40°C (Parker and Walker 2011) for one day. To apply the pH stress, liquid EMJH media were prepared at the different pH of 6, 7, 8, and 9 (pH-meter, Euteoh, Malaysia). Then, 1 ml of the mentioned culture was added to 4 ml of liquid EMJH medium with the above-mentioned pHs and incubated at 30°C for one day. For each of the stressors, the reference strain was also considered. Experiments were performed in three biological replicates. The reference strain, as mentioned isolates, was affected by different temperature and pH stresses.

Plant extract stress conditions. Plant collection. *Ph. amarus* plant was obtained from the ABS medicinal plant research center, Karippatti, Salem Tamilnadu, India, during December 2018. Then, it was washed several times with sterile distilled water and again sterilized, dried, and powdered.

Preparation of an aqueous extract. Ten grams of the plant powder were added to sterile distilled water and heated for 2 h. The liquid contents of the extract were centrifuged at 10,000 × g for 10 min. The supernatant was heated (2 h) and centrifuged, and the cycle was repeated again (Mohan et al. 2016). The liquid contents were first passed through the Whatman 1 filter paper and then filtered through a 0.2 µm filter and heated for 2 h. It was then dried in an oven for 6 h at 70°C (Fiaz et al. 2013).

Anti-leptospiral sensitivity test. The effect of aqueous extracts of *Ph. amarus* against pathogenic *Leptospira* species and reference *Leptospira* strain was investigated using a minimum inhibitory concentration (MIC) and a tube dilution technique (TDT) (Mohan et al. 2016). One ml of 5, 10, 20, 40, 80, 160, 320, 640, 1,280, 2,560, 5,120, 10,240 µg/ml concentrations was added to 1 ml of liquid EMJH medium, and the work was continued according to the above techniques for three isolates. Tubes were evaluated for anti-leptospiral activity, and the effect of plant extract stress on three selected isolates and a reference strain was investigated at 1- and 7-day intervals. Experiments were repeated in triplicate to evaluate the plant extract stress.

RNA extraction and cDNA synthesis. The RNA of three isolates of *Leptospira* and the reference strain was isolated and purified with and without shock using a hybrid-R™ (Gene ALL Korea-South Seoul) RNA extraction kit. cDNA synthesis was performed by the TwoStep kit HyperScript™ first-strand synthesis kit (Gene ALL Korea, South Seoul). cDNA was used as a template for PCR and real-time PCR.

Real-Time PCR. The real-time PCR reaction was performed on Step One and Step One Plus Real-Time PCR systems (Applied Biosystems-Thermo Fisher Scientific). Twenty µl of real-time PCR mixture was assayed, including 10 µl of 2 × real-time PCR SYBR Green Master mix (Amplicon, Denmark), 0.2 µl of forward primer, 0.2 µl of reverse primer, lipL32 primer (Table I), 2 µl of cDNA as a sample and 7.6 µl of dd water. Real-time PCR temperature program consisted of holding stage at 95°C for 15 min, cycling stage comprising 33 cycles (95°C for 40 s, 57°C for 1 min, 72°C for 30 s), melt curve stage at 72°C for 1 min, and 97°C for 5 s. All the experiments were repeated at least three times. The raw data was removed from the system as a CT. Analysis of relative gene expression data was conducted using real-time PCR (SYBR Green) and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical Analysis. SPSS (22) was used for statistical analysis. Variation among the groups was analyzed using the ANOVA test. A minimum range of variance $p < 0.05$ was reflected as significant.

Results

Results of macroscopic and microscopic observations of the samples. Different colony morphology of leptospiral growth was observed (Fig. 2). During negative staining, the observation of thin, helical organisms by optical microscopy at 100 × magnification revealed *Leptospira*'s presence in the samples *Leptospira* are usually curved in one or two ends (Fig. 3). Overall, of the 50 samples, 21 (42%) contained *Leptospira* based on colony formation and microscopic observation.

Detection of pathogenic and saprophytic *Leptospira* spp. by PCR. Of the 50 samples examined, 10 (20%) contained pathogenic species that were positive by PCR and specific primers for the 16S rRNA and *lipL32* genes, respectively. PCR amplification of the samples with the aforementioned primers was performed separately in both 430 bp and 474 bp regions, respectively. Amplification of a 474 bp fragment confirmed the presence of pathogenic *Leptospira* (Fig. 4). Eleven (22%) samples contained saprophytic species that were positive by PCR using the 16S rRNA gene-specific primer, and amplification of 430 bp-gene fragments was observed (Fig. 5).

16S rRNA PCR and sequencing analysis. Of the six samples selected randomly and sent for sequencing; three could not be sequenced. Elements of the nucleotide sequence of the other three samples in the NCBI site confirmed the existence of *Leptospira* (Table III).

The anti-leptospiral activity of the aqueous extract of *Ph. amarus*. Stress exerted by the aqueous extract of *Ph. amarus* plant was acceptable for all three isolates of pathogenic *Leptospira* and *L. interrogans* serovar

Table II
The sampling sites and positive cases reported in the study.

Number of positive cases of saprophytic *Leptospira*	Number of positive cases of pathogenic *Leptospira*	Number of samples	Site
3	2	5	Valiabad
2	2	5	Bagh e nazar
0	1	5	Mir shams-ol
1	1	5	Rud e posht
1	0	5	Alkaleh
1	0	5	Abkele sar
0	1	5	Lashkarak
1	1	5	Nematabad
1	1	5	Shiraj mahalleh-ye bozorg
1	1	5	Tilpordehsar
11 (22%)	10 (20%)	50	Total

Table III
The results of the sequencing.

Molecular detection	Max Score	Total Score	Query Cover	E Value	Per Ident	Accession
Leptospira perdikensis strain CES 16S ribosomal RNA gene	612	612	93%	3e-171	99.41%	MN086353.1
Leptospira ryugenii strain CES 16S ribosomal RNA gene	176	176	26%	5e-40	100%	MN086356.1
Leptospira sp. MS336 gene for 16S ribosomal RNA gene	593	593	100%	2e-166	100%	AB758752.1

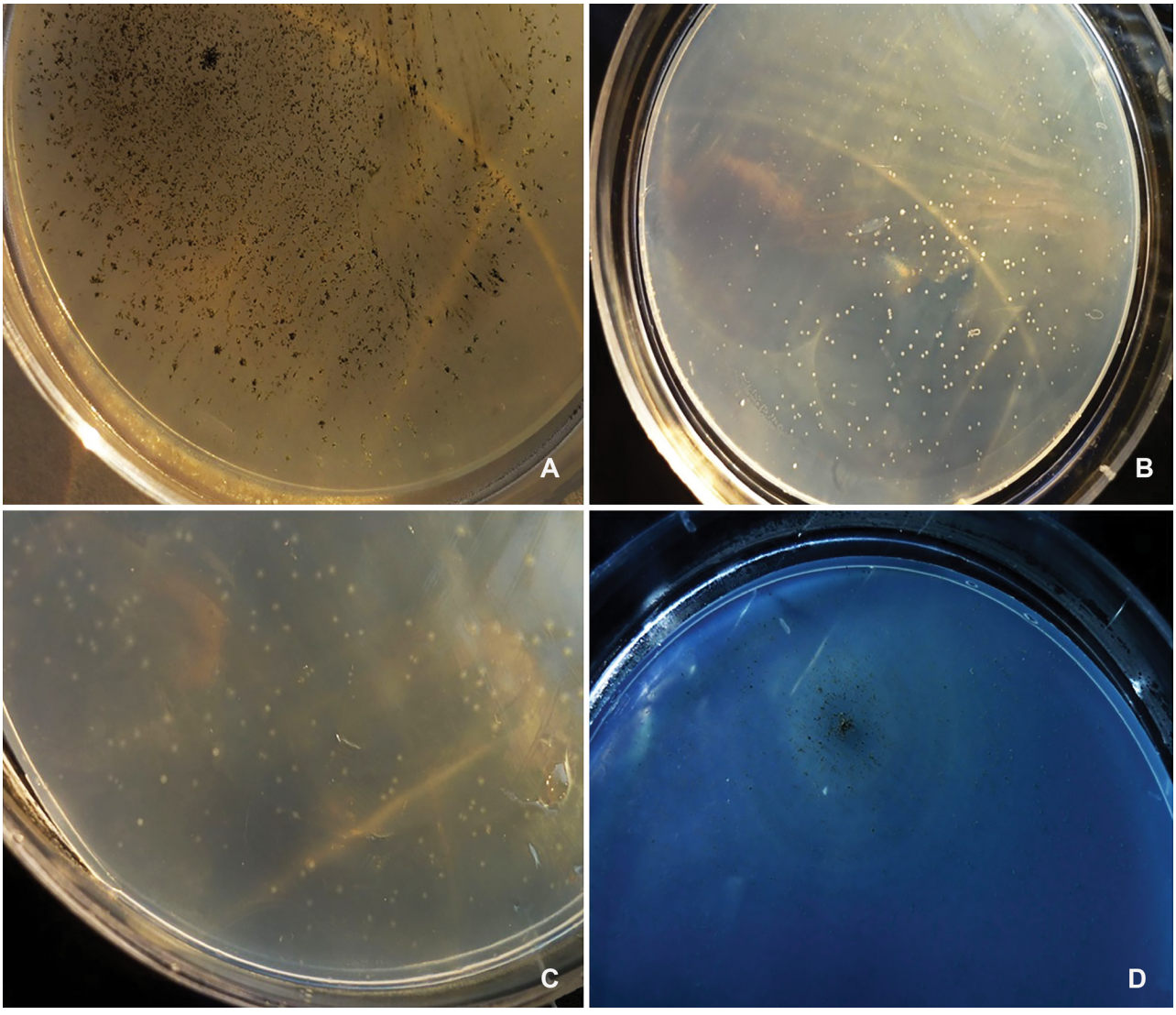

Fig. 2. *Leptospira* colonies.

(a) Formation of surface's black colonies, with round shape, needle tip size (at 30 days of incubation on EMJH agar), wooly margins, and hilly elevation related to *Leptospira* pathogen species. (b) Formation of sub surface's white colonies with round shape, small size, and some smaller colonies, size 1–2 mm (after 30 days of incubation on EMJH agar), smooth (entire) margins, flat elevation related to *Leptospira perdikensis*. (c) Formation of sub surface's very bright cream colonies with round shape, small size, 1–3 mm, (after 30 days of incubation on EMJH agar), smooth (entire) margins, with small to medium halo, flat elevation related to *Leptospira ryugenii*. (d) It had the characteristics of a colony (a), except that in this case, rings were seen to the same distance around the surface black colony related to *Leptospira* pathogen species.

Icterohaemorrhagiae and their growth was inhibited for seven days. A complete inhibition (100%) of aqueous extract of *Ph. amarus* plant was observed against isolates of pathogenic *Leptospira* No. 1, 2, and 3 at the concentrations of 10,240, 5,120, 2,560, 1,280, 640 µg/ml. For the reference strain, a similar inhibition level was visible at the concentrations of 10,240, 5,120, 2,560, 1,280, 640, 320 µg/ml, respectively. But the aqueous extract of *Ph. amarus* plant did not show the full inhibitory effect for three isolates of pathogenic *Leptospira* and reference strain of *Leptospira* on the first day of experiments.

The *lipL32* gene expression in *Leptospira* isolates by the real-time PCR and data analysis. The *lipL32* gene expression were successfully observed using real-time PCR, and the curve showed the *lipL32* gene amplification, indicating proper proliferation and function without any impediments. Samples without cDNA (i.e., negative) and those containing cDNA (i.e., positive) were assayed with other samples by RT-PCR.

Fold change associated to gene *lipL32*, according to the results of $2^{-\Delta\Delta CT}$ affected by temperature stress, pH, and *Ph. amarus* plant extract includes the following.

Fold change associated with the *lipL32* gene was in all three types of isolates affected by temperature stress of 30°C (equal to 1), 40°C, 35°C, and 25°C (less than 1). Thus, temperature stresses of 40°C, 35°C, and 25°C reduced the *lipL32* gene expression in the isolates and this reduction significantly led to a further downward

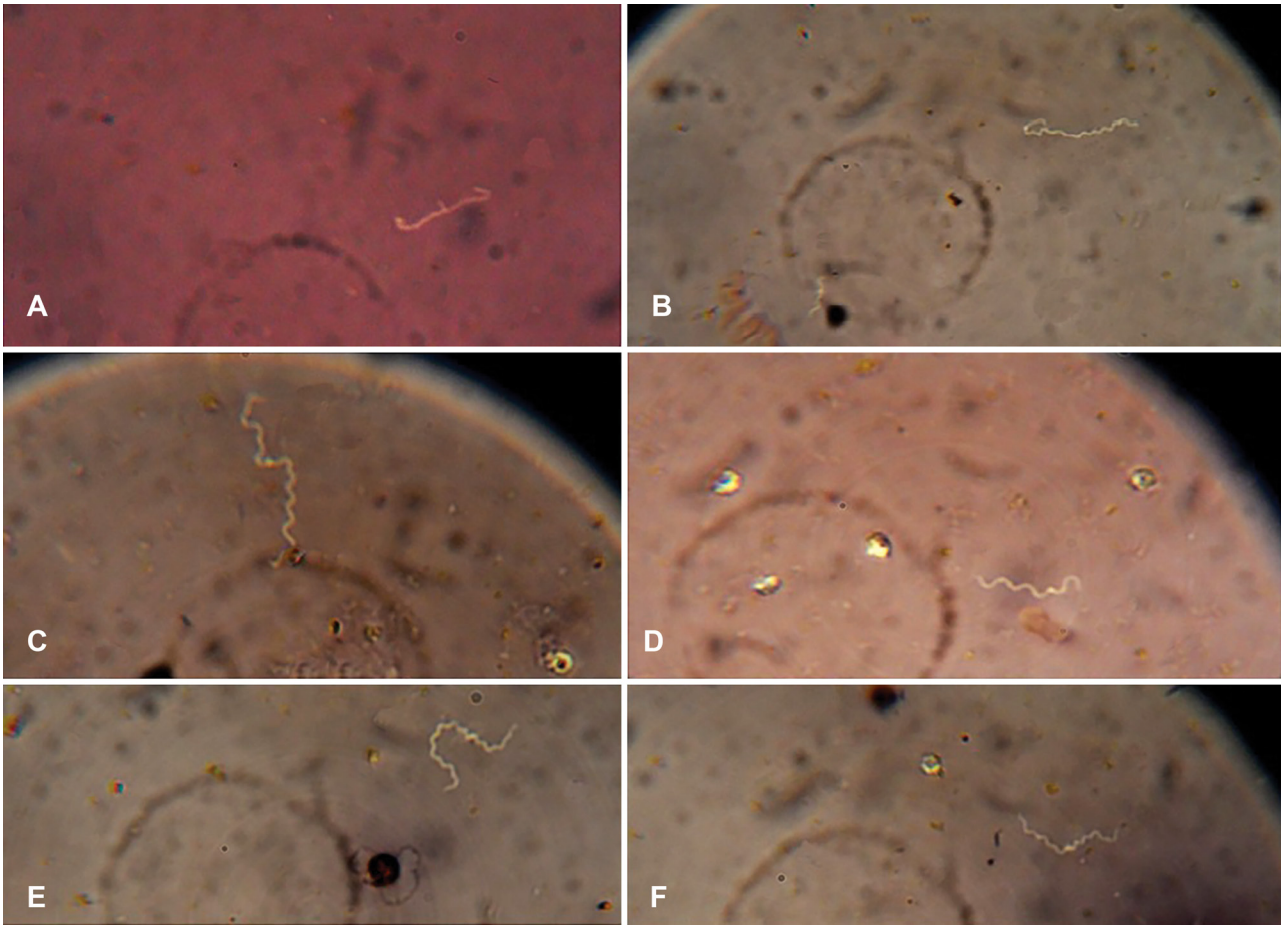

Fig. 3. Microscopic observation of *Leptospira* with a 100× magnification optical microscope

A: Multiplying *Leptospira*; the image relates to pathogenic *Leptospira* spp., B: The *Leptospira* bacterium that is twisted over itself is located on the right. In the lower-left a shorter *Leptospira* is visible; the image related to *Leptospira ryugenii*, C and E: Long *Leptospira*, D: The hook area of the *Leptospira* bacterium was clearly observed in a microscopic view; the image related to *Leptospira perdikensis*, F: Two *Leptospira* bacteria that are interconnected from one end and form a long separable leptospiral chain; the image related to *Leptospira* sp.

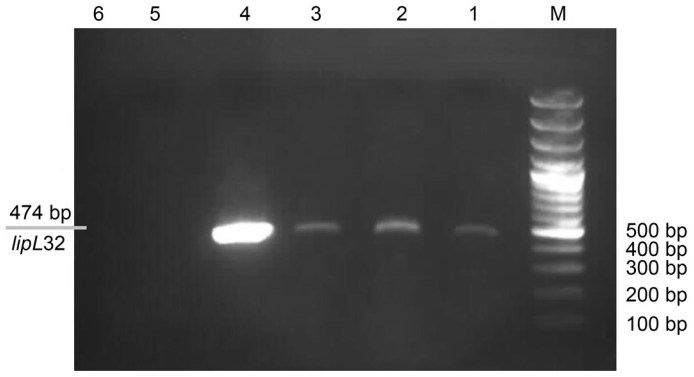

Fig .4. Agarose gel electrophoresis (1.5%) of PCR products of environmental samples.

Columns 1, 2, and 3, pathogenic *Leptospira* spp.; Column 4 – a reference strain; Column 5 – a negative control; Column 6 – *E. coli*; Column M – 100 bp DNA ladder.

trend in type 1 isolates (25°C = 0.12-fold; 35°C = 0.21-fold; 40°C = 0.17-fold) when compared to type 2 and 3 isolates (Fig. 6). In all three types of *Leptospira*, there was a significant difference in the CT values for the *lipL32* gene in control and the samples investigated under different thermal stresses ($p < 0.05$).

Fold change associated with the *lipL32* gene affected by pH stress was equal to 1 to type 2 and 3 isolates at pH = 7, and lower than 1 at pH = 8 and 9. Therefore, the stress of pH 8 and 9 reduced the *lipL32* gene expression in type 2 and 3 isolates, and this decrease was higher in type 2 isolates than in type 3 isolates. Isolates

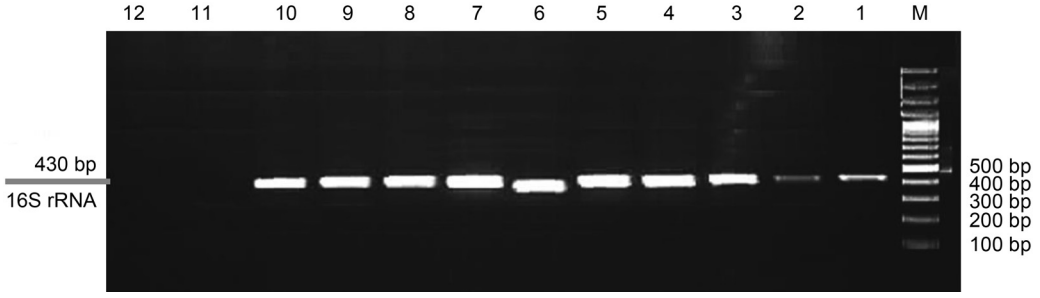

Fig. 5. Agarose gel (1.5%) analysis of a PCR products of environmental samples; Columns 1–9 – species of *Leptospira*; Column 10 – a reference strain; column 11 – a negative control; Column 12 – *E. coli E. coli*; Column M – 100 bp DNA ladder. Sampling sites that were positive for the presence of pathogenic *Leptospira* by both culture and PCR were reported in Fig. 1 and Table II.

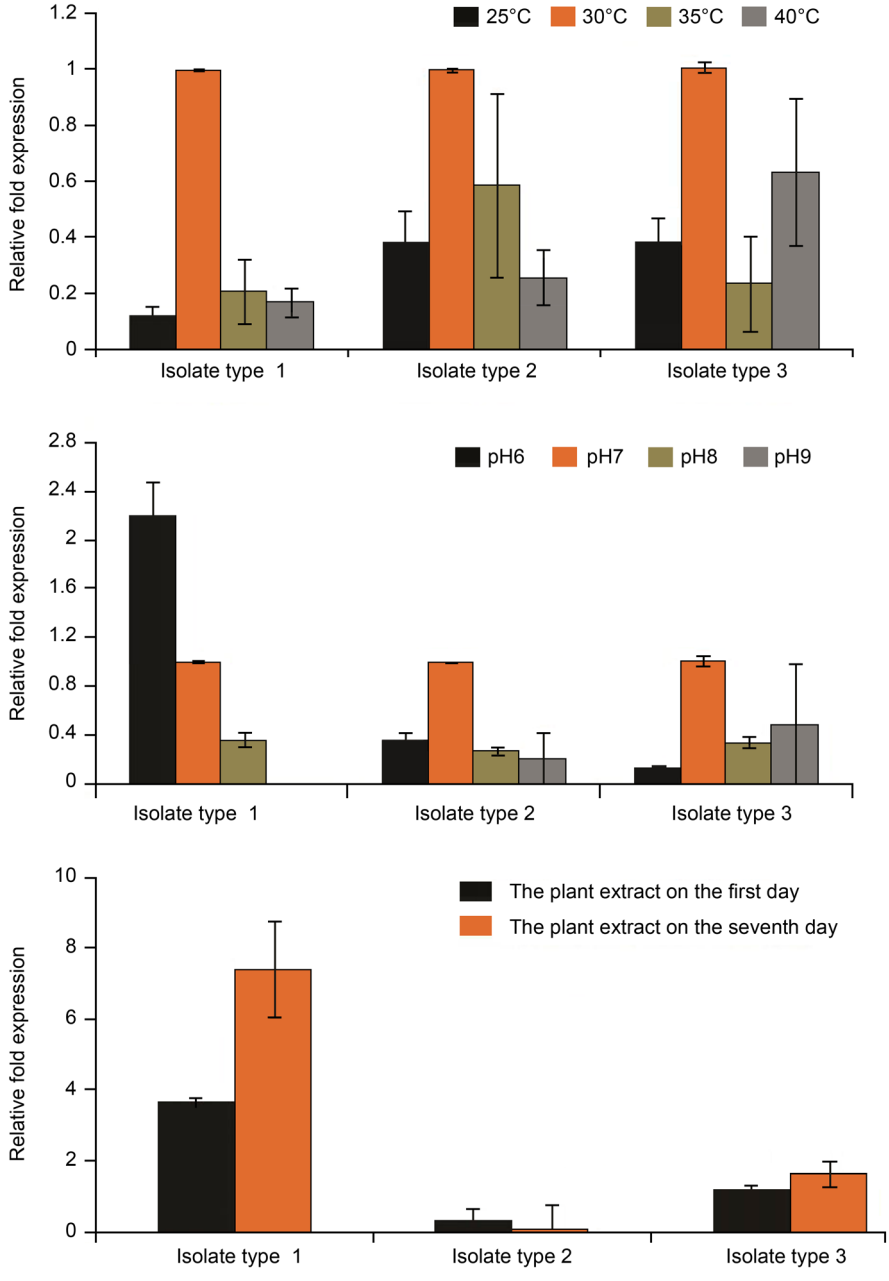

Fig. 6. Comparison of the *lipL32* gene expression in pathogenic *Leptospira* under the influence of temperature, pH, and *Ph. amarus* plant extract.

of type 1 did not show the expression at pH = 9 and demonstrated a decrease in the *lipL32* gene expression at pH 8. Isolates of type 1 were the only isolates that showed an increase in the *lipL32* gene expression (2.21-fold) at pH 6 (Fig. 6). In all the three isolates, there was a significant difference between the control *lipL32* gene CT and the *lipL32* gene CT under different pH conditions ($p < 0.05$).

Fold change associated with the *lipL32* gene expression affected by *Ph. amarus* plant extract was 0.31-fold on day one and 0.05-fold on day 7 in isolates of type 2. These results indicated a reduction in the *lipL32* gene expression under *Ph. amarus* plant extract's stress. A further decrease of the gene expression in isolates type 2 was observed on the seventh day; however, the fold change associated to the *lipL32* gene expression treated with *Ph. amarus* plant extract showed an increase in type 1 and 3 isolates. Fold change of this gene for isolates type 1 was 3.68-fold on first day and 7.41-fold of the seventh day, and for isolates of type 3 – 1.18-fold on first day and 7.41 of the seventh day (Fig. 6). Effect of stress imposed by the extract of *Ph. amarus* on the first and seventh days was significantly different in the three isolates ($p < 0.05$).

Ph. amarus plant extract, according to the MIC test, had strong anti-leptospiral activity and decreased the *lipL32* gene expression in isolates type 2.

Discussion

Due to the temperate climate of the northern regions of Iran, the prevalence of leptospirosis is high in these areas. In the study of the pollution status of stagnant waters and wetlands, more than 7% of the evaluated wetlands in Mazandaran were contaminated with pathogenic *Leptospira,* and leptospirosis is an endemic and occupational disease in these regions (Rafiei et al. 2012).

Studies have shown that pathogenic *Leptospira* species may be detected by real-time PCR because the *lipL32* gene is not present in non-pathogenic *Leptospira* species. Real-time PCR based on the *lipL32* gene is a tool used in the rapid diagnosis of acute leptospirosis, especially in cases with the potential for rapid mortality before a diagnosis could be performed by serology or culture (Stoddard et al. 2009).

In our study, a real-time PCR technique was used to evaluate the *lipL32* gene expression. The expression of the *lipL32* gene was affected by temperature, pH, or *Ph. amarus* plant extract. In the previous study by Fraser and Brown (2017), the effects of temperature and oxidative stress on the expression of virulence genes in *Leptospira borgpeterseneii* Jules and *Leptospira interrogans* Portlandvere species were investigated. Bacteria were grown in EMJH medium at 30°C before the transition to 37°C. A total of 14 virulence-related genes (i.e., *fliY*, *invA*, *lenA*, *ligB*, *lipL32*, *lipL36*, *lipL41*, *lipL45*, *loa22*, *lsa21*, *mce*, *ompL1*, *sph2*, and *tlyC*) were evaluated using Endpoint PCR. Transcriptional analysis of the *lenA*, *lipL32*, *lipL41*, *loa22*, and *sph2* genes was performed by quantitative real-time PCR. Temperature had reduced the *lipL32* gene expression in 30°C and 37°C for both Portlandvere and Jules serovars in the study mentioned above, and 30°C and 37°C were temperatures that also reduced the *lipL32* gene expression in our study.

Another study was conducted on the viability of *Leptospira* isolated from a human outbreak in Thailand in waters with different thermal and pH conditions (Stoddard et al. 2014). *Lepospira* spp. isolated from patients was genotyped using multilocus sequence typing during a multi-year outbreak in Thailand. The survival of ST34 isolates at different pH values, temperatures, and water sources was investigated. Most isolates survived until the end of the study, except for those subjected to temperatures higher than 37°C and pH = 8.65. In our study, the *lipL32* gene expression was assessed at different pH values, and the isolates of type 1 did not show any growth in pH = 9. The results of the electrophoresis of PCR products were negative and real-time PCR results did not show the *lipL32* gene expression when the isolates grew at pH = 9.

Today, due to the side effects of chemical drugs, herbal remedies gain interest. An important feature of medicinal plants is that treatment takes longer. In our study, the effect of *Ph. amarus* plant extract was higher on the *lipL32* gene expression on the seventh day than on the first day, especially in the isolates type 2, confirming that herbal drugs take time.

In 2016, a study using *Eclipta alba* and *Ph. amarus* was performed, and their activity against *L. interrogans* was evaluated (Mohan et al. 2016). In this study, the anti-leptospiral activity of plant extracts against *L. interrogans*, expressed as the MIC value following standard TDT was evaluated. The *Ph. amarus* extract (at the concentration of 160 μg/ml) had superior anti-leptospiral activity than the *E. alba* extract (at the concentration of 320 μg/ml). When compared with our study, the *Ph. amarus* extract at the concentration of ≥ 640 μg/ml completely inhibited the growth of the three selected *Leptospira* isolates on the seventh day. Methanolic and aqueous extracts of *E. alba* and *Ph. amarus* had anti-leptospiral activity and proved to be the best anti-leptospiral drugs *in vitro* (Chandan et al. 2012). Since no research has been conducted to evaluate the *Leptospira lipL32* gene expression under different physicochemical conditions, this study can be considered an innovative research in Iran examining the expression of the *lipL32* gene in pathogenic *Lepto-*

spira under different pH, temperature, or *Ph. amarus* extract stress conditions.

In this study, an increase or decrease in the *lipL32* gene expression can be attributed to the influence of a single factor or a combination of factors such as growth temperature, strain variation, pH conditions, the plant extract, and other unknown factors. On the other hand, it is well known that *Leptospira* bacteria favor neutral to slightly alkaline pH. The results of our study showed a reduction in the *lipL32* gene expression in pH = 9. The reason for choosing temperature 40°C *in vitro* was that we aimed to determine whether the *lipL32* gene expression might occur in patients with leptospirosis who had a fever of 40°C.

The decrease in the *lipL32* gene expression was associated with a 40°C thermal stress in isolates types 1, 2, and 3. According to our results and the findings of Fraser and Brown (2017) study, it can be stated that the *lipL32* gene expression may depend on various thermal conditions, genus and strain of *Leptospira*, and different pH levels.

The results of this study showed the inhibitory anti-*Leptospira* effect of *Ph. amarus* plant extract expressed as the MIC value, and the reduction of the *lipL32* gene expression in *Leptospira* isolates (Type 2) as it was demonstrated with real-time PCR technique. Therefore, it can be concluded that *Ph. amarus* plant can play an integral role in the prevention, control, and treatment of leptospirosis after examining its non-toxicity *in vivo* at the concentration used in this study.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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