Flowering time regulation by the miRNA156 in the beet (Beta vulgaris ssp. maritima)

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Abstract
Successful reproduction of the plants is considered as a critical challenge in meeting the nutritional needs of the growing world population. A major significant change in the plant's life cycle is the transition from vegetative to reproductive phase. The flowering and bolting processes are influenced by several genetic and physiological factors. Molecular and genetic studies have identified key factors in age-dependent gene regulatory pathways such as miR172-miR156-SPLs module in phase transition in plants. Unlike in model plants, the role of miR172-miR156-SPLs like regulatory networks remains unexplored in crops such as Beta vulgaris. In the present study, for the first time, miR156 from Beta vulgaris was identified and characterized. miR156 gene from Beta vulgaris was cloned and ectopically overexpressed under the control of strong 35S promoter in sugar beet explants. Subsequently, transcript levels of the miR156 and its known targets (SPL4 and SPL9) were quantified in the transgenic and control plants. Consistent with reported role of mir156 in downregulation of SPL4 and SPL9, miR156 overexpression plants exhibited delayed flowering and atypical rooting pattern indicative of abnormal root development and poor root foraging performance. Altogether, our results suggest that Beta vulgaris miR156 modulate phase transition by acting as negative regulator of SPL4 and SPL9.

Key message
The research confirmed that the overexpression of miR156 has an effective role in the reduction of SPL4 and SPL9 gene expression, and eventually results in beet flowering suppression.

Keywords Beta vulgaris · miR156 · SPL4 · SPL9 · Flowering pathway · Bolting

Introduction
The plant life cycle consists of several developmental stages, in which the transition from the vegetative to the reproductive phase is vital during course of development, particularly in flowering plants. Successful reproduction of the plants is considered as the major challenge for addressing nutritional needs of the growing human population. This process is influenced by various genetic and physiological factors. Several regulatory processes have been identified in Arabidopsis thaliana, including vernalization, photoperiod, Gibberellin, autonomous, and age-related paths (Sung and Amasino 2004; Kobayashi and Weigel 2007; Mutasa-Gottgens et al. 2009; Wang et al. 2009; Dohm et al. 2014). Age-related pathway is affected by miR172 and miR156.

MicroRNAs (miRNAs) are small (16–26 nucleotides) and non-coding RNAs present in eukaryotes (Lau et al. 2001; Llave 2002; Reinhart et al. 2002; Ambros 2004; Simpson 2004). The miRNAs are involved in complex cellular processes such as RNA silencing, regulation of gene expression, and cell death. The regulation of gene expression by the miRNAs is carried out both at transcriptional and post-transcriptional levels (Gandikota et al. 2007; Voinnet 2009; Kim et al. 2012; Sun 2012; Wang et al. 2014). In plants, miR156 is involved in developmental and biological
regulatory networks such as phase transition, nodulation, fertility, development of leaves, flowers, and fruits. miRNA is known to regulate expression of *Squamosa Promoter Binding Protein-Like* (SPLs), *WD40 repeat* (WD40), *TGACG Sequence-Specific Binding Protein 1* (TGA1), *Liguleless 1* (LG1) in the plants (Zhou and Luo 2013; Aung et al. 2015).

In Arabidopsis, there are numerous miR156s in the initial phase in which most of them mediate the cleavage of the mRNA transcript of SPL transcription factors. The SPLs regulates downstream genes by conserving the DNA binding domains. These transcription factors facilitate the transition towards the flowering stage by activating *LEAFY* (LFY), *FRUITFULL* (FUL), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (SOC1), *Activator protein 1* (AP1), and *AGAMOUS-LIKE 24* (AGL24) genes at the terminal bud (Wei et al. 2012). Many studies have demonstrated that over-expression of miR156 has led to a reduction in the levels of SPL3, SPL4, SPL5 and SPL9 expression and, consequently, a delay in flowering occurs in Arabidopsis (Wu and Poethig 2006; Gandikota et al. 2007). SPL3 and SPL9 are upstream activators of AP1, LFY and FUL. Thus, SPLs play crucial role in transition to identification of the meristem stage. Thus, maintenance of appropriate expression levels of SPLs is essential for transition to the flowering phase (Wigge et al. 2005; Yamaguchi et al. 2009; Wang et al. 2009). In addition, two miR156 targets (SPL9 and SPL10) can trigger the expression of miR172 in the leaves, which has a similar expression pattern to the miR156 and accumulates during maturity phase (Aukerman and Sakai 2003; Gandikota et al. 2007; Wu et al. 2009). miR156 has also an effective role in the reduction of SPL9 and miR172 genes’ expression that results in over-expression of APETALA2 and, finally, flowering suppression by the FT (Jiang et al. 2012; Cho et al. 2012).

miR156 is strongly expressed during the early stages of plant development; however, its expression gradually declines upon increasing age in Arabidopsis (Voinnet 2009). miR156 is conserved among different genus of flowering plants (Voinnet 2009; Huijser and Schmid 2011). The functional studies characterizing the role of miR156 have been performed in a range of plant species. Nevertheless, despite miR156 vital biological roles in plants, its developmental role in *Beta vulgaris* (B. Vulgaris L.) remains to be elucidated.

The sugar beet (*B. vulgaris*) is an herbaceous, dicotyledonous, and biannual plant from the Amaranthaceae family and is considered one of the most important flowering crops. In the sugar beet life cycle, the vegetative phase begins with the emergence of a leafy plant and a large root (the main source of the sucrose) in the first year. In the second year, the reproductive phase would be continued by stem elongation (bolting) and flowering after a period of low temperature (Biancardi et al. 2005; Chia et al. 2008). Different cultivars of sugar beet can be grown in different parts of the world, both in the spring (in temperate and cold regions) and autumn (in tropical and warm regions) seasons. Selection of the cultivations is influenced by the natural resistance of different cultivars to bolting (El-Mezawy et al. 2002). Because one of the main limiting factors for the cultivation of sugar beet in temperate regions is the long and weak lignin stems produced during the first year upon bolting. Exposure of some sugar beet plants to low temperatures leads to the production of some unwanted stems, which undesirably reduces the sugar percentage, root function, and purity of raw syrup (Mutasa-Gottgens et al. 2010; Pfeiffer et al. 2014; Hebrard et al. 2016). Full control of the bolting and flowering stages means sugar beet cultivation will be possible in autumn over the northern latitudes (Reeves et al. 2007). The switch from spring to autumn cultivation causes many positive consequences, such as an increase in the leaf and root growth and sugar content, less irrigation and water saving, limited weed growth, and fewer sugar beet diseases (Jaggard et al. 2009).

Due to the importance of the sugar beet’s vegetative growth, the study of the flowering process and the genes involved is vital for successful breeding. Therefore, in this study, we attempted to investigate the role of miR156 in reproductive phase transition in *B. vulgaris* for the first time. For this reason, the miR156 gene sequence from sea beet’s genome was identified and isolated. Subsequently, miR156 over-expression construct was generated and then introduced into the sugar beet explant. Finally, the influence of miR156 over-expression on the target genes was assessed followed by analysis of flowering and root growth variety of transgenic plants.

**Materials and methods**

**In silico search for the miR156 homologue and its target genes in the *B. vulgaris***

Previously known miR156 precursor gene sequences from eight different plants (*Ananas comosus, Arabidopsis thaliana, Camellia sinensis, Ceratopteris thalictroides, Citrus sinensis, Musa AAB, Oryza sativa, and Zea mays*) were compared in the National Center for Biotechnology Information (NCBI) GeneBank and miRBase databases. Because the sugar beet KWS2320 genotype was the only one annotated in NCBI when we began our research, we used BLASTN to identify refseq-genomic sequences of this plant associated with sequences of miR156 precursor genes from each of the plants mentioned. If there were several similar refseq-genomic sequences with the miR156 precursor sequences, each of them was individually considered. De novo assemblies of the sugar beet refseq-genomic sequences
and miR156 precursor sequences were carried out using the SeqMan Pro NGen (DNAStar, Inc., Madison, WI, USA). After the alignment, common sequences were considered as the miR156 precursors in the sugar beet. Verification of the identified sequences was performed using BLASTN in other plants' genomes. To identify the complete sequence of the genes in the sugar beet genome, various parts of the refseq-genomic sequences containing the gene sequences were thoroughly analyzed via alignment. Finally, the mature miR156 sequences were identified in precursor sequences and were aligned using the MegAlign Pro NGen (DNAStar, Inc., Madison, WI, USA).

For evaluation of the main targets of miR156 in sugar beet, the sequences of identified SPLs (SPL4 and SPL9) were first extracted from the NCBI GeneBank. To ensure that these genes are the target sequences and to identify the restrictions and complementary areas, two psRNATarget (https://plantgrn.noble.org/psRNATarget) and the RNAhybrid (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) software programs were used.

Plant materials and growth conditions

The seeds of Beta vulgaris ssp. Maritima (collected from the Shoush area of Iran) were obtained from the Sugar Beet Seed Institute (Located in Karaj, Iran), and were used in genetic transformation for all experiments. In the growth chamber, the seeds and plants were cultured in the Murashige and Skoog (MS) medium under long-day conditions with 16 h of light per day and a 24/16 °C day/night rhythm.

Generation of gene construct and explant transformation

Based on the identified sequence of the miR156 precursor gene in the Beta vulgaris genome, a 127-bp stem-loop fragment was amplified from the sea beat genome by the polymerase chain reaction (PCR) with the MIRF and MIRR primers (all primer sequences are given in Supplemental Table 1), introducing the NcoI and BamHI restriction sites. After confirmation by the sequencing P35S:BvmiR156 construct was generated by replacing the GUS gene with the BvmiR156 in the pGSA1285 vector. The pGSA1285-miR156 construct was confirmed by sequencing and then transformed into the Agrobacterium tumefaciens GV107 strain competent cells using the heat-shock method (Sambrook and Russell 2001). After preparing sugar beet leaf explants (Mirzaei-Asl et al. 2010), they were infected for five minutes and incubated at 25 °C for 48 h on coculture medium (MS medium and 1 mg/l 6-benzyl aminopurine), followed by incubation in selective medium (MS medium, 1 mg/l 6-benzyl aminopurine, 100–150 mg/l Kanamycin, 200 mg/l Cefotaxime). Thereafter, Kanamycin-resistant shoots were obtained by culturing in the selective medium. The BA hormone was removed from the selective medium after 8 months.

Analysis of transgenic plants

The genomic DNA was extracted from the leaves of both regenerated and non-transgenic plants (control) by the CTAB method (Murray and Thompson, 1980). To confirm the transgenicity of the plants, the PGSAF and PGSAR primers (Supplemental Table 1), designed from the pGSA1285 vector promoter and terminator, were used in PCR. The PCR reaction conditions included a five-minute pre-heat at 94 °C, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and a seven-minute final extension at 72 °C, and the PCR product was confirmed by sequencing.

Expression analysis of the miR156 and target genes

Total RNA was isolated from the young leaves of transgenic and non-transgenic plants using the RNX-Plus solution (Sinaclone Co.) according to instructions. Next, it was treated with the RNase-free DNase (Fermentase Co.) as per the instruction. An amount of 500 ng of RNA was used for the reverse transcription of miR156, SPL4, and SPL9 by the cDNA Synthesis Kit (Fermentase Co.). The RT-PCR reaction was performed with housekeeping genes primers of UB-quinitone (UbiF and UbiR) and actin-8 (ActinF and ActinR). The reaction conditions included a five-minute pre-heat at 94 °C, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and a five-minute final extension at 72 °C. The quantitative RT-PCR of the miR156 gene and its targets (SPL9 and SPL4) was performed using the SYBR-Green PCR Mastermix (Amplicon) and an applied biosystem Roche LightCycler 480 instrument. The Actin 8 gene was used as the internal control. The qPCR reactions were performed with three biological and technical replicates, separately. The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 30 s at 94 °C and 30 s at 58 °C. Transcript levels of miR156 and the target genes were analyzed for relative expression levels according to 2−ΔΔCT method (Livak and Schmittgen, 2001). The primers used for the RT-PCR and qRT-PCR are listed in the supplemental Table 1.

Flower and root development evaluation

After confirming the transgenic plants, their exact flowering time was compared with non-transgenic plants to determine the effect of the miR156 over-expression on flowering time. The root development under the miR156 over-expression effect was also evaluated in transgenic plants in comparison with non-transgenic plants.
Statistical analysis

Triplicate samples were collected for each transgenic line. The statistical analysis of the miR156, SPL4 and SPL9 genes expression was conducted using a one way-ANOVA test after evaluating "the normality residual exam" by the SAS software (SAS Institute, Cary, NC). A correlation analysis test was also performed using Minitab software. The P values < 0.05 were considered as significant.

Results

Identification of the BvmiR156 and its target genes in *B. vulgaris* genome

Overall, 39 miR156 precursor sequences (including 12 members of the miR156 family) from eight plant species were isolated from the NCBI GeneBank and miRBase databases, and then the mature miR156 were aligned from the precursor sequences. The number of bases in mature miR156 varied from 20 to 25 nucleotides in different plants. The most diversity of the miR156 sequence was found in O. sativa and C. thalictroides plants, whereas differences between the miR156 sequences of *A. comosus*, *A. thaliana*, *Camellia sinensis*, *Citrus sinensis*, *Musa AAB*, and *Z. mays* were not significant at the single nucleotide level. After the alignment of the miR156 precursor sequences for each mentioned plant with *Beta vulgaris* Refseq-genomic sequences, the homologous sequences were identified. The highest similarity was observed between the *B. vulgaris* sequence with an accession number of NC_025813 on chromosome two and the miR156 sequences of *A. comosus*, *Camellia sinensis*, *Citrus sinensis*, and *Z. mays*. In the meantime, 127 nucleotides were similar to miR156 precursor sequence of the *Camellia sinensis*, and 85–89 nucleotides were completely similar to the miR156 sequence in other three plants. Hairpin structures could also be predicted in the identified sequences (Fig. 1b). Thus, the identification of the mature miR156 sequence in the *B. vulgaris* Refseq-genomic sequences of the sugar beet was performed. The comparison of the identified miR156 precursor sequences with *A. comosus*, *Camellia sinensis*, *Citrus sinensis*, and *Z. mays* was performed and confirmed that all of them have relatively same sequences with 21 nucleotides (Fig. 1a).

Prediction and validation of the miRNA target sequences

Search for the SPL homologues in the *B. vulgaris* genome demonstrated nine SPL accessions. However, it was found that only five SPL genes (XM_010677198, XM_010674387, XM_010669328, XM_010681697, and XM_010684453) contain the complementary sequence with the miR156 gene sequence. Further research using the psRNATarget and
RNAhybrid software revealed that the miR156 has 19 complementary nucleotides with the SPL4 sequences in 928–947 region, as well as the SPL9 sequence’s 1286–1267 region. It may cause a cleavage in these areas due to the presence of mismatch (Fig. 2). SPL4 and SPL9 sequences were then amplified from the *B. vulgaris* genome using gene specific primers.

**Generation of transgenic plants with the BvmiR156b over-expression construct**

The presence of the BvmiR156 precursor fragment, under the control of *CaMV 35S* promoter, was confirmed in overexpression construct by genotyping, restriction-digestion, and sequencing. In this construct, the selectable marker *Neomycin Phosphotransferase II* gene (*NPT II*) was under the control of the pMAS 2' promoter. Thus, the resistant shoot was regenerated after kanamycin selection from the leaf explants infected with the *Agrobacterium* carrying pGSA-BvmiR156 vector. The regenerated shoots were separated. After transferring them to larger plates, the transgenicity was confirmed via PCR screening using promoter and terminator-specific primers. The sequencing results of amplified fragments in PCR also indicated that the transgene was steadily integrated into the plant genome (Supplemental Fig. 1). In sum, the transgenicity of 150 regenerated shoots could be approved.

**The miR156 expression pattern and its targets**

The green leaves from regenerated transgenic plants were used for the quantitative RT-PCR analyses. The level of pre-BvmiR156 transcript was higher in transgenic plants than in non-transgenic plants. The rate of increase in pre-BvmiR156 transcript ranged from 12.47× to 38.45× in the transgenic plants compared to the controls (Fig. 3).
For evaluation of the miR156 over-expression effects on its targets’ (SPL4 and SPL9) transcripts in the sea beet, qRT-PCR was used. The level of the BvSPL9 transcript ranged from 0.23 × to 0.80 × in transgenic plants compared to control plants. The results of this experiment also uncovered that the BvSPL4 transcript level ranged from 0.16 × to 0.08 × in transgenic beets compared to the controls (Fig. 4).

**Phenotypic characterization of transgenic plants**

Monitoring of transgenic and non-transgenic plants during the 13 months after the inoculation revealed noticeable morphological changes in terms of flowering and root development. In more detail, it was observed that the control plants were transferred to the reproductive stage through the generation of flowering stems after seven months; while, the transgenic plants were passing through the vegetative phase. Two months later, transgenic plants were still without any flowering stems, while the non-transgenic plants had several flowering stems and were passing through the reproductive growth stage. After 13 months of inoculation, none of the transgenic plants with over-expression of miR156 were transferred to the flowering stage, and even no flowering stem was shaped in each of them. However, during this period, the flowering and reproductive phases ended in a great number of non-transgenic plants (Fig. 5).

The evaluation of the effect of miR156 over-expression on the root growth in the sea beet showed a significant difference between the transgenic and non-transgenic plants. Twelve months after inoculation, all control plants were able to produce roots, whereas no evidence of root production was observed in the transgenic plants. The commencement of root production at the end of the month 13th revealed that the root production in plants with the miR156 over-expression has a remarkable delay compared to the control plants (Fig. 6).

**Discussion**

Although the involvement of the different miRNAs in the flowering process has been studied in many plants, there is no report concerning the effect of these factors on flowering time regulation in beet plants. According to the results of this study, the effect of the miR156 gene was demonstrated on flowering and bolting procedures in the sea beet. In this work, since the transgenic beet with over-expression of miR156 couldn’t transfer to reproductive phase, they didn’t have any progeny. The role of miR156 in flowering has already been demonstrated in Arabidopsis (Wang et al. 2009) and the results of our study further expanded the role in sea beet. Previous studies in eukaryotes suggest that different types of miRNAs are involved in complex processes of gene expression. The miRNAs are quite widespread and properly protected in the plant kingdom, and this probably indicates the existence of a common ancestor in the early evolution period (Zhang et al. 2006). Furthermore,
computational methods suggest that miRNAs make up 1\% of Arabidopsis and Drosophila coding and non-coding genes (Bartel and Chen, 2004; Zhang et al. 2005). These similarities are also visible in the sugar beet genome, where there may be approximately 274 miRNA-coding genes among 27,421 genes in the sugar beet genome. These genes contribute to various mechanisms; nevertheless, none of them has been identified in sugar beet, so far. In our study, in the miR156, only the NC-025813 sequence was identified through the comparison of sugar beet Refseq-genomic with the miR156 sequences in other plants, and no other similar sequences were detected in the genome of sugar beet. All recognized miR156 sequences are 21 bp. The sequence has been identified as the miR156 stem-loop structures, which are critical in the execution of their functions.

In several studies, it has been confirmed that the miR156 does not have any direct effect on flowering procedure and exerts its effect indirectly through other factors such as the SPLs. It should be noted that the action of the miR156-SPL

network in two stages of flowering stimulation and flowering meristem identification is notable (Gao et al. 2016). In Arabidopsis, the number of 11 out of 17 SPL have been recognized as the target sequences for miR156, in which these genes are able to create a junction with the mismatch nucleotides and disrupt the transition process towards the reproductive phase (Wang et al. 2009). In the sugar beet genome, we confirmed five SPL targets with complementary areas for miR156. The effect of the miR156 expression on the SPL4 and SPL9 expression was also demonstrated. These genes, as the downstream targets of miR156, are effective in the flowering process of plants (Schwarz et al. 2008; Wu and Liu 2009; Yamaguchi et al. 2009).

The over-expression extent of miR156 in different transgenic plants was varied compared to the control plants. In a study implemented by Aung (2015), three groups of transgenic alfalfa plants were identified in terms of the over-expression of miR156. Only in the group with high miR156 expression, the three SPLs (SPL6, SPL12, and SPL13) expression were affected (Aung and Abdelali Hannoufa 2014). In contrast, and based on the correlation results, it was perceived that over-expression of miR156 downregulated SPL4 and SPL9 in transgenic plants compared to the control plant.

Considering the SPL4 expression, decreasing the transcription level in transgenic plants was completely in line with the miR156 over-expression rate, while it was totally
different in case of SPL9. It means that the plants with the highest miR156 expression represented the lowest expression of the SPL4 compared to other transgenic plants. Previous studies have shown that the SPL4 expression is not affected by environmental conditions, and its expression is constant in both long and short-day conditions (Jung et al. 2011).

These changes most likely indicate that the expression of SPL9 in long-day conditions is influenced by factors other than miR156. Various studies have claimed that flowering regulators such as FLC, CO, FT, or SOC1 do not have any significant effect on miR156 expression in Arabidopsis. According to previous studies, FLC expression, as a flowering repressor, does not affect the SPL9 expression (Michaels and Amasino 2001). It has been shown that two gibberellins and photoperiod factors are effective on the SPLs expression (Zhou et al. 2016). In our experiments, the growth medium of transgenic plants lacked the exogenous gibberellins, and they were cultured under long-day conditions. Therefore, the SPL9 expression was influenced by the photoperiodic pathway factors in addition to miR156. Our results are also consistent with the findings of Wang et al (2009). They transformed the transgenic Arabidopsis from the short-day to the long-day condition and couldn’t detect any change in the expression pattern of miR156. Nonetheless, SPL9 expression increased in different parts of the lateral and leafy buds (Wang et al. 2009) (Fig. 7).

The flowering time is usually determined by two factors: calendar time and the number of leaves before flowering. In our study, after thirteen months of shoot regeneration, none of the transgenic plants with the over-expression of the miR156 were able to transition to the reproductive phase. Whereas one or several flowering stems developed in the control plants and their flowering ended during that period.

The effect of miR156 over-expression on rooting phenotype varies in different plants. Recent studies have uncovered that miR156 plays an important role in nitrogen fixation and root development in legumes. For example, over-expression of miR156 produced more (but smaller) roots in the transgenic rice (Xie et al. 2012). Transgenic Lotus japonicas with miR156 over-expression had significantly less branching and root length (Wang et al. 2015), whereas transgenic alfalfa with miR156 over-expression had longer roots and a higher number of nodes (Aung and Abdelali Hannoufa 2014). In our experiments, over-expression of miR156 caused a one-month delay in the root development of transgenic plants compared to control plants. It has been shown that miR172 is activated by the SPLs and is targeted by the miR156 and finally inhibits the AP2 (Yant et al. 2010). The studies accomplished by Wu et al. (2009) showed that over-expression of the SPL3, SPL4, and SPL5 had no effect on the transcription level of the miR172 in Arabidopsis, indicating that these genes do not play any role in cross talk between the miR156 and miR172. It has also demonstrated that SPL9 is a major activator of the miR172 and an accelerator of the link between the miR156 and miR172 (Wu et al., 2009). Thus, these results suggest that over-expression of miR156 represses SPL9 and, consequently, miR172 expression. Therefore, upregulation of the AP2 leads to the delay of root growth in transgenic plants (Fig. 7). It would also be very informative and challenging to determine other factors involved in this pathway.

Production of bolting resistant sugar beet is crucial to sugar beet cultivation in autumn over the northern latitudes. Our research revealed that over-expression of miR156 results in flowering suppression in the sea beet. This case must be evaluated on sugar beet for further research. However, bolting resistant sugar beet by over-expression of miR156 probably will not produce seeds because of transgenic plants have no flowers. In order to maintain, multiply and commercialize the transgenic bolting resistant cultivars, it is needed a system to control expression of a transgene, by using of a transactivation system (Van Roggen et al. 2009), an efficient controlled expression system for the expression
of desired genes in hybrid cultivars without expression in parental lines.

According to the findings, over-expression of miR156 had a suppressing effect on the root growth of transgenic beets under in vitro conditions. This effect needs further investigation on seeds of the transgenic sugar beet plants by using of a transactivation system.

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Author contributions MA and AM have designed the research. MA has conducted the experiment. All authors had contributed to data analysis. MA and AM have written the manuscript. All authors have read and approved the manuscript.

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Data availability The datasets generated during and/or analyzed through the current study will be available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The financial supports of this research were provided by Bu-Ali Sina University budgets.

Consent to participate All authors are consented to participate in the research.

Consent for publication All authors are consented for publication of the research.

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