Plant Omics Journal POJ

POJ 11(01):30-36 (2018) ISSN:1836-3644
doi: 10.21475/poj.11.01.18.pne1020

CrMYC1 transcription factor overexpression promotes the production of low abundance terpenoid indole alkaloids in *Catharanthus roseus*

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Abstract

Catharanthus roseus is known as the only source for the low-abundance anticancer agents namely vinblastine and vincristine. Fine tuning of accumulation of such secondary metabolites is highly governed by the regulatory genes. Among these genes, *Catharanthus roseus MYC1* (*CrMYC1*) is known as one of the key transcription factors regulating the biosynthesis of terpenoid indole alkaloid metabolites in *C. roseus*. In this study, *CrMYC1* coding sequence (AF283506) was isolated and cloned in PBI121 plant binary vector. Then, *CRMYC1* was transiently overexpressed in *C. roseus* leaves using agroinfiltration method. In addition to molecular analysis for confirming *CrMYC1* overexpression, the profile of some chief terpenoid indole alkaloids in control and transgenic plants was evaluated by HPLC to elucidate the role of *CrMYC1* in an increased in the anticancer components. The results indicated that overexpression of *CrMYC1* transcription factor can increase most important terpenoid indole alkaloids including vinblastine, vincristine, and catharanthine in *C. roseus*. HPLC analysis of catharanthine and vinblastine contents showed about 3 and 2.5 fold increases, respectively, while the increase in vinecristine was not significant compared to that of the control. Therefore, *CrMYC1* is introduced as an efficient candidate for manipulating TIA pathway in *C. roseus* and increasing at least the most valuable terpenoid indole alkaloids in this plant.

Keywords: catharanthine; metabolic engineering; regulatory gene; transient expression; vinblastine; vincristine.
Abbreviations: CrMYC1_Catharanthus roseus MYC1; TIA_terpenoidindole alkaloids; MEP_methyl D-erythritol 4-phosphate pathway; PRX1_peroxidase1; bHLH family_Helix loop Helix family; MeJ_methyl jasmonate; JA_Jasmonic acid; LB|_Luria-Bertani; OD_Optical density; npt II_Neomycin Phosphotransferase; STR_strictosidine synthase; TDC_tryptophan decarboxylase.

Introduction

Catharanthus roseus L. G. Don (Madagascar periwinkle) is an important dicotyledonous medicinal plant belonging to the Apocynaceae family. This plant is known as a source of producing more than 100 alkaloids some of which are very important natural pharmaceutical products. Among these alkaloids, Vinblastine, Vincristine, Ajmalicine, Serpentine, Vindoline, and Catharanthine are accounted for most important terpenoid indole alkaloids in this plant (Jacobs et al., 2004). Vinblastine and vincristine have been used as chemotherapeutics in the treatment of lymphoma and leukemia (Gidding et al., 1999). Ajmalicine and Serpentine are sometimes used as anti-hypertensive agents. In addition, it has been reported that major alkaloids such as vindoline have high antidiabetic activities particularly against type 2 diabetes (Nammi et al., 2003; Tiong et al., 2013). Generally, terpenoid indole alkaloids (TIA) are produced by combining the product of two independent pathways namely MEP and Shikmate that produce secologanin and tryptamine, respectively. This combination is made by a key enzyme known as *STR*, which generates strictosidine. Strictosidine as a major mediator embedded in the TIA pathway is converted to all different indole alkaloids such as ajmalicine, catharanthine, and vindoline in different branches. As catharanthine and vindoline are combined with *PRX1* enzyme, they produce higher indole alkaloids vinblastine and vincristine in a multi-step biochemical reaction (Zhu et al., 2014).

Despite the significance of this natural metabolite in Catharanthus, the application of these bio-products is limited due to their very low yields. The total amount of vinblastine produced by *C. roseus is* 0.01% of its dry weight. Still, this volume is even lower in case of vincristine (0.0003%) (Zhao and Verpoorte 2007). Numerous studies have been conducted for improving and increasing the amount of these significant natural metabolites in *C. roseus* (Zhao et al., 2013; Zhou et al., 2009). Biotechnology-based alkaloid enrichment programs provide a promising solution to overcome the obstacle of the TIA improvement in this plant (Mujib et al., 2012). Complexity in the TIA and its regulatory pathways that control TIA production can be considered as one of the limitations in the large-scale production of TIAs (Shanks 2005). In fact, TIAs in *C. roseus* are produced by a complex multi-step biosynthetic pathway known as TIA pathway. Moreover, their biosynthesis is

highly regulated by developmental, cellular, and molecular stages (Dutta et al., 2007). TIA production pathway is highly regulated at transcription level by different transcription factors, some of which working as transcriptional activators and some as repressors (Verma et al., 2014). However, very limited information is available about any of these regulators (Menke et al., 1999; Suttipanta et al., 2011; van der Fits et al., 2000; Zhang et al., 2011).

Among all efforts made to enhance the major alkaloid content in *C. roseus*, manipulation of a regulatory gene can be named as one of the most promising strategies (El-Sayed and Verpoorte 2007). Regulating a group of genes, which may lead to an increase in the metabolites through employing transcription factors, is the advantage of this strategy in comparison with the manipulation of one enzymatic gene in TIA pathway (Broun et al., 2006). *CrMYC1* is one of the putative TIA pathway regulators belongs to the bHLH transcription factors family. The effect of *CrMYC1* on the regulation of terpenoid indole alkaloid genes, enzymes, and the metabolites content has not been studied yet. *CrMYC1* expression is induced by methyl JA signaling pathway and fungal elicitor (El-Sayed and Verpoorte 2007; Memelink et al., 2001; Pauwels et al., 2009).

Investigating the effect of *CRMYC1* on Chief TIA yield through overexpressing this gene can be useful for enriching the most important metabolites in the TIA pathway. Hence, this research was conducted to explore the possibility of increasing the most important TIAs in *C. roseus* through transiently overexpression the *CRMYC*1 transcription factor as the first step in a long-term program.

Results

MYC1 expression in Catharanthus leaves using agroinfiltration method

CrMYC1 gene was transiently transferred to *C. roseus* leaves using agroinfiltration method by narrow insulin syringe (Fig. 1). According to the result of confirmation the transfer and expression of *CrMYC1* gene, this method was successful for expression of the gene in *C. roseus*, which has many limitations for regeneration after gene transfer.

Molecular confirmation of transiently overexpressed leaves by PCR and real-time PCR

As the Catharanthus plant has its own *CrMYC1* gene, to ensure the T-DNA transfer containing both *CrMYC1* and kanamycin resistant genes, *nptII* genes can be used and confirmed by the PCR. The PCR analysis of npt II genes confirmed the T-DNA transfer to leaves from *CrMYC1*+PBI121 constructs (Fig. 2). To compare the expression of *CrMYC1* in transgenic leaves with the control leaves and to confirm the overexpression of this gene at mRNA level, a quantitative real-time PCR was performed. The quantitative expression patterns of both leaves 48 and 72 hours after agroinfiltration of leaves for *CrMYC1* gene show an increase compared to those of the control leaves (Fig. 3). The result showed that the mRNA level of *CrMYC1* transcript, compared to the control line, has been increased 4 fold in overexpressed leaves after 48 hours of injection. However, the difference between the expression levels of *CrMYC1* at 72 hours and control was low (Fig. 3).

Analysis of alkaloids concentration in overexpressed versus control leaves

In order to determine the effect of *CrMYC1* overexpression on the level of indole alkaloids in *C. roseus*, the profiles of four different TIA alkaloids (Ajmalicine, catharanthine, vinblastine, and vincristine) were measured by HPLC in both overexpressed and control lines (Fig. 4). These alkaloids were identified by comparing the retention time and the UV spectra with standards. To ensure the peak purity, we used the AUTOchrom 3000 software. The standard curve of alkaloids was generated through the implementation of different levels of each standard employed as a reference. Next, the content of each alkaloid was quantified by comparing it with the standard curves. The content of each metabolite in overexpressed leaves was quantified and compared with those of controls. The values of all analyzed alkaloids are presented in Table 2. Different amounts of catharanthine, vinblastine, and vincristine were detected in both 48 and 72 hours overexpressed leaves in comparison with the control plants. The results showed a significant increase in the amount of Vinblastine and catharanthine after transient expression while the increase in vincristine was not significant (Fig. 5).

Discussion

A few studies have been accomplished on transient expression of TIA constructive enzymatic and reporter genes in *C. roseus*. Transient overexpression of *TDC* and *STR* gene in intact Catharanthus leaves have been accomplished and confirmed by Stephano Di Fiore et al. (2004), who demonstrated the transient accumulation of recombinant enzymes in Catharanthus leaves. However, they did not study the effect of transiently overexpressed *TDC* and *STR1* genes on TIA pathway intermediates or products. They concluded that agroinfiltration is a suitable tool to express the TIA genes in *C. roseus*. According to the result of this study, CrMYC1 was successfully overexpressed by using agroinfiltration method. Nevertheless, it should be considered that transient expression can be affected by other variabilities including physiological, growth, and developmental state of the plant itself. Thus, it is suggested to perform a transient expression on a large number of plants and in different growth stages whenever possible. In general, the transient expression system used in this study is introduced as a suitable testing tool for the further development of a stable metabolic engineering program by *CrMYC1* or other transcription factors.

Consistent with the hypothesis of this study, transient overexpression of *CrMYC1* leads to an increase in *CrMYC1* transcript. However, according to the small increase in *CrMYC1* transcript after 72 hours, the suitable time for transient expression is 48 hours after leaves injection. Nevertheless, it will be more useful to check for some other intermediate time points particularly for expression of enzymatic proteins. Finally, to check whether *CrMYC1* overexpression is successful for producing more amounts of TIA alkaloids, we examined the alkaloid levels.

Based on the HPLC analysis, Ajmalicine was not detected in any of extract samples. This result is in accordance with the most findings of the localization of Ajmalicine. It is

Table 1. Different Alkaloid contents of transient overexpressed and control lines in Catharanthus roseus leaves (mg/g dry wt.)

	Catharanthine	Vinblastine	Vincristine
Control	0.9575±0.051	0.7925±0.029	0.220±0.014
48hours	3.085±0.075	2.1625±0.050	0.350±0.021
72hours	2.175±0.202	1.3975±0.078	0.3625±0.054

Fig 1. Agroinfiltration of Catharanthus leaves with Agrobacterium tumefaciens containing PBI121+*CrMYC1* constructs.

Table 2. List of primers used in this study.

Primer name	Primer sequence
CrMYC1 for isolation	5'CGGGATCCAACAGTTGATGGTGGTGGTG3'
	5'CGGAGCTCGCTTGGCTTCTCAATGCTCT3'
CrMYC1 for real-time analysis	5'AATCCTTACAGCGCCAGGT3'
	5'AAAACCACCACCAATCTGCA3'
Elongation factorα	5'GCTTTACCTCCCAAGTCATCATC3'
	5'GGCTCCTTCTCAATCTCCTTACC3'
nptII	5'TTGGGTGGAGAGGCTATTCG 3'
	5'GAATCCAGAAAAGCGGCCAT3'

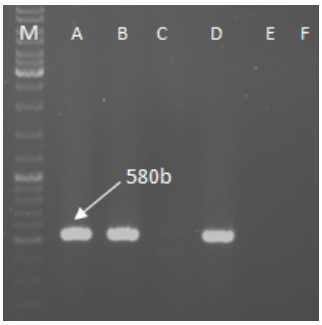

Fig 2. PCR analyses for the presence of the nptII gene. M) 100bp DNA marker, lane: A) 48h after agroinfitration, B) 72 h after agroinfiltration, and C) Control line. D) Plasmid as positive control. E) Negative control in PCR.

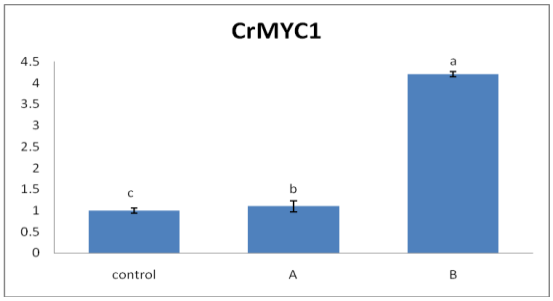

Fig 3. Real-time PCR for analysis *CrMYC1* mRNA level in transiently overexpressed lines in comparison with control: A) 48 h after injection and B: 72 h after injection. Values are presented as the mean and standard deviation of triplicate samples.

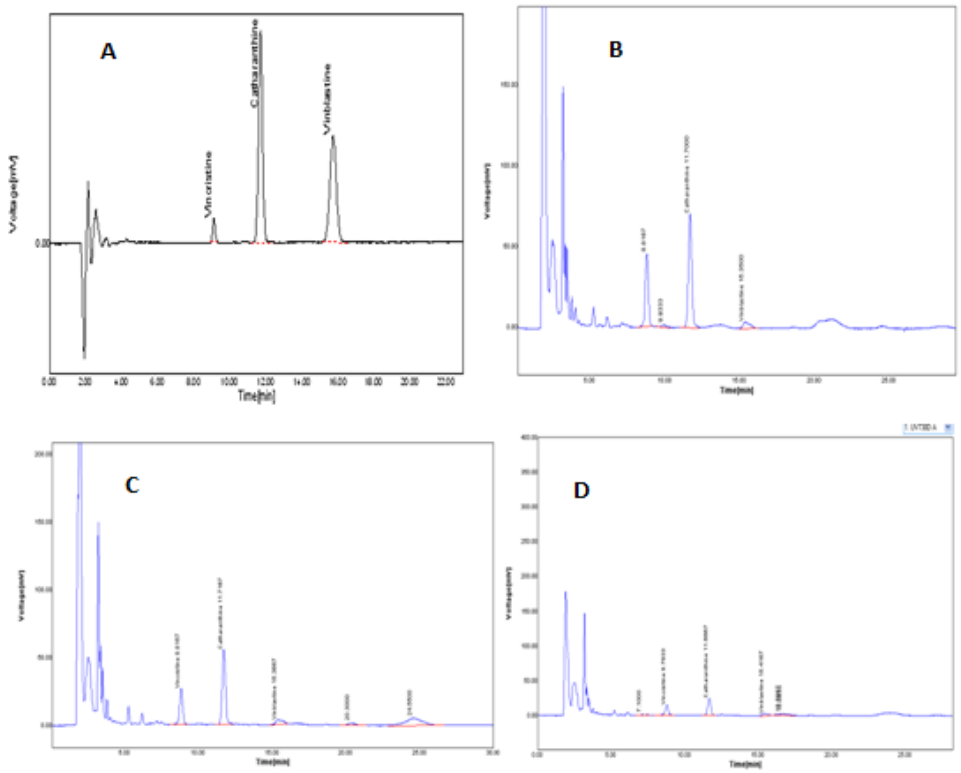

Fig 4. HPLC analyses of TIAs accumulation in transiently overexpressed leaves: A) Standard, B) 48 h after agroinfiltration, C) 72 h after agroinfiltration, and D) Control. The abscissas represent the retention time (min) and the y-axes represent the absorbance.

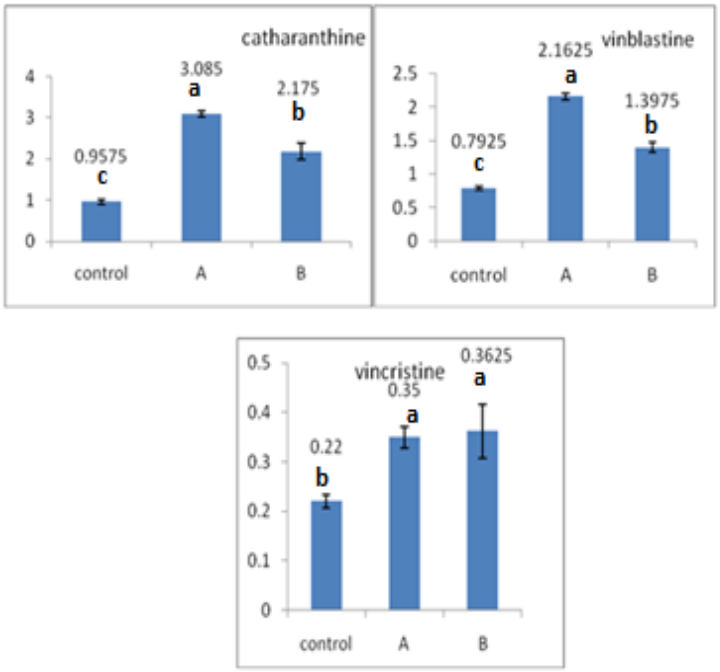

Fig 5. Catharanthine, vinblastine, and vincristine content in transiently overexpressed and control leaves: A) 48 h after agroinfiltration and B) 72 h after agroinfiltration.

Catharanthus roots as the main source (El-Sayed and Verpoorte 2007). However, Hirata *et al*. (1978) reported Ajmalicine in the culture of leaves and the stem tissue of Catharanthus at a low level of 0.02 mg/g of dried weight. The accumulation of all three alkaloid shows an increase in *CrMYC1* overexpressed lines (Table 1). Observing a significant increase in the amount of Vinblastine and catharanthine is probably due to the regulating mechanism of TIA pathway, which is governed by the *CrMYC1* regulator directly or indirectly. It means that *CrMYC1* is a potential transcription factor that induces expressions of some genes in the TIA pathway including *STR* and *PRX1*, which are responsible for catharanthine and vinblastine production. These findings confirm the result of Cathel *et al. (2003) and* Pre et al. (2000) *who* reported STR enzyme as a target for *CrMYC1* transcription factor since they found that this transcription factor was bound to G-box element in the promoter of *STR* protein as bait in the two hybrid yeast system. So, it can be concluded that overexpression in *CrMYC1* transcription factor causes an increase in the expression of some regulatory or enzymatic genes responsible for the catharanthine and vinblastine production. However, further chemical analyses on these enzymes can be also helpful to clarify if these enzymes or their functions increase through overexpression of *CrMYC1*. Nevertheless, determining an increase in the yield or function of the enzymes in the TIA pathway through overexpression of *CrMYC1* cannot confirm that they are the main targets of this transcription factor because *CRMYC1* may indirectly enhance the function of some TIA enzymes by targeting another one or more regulatory genes. Similarly, overexpression of *ORCA2* transcription factor in *Catharanthus roseus* hairy root resulted in a significant increase in catharanthine content but the amount of vinblastine and vincristine was below the detectable level (Li et al., 2013). However, there are some limitations for detailed investigation since the catharanthine production pathway is not recognized genetically and enzymatically. Besides, the nonsignificant increase in vincristine might be stem from the fact that the part of the pathway that contains the conversion of vinblastine to vincristine is not governed by *CrMYC1*. Hence, a slight increase in vincristine is observed just due to the increase in vinblastine as a precursor. According to the two different time points after transient expression, the results indicated that the accumulation of catharanthine and vinblastine was at the highest level after 48 hours while vincristine was at the highest accumulation in 72 hours after transient expression (Fig. 5). Accumulation of catharanthine and vinblastine were 3.08 and 2.16 mg/g of dry weight, respectively, indicating a 3-fold increase in transiently overexpressed lines compared to the control line. Interestingly, a close relationship between the highest mRNA level of *CrMYC1* and highest alkaloid (catharanthine and vinblastine) content was seen within a period of 48 hours after leaves injection. In other words, the 4-fold-change in *CrMYC1* transcript level in 48 hours after transient overexpression leads to the 3-fold-change in vinblastine and catharanthine amount. This coincident result implied the role and distinction of *CrMYC1* in improving the level of most important TIA in *C. roseus*.

Materials and Methods

Plant materials

C. roseus L. G. Don seeds were grown in the greenhouse under a controlled condition with natural daylight and 24/18°C day-night temperature. For gene isolation, one-month plants were used after MeJ treatment with the 0.6 mM concentration. Besides, mature leaves of two-month plants were used for agroinfiltration.

CrMYC1 isolation and cloning

Total RNA was extracted from MeJ exposed leaves by Denazist RNA extraction kit after 24 hours and first strand *cDNA* was synthesized by *cDNA* synthesis kit (Amplisense Company). The reference gene for isolation was already submitted to NCBI database by accession number AF283506. *CrMYC1* CDS was amplified and isolated by reverse transcription PCR (RT-PCR) using specific primers in Table 1. The 940 bp-isolated fragment was sequenced by Seqlab Company (Germany) and compared through using the Basic Local Alignment Search Tool (BLAST) service (http://www.ncbi.nlm.nih.gov/BLAST/) for the purpose of confirmation. The *CrMYC1* isolated gene was cloned into PBI 121 plant binary vector containing the *CaMV-35S* promoter and *NOS* terminator to generate the *CrMYC1* expression construct. *BamH*I and *Sac*I restriction enzymes were used for both *CrMYC1* and PBI vector digestion and subsequently, ligation was done for directional cloning procedure. Agrobacterium tumefaciens strain 15834 was transformed with PBI121-*CrMYC1* recombinant construct through the freeze-thaw method.

Transient overexpression of CrMYC1 in Catharanthus

A single transformed colony of recombinant *Agrobacterium tumefaciens* was cultured in the 20 ml LB media containing 50 mg/l kanamycin and grown up at 28℃ and 150 rpm for 16 h. The culture was centrifuged at 4000 rpm for 15 min. The pellet was resuspended in half of MS concentration and the OD was adjusted to 0.8. The bacterial suspension was used to overexpress *CRMYC1* transiently using the agroinfiltration method. The mature Catharanthus leaves were infected with recombinant bacteria using a 1 ml insulin syringe. The plants were kept in the greenhouse and the leaves were harvested 48 and 72 hours after infection for further analysis.

Molecular analysis of agroinfitrated plants

Genomic DNA was extracted from the control and infected leaves by following the CTAB method. The presence of *npt I* was confirmed by comparing the PCR amplification and specific primers in infected leaves and control plant leaves. Total RNA extraction (Denazist RNA extraction kit) and DNase treatment (Thermoscientific DNAse kit) were carried out for all samples to avoid RNA contamination. First strand *cDNA* was synthesized (Amplisense Company) for the real-time *CrMYC1* gene expression analysis. The relative real-time reaction was carried out using 1XSyber Green buffer by employing specific primer for *CrMYC1* and elongation factors α gene (Table 2). The elongation factor α gene was used as

an internal control for data normalization. The amplification reactions were performed in a lineGeneK thermal cycler (Bioer, China). The data were analyzed based on the threshold cycle (CT) method and the fold expression of *CrMYC1* mRNAs was calculated by the $2^{-\Delta\Delta CT}$ method (Larionov et al., 2005; Livak and Schmittgen 2001).

HPLC analysis of alkaloids

For the purpose of biochemical analysis, the samples were freeze-dried after harvesting the leaf samples and freezing in liquid nitrogen. The dry weight was measured after lyophilization. Next, 0.1gr of grounded tissue was used for alkaloid. Accurately weighed samples were extracted with methanol and sonicated for 10 min. The extracts were kept at room temperature for 12 hours. Afterward, the samples were filtered by a 0.2 µm filter and then injected. The HPLC analysis was performed using a YOUNGLIN Korean device equipped with the UV730D detector, 250 mm x 4.6mm Eclipse plus C18 column. The temperature of the column was maintained at 35℃. The injection volume was 20 µl and carried out manually. The mobile phase consisted of a mixture of methanol as Solvent A, acetonitrile as Solvent B, ammonium acetate with 25 mM concentration, and 0.1% trimethylamine as solvent C (15:45:40). The HPLC analysis was carried out at 1 mL/min flow rate (Ferreres et al., 2010; Siddiqui et al., 2011; Zhang et al., 2014).

The standard markers vincristine, vinblastine, ajmalicine, and catharanthine were purchased from Sigma (Sigma, USA). The samples were analyzed two times with two biological repeats for each (totally 4 times) and the results were presented as the mean ± standard deviation (SD).

Statistical analysis

All data in this research were statistically analyzed using the LSD test and the least difference between treatments was determined with a confidence interval of 95%. For real-time analysis, in addition to doing the experiments in a negative control and five standard samples, they were repeated at least 3 times under identical conditions. For HPLC analysis, the samples were analyzed two times with two biological repeats for each (totally 4 times) and the results were presented as the mean ± standard deviation (SD).

Conclusion

According to the findings of this research, *CrMYC1* is a suitable candidate for TIA enrichment in the metabolic engineering program of *C. roseus*. The overexpression of this gene causes an increase in the yield of the most important terpenoid indole alkaloid including catharanthine, vinblastine, and vincristine. Moreover, based on the results obtained from agroinfiltration method for transient gene expression in Catharanthus, this method is introduced as a reliable and short-term consuming system for expression of TIA genes as a primary step in gene transfer and manipulation of *C. roseus*.

Acknowledgement

The authors are grateful to the Biotechnology Institute of Shiraz University and Central Lab of Agriculture and Natural Resources College (University of Tehran) for technical support of this research. The authors declare no conflict of interest.

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