Exogenous application of rutin and gallic acid regulate antioxidants and alleviate reactive oxygen generation in *Oryza sativa* L.

Akanksha Singh¹ · Rupali Gupta¹ · Rakesh Pandey¹

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Abstract The effect of rutin and gallic acid on growth, phytochemical and defense gene activation of rice (*Oryza sativa* L.) was investigated. The seeds of rice were primed with different concentrations of rutin and gallic acid (10–60 $\mu\text{g mL}^{-1}$) to explicate the effect on germination on water agar plates. Further, to study the effect of most effective concentrations of gallic acid (60 $\mu\text{g mL}^{-1}$) and rutin (50 $\mu\text{g mL}^{-1}$), greenhouse pot experiment was set up to determine the changes in growth, antioxidant and defense parameters. The results revealed more pronounced effect of gallic acid on total chlorophyll and carotenoids as well as on total flavonoid content and free radical scavenging activities. Gene expression analysis of *OsWRKY71*, *PAL*, *CHS* and *LOX* genes involved in strengthening the plant defense further validated the results obtained from the biochemical analysis. Microscopic analysis also confirmed reduction in total reactive oxygen species, free radicals like H_2O_2 and O_2^- by exogenous application of gallic acid and rutin. The data obtained thus suggest that both gallic acid and rutin can affect the growth and physiology of rice plants and therefore can be used to develop effective plant growth promoters and as substitute of biofertilizers for maximizing their use in field conditions.

Keywords Antioxidants · Gallic acid · *Oryza sativa* L. · Reactive oxygen species · Rutin

Introduction

Farmer community around the globe often face the issue of low quality yield and loss of soil fertility. Further, ever increasing growing population is adding up to their menace. Therefore, in order to counter the above challenges and keeping in mind the socio-economic aspects in agriculture; development of innovative, environment-friendly and cost effective agronomy techniques to improve the crop yield is the need of hour. One of the emerging fields in this direction is allelopathic science which is not only environmentally friendly but also offers effective solution for the augmentation in crop yield (Wardle et al. 2011; Makoi and Ndakidemi 2012). Allelochemicals, which includes wide range of secondary compounds, are reported to interact with plants thereby positively influencing their growth and development at both cellular as well as molecular level (Hegab et al. 2008; Niakan et al. 2008). Recently, their potential has been successfully harnessed by few of the workers by using them as substitutes for bio-herbicides and biofertilizers (Machado 2007; Makoi and Ndakidemi 2012; Maqbool et al. 2013).

Among the various allelochemicals, phenolic compounds embody a varied collection of plant secondary metabolites like uncomplicated phenylpropanoids, flavonoids, tannins, coumarins, lignin and its precursors and benzoic acid derivatives (Vermerris and Nicholson 2006) etc. These compounds not only act as efficient free radical scavengers but also inhibit the lipid peroxidation process and membrane stabilizers (Arora et al. 2000; Michalak 2006; Verstraeten et al. 2003). They also have marked

Akanksha Singh and Rupali Gupta have contributed equally to this work.

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✉ Rakesh Pandey
r.pandey@cimap.res.in

¹ Department of Microbial Technology and Nematology,
CSIR- Central Institute of Medicinal and Aromatic Plants,
P.O. CIMAP, Lucknow 226015, India

ameliorative effects towards various abiotic stresses like heavy metals, chilling, osmotic stress and salinity (El-Tayeb et al. 2006; El-Soud et al. 2013; Li et al. 2011; Singh et al. 2013; Ozfidan-Konakci et al. 2015; Chauhan et al. 2017). Among the various class of plant phenolics, coumarin and its derivatives (Razavi 2011; Al-Amiery et al. 2012; Saleh et al. 2015), ferulic acid (Li et al. 2013), ellagic acid (El-Soud et al. 2013) etc. have attracted the interest of researchers for their effect on plant growth and many plant physiological processes. However, still number of plant phenolics with positive effects on various physiological processes linked with seed germination as well as plant growth and development remains unexplored. Amongst them, gallic acid and rutin are two allelochemicals which are extensively dispersed in plant kingdom. They have been mainly reported for their anticarcinogenic, antioxidative, antimutagenic and anti-inflammatory activities (Sharma et al. 2013; Choubey et al. 2015). However, to best of our knowledge the effect of these molecules as bio-priming agents on growth promotion, phytochemical and antioxidant activities remains unexplored. Keeping this in mind two different phenolic acids namely gallic acid and rutin were picked up for assessing their effect on inducing the defensive state and growth parameters of rice (*Oryza sativa* L.) in a dose dependent manner.

Materials and methods

Plant material and in vitro growth conditions

Surface sterilization of *O. sativa* seeds (cultivar BLB Pusa Basmati 1, India) was done for 5 min using sodium hypochlorite (2%) and washed with sterile distilled water several times. The sterilized seeds were divided into three groups; first and second group seeds were soaked in different concentration of rutin and gallic acid (10–60 $\mu\text{g mL}^{-1}$ in water) respectively, for 2 h at 22 °C while the third lot of seeds were immersed in similar set of conditions (unprimed). After treating the seeds, they were left for drying in air cabinet. Primed and unprimed seeds were then plated equidistantly on soft agar plates with moistened blotter discs to record the effect on seed germination.

In-vivo experiments

The best concentration of rutin and gallic acid obtained from the seed germination assay was selected for the greenhouse trial. Surface sterilized *O. sativa* seeds treated with rutin (50 $\mu\text{g mL}^{-1}$) and gallic acid (60 $\mu\text{g mL}^{-1}$) were transferred in pots (7.5 cm diameter) containing sterilized soil [pH 7.4, soil: compost (3:1) sieved through

2.5 mm mesh] under natural light conditions in a greenhouse. The sterilized soil was of sandy loam nature with EC 0.35 dS m^{-1} , 150 kg ha^{-1} available form of nitrogen (alkaline permanganate extractable), 7.5 kg ha^{-1} available P (0.40 M NaHCO_3 extractable), and 76 kg ha^{-1} potassium (neutral N ammonium acetate extractable). The experiment was designed with following three treatments: (a) control (healthy) plants (b) rutin (50 $\mu\text{g mL}^{-1}$) and (c) gallic acid (60 $\mu\text{g mL}^{-1}$) treated seeds. Four weeks after emergence of the seedlings, they were harvested and the data for different growth parameters (shoot and root length) were recorded. Random samples of fresh seedlings were uprooted from every individual group, crushed right away under liquid nitrogen and used for biochemical and gene expression analysis. Seven replications were maintained for each treatment from each set and the data from the replicated pots were then pooled for analysis.

Phytochemical and antioxidant activities after rutin and gallic acid priming

Total chlorophyll (“a” and “b”) and carotenoid contents were measured according to Singh et al. (2016) and Lichtenthaler and Wellburn (1983), respectively. Briefly, 100 mg of powdered leaf sample was extracted with $\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (9:1, v/v) mixture. The reaction mixture was centrifuged at 10,000g for 5 min and the optical density was read at 663, 645, 480 and 510 nm. The total phenolic content (TPC) was calculated using gallic acid (mg per g of dried sample) as standard (Gupta et al. 2017). A sodium carbonate solution (7%; 2.5 mL) and 0.5 mL of Folin Ciocalteu reagent were added to 1 mL methanolic extract of leaf tissue. Further, the reaction was placed in dark for 2 h and the absorbance was recorded at 765 nm. For, total flavonoid content (TFC), methanolic plant tissue (0.5 mL) extract was mixed with sodium nitrite solution (15%; 0.15 mL) and after 6 min the NaOH (4%; 2 mL) solution was added. The volume was made up to 5 mL using sterile water and the absorbance was recorded at 510 nm after 15 min (Gupta et al. 2016). Free radical scavenging activity was quantified by mixing 100 μL of the extracted sample, with 400 μL Tris-HCl (0.1 M, pH 7.4) and 500 μL of methanolic 1, 1-diphenyl-2-picryl-hydrazil solution (0.5 mM). The reaction was kept at room temperature (15 min) and the absorbance was recorded at 517 nm (Brand-Williams et al. 1995). The activity was estimated as: $\text{FRSA (\%)} = \frac{[(\text{control}_{\text{absorbance}} - \text{Treatment}_{\text{absorbance}}) (\text{control}_{\text{absorbance}})^{-1}]}{\times 100}$.

The total antioxidant capacity (TAC) was measured by adding 1 mL of reagent solution [sulphuric acid (3.3 mL), sodium phosphate (335 mg) and ammonium molybdate (78.416 mg) in 100 mL] with 100 μL of methanol extracted leaf tissue (Gillespie et al. 2007). The mixture

was kept at 100 °C for 1 h and the activity was estimated as: $TAC (\%) = [(Treatment_{absorbance} - control_{absorbance}) / (control_{absorbance})^{-1}] \times 100$.

Histochemical analysis of H_2O_2 and O_2^- , reactive oxygen species and callose deposition

Localization of H_2O_2 was conducted using the 3, 3-diaminobenzidine (DAB; Sigma) staining (Fryer et al. 2002). The DAB solution was freshly prepared in sterile water (pH 3.8 with KOH) to avoid auto-oxidation. Segments were kept in 1 mg mL⁻¹ DAB solution for 10 min and kept in the dark for 4 h at 30 °C in the same solution under very slow shaking. After staining for the above mentioned time, stained leaf segments were then bleached in mixture of acetic acid-glycerol-ethanol (1:1:3) (v/v/v) at boiling temperature for 5 min, and then incubated in glycerol-ethanol (1:4) (v/v) solution until final photographs were taken. H_2O_2 production was localized as a brown color owing to DAB polymerization. Three biological independent repeats were conducted for each experiment. Stained segments were observed under light microscope (Leica, Germany) to obtain images.

For detection of O_2^- , nitroblue tetrazolium (NBT; Sigma) was used. Leaves segments were kept in 0.1% NBT (w/v) prepared in NaN₃ (10 mM) in potassium phosphate buffer (10 mM; pH 7.8) for 20 min (Rao and Davis 1999). Thereafter, stained segments were bleached to remove chlorophyll in acetic acid-glycerol-ethanol (1:1:3) (v/v/v) solution at boiling temperature for 5–8 min. Further, leaf segments were then incubated in a glycerol-ethanol (1:4) (v/v) solution until photographs were taken.

The reactive oxygen species (ROS) generated in leaves was visualized using the fluorescent probe 5-(and 6)-carboxy-2',7'-dichloro dihydrofluorescein diacetate (DCF-DA) (Sigma). The primed and unprimed leaves were kept in for 3 min in DCF-DA (10 μM) in 2-morpholinoethanesulfonic acid; 10 mM; pH 6.1). Leaves were observed in epifluorescence microscope (excitation 480/40 nm, emission 527/30 nm) using a GFP filter set (Beneloujaephajri et al. 2013). Callose deposition was also analysed using aniline blue (0.01%). For callose visualization, leaf segments were further bleached as aforementioned and examined for the presence of cell wall deposits using a UV epifluorescence microscope (Ellinger et al. 2013).

Transcription analysis by real-time RT-PCR

Total RNA was isolated by trizol (Invitrogen) method as per the manual protocol. RNA was reverse-transcribed with random primers using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). *Actin* gene was taken as the internal control. All the primers involved in the experiment

were reported by Liu et al. (2007) and Hao et al. (2011) (Table S1). Real time RT-PCR was done as described by Hao et al. (2011). The PCR reactions were performed in triplicate. To analyze the real-time PCR data the $2^{-\Delta\Delta CT}$ method was used as previously reported by Livak and Schmittgen (2001).

Data analysis

The result was expressed as the mean of triplicates. Significant means were analyzed using Tukey's multiple comparison test at $P < 0.05$ by SPSS package (SPSS V16.0, SPSS Inc., Chicago, IL) and principal component analysis (PCA) was applied to select appropriate components in current investigation.

Results and discussion

The seed germination assay was set up to measure shoot and root length of rice primed with different concentration of rutin and gallic acid. Rutin and gallic acid showed the most significant results at 50 and 60 μg mL⁻¹ concentrations respectively (Fig. S1). Rutin at the concentration of 50 μg mL⁻¹ significantly ($P \leq 0.05$) stimulated plumule and radical length which showed an increase of 2.98 and 2.91 fold compared to the control seedlings, respectively (Table S2). However, gallic acid at a concentration of 60 μg mL⁻¹ significantly ($P \leq 0.05$) enhanced plumule (1.69 fold) and radical (2.65 fold) lengths as compared to the control seedlings, respectively (Table S2).

In the previous reports, a stimulatory or inhibitory effect of phenolic compounds on germination and early seedling growth have been observed depending on the compound taken, its concentration, and the plant selected (Reigosa et al. 1999). The results obtained here demonstrated that the effect of rutin and gallic acid is concentration-dependent which was more prominent on growth rather than germination of rice seedlings (Mata et al. 1998). Differential responses were obtained by Saleh et al. (2015) who showed the effect of these compounds on germination of faba bean seeds while Mata et al. (1998) observed the effect on growth parameters.

As illustrated in Fig. 2a, the rice seeds inoculated with rutin (50 μg mL⁻¹) and gallic acid (60 μg mL⁻¹) treatments, resulted in increase ($P \leq 0.05$) in plant growth than the control plants in greenhouse experiment. The growth parameters (shoot and root length) were significantly augmented when seeds were treated with rutin and gallic acid (Table 1). The highest increment in shoot and root length was found to be 1.28 and 2.05 fold, respectively higher in 60 μg mL⁻¹ gallic acid treatment followed by 50 μg mL⁻¹ rutin treatment (1.14 and 1.38 fold respectively) over the

Table 1 Effect of rutin (Ru) and gallic acid (Ga) on growth parameters of rice in greenhouse experiment

Treatments	Shoot length (cm)	Root length (cm)
Control	18.00 ± 0.58 ^b	1.95 ± 0.03 ^c
Rutin (50 µg/mL)	20.50 ± 0.87 ^{ab}	2.70 ± 0.17 ^b
Gallic acid (60 µg/mL)	23.00 ± 1.15 ^a	4.00 ± 0.11 ^a

Results are means of three replicates ± SD and means within a column followed by the same letter are not significantly different ($P \leq 0.05$)

control plants as showed in Table 1. The biochemical parameters data additionally supported the growth parameters results as pronounced increase was observed in rutin and gallic acid primed seedlings than the control plants. Total content of chlorophyll in leaves was increased ($P < 0.05$) in treatments primed with rutin and gallic acid (Fig. 1Ba). The augmentation in total chlorophyll and carotenoids by 2.20 and 2.26 fold, respectively was found in gallic acid treatment proceeded by rutin (1.26 and 1.53 fold, respectively) as compared to control (Fig. 1Ba, Bb). The accumulation of chlorophyll in plants is well known to be linked with the incorporation of pigment into highly organized lipoprotein arrangement in the chloroplast. In a study conducted by Singh and Chaturvedi (2014), ameliorative effect of cinnamic acid (CA) was observed in maize plants as contents of chlorophyll a, b and carotenoids were significantly higher in the presence of 0.05 mM CA under salinity stress in comparison to only salinity stressed plants. In the present investigation too it can be speculated that the increase in total chlorophyll and carotenoid contents in pretreated gallic and rutin rice seedlings probably might have resulted in higher photosynthetic rates due to higher photosynthetic pigments thereby improving the overall plant growth.

However, higher TPC was recorded in seedlings primed with rutin (13.52 mg gallic g⁻¹) followed by gallic acid (12.71 mg gallic g⁻¹) treatment, which were about 1.40 and 1.31 folds respectively higher than the control seedlings (Fig. 1Bc). The maximum TFC content was found in gallic acid (14.22 mg rutin g⁻¹) followed by rutin (11.10 mg rutin g⁻¹), which were 1.51 and 1.18 folds, respectively in elevated amount than the control plants (Fig. 1Bd). Priming by exogenous application of compounds or microbes is well known phenomenon for making the plants respond faster and stronger to abiotic and biotic challenge. Among the various pathways triggered, induction of bioactive secondary metabolites like flavonoids and phenolic acids play a noteworthy role in plant defense. Recently, El-Soud et al. (2013) established that pre-treatment of chickpea seedlings with ellagic acid resulted in higher phenylalanine ammonia lyase activity, a key enzyme acting as a connector between primary and

secondary metabolism under osmotic stress as compared to the untreated seedlings. Likewise, Saleh et al. (2015) reported induction of salicylic, syringic, gallic and ferulic acids in coumarin primed wheat seedlings. The enhanced TPC and TFC in the present investigation thus, provide an evidence of direct or indirect role played by rutin and gallic acid in regulation of shikimic acid biosynthetic pathway.

Further, the gallic acid treated seedlings ($P < 0.05$) increased FRSA (1.08 fold) followed by treatment having rutin as compared to the control plants (Fig. 2a). However, insignificant value of TAC was found in rutin treatment, which was 1.13 fold higher than the control (Fig. 2b). The obtained results can be very well corroborated with the increased accumulation of total phenolic content as they are well known antioxidants and improve the nonenzymatic antioxidant capacity of plants by their FRSA (Karamac et al. 2005; Michalak 2006).

Reduction in hydrogen peroxide content was observed in leaf tissues of treatments having rutin and gallic acid (Fig. 3A). This visible reduction was observed by DAB staining (Fig. 3A). The gallic acid (60 µg mL⁻¹) treatment showed least brown stain deposit in comparison with the control and rutin (50 µg mL⁻¹) treated plants (Fig. 3A). In the control plants, superoxide content was highest on the leaf surface (Fig. 3B1). Visual observations of superoxide radical in leaves can be correlated with the H₂O₂ results as strong blue coloration was found in the leaves of control treatment plants. However, in comparison to the control plants, the plants treated with rutin and gallic acid showed less blue pigmentation mostly in veins and sparsely in the interveinal regions (Fig. 3B2, B3). Likewise, histochemical analysis of ROS in rice leaves using DCF-DA also revealed maximal expanse of ROS localization in the midrib region of healthy plants than the treated ones (Fig. 3C1). In contrast to the control, lesser ROS was found in gallic acid treatment followed by rutin (Fig. 3C2, C3). ROS such as H₂O₂ and O₂⁻ are usually produced in plants as toxic by products of aerobic metabolism whose concentration increases when plants are exposed to a variety of both biotic and abiotic stresses (Bailey-Serres and Mittler 2006). Similarly, Chauhan et al. (2017) reported increased production of H₂O₂ and O₂⁻ in both root and shoot during arsenic stress. The excess production and accumulation of ROS is linked with lipid peroxidation (Foyer et al. 1994) which can damage ultra structure, disturb the normal plant metabolism, and can even lead to cell death. Application of phenolic acids for alleviation of different kinds of stress is being taken up by the research community globally as in some of the recent investigations priming of cucumber seedlings with phenolic acids like ferulic and cinnamic acid attenuated dehydration-induced oxidative stress via expression of antioxidant enzymes, lessening of H₂O₂, MDA contents and by increasing total antioxidant capacity

Fig. 1 Comparison of growth and biochemical parameters between different treatments (a) effect of different treatments on plant growth promoting traits of rice and (b) (a) total chlorophyll (mg g⁻¹ fw), (b) carotenoid content (mg g⁻¹ fw), (c) total phenolic (mg g⁻¹ dw) and (d) total flavonoid content (mg g⁻¹ dw) of rice after treatment

with rutin, gallic acid and control. Bars indicate the standard errors of the means from three replicates. Columns with different letters are statistically different according to the Tukey's test ($P < 0.05$) among treatment

(Sun et al. 2012; Li et al. 2013). Likewise, application of ferulic acid, ellagic acid and proline mitigated various stresses by reducing ROS levels in plants (Ozden et al. 2009; El-Soud et al. 2013). The reduced levels of ROS and free radicals like H₂O₂ and O₂⁻ could be possibly by the triggering of the antioxidant enzymes, total phenolics and flavonoids directly due to exogenic treatment of gallic acid and rutin.

Plants have been bestowed with pre-invasive structural defenses and local modifications of cell walls that protect them from the attacking pest and pathogens (Van Kan 2006). However, regardless of the innate defense plant must rapidly regulate gene expression to adapt its physiology to changing environmental conditions. Interestingly, callose, a structural barrier localized by an intense blue-

green fluorescence after aniline blue staining, was also found to be majorly distributed in the interveinal regions cells of rice leaves (Fig. 3D). However, in comparison to the control plants, the plants treated with gallic acid showed high callose deposition mostly in veins and sparsely in the interveinal regions (Fig. 3D2, D3), indicating the role of gallic acid in strengthening the defense response in plants.

RT-qPCR was used to determine the expression of defense gene in rice plants primed with rutin, gallic acid and control (Fig. 4). Results indicated that rice plants primed with rutin and gallic acid treatments showed the highest level of *OsWRKY71*, *PAL* and *CHS* transcription compared to the control plants (Fig. 4). *OsWRKY71* expression was up-regulated (4.8 and 3.15 fold) in rutin

Fig. 2 Effect of rutin (Ru) and gallic acid (Ga) inoculation on **a** free radical scavenging activity and **b** total antioxidant capacity of rice. Bars indicate the standard errors of the means from three replicates. Columns with different letters are statistically different according to the Tukey's test ($P < 0.05$) among treatment

and gallic acid treatments, respectively with respect to control. Gallic acid treatment showed the highest level of *PAL* (5.33 fold) and *CHS* (4.19 fold) transcription compared to the control plants, suggesting its role in promoting the defense response by activation of these two genes (Fig. 4). However, there was not much significant difference in the *LOX* transcript levels treated with rutin and gallic acid as compared to control. Among the different defense response factors generated in plants, WRKY factors function in synchronizing a wide array of stress responses under biotic and abiotic stresses in several plants (Kim et al. 2000; Asai et al. 2002). OsWRKY71 has been reported to be induced by defense signalling molecules like methyl jasmonate (MeJA), SA, and 1-aminocyclo-propane-1-carboxylic acid (ACC). Thus, its induction in rutin and gallic acid treatment thus suggests the plausible role played by OsWRKY71 in strengthening the defense response.

Likewise, *PAL* and *CHS* are the key enzymes of phenylpropanoid pathway producing phenolic compounds and flavonoid/isoflavonoid biosynthesis pathway respectively (Dixon and Paiva 1995; Dao et al. 2011). In the present investigation, higher transcription of *PAL* and *CHS* genes in gallic acid and rutin pretreated seedlings are in correlation with higher induction of total phenolic and flavonoid content. Present findings are well supported by observations recorded by El-Soud et al. (2013) who observed that ellagic acid primed seedlings showed maximum *CHS* activity and flavonoid content under 0.4 MPa. Similarly, when salicylic acid was applied to common bean plantlets increase in *CHS* and *PAL* activities was observed thereby enhancing the defensive state of bean plants (Campos et al. 2003). Further, decrease in *LOX* transcript levels in comparison to the control plants can be related with the higher accumulation of phenolic compounds which are reported to inhibit lipid

Fig. 3 Effect of rutin (Ru) and gallic acid (Ga) inoculation on **a** hydrogen peroxide detected by DAB staining ($\times 10$, scale bar 100 μm), **b** superoxide radical detected by NBT staining ($\times 10$, scale bar 100 μm), **c** ROS production ($\times 10$, scale bar 100 μm) and **d** callose deposition ($\times 20$, scale bar 200 μm) in leaves of rice. Black arrows denote H_2O_2 production, superoxide generation and callose deposition in different treatments

Fig. 4 The expression of target genes in the rice plants inoculated with rutin, gallic acid and control analyzed by quantitative RT-PCR. Transcript levels of target genes were calculated relatively to the *actin* gene. Error bars represent the standard error of the mean from three independent experiments

peroxidation and help in maintaining the membrane integrity by trapping the lipid alkoxyl radical (Milic et al. 1998; Verstraeten et al. 2003).

Moreover, the data was validated by PCA as grouping of treatments revealed formation of three different clusters:

first having only control, second having rutin and third having gallic acid treatment. A separate group formed by rutin and gallic acid treatments where all plant growth parameters were significantly ($P < 0.05$) enhanced. The PC1 contributed 78.60%, and PC2 contributed 21.40% of the total variance (Fig. S2).

Conclusions

The result of this study suggest that rutin and gallic acid not only affected the growth of rice seedlings but also ameliorated the plants defensive state via enhancement in TPC, TFC, callose and reduction in total ROS. Since, modern agriculture is constantly challenged with the need to lessen the health risks and environmental damage caused by synthetic chemicals and growth promoters, the present study highlights the prospective use of natural molecules in survival and triumphant establishment in competitive environments. Also the findings obtained may be translated into endeavours aimed to develop effective plant growth

promoters and substitute of biofertilizers, thus maximizing their use in field conditions.

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Compliance with ethical standards

Conflict of interest There exists no potential conflict of interest among the authors.

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