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Original Article

Differential expression of flavone synthase in *Bidens pilosa* tissues and its relevance to antioxidant-based CBRNE mitigation strategies

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Abstract—Flavone synthase (FNS) is a key enzyme involved in the conversion of flavanones into flavones, a class of secondary metabolites that contribute to plant defense through their antimicrobial and antioxidant properties. These bioactive compounds play an important role in mitigating oxidative stress induced by environmental factors, including ultraviolet (UV) radiation and other stressors relevant to Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNE) scenarios. *Bidens pilosa* L. is a herbaceous plant recognized for its rich flavonoid content, particularly in its leaves and stems, making it a promising source of bioactive phytochemicals. This study aimed to evaluate tissue-specific *FNS* gene expression in *B. pilosa* to assess its flavonoid biosynthetic potential and its prospective relevance to CBRNE protective phytotechnology. Total RNA was isolated from fresh leaf and stem tissues using the phenol–chloroform method, followed by cDNA synthesis through reverse transcription. Gene expression analysis was conducted using real-time quantitative PCR (RT-qPCR), with *FNS* as the target gene and actin as the reference gene. The results revealed differential *FNS* expression between leaf and stem tissues, indicating variations in flavonoid biosynthesis and potential flavonoid accumulation. These findings provide a molecular basis for evaluating flavonoid-producing capacity in *B. pilosa* and support the exploration of plant-derived antioxidant resources for future applications in protective phytotechnology and resilience against oxidative stress associated with CBRNE-related environmental challenges.

Keywords— *Bidens pilosa* L., *Biosynthesis, Flavone synthase, Gene expression, Real-time PCR*

1. INTRODUCTION

Bidens pilosa L. is a plant belonging to the Asteraceae family that has been widely utilized as a traditional medicinal herb, particularly in Asia, Africa, and Latin America, due to its rich content of secondary metabolites, especially flavonoids. This plant is known to exhibit various pharmacological activities, including anti-inflammatory, antibacterial, antioxidant, and anticancer properties. Recent studies have shown that *Bidens pilosa* contains a wide range of bioactive compounds, including flavonoids, polyacetylenes, phenolic acids, terpenoids, alkaloids, and lipids, which contribute to its antioxidant, anti-inflammatory, and immunomodulatory activities [1].

Flavonoids are a group of plant secondary metabolites capable of scavenging free radicals and inhibiting oxidative stress in plants. In *Bidens pilosa*, flavonoids function as natural antioxidant compounds by donating electrons to stabilize reactive oxygen species *(ROS).* Antioxidant activity plays an essential role in preventing cellular damage, DNA mutations, lipid peroxidation, and chronic inflammatory processes associated with cancer development [2]. The flavonoids produced are subsequently accumulated in plant tissues and extracted into BPA (*Bidens pilosa extract*), which can be utilized as an herbal remedy for cancer treatment. Flavonoids present in BPA are capable of suppressing the formation of pro-tumor immune cells, such as M2 tumor-associated macrophages (M2-TAMs) and regulatory T cells (Tregs), while simultaneously enhancing antitumor immune cell activity [3].

In addition, flavonoids also function as antifungal agents, as these compounds tend to bind to proteins and thereby interfere with fungal metabolic processes [4]. Inhibition of fungal cell wall synthesis is caused by phenolic flavonoid compounds, which damage fungal cell membranes, neutralize and inhibit fungal enzymes, disrupt hyphal tip formation, and inhibit fungal nucleic acid and protein synthesis [5].

Flavonoid biosynthesis in *Bidens pilosa* involves the phenylpropanoid biosynthetic pathway through the activity of the *Flavone Synthase* (FNS) gene. This gene encodes the flavone synthase enzyme, which functions to convert flavanones into flavones, one of the major

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classes of flavonoids. Molecular biology–based approaches, such as RNA isolation, cDNA synthesis, and gene expression analysis using real-time PCR (RT-qPCR), are highly effective methods for evaluating FNS gene expression activity in *Bidens pilosa*. The use of the *FNS gene* as the target gene and the actin gene as the internal control enables accurate expression quantification and can be used to estimate flavonoid biosynthetic potential in specific tissues. The *FNS gene* serves as the target gene whose expression level is measured. *FNS gene* expression is influenced by high light intensity and UV radiation exposure, both of which can enhance flavonoid accumulation as a protective mechanism against oxidative stress in plants [6]. By understanding the expression pattern of *FNS* in *Bidens pilosa*, this study is expected to provide a strong molecular basis for improving the utilization of this plant as a natural flavonoid source and to support strategies for medicinal plant development and metabolic engineering.

2. EXPERIMENTAL SECTION

2.1. Materials

Leaf and stem tissues of *Bidens pilosa* L. were obtained from the environment of Universitas Pertahanan RI, located in Sentul, Bogor. The materials used in this study included liquid nitrogen, chloroform, 75% ethanol, 90% ethanol, ddH_2O, gloves, masks, labels, TRIzol Reagent, chloroform, 5× Reaction Buffer, RiboLock RNase Inhibitor, 10 mM dNTP Mix, RevertAid M-MuLV Reverse Transcriptase, oligo(dT), SensiFAST SYBR Green Master Mix, forward and reverse primers for the actin gene, and forward and reverse primers for the *Flavonoid Synthase (FNS) gene*.

2.2. Intrumentation

The equipment used in this study included a cool box, a −80 °C freezer, biological sample storage containers (sterile tubes), sterile knives, permanent markers, a vortex mixer, a Real-Time PCR thermal cycler, mortar and pestle, 1.5 mL microtubes, micropipettes with sterile tips, a centrifuge, a thermal cycler, a Real-Time PCR machine, a NanoDrop spectrophotometer, tube racks, a Thermo Scientific freezer at −40 °C, and other standard laboratory glassware.

2.3. RNA Isolation

RNA isolation was performed using a TRIzol Reagent–based RNA isolation method. According to a study conducted by Simbolon (2023), the TRIzol Reagent method is more effective for RNA extraction than the RNAqueous Phenol-free Total RNA Isolation method. The plant tissues used for RNA isolation were fresh leaf and stem tissues of *Bidens pilosa* L.. Leaf and stem samples were cut into small pieces and ground using a mortar with the aid of liquid nitrogen until a fine powder was obtained. The homogenized samples were transferred into labeled 1.5 mL tubes according to sample type. A total of 200 µL of TRIzol was added to each sample, followed by vortexing and incubation for 2 minutes. Subsequently, 200 µL of chloroform was added, and the samples were gently inverted for 1 minute, centrifuged at 12,000 rpm for 2 minutes, and incubated again for 2 minutes. The supernatant was transferred into a new tube, followed by the addition of 200 µL of 100% isopropanol. The samples were incubated for 10 minutes before centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the resulting pellet was washed with 200 µL of 75% ethanol, vortexed, and centrifuged at 7,500 rpm for 5 minutes at 4°C. After removal of the supernatant, the pellet was dried in an oven at 80°C for 30 seconds, dissolved in 20 µL of ddH_2O, and homogenized by gentle tapping. The samples were subsequently analyzed using a NanoDrop spectrophotometer and stored overnight at −40°C in a Thermo Scientific freezer.

2.4. cDNA Synthesis

cDNA synthesis was performed using total RNA isolated from *Bidens pilosa* L. tissues. Total RNA was extracted using TRIzol reagent, and RNA concentration and purity were measured using a NanoDrop spectrophotometer based on the A260/A280 absorbance ratio. RNA samples with a purity ratio ranging from 1.8 to 2.0 were subsequently used for cDNA synthesis. The RNA samples were treated with DNase I to eliminate genomic DNA contamination. A total of 1 µg of total RNA was mixed with oligo(dT) primers or random hexamers and nuclease-free water, followed by incubation at 65°C for 5 minutes to denature RNA secondary structures and immediately cooled on ice [7].

cDNA synthesis was carried out using a reverse transcriptase kit containing reverse transcriptase enzyme, dNTP mix, reaction buffer, RNase inhibitor, and oligo(dT) primers. The reaction mixture was incubated at 25°C for 5 minutes, followed by incubation at 42°C for 30 minutes to allow reverse transcription of RNA into cDNA. The reaction was terminated by incubation at 85°C for 5 minutes to inactivate the reverse transcriptase enzyme. The resulting cDNA was subsequently diluted using nuclease-free water and stored at −20°C before being used as a template for gene expression analysis using quantitative Real-Time PCR (qRT-PCR), including analysis of *FNS (Flavone Synthase)* gene expression, which plays a role in flavonoid biosynthesis [8].

2.5. Preparation of PCR mIX

PCR mix preparation was carried out under sterile conditions using nuclease-free tubes placed on ice to prevent degradation of the reaction components. The PCR mixture, with a total volume of 20 µL, consisted of 10 µL of 2× PCR Master Mix, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 2 µL of cDNA template, and 6 µL of nuclease-free water. The Master Mix generally contained DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer required for the DNA amplification process.

For gene expression analysis using quantitative *Real-Time PCR (qRT-PCR*), the reaction mixture consisted of 10 µL of 2× SYBR Green Master Mix, 0.4 µL of forward primer, 0.4 µL of reverse primer, 2 µL of cDNA template, and nuclease-free water to achieve a final volume of 20 µL. All components were thoroughly mixed using a

micropipette and briefly centrifuged before loading into the thermal cycler. The PCR amplification program generally consisted of a pre-denaturation step at 94–95°C for 3–5 minutes, followed by 35–40 cycles of denaturation at 94–95°C for 15–30 seconds, annealing at 55–60°C for 30 seconds, and extension at 72°C for 30–60 seconds. A final extension step was performed at 72°C for 5–10 minutes [9].

2.6. Gene Expression Analysis Using RT-qPCR

The template used in this study was cDNA synthesized from the total RNA of *Bidens pilosa* L. This analysis was conducted to determine the expression level of the target gene *Flavone Synthase (FNS),* with the actin gene serving as the internal reference gene. The qPCR reaction mixture was prepared in a total volume of 20 µL, consisting of 10 µL of 2× SYBR Green Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of cDNA template, and 6 µL of nuclease-free water. All components were mixed in a sterile PCR tube and briefly spin-downed to ensure that the mixture collected at the bottom of the tube and was homogenously mixed.

The PCR tubes were subsequently placed into a Real-Time PCR instrument and amplified under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles consisting of denaturation at 95°C for 5 seconds and annealing at 60°C for 30 seconds. Gene expression analysis was performed based on the threshold cycle (Ct) values obtained from each sample. The obtained Ct values were used to calculate the relative expression level of the target gene using the comparative Ct (2^-ΔΔCt) method. The Ct value of the target gene was normalized against the actin reference gene to minimize variations caused by differences in cDNA template quantity among samples. The analysis results were expressed as relative expression levels compared with the control [10].

3. RESULT AND DISCUSSION

Bidens pilosa was utilized for the analysis *of Flavone Synthase (FNS)* gene expression by RT-qPCR. The stem and leaf tissues were selected as the experimental samples for gene expression analysis (**Figure 1**).

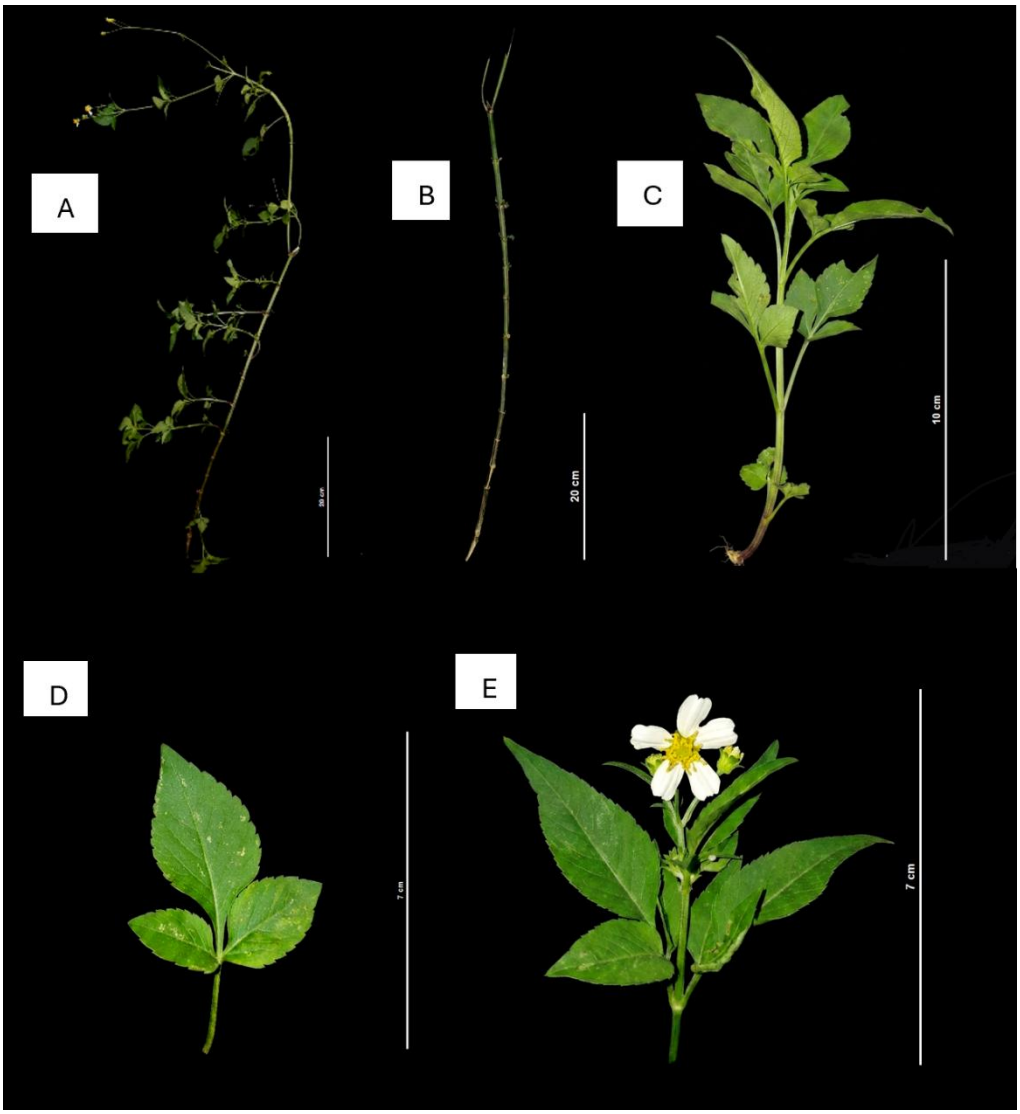

Figure 1. Morphology of *Bidens pilosa*. (A) Whole *Bidens pilosa* plant, scale bar = 20 cm , (B) Stem, scale bar = 20 cm, (C) Leaf with petiole, scale bar = 10 cm, (D) Leaf, scale bar = 7 cm, (E) Flower, scale bar = 7 cm.

Based on the quantitative qPCR analysis, the relative expression of the *FNS* gene varied between the two plant organs analyzed. Relative gene expression levels were determined using the 2^−ΔΔCt method, in which leaf tissue was used as the calibrator and assigned a baseline expression value of 1.00 (**Figure 2**).

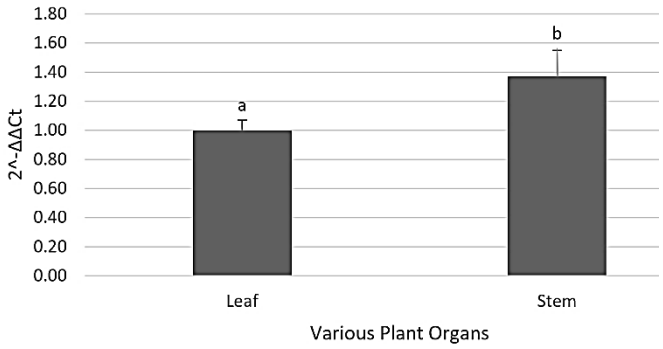

Figure 2. Detailed Analysis of qPCR Ct Values

The relative expression level of the *FNS* gene in the stem tissue of *Bidens pilosa* showed a 1.37-fold increase compared to that in the leaf tissue (**Table 1**). Based on the post hoc statistical analysis, the different letter notations assigned to the leaf tissue (a) and stem tissue (b) indicate a statistically significant difference ($p < 0.05$) in the expression levels of the *FNS* gene between the two organs [13].

Table 1. Duncan's Multiple Range Test (DMRT) Analysis

Sample	FNS Gene (*Bidens pilosa.)*
Leaf	1.00 ± 0.18 a
Stem	1.37 ± 0.07 b

Table 2. Sample Concentration A260/A280

Sample (*Bidens pilosa)*	Pre tillering			
	Concentration (ng/µL)	A260	A280	A260/A280
L1	2765	68.12	63.82	1.5
L2	983.2	24.58	24.88	0.98
S1	1175	29.38	28.46	1.03
S2	551.2	13.04	13.78	1.05

L: Leaf, S: Stem

The qPCR Ct value analysis shown in **Figure 3** indicated that the actin gene, used as the reference gene, exhibited stable Ct values in both tissues, namely 40.00 ± 0.00 in leaves and 27.66 ± 0.00 in stems. In qRT-PCR analysis, the Ct (Cycle threshold) value represents the PCR cycle number at which the fluorescence signal exceeds the detection threshold. Lower Ct values indicate a higher amount of initial cDNA template. Stable actin Ct values suggest that actin expression remained relatively constant among samples; therefore, the observed variation in the target gene expression was attributed to biological differences in the target gene rather than variations in RNA quantity, cDNA quality, or technical errors. This condition indicates that actin is a suitable reference gene for normalizing FNS gene expression data [13].

In contrast, the FNS gene showed a Ct value of 39.55 ± 0.00 in leaves and 34.01 ± 10.38 in stems. These results indicate that the amount of cDNA template in stem tissues was higher than that in leaves. The quantity of cDNA template influences gene expression levels, where a greater amount of cDNA corresponds to higher gene expression. Biologically, *Bidens pilosa* demonstrated higher flavonoid biosynthetic activity in stem tissues than in leaves.

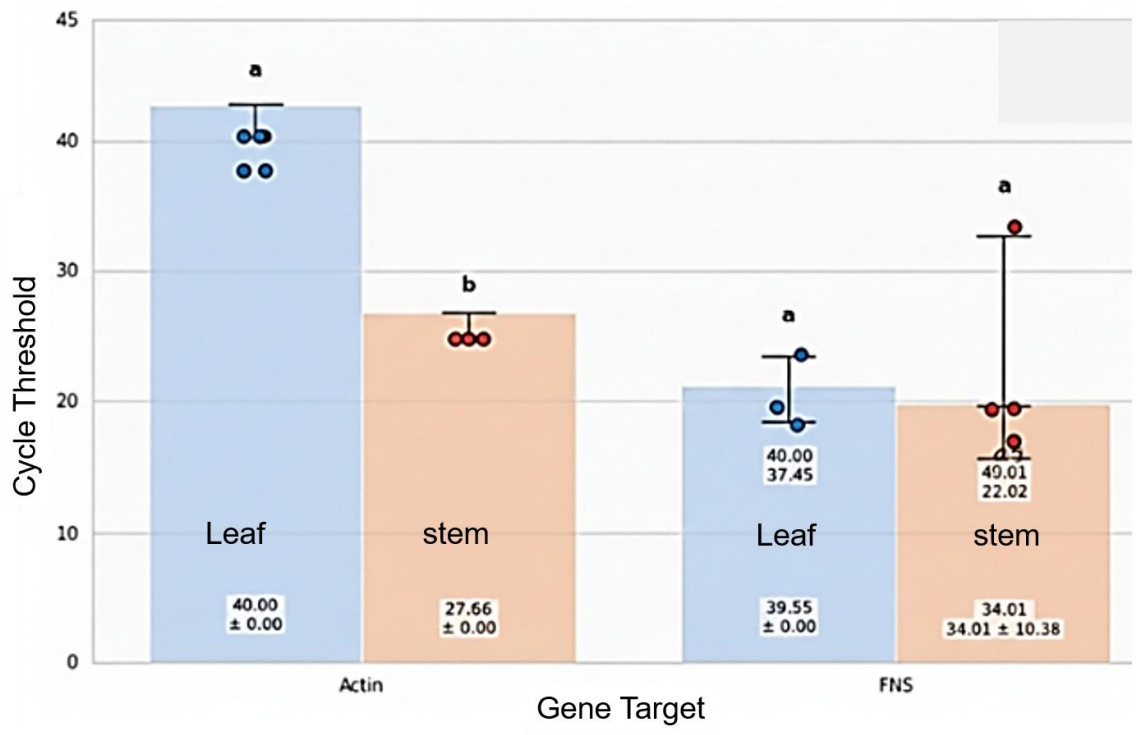

Figure 3. Ct value analysis of the actin and FNS genes in bidens pilosa using qPCR

Physiologically, the higher expression of the *FNS* gene in the stem may be attributed to the more active phenylpropanoid metabolism occurring in stem tissues. The phenylpropanoid pathway produces various defense-related compounds, including lignin and flavonoids, which play essential roles in maintaining stem integrity and structural stability [11-12]. According to Cao (2024), another contributing factor is the differential transcriptional regulation between stem and leaf tissues, where transcription factors such as MYB, bHLH, and WRKY exhibit greater activity in the stem, thereby enhancing *FNS* gene expression compared with that in the leaves [13]. The *FNS* gene encodes a key enzyme that catalyzes the biosynthesis of flavones, an important class of secondary metabolites involved in both plant defense mechanisms and anatomical development [14].

The presence of flavonoids in *Bidens pilosa* has been demonstrated to provide effective protection against pest attacks. In a study conducted by Annisa (2022), flavonoids extracted from *Bidens pilosa* significantly increased the mortality of armyworms and thrips while reducing the intensity of pest infestations in crops. Their mode of action involves disrupting the paraffin layer of the insect cuticle, ultimately leading to pest mortality [15]. Furthermore, flavonoids produced by *Bidens pilosa* contribute to agricultural security by serving as environmentally friendly botanical pesticides, thereby reducing dependence on synthetic pesticides that may adversely affect ecosystems. Through effective pest control, these flavonoids help protect crop yields from damage and crop failure, consequently enhancing agricultural productivity and food security.

4. CONCLUSION

The RT-qPCR analysis demonstrated differential expression of the Flavone Synthase (FNS) gene between Bidens pilosa tissues, with stem tissues exhibiting higher expression levels than leaves. This result suggests that flavonoid biosynthesis and phenylpropanoid metabolism are more active in stems, likely reflecting their role in maintaining structural integrity and supporting plant defense functions. As a key enzyme in flavonoid biosynthesis, FNS contributes to the production of secondary metabolites with antifungal, antibacterial, and antioxidant properties that enhance the plant's natural defense mechanisms. The elevated expression of FNS in stems highlights the potential of Bidens pilosa as a valuable source of bioactive flavonoids for agricultural applications, including the development of natural pesticides to improve crop protection and productivity. Furthermore, flavonoids obtained from Bidens pilosa extracts (BPE) have demonstrated inhibitory effects on tumor cell growth, indicating their potential as herbal therapeutic agents. In the context of CBRNE mitigation strategies, the antioxidant properties associated with flavonoid biosynthesis provide a scientific basis for exploring Bidens pilosa-derived compounds as natural resources that may contribute to protection against oxidative stress induced by chemical and radiological exposure, thereby supporting the development of antioxidant-based CBRNE countermeasures.

5. AUTHOR'S DECLARATION

5.1. Supporting Information

This manuscript does not include supplementary materials. All methods, analytical procedures, and results necessary for understanding and evaluating the study are contained within the main article. Access to the qPCR data supporting the findings may be provided by the authors upon reasonable request.

5.2. Acknowledgements

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5.3. Conflict of Interest

State that the authors have no conflict of interest.

5.4. Author Contributions

ABC conducted the experiment, XY conducted the DFT calculations, ABC and XY wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

5.5. AI Statement

Artificial intelligence applications served only as auxiliary tools for manuscript editing and graphical abstract preparation. No AI system was involved in the development of scientific arguments, interpretation of results, or formulation of conclusions; these tasks were performed and verified solely by the authors.

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