

Oxyhydrogen nanobubbles attenuate angiogenesis, inflammation, and immunosuppression in Wistar rats hepatocellular carcinoma model

Aulanni'am^{1,*}, Natasya Adiba Zahrah², Sri Widyarti²,
Olly Indrajani³, Dyah Kinasih Wuragil⁴, Wibi Riawan⁵,
Dodik Prasetyo⁴, Syifa Mustika⁶, Aditya Hernowo³,
Sutiman Bambang Sumitro^{1,3}, Ahmad Lubab⁷

1. Department of Chemistry, Faculty of Sciences, Technology, and Mathematics, Universitas Brawijaya, Malang, 65145, Indonesia.
2. Department of Biology, Faculty of Sciences, Technology, and Mathematics, Universitas Brawijaya, Malang, 65145, Indonesia.
3. Inovasi Molekul Indonesia (IMI), Malang, 65151, Indonesia.
4. Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, 65151, Indonesia.
5. Department of Biochemistry, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia.
6. Department of Internal Medicine, Faculty of Medicine, Universitas Brawijaya, Malang, 65145, Indonesia.
7. Department of Mathematics, Faculty of Science and Technology, Universitas Islam Negeri Sunan Ampel Surabaya, Surabaya, 60237, Indonesia.

* Corresponding author at:
Department of Chemistry,
Faculty of Sciences, Technology,
and Mathematics, Universitas
Brawijaya, Malang, 65145,
Indonesia.
E-mail: aulani@ub.ac.id
(Aulanni'am Aulanni'am).

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ABSTRACT. Hepatocellular carcinoma (HCC) remains a major cause of cancer-related mortality worldwide, while currently available first-line therapies are often constrained by systemic toxicity, resistance, and limited single-target mechanisms. Therefore, safer and broader-spectrum therapeutic strategies are urgently needed. This study evaluated the antitumor efficacy of oxyhydrogen nanobubbles (HHOnbs), an innovative multigas nanosystem that simultaneously delivers O₂, H₂, and low-dose H₂O₂, and hypothesized that HHOnbs would suppress HCC progression through multitarget modulation of the tumor microenvironment. A true experimental study was conducted using diethylnitrosamine/carbon tetrachloride (DEN/CCl₄)-induced HCC in male Wistar rats. Six groups were assigned: negative control, positive control, HHOnbs (200 million particles/mL; 0.1 mL per intravenous injection) administered every 2 days for a total of 12 or 24 treatments, and Lenvatinib (1.2 mg/kg body weight, orally) administered in 3- or 5-dose regimens. Antitumor activity was evaluated by immunohistochemical analysis of CD31, CD68, and FoxP3 expression, representing angiogenesis, macrophage-mediated inflammation, and regulatory T-cell-associated immunosuppression, respectively. In addition, *TERT* gene amplification was quantified using PCR. Compared with the positive control, HHOnbs significantly reduced CD31 by 42.07–43.55%, CD68 by 42.61–48.02%, and FoxP3 by 44.96–48.71% (all $p < 0.001$), with greater effects observed at higher treatment frequencies. *TERT* gene amplification was suppressed by up to 47.89% in the 24-administration group. These effects were comparable to those of Lenvatinib, while HHOnbs showed more consistent frequency-dependent responses across all biomarkers. In conclusion, HHOnbs demonstrated significant multitarget antitumor

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activity in experimental HCC and represent a promising nanotherapeutic candidate for liver cancer management.

KEYWORDS: Hepatocellular carcinoma, oxyhydrogen nanobubbles, *TERT*, tumor microenvironment

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer. Currently, it ranks among the six most commonly diagnosed malignancies and the third leading cause of cancer-related mortality worldwide, accounting for more than 800,000 deaths annually [1]. Despite meaningful advances in surveillance and locoregional therapies, the majority of patients present with advanced-stage disease, for which systemic treatment options remain limited in both efficacy and tolerability. First-line agents such as Sorafenib and Lenvatinib, while approved for advanced HCC, are associated with high rates of palmar-plantar erythrodysaesthesia, diarrhea, hypertension, and grade ≥ 3 toxicities, leading to discontinuation rates of 30–40% in real-world settings [2, 3]. These limitations underscore the urgent need for novel therapeutic modalities that combine multitarget activity with an improved safety profile.

A critical driver of HCC progression and therapeutic resistance is the tumor microenvironment (TME), an immunosuppressive and pro-angiogenic niche shaped by interacting cellular and molecular components, including tumor-associated macrophages, regulatory T cells (Tregs), and hypoxia-driven vascular remodeling [4]. Elevated expression of CD31, a marker of endothelial angiogenesis; CD68, associated with macrophage-mediated inflammation; and FoxP3, a master regulator of Treg-mediated immune evasion, collectively reflects the degree of TME reprogramming in HCC and is independently associated with poor prognosis [5]. Targeting multiple TME components simultaneously, rather than a single pathway, may therefore offer superior therapeutic outcomes.

In addition to alterations in the tumor microenvironment, HCC progression is associated with promoter mutations in the telomerase reverse transcriptase (*TERT*) gene, which encodes the catalytic subunit of the telomerase enzyme. *TERT* promoter mutations have been identified in approximately 65% of HCC cases and are considered the most common somatic genetic alteration in HCC [6], particularly in hepatitis-associated tumors [7]. Given its central role in HCC development, *TERT* expression represents an important molecular marker for evaluating the therapeutic response to novel anticancer strategies.

Gas-based therapy has emerged as a promising biomedical strategy owing to its favorable biocompatibility, controllability, and ability to modulate pathological microenvironments. Gaseous molecules, particularly O_2 and H_2 , have emerged as promising therapeutic agents for suppressing tumor progression. Molecular O_2 has been shown to directly downregulate p53 protein expression in A549 lung cancer cells [8]. In parallel, molecular H_2 significantly inhibits tumor growth by reducing tumor volume and Ki-67 expression, while also attenuating cancer cell proliferation, invasion, migration, and colony-forming capacity in Hs38.T and PA-1 cell lines [9]. Furthermore, although H_2O_2 is classified as a reactive oxygen species (ROS), low concentrations of H_2O_2 can induce cell cycle arrest by modulating the expression of oxidative stress-related genes in MCF-7 breast cancer cells [10] and A549 lung cancer cells, primarily through the downregulation of cyclin D1 and cyclin E expression [11].

The clinical application of therapeutic gases is constrained by rapid diffusion loss, unpredictable biodistribution, and insufficient accumulation at tumor sites [12]. Therefore, an effective drug delivery strategy is required to enable the therapeutic application of gaseous molecules. Nanobubbles (NBs), nanoscale gas-encapsulating carriers typically below 200 nm in diameter, have attracted growing interest as delivery platforms for gaseous therapeutics because of their high internal pressure, large gas–liquid interfacial area,

and prolonged colloidal stability [13]. Their nanoscale dimensions facilitate passage through capillary networks, enhanced tissue penetration, and efficient cellular uptake. Oxyhydrogen nanobubbles (HHOnbs) represent a novel multigas nanobubble platform capable of co-delivering O₂, H₂, and low concentrations of H₂O₂ within a single nanocarrier [14]. Furthermore, the gaseous components contained in HHOnbs are naturally occurring molecules in the human body. Consequently, their administration is less likely to elicit immune recognition as foreign substances, thereby contributing to their favorable biocompatibility. In addition, this therapy has been associated with enhanced physiological resilience by improving the body's capacity to maintain redox homeostasis and adapt to oxidative stress.

HHOnbs therapy has been evaluated in patients with hypertension and has been shown to normalize blood pressure without causing significant adverse effects, thereby demonstrating its therapeutic efficacy and favorable safety profile [15]. Molecular O₂ alleviates tumor hypoxia by enhancing oxygen availability [16], while molecular H₂ acts as a selective antioxidant that scavenges excessive ROS and maintains intracellular redox balance [17]. Meanwhile, the low concentration of H₂O₂ selectively induces oxidative stress in cancer cells, which are inherently more vulnerable to redox perturbations than normal cells, thereby providing a basis for selective anticancer activity [18]. Preliminary *in vivo* evidence has suggested antitumor activity of HHOnbs in HCC models. However, their comparative efficacy relative to standard-of-care therapy, frequency-dependent effects, and modulation of multiple tumor-associated biomarkers have not been systematically characterized [19].

Based on these considerations, we hypothesized that HHOnbs would suppress HCC progression through multitarget regulation of the tumor microenvironment and would produce antitumor effects comparable to or superior to those of standard systemic therapy. The present study aimed to address these gaps by evaluating the antitumor efficacy of HHOnbs at two treatment frequencies in a chemically induced HCC model using Wistar rats and by comparing their effects on TME biomarkers CD31, CD68, FoxP3, and *TERT* band intensity with those of the standard first-line agent Lenvatinib. By providing a comprehensive *in vivo* characterization, this study seeks to establish HHOnbs as a mechanistically distinct and potentially superior alternative for HCC treatment.

MATERIALS AND METHODS

Ethical approval

This study received ethical approval from the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Brawijaya (Approval No. 188-KEP-UB-2024).

Study period and location

The research was conducted from February to September 2025. All research activities were conducted at Universitas Brawijaya, with the following locations used for specific procedures. Sample analyses were performed at the Biochemistry Laboratory, Faculty of Sciences, Technology, and Mathematics; the Central Biomedical Laboratory, Faculty of Medicine; and the Animal Disease Diagnostic Laboratory, Faculty of Veterinary Medicine.

Animals and experimental design

A true experimental *in vivo* design was employed. The study included six groups, comprising two control groups and four treatment groups. Prior to allocation, animals were stratified according to body weight to reduce baseline variability. Each rat was housed individually and assigned a unique cage identification number. Randomization was performed using a computer-generated randomization sequence, and allocation concealment was maintained through coded cage identifiers that did not disclose treatment assignments to personnel involved in outcome assessment.

The control groups consisted of negative control (NC) group of healthy rats without HCC induction and positive control (PC) group of HCC-induced rats without treatment. The treatment groups consisted of T1, HHOnbs administered 12 times; T2, HHOnbs administered 24 times; T3, lenvatinib administered 3 times; T4, lenvatinib administered 5 times. The minimum sample size for each group was determined using Federer's formula: $(t - 1)(n - 1) \geq 15$, where t represents the number of groups and n denotes the number of animals per group. Based on the sample size calculation, the minimum number of animals required per group was four ($n = 4$). Accordingly, a total of 24 rats were allocated equally among the six experimental groups, with four animals per group. Sample size determination was based on this established approach for animal experiments, balancing statistical requirements with ethical considerations to minimize animal use.

HCC induction

HCC was established using a two-step DEN/CCl₄ protocol adapted from previous reports [20]. During the initiation phase, rats aged 2–3 weeks received a single intraperitoneal injection of DEN (Sigma-Aldrich, Cat. No. N0258) at a dose of 50 mg/kg body weight, diluted in 2 mL of vehicle solution. During the promotion phase, when the animals reached 4–5 weeks of age, CCl₄ (Merck, Cat. No. 1.02222.2500), diluted in corn oil (Sigma-Aldrich, Cat. No. C8267) at a ratio of 1:9 (v/v), was administered at 0.15 mL per dose. The treatment was repeated 24 times at 2-day intervals.

Confirmation of HCC induction was performed by euthanizing one rat from the control group and one rat from the HCC-induced group for immunohistochemical assessment of Ki-67 expression. Upon confirmation of HCC development, the treatment protocol was initiated. If HCC was not confirmed, the animals underwent one additional round of CCl₄ induction before subsequent histopathological and immunohistochemical reassessment.

Physicochemical characterization of HHOnbs

The HHOnbs formulation used in this study was supplied in pharmaceutical-grade quality by a licensed intravenous solution manufacturer, Inovasi Molekul Indonesia (IMI). NBs were suspended in sterile water for injection and produced using a proprietary electrolytic gas infusion technology. In this process, medical-grade H₂ and O₂ were combined at a 2:1 molar ratio, resulting in the intrinsic generation of low concentrations of H₂O₂ during NB formation [21]. The physicochemical characteristics of the HHOnbs formulation used in this study were based on previously reported data [16]. Nanoparticle tracking analysis (NTA) showed a mean particle diameter of approximately 116 nm (mode: 78 nm; median: 89 nm). The formulation also exhibited excellent stability under thermal and pH stress conditions, supporting its suitability for biomedical applications [22].

The HHOnbs therapy

The HHOnbs concentration administered in this study was 200 million particles/mL. Each animal received 0.06 mL per injection, which was rounded to 0.1 mL for administration. Treatments were delivered intravenously at 2-day intervals. HHOnbs treatment was initiated only after completion of the HCC induction protocol and confirmation of HCC development in the experimental animals.

Two treatment frequencies (12 and 24 administrations) were selected to evaluate whether repeated exposure to HHOnbs would produce a frequency-dependent biological response. The 24-administration regimen represented an extended exposure schedule relative to the 12-administration regimen, enabling assessment of potential cumulative therapeutic effects.

Lenvatinib therapy

Following completion of the HCC induction protocol and confirmation of tumor development, rats in the treatment groups received Lenvatinib according to their respective treatment schedules. Lenvatinib

(Lenvima, 10 mg) was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) at a ratio of 1:5 (v/v) [23], and administered orally (p.o.) to the Lenvatinib treatment groups at a dose of 1.2 mg/kg body weight in a total volume of 1 mL. Lenvatinib was administered three or five times in the respective treatment groups.

The treatment schedules for HHOnbs and Lenvatinib were intentionally designed to account for their distinct pharmacological properties and mechanisms of action. Therefore, differences in administration frequency were adopted to optimize the therapeutic activity of each intervention rather than to directly compare equivalent dosing schedules. Repeated administration of Lenvatinib was also intended to maintain sustained kinase inhibition while minimizing the potential for treatment-related toxicity and resistance associated with prolonged exposure.

Immunohistochemistry

Liver tissues were collected, fixed in 10% buffered formaldehyde, paraffin-embedded, and sectioned at 4 µm. Sections were deparaffinized in xylene, rehydrated through graded ethanol, and stored at 4°C overnight before staining. Immunohistochemical analysis was performed using a commercial kit (ScyTek). Endogenous peroxidase activity was blocked with 3% H₂O₂, after which the slides were incubated in blocking serum in a humidified chamber at 4°C overnight.

Tissue sections were then incubated with primary antibodies against CD68 (1:100; Santa Cruz Biotechnology, Cat. No. sc-20060), FoxP3 (1:100; Cat. No. sc-53876), and CD31 (1:100; Cat. No. sc-37676) for 1 h, followed by incubation with the secondary antibody and horseradish peroxidase (HRP), each for 1 h. Immunoreactivity was visualized using diaminobenzidine (DAB), and nuclei were counterstained with Mayer's hematoxylin. Slides were air-dried, mounted with Entellan, and examined microscopically for further analysis.

The TME-index measurement was based on the HCC score classification, with slight modifications [5]. The TME-index was assessed using 15 randomly selected microscopic fields at 400× magnification. The area of each field was quantified using ImageJ software to determine the proportion of positively stained immune cells. Based on the percentage of positive cells, samples were assigned the following scores: 1 (0–10%), 2 (11–25%), 3 (26–50%), and 4 (51–75%).

The overall TME-index was calculated by summing the scores of the assessed parameters. Staining intensity was scored as follows: 0 (no staining), 1 (light yellow), 2 (yellowish brown), and 3 (dark brown). The overall TME-index was subsequently classified as negative (0), weakly positive (1–2), moderately positive (3–4), or strongly positive (5–7). All images were analyzed by three investigators under blinded conditions to minimize scoring variability. The use of digital image analysis reduced subjective interpretation by applying identical quantification parameters across all specimens.

TERT gene amplification by PCR

Total DNA was extracted from rat liver tissues using the DNeasy Blood & Tissue Kit (Cat. No. 69504). PCR amplification was performed using GoTaq Green Master Mix in a total reaction volume of 20 µL with *TERT*-specific primers: forward 5'-CCT GAA AGA GCT GGT GTC CA-3' and reverse 5'-CGA CTG ACA CAC AGG GAC TC-3', yielding a 177-bp product. Thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 5 min.

PCR products were separated by 1.5% agarose gel electrophoresis and visualized under UV illumination. Band intensity was quantified using ImageJ software and expressed as relative integrated density values. All samples were processed under identical amplification, electrophoretic, and imaging conditions to permit relative comparison among experimental groups. No direct calculation of telomerase enzymatic activity was performed.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics. All variables satisfied the assumptions of normality (Shapiro–Wilk test, $p > 0.05$) and homogeneity of variance (Levene's test, $p > 0.05$). Variables meeting the assumptions of normality and homogeneity were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test for multiple-group comparisons. For data that did not satisfy these assumptions, the Kruskal–Wallis test was applied, followed by appropriate pairwise post hoc comparisons with Bonferroni adjustment. Statistical significance was defined as $p < 0.05$.

RESULTS

Ki-67 expression for cancer confirmation

Confirmation of successful cancer induction is a critical step in preclinical drug development studies to ensure the internal validity and biological relevance of the experimental model. Figure 1a presents the immunohistochemical staining of Ki-67 in liver tissues obtained from rats subjected to DEN/ CCl_4 -induced HCC. Ki-67 is a nuclear protein expressed in actively proliferating cells during the G1, S, G2, and M phases of the cell cycle [24]. Owing to its consistent expression throughout the active phases of cell division, Ki-67 is widely recognized as a reliable and robust marker of cellular proliferation [25]. In HCC, elevated Ki-67 expression has been associated with more aggressive tumor behavior and poorer overall survival, underscoring its prognostic significance [26].

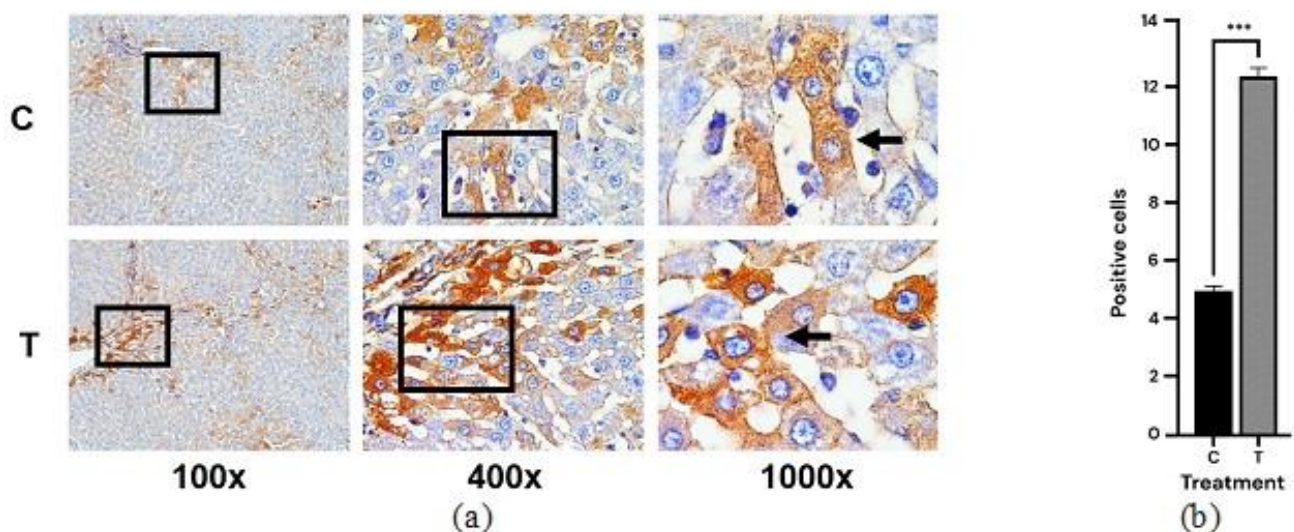


Figure 1. Ki-67 expression. (A) Representative immunohistochemical staining of Ki-67 in liver tissue at 100 $\times$, 400 $\times$, and 1,000 $\times$ magnifications. (B) Total number of Ki-67-positive cells. *** $p < 0.001$. C, control group; T, DEN- CCl_4 -induced group.

The mean Ki-67 expression was higher in the treatment group (12.300 ± 0.843) than in the control group (4.900 ± 0.585), indicating enhanced cellular proliferative activity following DEN/ CCl_4 induction (Figure 1b). This increase in Ki-67 expression suggests that the DEN/ CCl_4 induction protocol successfully promoted hepatocarcinogenesis in the experimental rats. These findings are consistent with previous reports demonstrating that DEN/ CCl_4 -induced rats develop hepatic fibrosis, chronic inflammation, and histopathological features that closely resemble human HCC, thereby supporting the validity of this experimental model [27].

CD31 expression

A total of 24 rats were included in the study and completed all experimental procedures. Each experimental group consisted of four animals ($n = 4$), and all animals were included in the final analyses of CD31, CD68, FoxP3, and *TERT* band intensity. The Kruskal–Wallis test showed a significant difference in CD31 expression among the six groups ($H(5) = 63.775$, $p < 0.001$, $\varepsilon^2 = 0.397$). CD31 expression, as a marker of angiogenesis, showed a clear increase in the positive control group compared with the negative control group, with TME-index values rising from 2.518 ± 0.111 to 5.034 ± 0.175 , indicating enhanced vascularization under HCC conditions (Table 1). This increase was statistically significant ($p < 0.001$) (Figure 2).

Table 1. CD31 expression across experimental groups. Values are expressed as mean $\pm$ SD

Group	Area positive cells (%)	Area score	Color intensity score	TME-index	<i>p</i> -value
NC	10.830	1.370	1.148	2.518 ± 0.111	0.000
PC	16.201	2.482	2.551	5.034 ± 0.175	
T1	11.879	1.458	1.458	2.916 ± 0.198	
T2	11.816	1.368	1.473	2.842 ± 0.206	
T3	4.339	1.000	1.681	2.681 ± 0.166	
T4	5.285	1.043	1.739	2.782 ± 0.153	

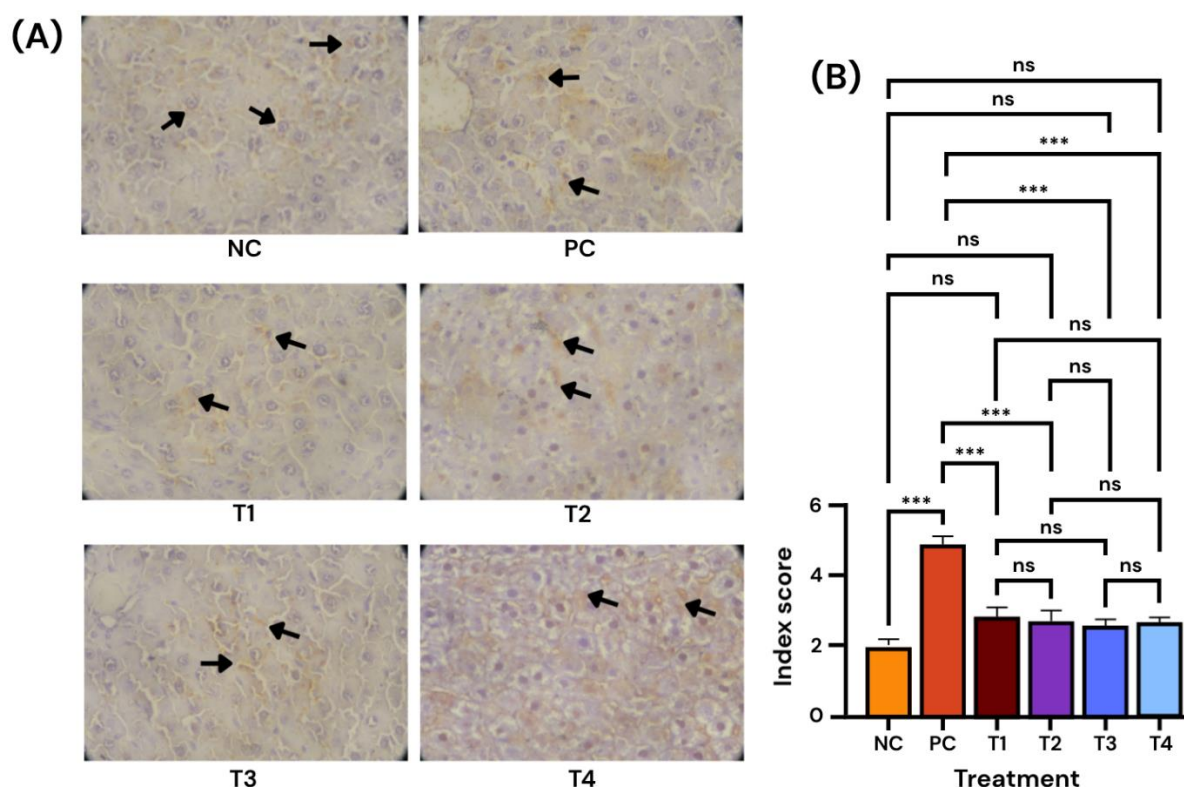


Figure 2. CD31 expression in liver tissue. (A) Representative immunohistochemical staining of CD31 at 400 $\times$ magnification. (B) CD31 expression was statistically analyzed using one-way ANOVA followed by a post hoc test. *** $p < 0.001$; ns, not significant.

Administration of HHOnbs reduced CD31 expression in both treatment groups, with TME-index values of 2.916 ± 0.198 (12 administrations) and 2.842 ± 0.206 (24 administrations). Similarly, Lenvatinib treatment decreased CD31 expression, with TME-index values of 2.681 ± 0.166 (3 doses) and 2.782 ± 0.153 (5 doses). Overall, both HHOnbs and Lenvatinib significantly attenuated angiogenesis compared with the positive control group ($p < 0.001$), with comparable efficacy between the two treatments. HHOnbs exhibited more consistent effects across different administration frequencies.

Administration of HHOnbs resulted in a consistent reduction in CD31 expression, indicating suppression of tumor-associated angiogenesis. This effect can be mechanistically linked to the oxygen component of HHOnbs, which alleviates tumor hypoxia. Reduced hypoxic stress is known to downregulate HIF-1 α signaling, thereby decreasing vascular endothelial growth factor (VEGF) expression and subsequent neovascularization [4, 28]. By attenuating angiogenesis, HHOnbs may limit nutrient and O₂ supply to tumor cells, ultimately restricting tumor expansion.

CD68 expression

The Kruskal–Wallis test showed a significant difference in CD68 expression among the six groups ($H(5) = 59.515$, $p < 0.001$, $\varepsilon^2 = 0.482$). CD68 expression, a marker of macrophage-associated inflammation, was markedly higher in the positive control group than in the negative control group, with TME-index values increasing from 2.416 ± 0.119 to 5.421 ± 0.192 (Table 2), indicating enhanced inflammatory activity under HCC conditions ($p < 0.001$) (Figure 3). HHOnbs treatment reduced CD68 expression in both groups, with TME-index values of 3.111 ± 0.137 (12 administrations) and 2.818 ± 0.156 (24 administrations). The reduction was more pronounced at the higher administration frequency. Similarly, Lenvatinib treatment decreased CD68 expression, with TME-index values of 3.142 ± 0.158 (3 doses) and 2.866 ± 0.215 (5 doses). Both HHOnbs and Lenvatinib significantly attenuated macrophage-associated inflammation compared with the positive control group ($p < 0.001$).

HHOnbs significantly modulated macrophage-associated inflammation, as reflected by decreased CD68 expression. The H₂ component of HHOnbs selectively neutralizes excessive ROS, thereby reducing oxidative stress and inhibiting the activation of pro-inflammatory pathways such as NF- κ B, consequently decreasing the recruitment and activation of tumor-associated macrophages [29]. HHOnbs may therefore disrupt the chronic inflammatory milieu that facilitates tumor progression.

Table 2. CD68 expression across experimental groups. Values are expressed as mean $\pm$ SD

Group	Area positive cells (%)	Area score	Color intensity score	TME-index	<i>p</i> -value
NC	3.137	1.083	1.333	2.416 ± 0.119	0.000
PC	39.286	2.947	2.473	5.421 ± 0.192	
T1	15.148	1.833	1.277	3.111 ± 0.137	
T2	11.279	1.500	1.318	2.818 ± 0.156	
T3	15.400	1.761	1.380	3.142 ± 0.158	
T4	10.691	1.466	0.533	2.866 ± 0.215	

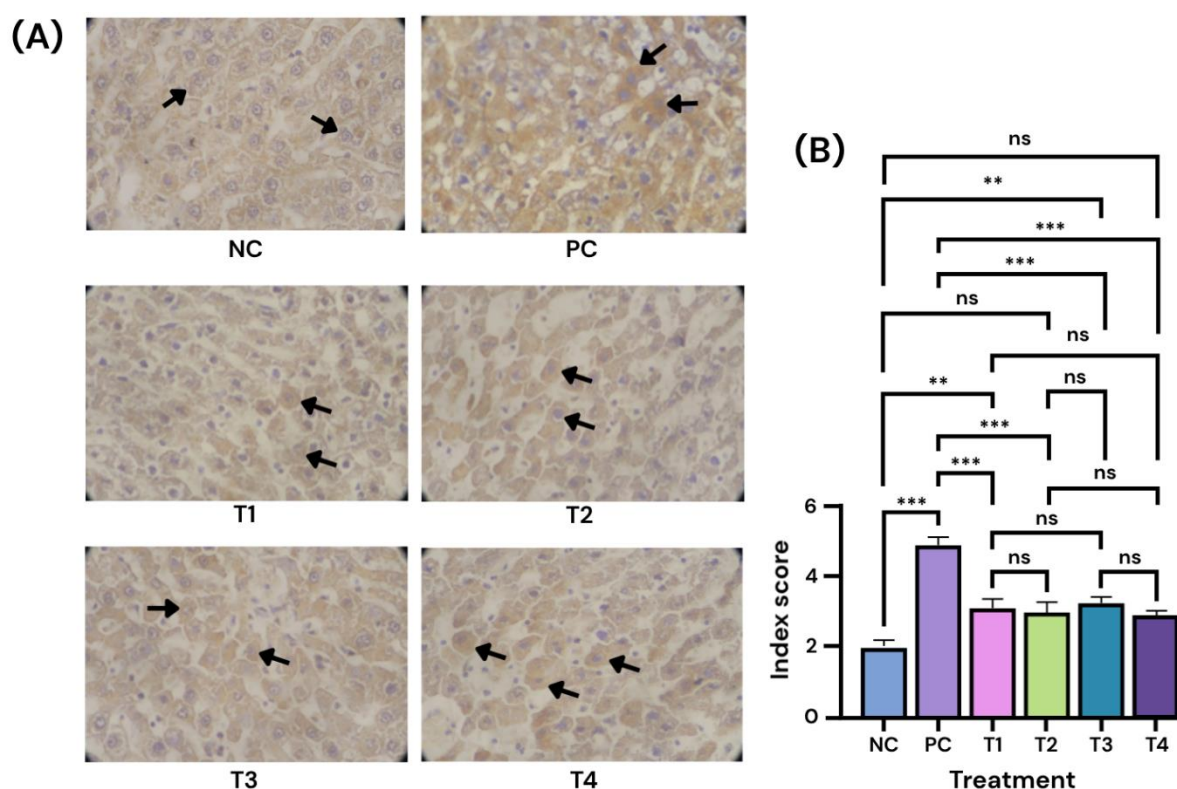


Figure 3. CD68 expression in liver tissue. (A) Representative immunohistochemical staining of CD68 at 400× magnification. (B) CD68 expression was statistically analyzed using one-way ANOVA followed by a post hoc test. ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

FoxP3 expression

The Kruskal–Wallis test showed a significant difference in FoxP3 expression among the six groups ($H(5) = 78.987$, $p < 0.001$, $\varepsilon^2 = 0.540$). FoxP3 expression, as a marker of immunosuppression indicating the accumulation of Treg cells, was substantially elevated in the positive control group compared with the negative control group, with TME-index values increasing from 3.030 ± 0.159 to 6.888 ± 0.076 (Table 3), indicating enhanced immunosuppressive activity under HCC conditions ($p < 0.001$) (Figure 4). HHOnbs treatment reduced FoxP3 expression in both groups, with TME-index values of 3.791 ± 0.216 (12 administrations) and 3.533 ± 0.236 (24 administrations). Lenvatinib treatment also decreased FoxP3 expression, with TME-index values of 4.617 ± 0.140 (3 doses) and 3.526 ± 0.159 (5 doses). HHOnbs demonstrated a more consistent effect across treatment frequencies than Lenvatinib.

Table 3. FoxP3 expression across experimental groups. Values are expressed as mean $\pm$ SD

Group	Area positive cells (%)	Area score	Color intensity score	TME-index	<i>p</i> -value
NC	10.098	1.393	1.636	3.030 ± 0.159	0.000
PC	63.301	4.000	2.888	6.888 ± 0.076	
T1	22.537	2.083	1.708	3.791 ± 0.216	
T2	12.668	1.666	1.866	3.533 ± 0.236	
T3	28.135	2.588	2.029	4.617 ± 0.140	
T4	13.803	1.631	1.894	3.526 ± 0.159	

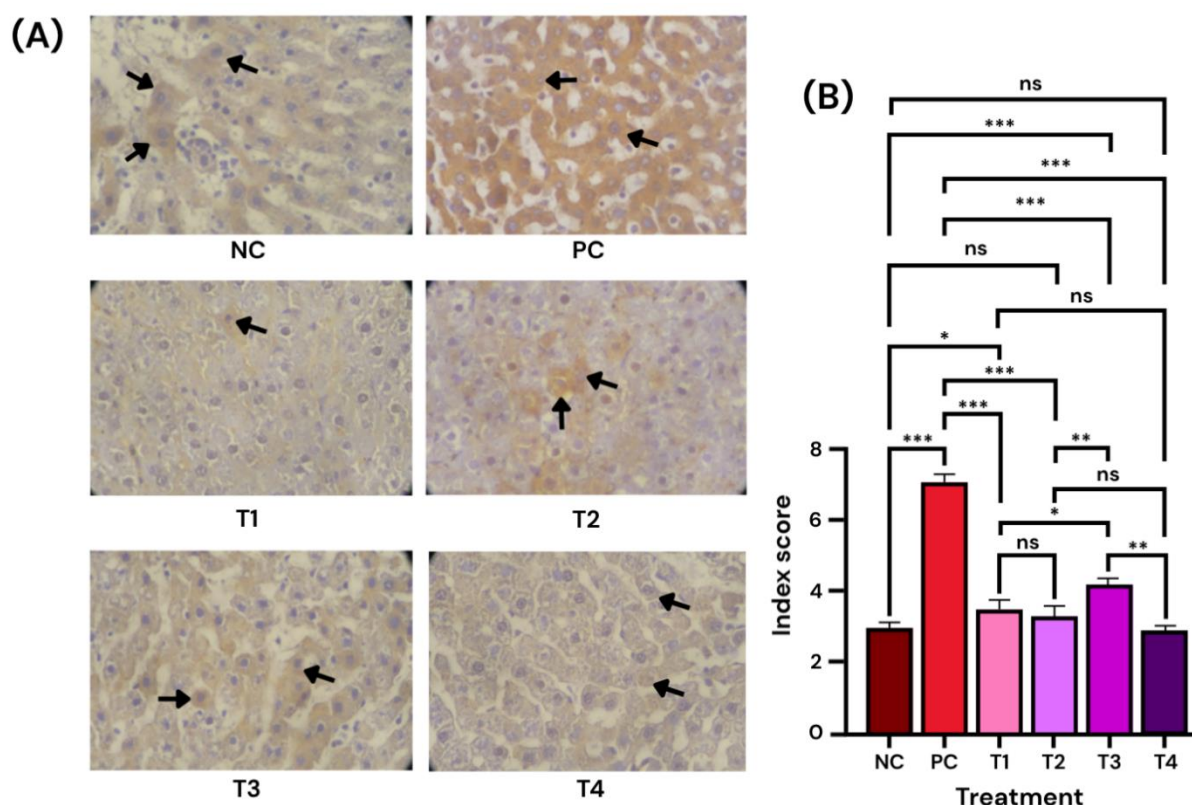


Figure 4. FoxP3 expression in liver tissue. (A) Representative immunohistochemical staining of FoxP3 at 400× magnification. (B) FoxP3 expression was statistically analyzed using one-way ANOVA followed by a post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

TERT band intensity

The Kruskal–Wallis test showed a significant difference in *TERT* band intensity among the six groups ($H(5) = 14.315$, $p < 0.05$, $\varepsilon^2 = 0.518$). *TERT* band intensity was markedly elevated in the positive control group compared with the negative control group, with mean intensity values increasing from 83.261 to 251.054 (Table 4), indicating enhanced proliferative capacity of tumor cells under HCC conditions ($p < 0.05$) (Figure 5). HHOnbs treatment reduced *TERT* band intensity to 189.661 (12 administrations) and 130.813 (24 administrations). Similarly, Lenvatinib decreased *TERT* band intensity to 204.640 (3 doses) and 124.687 (5 doses). Both treatments significantly suppressed *TERT* band intensity compared with the positive control group ($p < 0.05$), with stronger effects observed at higher treatment frequencies.

Table 4. *TERT* band intensity across experimental groups. Values are expressed as mean $\pm$ SD

Group	Mean intensity	<i>p</i> -value
NC	83.261 $\pm$ 18.844	0.014
PC	251.054 $\pm$ 30.351	
T1	189.661 $\pm$ 7.117	
T2	130.813 $\pm$ 28.522	
T3	204.640 $\pm$ 30.626	
T4	124.687 $\pm$ 24.327	

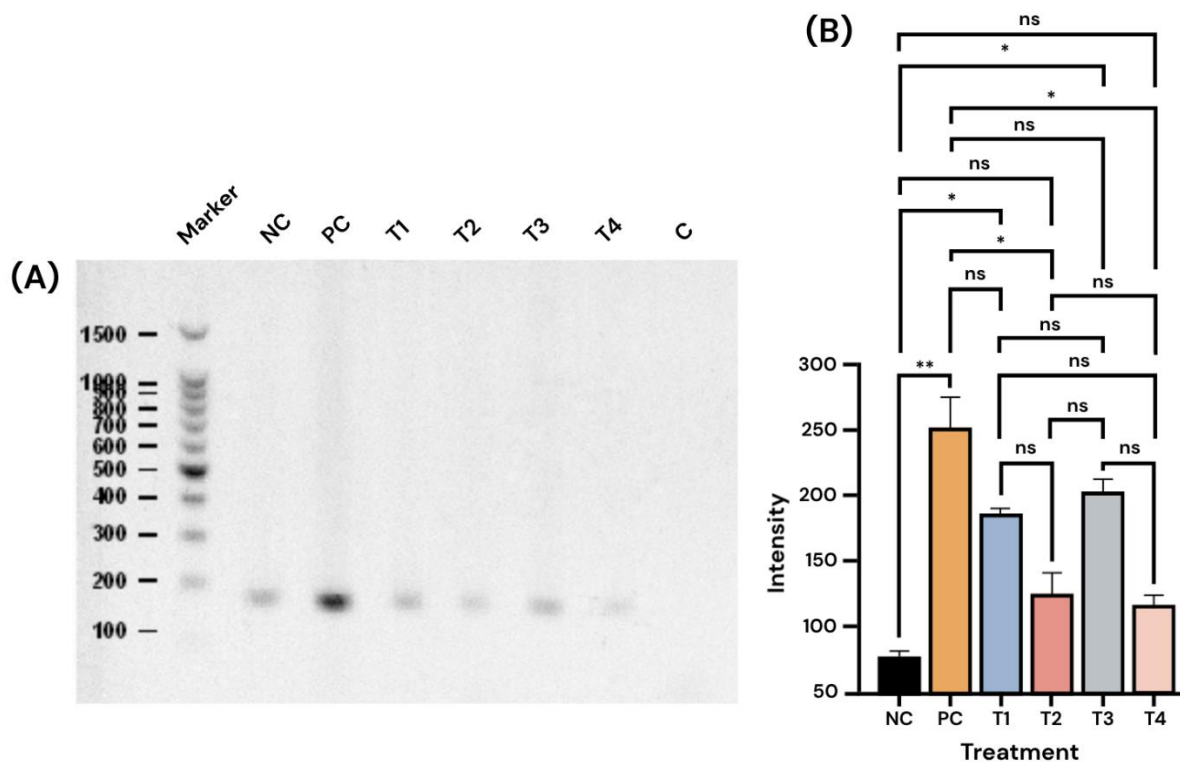


Figure 5. *TERT* band intensity. (A) Representative visualization of *TERT* PCR bands. (B) Relative *TERT* band intensity was statistically analyzed using one-way ANOVA followed by a post hoc test. * $p < 0.05$; ** $p < 0.01$; ns, not significant.

DISCUSSION

The present study provides *in vivo* evidence that HHOnbs modulate multiple biomarkers associated with HCC progression in a chemically induced HCC model. HHOnbs reduced the expression of CD31, CD68, FoxP3, and *TERT* band intensity, suggesting potential effects on angiogenesis, macrophage-associated inflammation, immunosuppressive activity, and proliferative signaling. Across all assessed biomarkers, HHOnbs exhibited a relatively consistent frequency-dependent response pattern comparable to the trends observed in the Lenvatinib-treated groups.

The reduction in CD31 expression following HHOnbs administration suggests a potential attenuation of tumor-associated angiogenesis. Although the molecular mechanisms were not directly investigated in the present study, previous studies have reported that improved molecular O_2 availability may reduce hypoxia-related signaling pathways involved in angiogenic responses [4, 16, 28]. Nevertheless, CD31 expression in the Lenvatinib-treated groups tended to be lower than that observed in the HHOnbs-treated groups, although the difference did not reach statistical significance. This finding may be attributed to the distinct mechanisms of action of the two therapies. Lenvatinib exerts direct antiangiogenic effects by targeting multiple receptor tyrosine kinases, including vascular endothelial growth factor receptors (VEGFRs) and fibroblast growth factor receptors (FGFRs), which play critical roles in tumor proliferation and angiogenesis [30]. In contrast, HHOnbs primarily function by enhancing oxygen delivery and modulating oxidative stress through their antioxidant properties [16, 21]. Therefore, the observed decrease in CD31 expression is consistent with a possible antiangiogenic effect of HHOnbs, although direct validation of the underlying pathway is required.

The reduction in FoxP3 expression observed following HHOnbs treatment suggests decreased immunosuppressive activity within the tumor microenvironment. FoxP3 is widely recognized as a marker of regulatory T cells, which contribute to immune evasion and disease progression in HCC [5]. Although the pathways responsible for this reduction were not examined directly, previous studies have shown that oxidative stress, inflammatory signaling, and hypoxic conditions can influence Treg accumulation and function

[29, 31]. Therefore, the present findings are compatible with a potential immunomodulatory effect of HHOnbs, but the underlying molecular mechanisms remain to be elucidated. These convergent mechanisms may explain why HHOnbs demonstrated more consistent frequency-dependent reductions in FoxP3 expression (44.96% at 12 administrations and 48.71% at 24 administrations) compared with the greater variability observed with Lenvatinib (32.97% at 3 doses vs. 48.79% at 5 doses).

HHOnbs also reduced *TERT* band intensity in a frequency-dependent manner. Given the established role of telomerase in maintaining replicative capacity in malignant cells [32], this finding suggests a potential influence of HHOnbs on pathways related to tumor cell proliferation. Mechanistically, this effect may be attributed to the suppression of upstream signaling pathways, such as HIF-1 α and NF- κ B, both of which are known to regulate *TERT* expression [33, 34]. However, because molecular mechanisms and functional proliferative outcomes were not directly assessed, the present data should be interpreted as evidence of altered telomerase-related signaling rather than direct inhibition of tumor cell survival.

Overall, no statistically significant differences were observed between the HHOnbs- and Lenvatinib-treated groups across the evaluated biomarkers. Both treatments reduced the intensities of CD31, CD68, FoxP3, and *TERT* PCR bands compared with the untreated HCC group. However, several limitations should be acknowledged. First, this study was not designed to determine therapeutic equivalence, non-inferiority, or superiority. Thus, the findings indicate similar biomarker modulation rather than comparable efficacy. Second, *TERT* was analyzed by conventional PCR using genomic DNA; therefore, PCR band intensity reflects DNA amplification rather than *TERT* expression or telomerase activity. Third, HHOnbs were evaluated only as monotherapy, limiting conclusions regarding their role as a standalone treatment or alternative to current first-line HCC therapies. Finally, only two HHOnbs administration frequencies were evaluated, precluding assessment of long-term efficacy and safety. Future studies should include mechanistic investigations, quantitative assessment of *TERT* expression and telomerase activity, extended treatment regimens, combination strategies with standard therapies, and adequately powered comparative efficacy studies.

CONCLUSIONS

This study provides *in vivo* evidence that HHOnbs modulate multiple tumor-associated biomarkers in a chemically induced HCC model. HHOnbs significantly reduced CD31, CD68, and FoxP3 expression and decreased *TERT* band intensity relative to untreated HCC controls. These findings indicate that HHOnbs influence biomarkers associated with angiogenesis, inflammation, immunoregulation, and other biological processes relevant to HCC. No statistically significant differences were observed between the HHOnbs-treated and Lenvatinib-treated groups for the evaluated biomarkers. However, the present study was not designed to establish therapeutic equivalence, non-inferiority, or superiority between treatments, nor did it evaluate clinically relevant outcomes such as tumor burden, survival, recurrence, metastasis, or overall disease progression. Furthermore, the molecular mechanisms underlying the observed biomarker changes were not directly investigated. Therefore, HHOnbs should be regarded as a promising investigational nanotherapeutic approach that warrants further preclinical evaluation. Future studies should incorporate direct assessment of molecular pathways, tumor burden, survival outcomes, pharmacokinetic characterization, comprehensive toxicity evaluation, and long-term safety analyses to better define the therapeutic potential of HHOnbs in HCC.

DECLARATIONS

Availability of data and materials

All the generated data are included in the manuscript.

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Declaration of generative artificial intelligence (AI)

The authors declare that no generative AI tools were used to write, generate, or create the manuscript content, including tables and figures. AI-assisted tools were used only for translation, grammar correction, and language improvement using Grammarly. All scientific content, analyses, and interpretations were produced solely by the authors.

Authorship contributions

Aulanni'am Aulanni'am contributed to writing—original draft preparation and conceptualization. Natasya Adiba Zahrah contributed to data curation, investigation, and methodology. Sri Widyarti contributed to writing—review and editing and conceptualization. Dyah Kinasih Wuragil contributed to methodology and resources. Wibi Riawan contributed to data curation, methodology, resources, and investigation. Olly Indrajani contributed to funding acquisition, conceptualization, and resources. Ahmad Lubab contributed to software and data curation. Aditya Hernowo contributed to funding acquisition, conceptualization, and resources. Sutiman Bambang Sumitro contributed to funding acquisition, conceptualization, and resources.

Conflicts of interest

The authors declare no conflicts of interest.

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