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Comparative *in vitro* antioxidant study of saponin fraction and crude leaf extract of *Crateva religiosa*: insights from traditional medicine

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Oxidative stress is caused by an imbalance between free radicals and antioxidants. It is implicated in numerous disorders, including inflammatory, digestive, respiratory, skin, and reproductive diseases. Addressing this imbalance is crucial for mitigating disease progression. This study explores the antioxidant potential of *Crateva religiosa* leaf crude and saponin fraction against various free radicals, such as DPPH, metal ions (Fe^{2+}), superoxide anion (O_2^-), and nitric oxide ($NO\cdot$), using scavenging assays and comparing with standard antioxidants. Total phenolic, flavonoid, and saponin contents were determined, along with reducing power. Leaf-derived saponins of *C. religiosa* are studied for the first time. Crude leaf extract exhibited high phenolic (17.75±0.0007 mg GAE/g) and flavonoid (136±0.003 mg QE/g) contents. Saponin content was 3.77±0.71% in leaves. Crude extract (472.48±0.54 µg/mL) had more DPPH scavenging activity than saponin fraction (581.68±0.14 µg/mL). The crude extract shows comparable activity to the saponin fraction in metal ion scavenging. However, the IC_{50} value of the saponin fraction shows good activity in scavenging nitric oxide and superoxide anion. It also has a higher reducing ability than crude extract. The crude extract and saponin fraction demonstrated strong free radical scavenging activity, correlating with their rich phenolic, flavonoid, and saponin content. These findings highlight the potential of the saponin fraction of *C. religiosa* leaf as a source of natural antioxidants to mitigate metal ion, superoxide, and nitric oxide radical-induced diseases.

Keywords: Antioxidant, *Crateva religiosa*, Free radicals, Leaf, Plant extracts, Traditional medicine

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Introduction

Endogenous metabolic processes and exogenous physicochemical stimuli generate free radicals. A balance between free radicals and antioxidants is necessary for proper physiological function[1]. Free radicals exhibit oxidative potential to interact with macromolecular cellular constituents such as DNA and cell membranes, damaging cells and tissues, causing oxidative stress. Excessive oxidative stress can cause lipids and proteins to oxidise, changing their structure and function in the process.

Sacred garlic pear (*Crateva religiosa*) belongs to the *Capparaceae* family. The bark and leaves of the tree were used as medicines for various ailments such as urinary, inflammatory, digestive, respiratory, liver, and gynaecological diseases. The herb was used to treat benign prostatic enlargement, kidney stones, urinary tract infections, and problems related to the prostate. The bark has laxative, carminative, antibacterial, and anti-inflammatory properties[2]. *C. religiosa* is known for its wound-healing effect[3], antimicrobial activity[4], antioxidant potential[5], antiarthritic effect[6], and analgesic potential[7]. It contains saponins, flavonoids, alkaloids, triterpenes, tannins, plant sterols, etc[8]. In traditional medicine, *C. religiosa* is included in formulations to support female reproductive health. The herb is a component of certain Ayurvedic remedies designed to restore hormonal balance and promote regular menstrual cycles[9]. While these findings are promising, there are no reports regarding the efficacy of *C. religiosa* in oxidative stress-related female reproductive disorders. Therefore, more targeted research is necessary to confirm its antioxidant potential benefits in relation to female reproductive health.

This study focuses on the *C. religiosa* leaf and its saponin-enriched fraction. In most cases, saponins are described at the group level, and their roles in antioxidant and medicinal activity for each radical are not highlighted. The bioactivity of the saponin

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fraction can be explained by several biological mechanisms linked to saponin chemical structure. The mechanisms include direct free radical scavenging, enhancement of endogenous antioxidant defences, and their ability to donate electrons or hydrogen atoms, thereby neutralising reactive species. Saponins possess glycosidic bonds and several hydroxyl (–OH) groups, which allow them to directly interact with and neutralise free radicals. This action involves donating electrons or hydrogen atoms from these groups to the free radicals, rendering them non-reactive and reducing oxidative stress[10]. Saponins have been shown to activate cellular antioxidant defence mechanisms, such as upregulating nuclear factor erythroid 2–related factor 2 (NRF2). NRF2 activation leads to increased expression of protective enzymes such as glutamate cysteine ligase (GCL), which are crucial for glutathione synthesis and overall cellular antioxidant capacity. Saponins also suppress inflammatory pathways (for example, by inhibiting NF-κB signaling and reducing the expression of inflammatory genes like COX-2). By reducing inflammation-associated oxidative stress, total cellular reducing power is increased[11]. The current study aimed to assess the antioxidant activity of the *C. religiosa* leaf-derived saponin fraction.

Materials and Methods

Chemical reagents

Analytical-grade chemicals and Milli-Q water were used for the entire analysis. All organic solvents (analytical grade), sodium carbonate, sodium nitroprusside, and ascorbic acid were purchased from Merck, India. Folin-Ciocalteau reagent, aluminum chloride, 2,2-bipyridyl, trisHCl buffer, trichloroacetic acid, NADH, and phenazinemethosulfate (PMS) were purchased from Spectrochem Private Limited. Gallic acid, quercetin, and Griess reagent were purchased from Sigma-Aldrich. Ferrous sulfate, hydroxylamine-HCl, potassium ferricyanide, ferric chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Rankem. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from SRL (Sisco Research Laboratory), and nitro blue tetrazolium (NBT) was purchased from TCI (Tokyo Chemical Industry).

Plant sample collection and identification

The leaves of *C. religiosa* were collected in September 2024 from the Regional Ayurveda Research Institute, Thiruvananthapuram. The samples were identified by the taxonomist Christil Lila R, Senior Research Officer (Botany), Pharmacognosy Unit, Ayurveda Research Institute, Poojapura, Thiruvananthapuram, with Voucher specimen No: HLL/05/2024 and deposited at Natural Products Laboratory, CRDC HLL, for future reference. The dried samples were ground and stored in an airtight container.

Preparation of *C. religiosa* leaf extract

The powdered samples were extracted in 80% ethanol using the refluxing method. The sample (50 g) was extracted with 500 m of solvent at 80°C for 3 hours, and repeated 2 times. The dried extracts were collected using a rotatory evaporator and stored at 4°C in airtight containers for further analysis, and the percentage yield was calculated using the given formula.

$$\%\ Yield = \frac{Weight\ of\ dried\ extract}{Weight\ of\ plant\ powder} \times 100$$

Extraction of the saponin-rich fraction from *C. religiosa* leaf extract

The aqueous-alcoholic extract was defatted with 200 mL of hexane for 8 hours and dried at 40°C for a day. The alcohol fraction was distilled off, and the aqueous fraction was cooled to room temperature. The aqueous fraction was re-extracted with butanol in a 1:3 ratio three times. The butanol fraction was concentrated using a rotary evaporator at 40°C and then dried. The crude saponin extract thus obtained was weighed[12].

Phytochemical screening

C. religiosa leaves were subjected to phytochemical screening for the detection of saponins, cardiac glycosides, anthraquinones, flavonoids, tannins, alkaloids, terpenoids, quinones, glycosides, triterpenoids, phenols, steroids, carbohydrates, and proteins[13].

Determination of Total phenolic content

Total phenolic content was determined using the Singleton and Rossi[14] and Lalhminghlui and Jagetia[15] procedures with slight modifications. Add 5 mL dilute solution (10-fold dilution) of Folin-Ciocalteu reagent to 1mL sample, 4 mL of 7.5% sodium carbonate, and mix well in a vortex shaker. After 30 minutes of incubation at 20°C, measure the absorbance at 765 nm. Gallic acid was used as a standard.

Determination of Total flavonoid content

Total flavonoid content was determined using a colourimetric assay procedure, as mentioned in Jia Zhishen *et al.*[16]. Add 4 mL of distilled water,

0.3 mL of 5% sodium nitrite, and 0.3 mL of 10% aluminum chloride to the sample. After 5 minutes, add 2 mL of 1 M sodium hydroxide and make up to 5 mL with distilled water. After incubation for 30 minutes at room temperature, the absorbance was measured at 510 nm. Quercetin was used as the reference standard. The total flavonoid content of the extracts was expressed as mg catechin equivalents per gram of sample (mg/g).

Determination of saponin content

The saponin content was determined using the procedure described by Mora-Ocación *et al.*[17] with slight modifications. A 50 g sample was defatted and extracted with 200 mL of 20% aqueous ethanol in a hot-water bath (55°C, 4 h). The extract was filtered, and the alcohol fraction was distilled. The aqueous fraction was re-extracted in butanol (1:3) using a separating funnel. The butanol fraction was evaporated and collected. The saponin content was determined as,

$$Saponin\ (mg\%) = \frac{Weight\ of\ residue\ \times\ 100}{Weight\ of\ sample}$$

Determination of DPPH radical scavenging capacity

The DPPH free radical scavenging activity of each extract was determined using the method described by Leong and Shui[18] and Wong *et al.*[19] with slight modifications. An aliquot of each extract dilutions was prepared with methanol and mixed with 4 mL of methanolic DPPH solution (0.1 mM). The mixture was shaken and incubated for 30 minutes in the dark conditions. The absorbance was measured at 517 nm with a Pharma-Spec UV-1700 UV/Vis Spectrophotometer (Shimadzu Scientific Instruments). Ascorbic acid was used as a reference standard. The percentage of DPPH radical scavenging activity of the extracts was calculated using the following formula:

$$\%\ RSA = \frac{Absorbance\ of\ control - Absorbance\ of\ sample\ \times\ 100}{Absorbance\ of\ control}$$

Determination of metal ion chelating capacity

The metal ion chelating potential of the plant extract was measured using the procedure mentioned in Huyut *et al.*[20]. 0.25 mL (1 mM) of ferrous sulphate solution was added to 0.25 mL of extract (200–1000 µg/mL). Later, 0.25 mL (0.1%) 2,2-bipyridyl solution and 1 mL trisHCl-buffer (pH=7.4) were added, followed by 0.4 mL hydroxylamine-HCl and 2.5 mL ethanol. The reaction mixture was adjusted to a final volume of 5 mL with distilled water and incubated for 10 minutes at room temperature. Fe2+ combines with the chelating agent to form a blue-coloured complex, which is measured by spectrophotometry at 521 nm. The chelating activity of the extract was determined using the given equation.

$$\%\ RSA = \frac{Absorbance\ of\ control - Absorbance\ of\ sample\ \times\ 100}{Absorbance\ of\ control}$$

Determination of superoxide anion scavenging capacity

The superoxide anion scavenging activity of the plant extract was determined by an NBT reduction assay based on Robak and Gryglewski's[21] and Sharma and Singh's[22] procedure with slight modifications. The assay mixture containing 2.4 mL PBS (pH 7.4), 1.5 mL of NBT (in phosphate buffer), 1.5 mL of NADH solution, and 0.6 mL of plant extract (200-1000 µg/mL) was mixed. The reaction was initiated by adding 1.5 mL PMS and incubated at 30°C for 10 minutes. The absorbance of the mixture was measured at 560 nm after the incubation time to quantify the formazan produced. Ascorbic acid was used as a reference compound. Percentage inhibition was calculated by comparing the results of the control and test samples using the given equation.

$$\%\ RSA = \frac{Absorbance\ of\ control - Absorbance\ of\ sample\ \times\ 100}{Absorbance\ of\ control}$$

Determination of nitric oxide ion scavenging capacity

Nitric oxide radical scavenging activity of plant extracts was measured according to the method of Sreejayan and M N A Rao[23] and Martins-Gomes *et al.*[24,] with some modifications. Extracts with different concentrations (40-200 mg/mL) were mixed with 3 mL of 10 mM sodium nitroprusside in phosphate buffer. The resulting solution was then incubated at 25°C for 60 min. A similar procedure was repeated with methanol as a blank and ascorbic acid as a control. After incubation, 5 mL of Griess reagent was added, and absorbance was measured at 540 nm. The percentage inhibition of nitric oxide was compared with the reference control and ascorbic acid.

$$\%\ RSA = \frac{Absorbance\ of\ control - Absorbance\ of\ sample\ \times\ 100}{Absorbance\ of\ control}$$

Determination of reducing power ability

Reducing potential of the extracts was estimated using the method of Oyaizu[25] and Tonisi *et al.*[26] with

Table1 — Percentage yield of *C. religiosa* leaf extract and saponin-rich fraction

	Percentage yield of extracts	
	80% Ethanol crude extract	Saponin-rich fraction
C. religiosa (Leaf)	15.82 %	3.77 %

slight modifications. 1 mL methanolic extract (40 to 200 µg/mL) was mixed with 2.5 mL of (pH 6.6) phosphate buffer and 2.5 mL of (1%) potassium ferricyanide. The mixture was incubated at 50°C in a water bath for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of solution 2.5 mL was mixed with 2.5 mL distilled water and a freshly prepared 0.5 mL of 0.1% ferric chloride solution. The absorbance was measured at 700 nm in a UV spectrometer. Ascorbic acid at various concentrations (10 to 100 µg/mL) was used as a standard.

Statistical analysis

Results were expressed as mean ± SD of three independent experiments in triplicate (n = 3). The correlation coefficient (r) was calculated using the Pearson method in Microsoft Excel. The resulting data were evaluated using One-way Analysis of Variance (One-way ANOVA) to determine the significance of the difference. Results were considered statistically significant for $p < 0.05$.

Results and Discussion

Extraction yield of plant extracts

Aqueous ethanolic extract showed a greater yield than the saponin fraction (Table 1). This can be attributed to its ability to extract polar and non-polar compounds[27]. Mixing alcohols with water increases the dielectric constant of the prepared mixture; thus, it can extract oil and non-oil compounds[28]. This may be the reason for the high yield of aqueous ethanolic extracts.

Phytochemical analysis

Qualitative screening of phytochemicals in the samples reveals that most extracts contain saponins, flavonoids, terpenoids, carbohydrates, and phenols (Table 2). The results agree with previous work by Faheem *et al.*[29]. The presence of alkaloids and carbohydrates in this study may be attributed to the aqueous phase of the plant extract.

Total phenolic content

Phenolic compounds are among the leading chemical classes recognised for their ability to function as natural antioxidants. The study revealed

Table 2 — Phytochemical screening of *Crateva religiosa* leaf extract

Phytochemical test	*C. religiosa* (Leaf)
Frothing test for saponins	+
Test for cardiac glycosides	-
Test for anthraquinones	-
Test for flavonoids	+
Ferric chloride test for tannins	-
Test for alkaloids	+
Test for terpenoids	+
Test for quinines	+
Test for glycoside	-
Test for phenols	+
Test for steroids	+
Test for carbohydrates	+
Test for proteins	-

significant variations in phenolic content across different extracts of *C. religiosa* compared with standard (Fig. S1). The total phenolic content in aqueous ethanolic extract of *C. religiosa* leaf is 17.75±0.0007 mg GAE/g. Faheem *et al.*[29] reported that the phenolic contents of the ethanolic extract of *C. magna* leaf was 23.1±0.7 mg GAE/g, which is higher than our study. However, the data obtained in our study are in good agreement with Mohanapriya *et al.*' s[30] results.

Total flavonoid content

The total flavonoid is higher in 80% ethanolic extract is determined as 136±0.003mg QE/gof in *C. religiosa* leaf compared with Quercetin (Fig. S2). Flavonoid content is similar to the value reported by Mohanapriya *et al.*[30].

Saponin content

C. religiosa leaf has 3.77±0.71 mg% of saponin content. Wagay *et al.*[8] reported that the bark of *C. religiosa* G. Forst. contained 1.28±0.25% saponin, which is consistent with the present study. The saponins identified in *C. religiosa* are glycosides that typically have a triterpenoid or steroidal aglycone (sapogenin) attached to sugar moieties. These compounds contribute to the plant's medicinal properties, including anti-inflammatory, analgesic, diuretic, and hepatoprotective effects[31]. However, related compounds such as triterpenoid saponins and glycosidic saponins have been detected in other parts or

closely allied species, implying that similar saponin structures—triterpene or steroidal types—are likely present in the leaf extract as well. Individual saponin structures like oleanane- and lupane-type saponins are common in the *Capparidaceae* family, to which *C. religiosa* belongs.

DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical used to evaluate the antioxidant ability of extracts. The calibration curve (Fig. S3) was plotted with ascorbic acid as a standard, with an R^2 value of 0.993. Aqueous ethanolic (472.48±0.54 µg/mL) had more DPPH scavenging activity than the saponin fraction (581.68±0.14 µg/mL) (Table 3). Studies reveal that low polar extracts show high DPPH activity, such as dichloromethane (24±1), petroleum ether (54±1.7), and ethyl acetate (88±1.3), which show higher activity than the present study[32]. High polar extracts such as ethanol (84.16±10.72) and water (112.27±17.23). Alcoholic extract of the leaf can scavenge DPPH radicals more efficiently. They contain active scavengers like phenols and flavonoids. Faheem *et al.*[29] reported the IC_{50} value of ethanolic leaf extract (1-200 mg/mL) of *C. magna* as 112.27±17.23 µg/mL, higher activity than the present study. Leaves contain isovitexin, proanthocyanidins, myricetin, phenolic acids, p-hydroxyl benzoic acid, vanillic acid, ferulic acid, and sinapic acid[33].

Metal ion chelating assay

Excessive absorption of metals through several pathways can lead to serious consequences, including irreversible effects. In the current study, metal chelation by bipyridine was performed. Ethylenediaminetetraacetic acid (EDTA) was used as a standard reference, and the calibration curve (Fig. S4) was plotted with an R^2 value of 0.996. The crude extract (27.60±0.31µg/mL) of *C. religiosa* leaf shows comparable activity to the saponin fraction (39.76±1.09 µg/mL) (Table 4). Specific data on the metal ion scavenging activity of *C. religiosa* leaf extract and its saponin fraction is limited, but studies indicate the presence of significant antioxidant potential, implying these extracts can scavenge transition metal ions responsible for oxidative damage. The metal ion scavenging activity of the saponin fraction of *C. religiosa* leaf was first reported. Some saponins can chelate transition metal ions such as Fe^{3+} and Cu^{2+}, thereby preventing these metals from catalysing free radical production. By inhibiting metal-catalysed formation of harmful oxidative species, saponins further enhance reducing capacity[34]. Wagay *et al.*[8] reported the presence of a high concentration of triterpenes, such as lupenone, in the chloroform extract of *C. religiosa* G. Forst. bark, which may be a reason for high metal scavenging activity in less polar extracts.

Superoxide anion scavenging assay

Plant samples show a concentration-dependent increase in the inhibition of superoxide anion. A standard curve (Fig. S5) was plotted using reference standard ascorbic acid with an R^2 value of 0.997. Both the crude extract and the saponin fraction effectively scavenge superoxide anions, helping to neutralise reactive oxygen species involved in oxidative stress. The saponin fraction exhibits high scavenging potential with an IC_{50} value of 7.09±0.39 µg/mL than crude leaf extract (IC_{50} =91.90±0.25) (Table 5). Superoxide scavenging activity of saponin fraction is reported for the first time. From the data in this study, it is clear that saponins from *Crateva* shows an activity in superoxide-mediated diseases such as cardiovascular, inflammatory, diabetic, neurodgenerative diseases etc. Excess superoxide anion free radicals are associated with several diseases, which is due to their role in oxidative stress and cellular damage. Superoxide anion acts as a precursor to other reactive species like peroxynitrite, further amplifying oxidative stress and tissue injury in these diseases[35]. Vijaya *et al.*[36] reported the IC_{50} value of ethanolic bark extract of *C. magna* as 35.22 mg/mL (3522 µg/mL), which showed a lower activity than the current study. Rather than aqueous-alcoholic

Table 3 — IC_{50} values of DPPH scavenging assay of crude and saponin fraction of *C. religiosa* leaf

Extracts	IC_{50} Value (µg/mL)	
	Crude extract	Saponin fraction
C. religiosa leaf	472.48±0.54	581.68 ±0.14

Table 4 — IC_{50} values of metal ion chelating assay of crude and saponin fraction of *C. religiosa* leaf

Extracts	IC_{50} Value (µg/mL)	
	Crude extract	Saponin fraction
C. religiosa leaf	27.60±0.31	39.76±1.09

Table 5 — IC_{50} values of superoxide anion scavenging assay of crude and saponin fraction of *C. religiosa* leaf

Extracts	IC_{50} Value (µg/mL)	
	Crude extract	Saponin fraction
C. religiosa leaf	91.90±0.25	7.09±0.39

extracts, enriched fractions such as saponin fractions can effectively scavenge the superoxide radical generated by NBT reduction.

Nitric oxide ion scavenging assay

The antioxidant activity of plant extracts was determined using a nitric oxide radical scavenging assay. Ascorbic acid was used as a reference standard for plotting the calibration curve (Fig. S6) with an R^2 value of 0.995. The activity of *C. religiosa* leaf extract was not within a detectable range. However, the IC_{50} value of the saponin fraction shows activity in scavenging nitric oxide (71.52±0.57) (Table 6). Polar to non-polar extracts of *C. religiosa* show high activity in scavenging NO ions. These extracts are high in polyphenols such as flavonoids and steroidal triterpenes such as lupeol, drebyssogenin, and lupenone. NO radicals have wide-reaching effects on the nervous, cardiovascular, immune, respiratory, metabolic, and skin systems, with excess production leading to cellular damage and disease progression[37,38,39].

Reducing power assay

The reducing ability of plant extracts was determined and standardised using reference ascorbic acid. The calibration curve (Fig. S7) was plotted with an R^2 value of 0.966. *C. religiosa* saponin fraction showed higher reducing power compared to crude extract. Absorbances of the extracts increased with the concentration (Table 7). A higher absorbance indicated a higher reducing power[36], which supports the current results. This has been supported by the fact that aqueous-alcoholic extracts have the highest phenolic content and are good reducing agents. Fe (III) reduction is often used in this assay as an essential marker of electron-donating activity, an important regulator of phenolic antioxidant action. It is associated with reductones (carbonyl group adjacent to enediol), demonstrating their antioxidant action by donating a hydrogen atom to break the radical chain[40]. The higher reducing ability of the saponin fraction indicates that saponins themselves are potent antioxidants. Fractionation removes other inactive or less active components, thereby distinctly amplifying the antioxidant power compared to the crude extract. This finding highlights the potential of using saponin-rich fractions in developing antioxidant therapies or supplements, as they provide more potent biological activity than crude extracts[41].

Table 6 — IC_{50} values of nitric oxide ion scavenging assay of crude and saponin fraction of *C .religiosa* leaf

Extracts	IC_{50} Value (µg/mL)	
	Crude extract	Saponin fraction
C. religiosa leaf	0.004 (non-detectable)	71.52±0.57

Table 7 — Absorbance values of reducing power assay of crude and saponin fraction of *C. religiosa* leaf

Concentration (µg/mL)	Absorbance (nm)	
	Crude extract	Saponin fraction
10	0.080	0.228
50	0.095	0.381
100	0.122	0.394

Conclusion

Antioxidants inhibit free radicals from interacting with other molecules, which aids in maintaining human health. In the present study, we evaluated the effect of natural antioxidants in crude leaf extract and saponin fraction of *C. religiosa* against free radicals. Results obtained from the study imply that apart from DPPH radical, other radicals are efficiently scavenged by the saponin fraction. The enriched fraction has comparatively more activity than the crude extract. *C. religiosa* has an excellent ability to scavenge metal ions, superoxide anion, and nitric oxide ions, which are relevant in female reproductive health. It is evident that total phenolic, flavonoid, and saponin content significantly affects the antioxidant potential. Antioxidants bind to free radicals, neutralizing them and thus preventing them from causing damage. Moreover, antioxidants help in maintaining and improving female reproductive health by combating oxidative stress and thereby improving oocyte quality, enhancing fertility, balancing hormones, and overall reproductive function. Antioxidants are essential for a healthy lifestyle and can help protect the body from various health problems.

Conflict of interest

The authors declare no conflict of interest.

AI use disclosure

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) as an AI-assisted language tool to improve grammar, sentence structure, and overall readability of the text. The AI tool was not used for experimental design, data generation, data analysis, interpretation of results, preparation of figures, or formulation of scientific conclusions. All AI-assisted content was carefully reviewed and verified by the authors, who take full responsibility for the originality, accuracy, and integrity of the manuscript.

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