

Evaluation of the Antioxidant and Cytotoxic Activities of *Carthamus caeruleus* L. Rhizome Extracts

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ABSTRACT

The genus *Carthamus* is recognized as an essential source of traditional medicine due to its immune-enhancing, antioxidant, and anti-cancer properties. This study aims to compare and determine the antioxidant and cytotoxic activities of the hydromethanolic and chloroformic extracts of the rhizome of *Carthamus caeruleus* L. The antioxidant activity was assessed using three radical scavenging methods, namely 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS^{•+}), nitric oxide scavenging (NO[•]) and non-enzymatic radical scavenging (SO[•]). The potential cytotoxic was evaluated on several tumor cell lines, including gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo2), breast adenocarcinoma (MCF-7), lung carcinoma (NCI-H460), cervical adenocarcinoma (HeLa), and one non-tumor cell line of African green monkey kidney (VERO). The hydromethanolic extract was significantly more potent than the chloroformic extract for both activities evaluated, exhibiting IC₅₀ values of 0.14 ± 0.008, 1.39 ± 0.27, and 0.89 ± 0.074 µg/mL in ABTS^{•+}, NO[•] and SO[•] assays, respectively. As for cytotoxic potential, the hydromethanolic extract was particularly active against the NCI-H460 cells (IC₅₀ value of 177.69 ± 1.90 µg/mL). These results suggest that the hydromethanoic extract of the rhizome of *C. caeruleus* could be used as an antioxidant and cytotoxic agent, supporting its traditional use as a treatment for burns.

Keywords: *Carthamus caeruleus* L.; antioxidant activity; cytotoxic potential; radical scavenging.

INTRODUCTION

The human organism has various endogenous or exogenous antioxidant mechanisms for regulating the redox systems that naturally exist and play important physiological functions. These are essential defenses against free radicals, damaging cells and tissues.¹ Oxidative stress occurs when these mechanisms fail, and the organism cannot defend itself against the oxidative damage caused by reactive oxygen species (ROS) due to an imbalance between the production of these substances and the effectiveness of antioxidants in neutralizing them, resulting in ROS accumulation.² Free radicals are molecular entities that exist independently and possess an unpaired electron in their atomic orbit. These radical substances are volatile and reactive, either donating or accepting an electron, thus functioning as oxidants or reducers.³ Numerous diseases exhibit heightened oxidative stress, including cancer and atherosclerosis, which are among the most common free radical-related diseases. Several scientists have suggested the pivotal involvement of free radicals in carcinogenesis, being known to cause damage in the DNA chains that lead to mutations and ultimately give rise to cancer.⁴ Cancer is one of the leading causes of death worldwide.⁵ The exploitation of natural, synthetic, or biological resources to reverse, delay, or prevent cancer development is emerging as a significant therapeutic strategy in the fight against this disease. This approach, known as cancer chemoprevention, offers considerable hope for reducing cancer incidence and mortality. Studies have revealed promising anti-cancer properties in various medicinal plants, with *Annona muricata* showing potential against tongue and breast cancer, *Cannabis sativa* for colorectal cancer, and *Curcuma longa* offering hope for colorectal, breast, and lung cancers.⁶ *Carthamus caeruleus* L., the genus *Carthamus* within the Asteraceae family, has been employed in traditional medicine to treat burns and skin issues. The biological properties of *Carthamus caeruleus* rhizome are attributed to its diverse phytochemical composition, which includes catechins, gallic tannins, anthraquinones, mucilages, coumarins, steroids, and glycosides.⁷ The *Carthamus* genus comprises 25 species from northwest India westward and around the Mediterranean Sea.^{8,9} Plants belonging to this genus possess various pharmacological properties; for instance, *Carthamus tinctorius* exhibits anticoagulant and antithrombotic effects, antioxidative, anti-aging, anti-fatigue, anti-inflammatory, anti-hepatic fibrosis, and anti-cancer properties.¹⁰ Another example is *Carthamus oxycantha*, used for its anti-scarring and anti-inflammatory purposes.¹¹ The rhizome of *Carthamus caeruleus* is traditionally used by the population for healing and treating burns of varying degrees.¹¹ It is particularly effective for skin problems and burns due to a cream prepared from this rhizome. A study conducted by Benhamou et al.¹² evaluated the healing efficacy of the cream on Wistar rats, in which second-degree burns and wounds were induced. The results showed that applying the rhizome-based cream led to significant healing and regeneration of the burned skin and a notable reduction in wound surface area. After 15 days, the diameter of the wounds in rats treated with the cream had decreased by 85.66%, surpassing the results obtained with reference ointments and products, which showed a reduction of 75.12%. Histopathological examination validated the cream's efficacy by revealing significant epithelial tissue regeneration, indicating optimal scar tissue epithelialization. The antimicrobial properties of *Carthamus caeruleus* rhizomes and leaves were evaluated by Saffedine et al.¹¹ Their research demonstrated that the methanol and ethanol fractions of the rhizomes exhibit more potent antibacterial activity than those of the leaves against the tested microorganisms. The rhizome fractions showed significant activity against *Candida albicans*, *Staphylococcus aureus*, *Bacillus cereus*, and *Acinetobacter bowii*, while the leaf fractions only exhibited effects against *Acinetobacter bowii* and *Staphylococcus aureus*.

Carthamus caeruleus is poorly explored in the scientific literature. The paucity of research on this species underlines the importance of further investigation to better understand its unique characteristics and potential applications. This study investigates the in vitro antioxidant and antitumor capacity of hydromethanolic and chloroformic extracts obtained from *C. caeruleus* rhizomes for the first time. Antioxidant potential was evaluated using ABTS^{•+}, NO[•] and non-enzymatic O₂^{•-} scavenging essays. Additionally, the study investigated the antiproliferative effects of these extracts on human tumor cells and normal cells.

MATERIAL AND METHODS

Plant material

C. caeruleus was collected in May 2023 from Medjadja (36°15'47.9 "N 1°23'50.9" E) region in Chlef, north-western Algeria. The esteemed Professor Saadi Abdelkader of the Faculty of Life and Natural Sciences at Hassiba Benbouali University in Chlef, Algeria, confirmed the botanical identification and classification of the plant, which was a crucial step in our research. The rhizomes of the *C. caeruleus* L. plants were thoroughly rinsed and washed to remove any dirt or debris. After drying naturally, the rhizomes were crushed into a fine powder suitable for extraction.

Extraction procedure

In line with the established method of Wijaya et al.¹³, 100 g of rhizomes were macerated for 24 hours under continuous stirring in 500 ml of each solvent (chloroform or methanol 80%). The resulting extracts were then filtered using Whatman N°. 1, and the solvents were evaporated at 50°C using a rotary evaporator. The resulting extract was stored at four °C until further analysis, ensuring the preservation of its properties.

Assessing the antioxidant potential of the extracts through in vitro assays

ABTS^{•+} scavenging

A solution of ABTS consisting of 7 mM ABTS and 2.45 mM potassium persulfate was prepared to assess the antioxidant potential by dissolving the reagents in distilled water. This solution was then incubated in the dark at room temperature for 12 to 16 hours to allow the generation of the ABTS radical, a stable compound whose ability to decolorize formed the basis of our test. The stock solution was diluted in ethanol to adjust its absorbance to 0.70 ± 0.02 at 734 nm. Then, 100 µL of each concentration of extract or standard (ascorbic acid) was added to 1 ml of diluted ABTS solution. After 20 minutes of incubation in the dark, the absorbance of each solution was measured at 734 nm, and the percentage of inhibition of ABTS^{•+} was determined, following equation 1, and the IC₅₀ was estimated as the concentration of extract/standard able to scavenge ABTS^{•+} in 50%.¹⁴

$$\% \text{ radical scavenging} = \frac{(X-Y)}{X} \times 100 \quad (1)$$

X = absorbance of the control (absence of extract/standard); **Y** = absorbance of the extract/standard.

NO[•] radical scavenging

The extracts were incubated with sodium nitroprusside in phosphate buffer under light for 15 minutes. Griess reagent was added, and the mixture was then incubated in the dark for 10 minutes. The absorbance was measured at 562 nm, and a calibration curve was established by plotting the percentage of inhibition against extract concentrations.¹⁵ The IC₅₀ was determined as the concentration inhibiting 50% of NO[•]. The rate of NO[•] scavenged was calculated using equation 1, where **X** represents the absorbance of the control, and **Y** represents the absorbance of the extract.

Superoxide anion radical scavenging

According to Pereira et al.,¹⁵ 1475 µL of each extract or standard solution was combined with 100 µL of β-NADH (300 µM), 75 µL of NBT (200 µM), and 50 µL of PMS (15 µM). After a 5-minute incubation, the

reaction mixture's absorbance was measured at 560 nm. The $O_2^{\cdot-}$ scavenging activity was then determined using equation (1). Gallic acid served as a standard for comparison.

Cytotoxic potential

Cell Lines

This study used different human tumor cell lines (HeLa-cervical adenocarcinoma, AGS-gastric adenocarcinoma, CaCo2-colorectal adenocarcinoma, MCF-7-breast adenocarcinoma, NCI-H460-lung carcinoma) and non-tumor cell lines (VERO-African green monkey kidney). The ECACC (European Collection of Authenticated Cell Cultures) supplied the cell lines for all experiments.

Except for VERO, all cell lines were cultured in RPMI-1640 medium containing 100 U/mL penicillin, 10% fetal bovine serum, 100 mg/mL streptomycin, and two mM glutamine while VERO cells were grown in a DMEM medium enhanced with glutamine, 10% fetal bovine serum, antibiotics and glutamine.

The cells were cultivated in flasks at 37°C and a carbon dioxide level of 5% in a moist environment. Experiments were commenced solely when the cell confluence reached 70-80%.

The Sulforhodamine B (SRB) colorimetric assay

The SRB assay is widely used to assess cytotoxicity in cellular research, making it the preferred method for cost-effective, high-throughput screening.¹⁶

Each extract concentration (400 – 6.25 µg/mL; 10 µL) was combined with 190 µL of cell suspension from the tested cell lines. All cell lines were tested at a density of 10,000 cells/well except for Vero cells, which were tested at 19,000 cells/well. This mixture was then incubated in 96-well microplates at 37°C and a CO₂ concentration of 5% under a humidified atmosphere. Following the incubation period, the cells were treated with 100 µL of pre-cooled 10% (w/v) trichloroacetic acid (TCA) at four °C for 1 hour to fix them. The plates were then washed with water and dried. Subsequently, 100 µL of 0.057% (w/v) sulforhodamine B (SRB) solution was added and incubated at room temperature for 30 minutes. The plates were washed three times with 1% acetic acid to remove non-binding SRB and allowed to dry. Finally, adherent SRB was solubilized with 200 µL of 10 mM Tris. The absorbance measurement was performed at 540 nm using a Biotek ELX800 microplate reader. The results are expressed in terms of the concentration of extract with the ability to inhibit cell growth by 50% - GI₅₀. As a positive control, ellipticine was used.¹⁷

Statistical analysis

All results were represented as mean value ± standard deviation of three repetitions. The results were analyzed statistically by implementing a one-way ANOVA with complete randomization (Stat Box 6.4) after the NEW-MAN and KEULS tests were used to compare the means of the two groups. The disparity was deemed statistically significant, with a confidence level of 95%. (P<0.05).

RESULTS

Antioxidant activities

As shown in Table 1, the hydromethanolic rhizome extract displayed superior antioxidant activity compared to the chloroform extract in all three tested essays, albeit less active than the standard reference. Regarding the ABTS^{•+} scavenging ability, the rhizome extracts exhibited IC₅₀ values of 0.14 ± 0.01 µg/mL and 0.19 ± 0.04 µg/mL, respectively, while that of ascorbic acid was of 0.003 µg/mL. In the same line, the hydromethanolic extract exhibited higher NO[•] scavenging properties (1.39 ± 0.27 µg/mL) than chloroformic root extract (IC₅₀ of 1.85 ± 0.24 µg/mL), which were approximately 19 to 25 higher than that of the standard compound.

The differences between the two extracts were even more pronounced in their ability to inhibit superoxide radicals, as the chloroformic extract’s effectiveness was about one-third that of the hydromethanolic extract. Compared to gallic acid, the IC₅₀ values of the chloroformic and hydromethanolic extracts were approximately 50-fold and 14-fold higher, respectively.

Samples	ABTS ^{•+}	NO [•]	O ₂ ^{•-}
HMeOH_R	0.14±0.01 ^a	1.39± 0.27 ^a	0.89± 0.07 ^a
Chloro_R	0.19±0.04 ^b	1.85±0.24 ^b	2.93±0.09 ^b
Standard	0.003±0.00 ^c	0.075±0.017 ^c	0.06 ± 0.013 ^c

Hydromethanolic extract of rhizome (HMeOH_R);chloroformic extract of rhizome (Chloro_R). Values expressed as mean ± SD The different letters (a-b) in the same column indicated a significant difference (p<0.05).Standard in ABTS^{•+} and NO[•] was ascorbic acid; Standard in O₂^{•-} test was gallic acid.

Table 1. Assessment of antioxidant potential in hydromethanolic and chloroformic extracts of *C. caeruleus* L. rhizome expressed in IC₅₀ (µg/mL).

Cytotoxic activity

The hydromethanolic extract exhibited significantly higher cytotoxicity against the tested cell lines than the chloroformic extract, which was less effective at suppressing the cell lines. These findings indicate that the hydromethanolic extract has potential as an anti-cancer agent, as it displayed promising cytotoxic activity against multiple cancer cell lines. The extracts showed the most potent activity against NCI-H460 cells, with GI₅₀ values of 177.69 ± 1.90 µg/mL and 210.99 ± 12.76 µg/mL, respectively. The hydromethanolic extract exhibited cytotoxic potential with IC₅₀ values of 189.68 ± 4.83 µg/mL for Caco2 cells, 205.84 ± 10.1 µg/mL for HeLa, 213.83 ± 8.57 µg/mL for AGS cells, 226.51 ± 14.1 µg/mL for MCF7 cells. In comparison, chloroform extract showed GI₅₀ values of 224.76 ± 9.06 µg/mL, 240.96 ± 5.33 µg/mL, 249.55 ± 8.92 µg/mL, 255.02 ± 23.59 µg/mL, and 347.65 ± 5.48 µg/mL for Caco2, AGS, MCF7, HeLa and VERO cells, respectively.

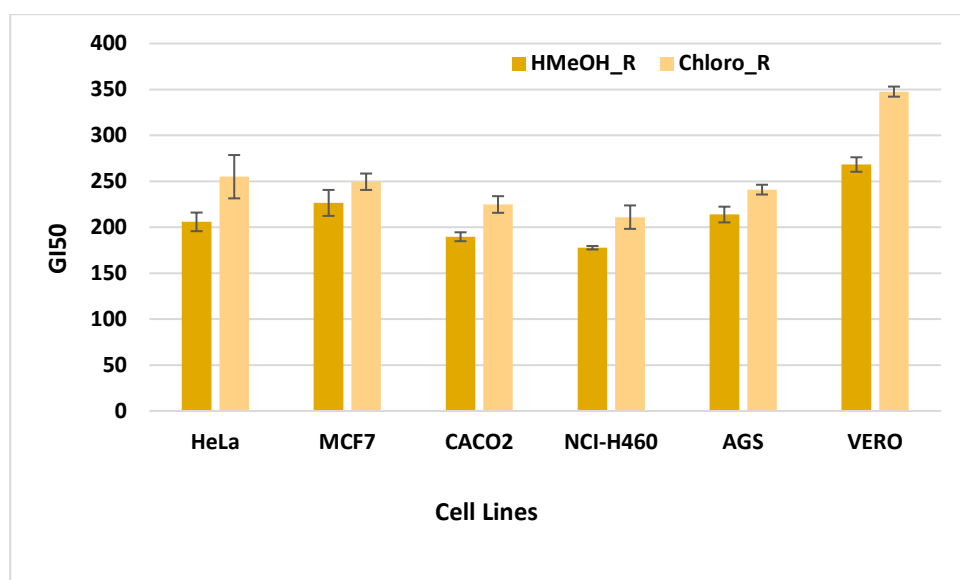


Figure 1. Assessment of the cytotoxic potential of hydromethanolic extract of rhizome (HMeOH_R); chloroformic extract of rhizome (Chloro_R) on tumor cell lines and non-tumor cell lines GI50 (50% of cell proliferation inhibition) in µg/mL. Values expressed as mean ± SD. Ellipticine values in µg/mL - HeLa: 1.01 ± 0.03 ; MCF-7: 1.02 ± 0.02 ; CaCo2: 1.21 ± 0.02 ; AGS: 1.23 ± 0.03 ; VERO: 1.41 ± 0.06 .

DISCUSSION

A redox state imbalance known as oxidative stress, which results in oxidative burden, is a significant biological phenomenon in the progression of cancer.¹⁸ a limited number of studies have been undertaken to investigate these activities in the *Carthamus* genus, primarily focusing on species such as *Carthamus tinctorius*, *Carthamus oxycantha*, and *Carthamus pinnatus*. Our study compared the antioxidant and cytotoxic effects of *C. caeruleus* and revealed a significant difference between the hydromethanolic and chloroformic extracts, with the first demonstrating superior performance in both tasks. The distinct characteristics of the used solvents can account for this difference, as methanol is more efficient in extracting moderately polar and polar phenolic compounds due to its high polarity.¹⁹ Pryzbylski et al.²⁰ revealed that the antioxidant potential of buckwheat extracts varied depending on the solvent's polarity, with the methanolic extract exhibiting relatively higher antioxidant ability. Baghiani et al.²¹ evaluated the antioxidant activity of methanolic and chloroformic fractions using DPPH and β-carotene/linoleic acid-free radical scavenging tests. They observed that the chloroformic fraction demonstrated superior antioxidant activity to the methanolic fraction, with an IC₅₀ of 53.26 µg/mL.

Additionally, they reported an inhibition percentage of $81.008\% \pm 2.03$ for the chloroformic fraction, indicating that it was more effective than the synthetic antioxidant BHT. Our findings differ from those of Baghiani et al.²¹; we found that the antioxidant activity of the methanolic extract was superior to that of the chloroformic extract. Variations in the extraction techniques or study-specific experimental setups could account for this disparity. Toubane et al.²² examined the antioxidant activity using DPPH and found an IC₅₀ value of 4.56 mg/mL for the methanolic extract of rhizome. Our results are lower than those of previous studies. This difference could be attributed to variations in the specific antioxidant assay methods or extraction procedures.

Our findings are inferior to the research conducted on the *Carthamus* genus, where a study was carried out on the two species *C. tinctorius* and *C. pinnatus*, evaluating the antioxidant activity using the ABTS technique. The honey of *C. tinctorius* showed an IC₅₀ value of 81.88 ± 0.54 µg/mL for ABTS radical scavenging activity,²³ while the aerial parts of *C. pinnatus* demonstrated the extract's ability to scavenge 50% of ABTS radicals at a concentration of 0.736 mg/mL.²⁴

Nitric oxide is a relatively stable molecule that reacts with oxygen to form stable products, such as nitrates and nitrites.²⁵ Our results are comparable with those of studies presented by Paramesha et al.²⁶ where the methanolic extract of *C. tinctorius* leaves exhibited significant antioxidant potential, with an IC₅₀ value of 12 µg/mL. Another study on the hydromethanolic extract of *C. tinctorius* showed that at a concentration of 20 µg/mL, it stopped the production of superoxide anion radicals by $74.2 \pm 3.7\%$.²⁷

The antioxidant potential of *Carthamus caeruleus* rhizome could be attributed to its abundance of secondary metabolites, as noted in studies by Ouda et al., Dahmani et al., and Benhamou et al.¹² The rhizome is rich in flavonoids, tannins, saponins, terpenoids, steroids, glycosides, mucilage, and coumarins, which exhibit various pharmacological and medicinal properties, including antiviral, anti-cancer, antioxidant, and antiallergic effects. According to Zhou et al.,²⁸ flavonoids, particularly flavonol glycosides like kaempferol and quercetin, are considered the characteristic and active constituents of *C. tinctorius*. These flavonoids are renowned for their potent antioxidant properties.

Numerous research institutions worldwide are conducting extensive primary screening of plant extracts to find potential anti-cancer agents from plant sources.²⁹ To the best of our knowledge, no prior research has been done on the cytotoxic potential of the species *C. caeruleus*. Previous studies have been carried out on the *Carthamus* genus. The cytotoxic potential of the hydromethanolic extract from the rhizomes of *C. oxycantha* was tested against five cell lines, namely T47D, MDA, MB231, CaCo2, EMT6/P, and VERO, yielding IC₅₀ values (mg/mL) of >5, >5, 2.98 ± 0.008 , >5, and 4.7 ± 0.035 , respectively.³⁰

Based on these results, the hydromethanolic extract from the rhizomes of *C. caeruleus* exhibited higher cell proliferation inhibition than the hydromethanolic extract from the roots of *C. oxycantha*.

Arpornsuwan et al.³¹ showed that dichloromethane, methanol, and hexane extracts of *C. tinctorius* flowers demonstrated efficacy against BHK, Hep2, and SW620 cell lines, with superiority of the dichloromethane extract, displaying IC₅₀ of 0.6, 0.5 and 0.15 mg/mL respectively. An investigation was conducted on the aerial parts of *C. tenuis* against MCF-7, HT-29, PC-3, and CaCo2 cell lines, yielding IC₅₀ values of 25.52 µg/mL, 17.37 µg/mL, 25.77 µg/mL and 24.49 µg/mL, respectively.³²

Our results are lower concerning the cytotoxic potential compared to the study by Rezazadeh et al.³³ where the extract of the aerial parts of *C. tinctorius* exhibited a reduced effect on the MCF-7 breast cancer cell line (173.39 ± 21.76 µg/mL). The hydro-methanolic extract of *C. caeruleus* rhizome exhibits promise against cancer. It inhibits nitric oxide (NO), a molecule crucial for cancer growth.³⁴ Studies by Dahmani et al.³⁵ identified N-hexadecanoic acid (palmitic acid) and mono(2-ethylhexyl) phthalate (MEHP) in the rhizome. These compounds hold potential for treating various conditions, including cancer. N-hexadecanoic acid, a common fatty acid, may exert its anti-cancer effect by interfering with DNA replication, hindering cancer cell proliferation.³⁶ In vivo studies have demonstrated the extract's safety, with Ouda et al.⁷ reporting no toxicity in rats following oral administration of aqueous rhizome extract at doses of 300, 500, and 1000 mg. Similarly, Dahmani et al.³⁵ found no mortality in mice administered hydro-methanolic rhizome extract orally, although a slight decrease in appetite, alertness, and mobility was observed at a high dose of 250 mg/kg. These findings

support the safe use of *C. caeruleus* rhizome for therapeutic purposes and warrant further investigation of its potential as an anti-cancer agent.

CONCLUSIONS

C. caeruleus is a plant known in traditional medicine for treating burns. According to this study, the rhizome of *C. caeruleus* has significant potential to inhibit oxidative stress and halt cell proliferation, particularly its hydromethanolic extract. Further research is needed to understand how its rhizome produces these effects. Studying the chemical composition of the rhizome is crucial to identifying the key components responsible for these actions. In addition, studying potential synergies with other natural compounds and testing its efficacy in vivo are essential steps toward revealing its therapeutic potential in cancer treatment.

Supplementary Materials: The following are available online at www.revistabionatura.com/xxx/s1, Figure1: Assessment of the cytotoxic potential of hydromethanolic extract of rhizome (HMeOH_R); chloroformic extract of rhizome (Chloro_R) on tumor cell lines and non-tumor cell lines GI50 (50% of cell proliferation inhibition) in $\mu\text{g/mL}$. Values expressed as mean $\pm$ SD. Elipticine values in $\mu\text{g/mL}$ - HeLa: 1.01 ± 0.03 ; MCF-7: 1.02 ± 0.02 ; CaCo2: 1.21 ± 0.02 ; AGS: 1.23 ± 0.03 ; VERO: 1.41 ± 0.06 .

Author Contributions: Karima Djafri : Conceptualization; methodology; software; writing; preparation of the original version; formal analysis; investigation; revision and editing. Fatima Zohra Bouarsa: supervision, validation; visualization; writing and editing. Abdellah Noui: co-supervision, validation, visualization; writing and editing. Abdelkader Saadi: Conceptualization; Visualization; Writing; Reviewing and Editing. Susana M. Cardoso: Methodology; Resources; Validation; Data curation. Marcelo D. Catarino: Methodology; Supervision; Reviewing and Editing. Ricardo C. Calhelha : Data curation; resources. Revising the final version of the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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