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Phytochemical characterization and immunomodulatory effects of aqueous and ethanolic extracts and essential oil of Moroccan *Laurus nobilis* L. (Lauraceae) on human neutrophils

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ABSTRACT

Introduction: The leaves of Moroccan bay laurel (*Laurus nobilis* L.) have been used in several forms of extracts to cure rheumatic pain due to their anti-inflammatory properties. Our work aimed to evaluate the effects of aqueous and ethanolic extracts, as well as the essential oil (EO) from laurel, on the microbicidal activity of human neutrophils when compared to the effect of eucalyptol.

Methods: The extracts (ethanolic and aqueous) were subject to phytochemical profiling and high-performance liquid chromatography (HPLC) analyses. The EO obtained by hydrodistillation from laurel was analyzed by gas chromatography-mass spectrometry (GC-MS). The immunomodulatory effects on neutrophil microbicidal activity of the extracts, EO, and eugenol were carried out by 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltétrazolium (MTT) assay.

Results: The phytochemical analysis of the extracts revealed the presence of flavonoids, coumarins, phenols, flavone aglycones, and tannins. HPLC analysis showed the presence of numerous phenolic molecules such as syringic acid, ferulic acid, gallic acid, caffeine, and quercetin. The chemical composition of EO revealed that the major components were eucalyptol (44.14%), α-terpinyl acetate (11.11%), and β-phellandrene (6.74%). Aqueous and ethanolic extracts and EO revealed a significant and dose-dependent ability to inhibit neutrophils microbicidal activity with maximal inhibition at 200 µg/mL concentration with 30.42%, 24.7%, and 38.13%, respectively ($P < 0.001$).

Conclusion: The obtained results revealed the immunomodulatory properties of laurel as a potential natural anti-inflammatory agent that would also allow the development of new anti-inflammatory drugs.

Implication for health policy/practice/research/medical education:
The extracts and essential oil (EO) of bay laurel, as well as eucalyptol, revealed a good potential immunomodulatory effect on human polymorphonuclear neutrophils (PMNs) microbicidal activity. *In vivo* assays are solicited to obtain more evidence for the observed effect. Molecules present in bay laurel extracts and EO might be used as an alternative to drugs to attenuate inflammatory problems.

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Introduction

In the innate immune system, inflammation is the response to damaging stimuli (pathogens, irradiation, damaged cells, or toxic compounds) (1), leading to its elimination and permitting the curative process (2). Many cells are involved in this type of immune response. Polymorphonuclear neutrophils (PMNs) represent 70% of the circulating leukocyte population in the bloodstream.

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They are considered one of the principal effectors of the innate response; they are among the first cells to be present at the inflammatory site and are capable of phagocyte and degrading a wide range of pathogens (3). The microbicidal function of PMNs is ensured by the production of antibacterial oxidants, peptides, and proteases (4). Moreover, major dysfunction of the primary function of these cells by prolonged activation, excessive and unnecessary accumulation, over-activation, or disruption can cause several health problems (3).

Recently, many researchers focused their research on numerous studies on medicinal plants (MP), one of them being an ethnopharmacological investigation permitting the identification of their chemical composition, toxicity, and biological properties leading them to discover novel therapeutic molecules (5). For ages, people have treated inflammatory diseases using MP (6). Research suggests that it is linked to the presence of natural products like polyphenols, terpenes, and tannins (7). It has been found that these biomolecules may regulate the immune response by inhibiting or stimulating cellular signaling pathways of the inflammatory response (8).

Among the Mediterranean countries, Morocco has a particular geographical position granting it a wide strip of very diverse vegetation, in particular MP. In Morocco, the use of MP is a very old practice, and this empirical knowledge has been handed over verbally to generations and has been enriched through the country's strategic approaches. *Laurus nobilis* L. (bay laurel) from the *Lauraceae* family is an aromatic MP located in the Rif of Morocco known for its healing properties (9). It is used in Mediterranean gastronomy (10) for seasoning and folk medicine, in addition to the use of its fruits to relieve and treat many diseases, such as impaired digestion, viral infections, rheumatism, and other health problems (11). Many researchers reported the antimicrobial (12), antioxidant, anticancer (13), anti-inflammatory (14,15), and antifungal (16) effects of bay laurel extracts and essential oil (EO). These activities give bay laurel extracts and EO numerous utilization in various industries. The isolated components from the leaves showed great interest compared to those of the other parts of the plant. The presence of eucalyptol, α-pinene, methyl eugenol, α-terpineol, and β-pinene have been suggested as major compounds in bay laurel EO responsible for the antioxidant, antibacterial, and anti-inflammatory effects (17,18). Despite several studies engaged in the phytochemical analysis of EO and natural products from the extracts of bay laurel collected from different countries and origins, their immunomodulatory effects on human PMNs are rare, and there are no Moroccan species described in the literature. This study aims to evaluate the immunomodulatory effects of aqueous and ethanolic extracts and EO of Moroccan *L. nobilis* on human PMNs microbicidal activity in comparison to eucalyptol.

Material and Methods

Preparation of plant material and extracts

The plant *L. nobilis* was purchased from a local market in Casablanca, Morocco. It was collected during the full flowering period on May 3, 2019 in Taza – Morocco (Latitude 33.938848 and Longitude -4.305174). The identification of the plant was realized as described in our previous work (19). A specimen was deposited under certificate number LN 05062019 for *Laurus nobilis* L. Briefly, the leaves aqueous and ethanolic extracts were prepared respectively, by infusion and maceration of the powdered leaves of *L. nobilis* (50 g each) with 500 mL of the appropriate solvents. The extraction procedure was further continued according to the described scheme in our previous work (19).

Extraction of essential oil

Extraction of EO was realized by hydrodistillation from laurel for 3 hours using a Clevenger-type apparatus. The EO was kept at -20°C in the dark until use.

Phytochemical screening

To identify the different classes of natural products present in *L. nobilis* extracts, a qualitative phytochemical screening was realized according to the methods described in the literature (20,21).

The presence of phenols was confirmed by a purple, green, red, or blue coloration after adding Iron III chloride ($FeCl_3$) to samples. A yellow coloration after adding 1% aluminum chloride ($AlCl_3$) to samples indicates the presence of flavonoids. The red-orange color after adding metallic magnesium and pure hydrochloric acid (HCl) to the heated samples confirms the presence of flavones aglycones. Saponins were detected by the formation of foam persisting for at least 15 minutes after vigorously mixing samples with distilled water. Green and dark blue precipitation after the addition of 3 drops of 1% $FeCl_3$ solution confirm the presence of catechol tannins and gallic tannins, respectively. A yellow coloration after adding 10% NaOH to samples was an indicator of coumarins. Triterpenes and sterols were detected by dissolving dried samples in 0.5 mL of acetic anhydride 0.5 mL of chloroform then 0.5 mL of pure sulphuric acid was added. A red-brown coloration was the triterpenes indicator and green or violet coloration was the sterols indicator.

Determination of total phenolic and flavonoids content

To quantify total phenolic content (TPC) and total flavonoid content (TFC), Folin-Ciocalteu and aluminum colorimetric methods were used, respectively (22,23). Phenol and flavonoid contents were expressed in milligrams per gram of gallic acid (mg GAE/$g_{extract}$) and quercetin (mg of QuE/$g_{extract}$) equivalents, respectively, from calibration curves.

Identification of phenolic compounds by HPLC

The quantitative high-performance liquid chromatography (HPLC) assay of *L. nobilis* extracts was carried out using the HPLC technique as described in our previous work using the same equipment and a fully validated chromatographic procedure (19).

Essential oil analysis

The chemical analysis of EO was realized using GC/MS technique as described in our previous work (19).

PMNs microbicidal activity

The microbicidal activity of PMNs was realized according to methods established by Stevens et al (24).

Isolation of PMNs

To isolate PMNs, human heparinized venous blood was obtained from healthy donors following a protocol approved by the Institutional Review Board for Human Subjects of the National Institute of Allergy and Infectious Diseases (NIAID) (Bethesda, MD, USA). PMNs were isolated as previously described (25). The viability of PMNs was 95% determined by the trypan blue dye method. PMNs were resuspended in RPMI 1640 and then maintained on ice until use.

In vitro treatment of PMNs

Fifteen microliters of extracts, and EO of *L. nobilis* (50, 100, 150, and 200 µg/mL) and eucalyptol (Elevations terpenes) at (5, 10, 15, and 20 µg/mL) were added to 50 µL of PMNs solution (10^7/mL) in RPMI 1640 supplemented with 5% of fetal bovine serum (FBS) and incubated for 30 minutes at 37°C before use. The PMNs viability was not affected by the range of concentration used; however, PMNs viability decreased with concentrations above the aforementioned values. To dissolve EO and eucalyptol, a 0.1% of dimethylsulfoxide (DMSO) solution was used, which did not affect the PMNs function (26).

Colorimetric microbicidal assay

Bacteria (Methicillin-resistant *Staphylococcus aureus* ATCC 43300) were opsonized with 10% of autologous inactivated human serum for 20 minutes at 37°C. Briefly, 50 µL of bacteria (10^8 CFU/mL) were mixed with untreated PMNs (10/1) and let incubated under agitation at 37°C for 1 hour. A control sample containing 50 µL of bacteria and 50 µL of untreated PMNs was incubated in parallel. After incubation, 50 µL of Triton X-100 (0.2 %) was added to lyse PMNs, 50 µL of MTT solution (2 mg/mL) was added in each sample, and then incubated at ambient temperature for 10 minutes. All samples were centrifuged at 1600 g for 5 minutes. After removing the supernatant, 150 µL of DMSO was added to the samples and left incubated at ambient temperature for 10 minutes under shaking. In the end, PBS (50 µL) was added to the samples to completely dissolve the residual formazan. Produced formazan by viable bacteria was quantified at 560 nm. The results were expressed as:

$$\%\ of\ killed\ bacteria\ = 1 - \frac{(OD\ sample) - (OD\ 90\%\ killing)}{(OD\ 0\%\ killing) - (OD\ 90\%\ killing)} \times 90\%$$

Statistical analysis

All experiments were realized in triplicate. Results were presented as mean ± standard deviation. The comparison test was carried out by a one-way ANOVA test followed by Tukey's multiple for multiple groups. Statistical tests were performed by GraphPad Prism 8.0.2. Differences were significant when $P < 0.05$.

Results

Screening analysis

We studied the chemical composition of *L. nobilis* extracts and the results are presented in Table 1. Both extracts contained phenols, flavone aglycones, flavonoids, tannins, and coumarins. The ethanolic extract revealed the presence of saponins while being absent in the aqueous extract and no triterpenes were registered in both extracts.

Total phenolic and flavonoid content

The results of the quantification of the total phenolic and flavonoid content of *L. nobilis* extracts are presented in Table 2. The aqueous extract showed a higher level of TPC than the ethanolic extract with 13.32 ± 1.24 mg and 7.27 ± 1.45 of GAE/g extract, respectively. For TFC level, there was no difference between aqueous and ethanolic extract with 0.63 ± 0.69 mg, and 0.64 ± 0.28 mg of QE/g extract, respectively.

HPLC analysis

The aim of this study was the analysis and determination of biomolecules present in *L. nobilis* extracts using HPLC. The identified molecules by HPLC in the extracts are given in Table 3. In the ethanolic extract, we identified quercetin, cafein, rutin, ferulic, gallic, and syringic acids,

Table 1. Qualitative screening of *Laurus nobilis* extracts

Chemical constituents	*L. nobilis*	
	Ethanolic	Aqueous
Flavonoids	+	+
Coumarins	+	+
Phenols	+	+
Saponins	+	-
Flavones aglycones	+	+
Triterpenes	-	-
Sterols	+	+
Catechic tannins	+	+
Gallic tannins	-	-

– Absence, + Present.

Table 2. Total phenol and flavonoid content of Moroccan *Laurus nobilis* extracts

	Sample	Extraction yield (%)	Phenolic content (mg GAE/$g_{extract}$)	Flavonoid content (mg QuE/$g_{extract}$)
L. nobilis	Ethanolic	17.44	7.27 ± 1.45*	0.64 ± 0.28ns
	Aqueous	18.22	13.32 ± 1.24*	0.63 ± 0.69ns

Results are presented as mean ± standard deviation (n = 3), * $P < 0.001$ Phenolic content of aqueous extract compared to phenolic content of ethanolic extract; ns: $P = 0.08$ Flavonoid content of aqueous extract compared to the flavonoid content of the ethanolic extract. QE: Quercetin equivalent; GAE, Gallic acid equivalent.

while in the aqueous extract we detected rutin, cafein, syringic, gallic and ferulic acids.

Essential oil analysis

GC/MS analysis permitted the identification of 34 compounds representing 92.9% of the total EO composition (Table 4). The major compounds were as follows: eucalyptol (44.14%), α-terpinyl acetate (11.11%), and β-phellandrene (6.74%).

PMNs microbicidal assay

We studied the effect of *L. nobilis* extracts, EO, and eucalyptol on human PMNs microbicidal activity. As shown in Figure 1, pretreatment with extracts, EO, and eucalyptol caused a significant decrease in PMNs function in a dose-dependently ($P < 0.001$). The highest decrease in this activity was obtained at 200 µg/mL with a reduction of 38.13%, 30.42%, and 24.7% for the EO, the aqueous extract and the ethanolic extract, respectively (Figure 1A, B, C). Meanwhile, the use of eucalyptol (20 µg/mL), revealed a higher inhibition than that obtained with EO reaching 44.56% (Figure 1D).

Discussion

According to the literature, degranulation and oxidative burst are the main functions of PMNs to destroy pathogens (26), and also control cellular homeostasis (27). Nevertheless, the immoderate and exorbitant activation of PMNs by a dysregulation of the immune system may

Table 3. HPLC profile of Moroccan *Laurus nobilis* extracts

Components	Retention time (min)	*L. nobilis*	
		Ethanolic	Aqueous
Gallic acid	3.38	+	+
Caffeic acid	8.22	-	-
Catechine	9.10	-	-
Cafein	9.60	+	+
Syringic acid	14.11	+	+
Ferulic acid	19.63	+	+
Coumarin	20.96	-	-
Rutin	23.33	+	+
Vanillin	24.06	-	-
Quercetin	24.18	+	-

Table 4. Chemical constituents of Moroccan *Laurus nobilis* essential oil detected by gas chromatography/mass spectrometry

Components	Kovats index		Area %
	KI Exp[a]	KI Lit[b]	
Benzene	654	655	0.36
2,4-Dimethylhexane	734	732	0.84
Isovaleric acid	827	826	0.03
2-Heptanol	894	895	0.03
Isobutyl isobutyrate	908	905	0.08
α-Thujene	924	922	0.27
α-Pinene	932	930	3.98
Trans-1,2-bis-(1-methylethenyl) cyclobutane	934	933	2.43
Camphene	946	944	0.44
β-Pinene	974	975	3.86
β-Myrcene	988	989	0.45
δ-Carene	1001	1000	0.32
β-Phellandrene	1002	1002	6.74
p-Cymene	1020	1018	2.51
Eucalyptol	1026	1025	44.14
β-cis-Ocimene	1032	1032	0.28
cis-Sabinene hydrate	1041	1040	0.19
γ-Terpinene	1054	1051	0.74
Linalool	1095	1096	3.56
Terpinen-4-ol	1174	1175	2.31
α-Terpineol	1186	1184	1.89
Anisole, p-allyl-	1198	1196	0.14
Nerol	1227	1227	0.18
3,8-Dimethylundecane	1228	1228	0.08
Bornyl acetate	1284	1283	0.46
α-Terpinyl acetate	1346	1345	11.11
2-Dodecanone	1388	1389	0.11
Caryophyllene	1417	1416	0.04
(E) Methyl isoeugenol	1451	1450	2.77
cis-Aconitic anhydride	1524	1523	0.02
10-Oxocyclodec-2-enecarboxylic acid, methyl ester	1680	1679	0.03
1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate	2157	2155	0.16
3-Allyl guaiacol (CAS Number.: 1941-12-4)	2169	2170	0.61
1H-Inden-5-ol, 2,3-dihydro-3-(4-hydroxyphenyl)-1,1,3-trimethyl-	2331	2330	1.72
Total			**92.9**

[a] Experimental linear retention index. [b] Relative retention index in literature.

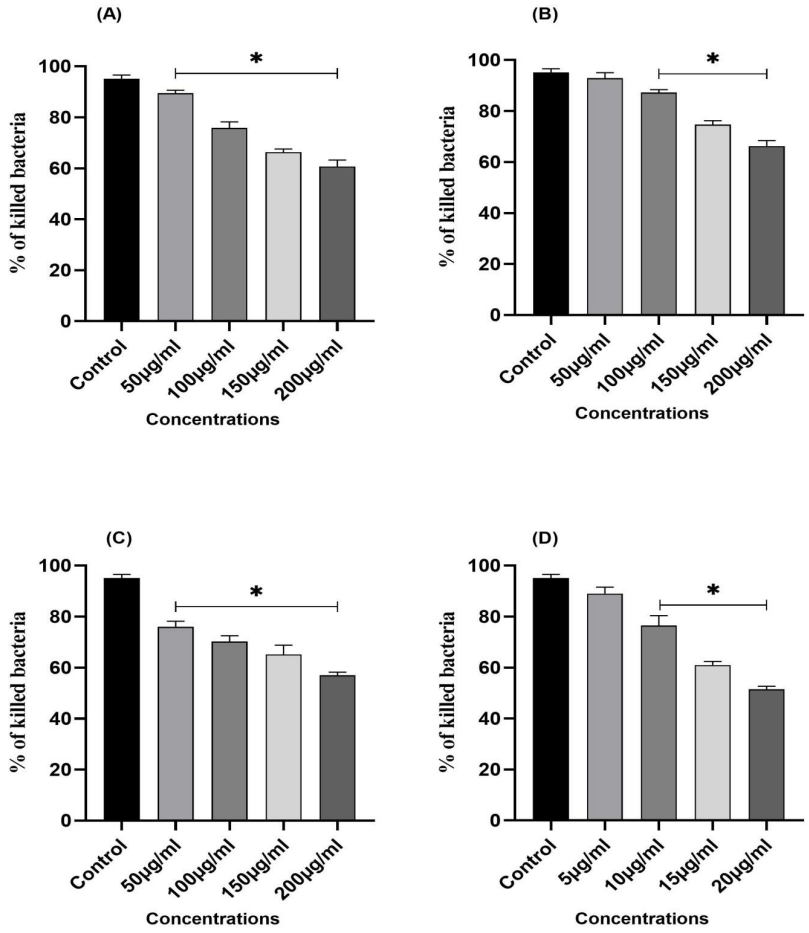

Figure 1. *In vitro* effect of L. nobilis on human PMNs microbicidal activity. (A) aqueous extract, (B) ethanolic extract, (C) essential oil, and (D) eucalyptol. Data are expressed as means ± standard deviation (n = 3) level of the percentage of killed bacteria. * $P < 0.001$ compared to the control group (untreated PMNs) by ordinary one-way ANOVA test.

cause inflammatory diseases and lead to damage to the human biological system (28).

In the current work, we studied the chemical profile of Moroccan *L. nobilis* extracts (aqueous and ethanolic) and we found that they contained flavonoid and phenolic molecules. Previous studies have revealed the attendance of these biomolecules in these extraction forms. Silva et al analyzed the aqueous extract of *L. nobilis* and showed the presence of flavonoid and phenolic molecules (29). Pugazhenti et al identified the composition of Indian laurel and found that both ethanolic and aqueous extracts contained flavonoid and phenolic compounds (30). This is in concordance with our results obtained on the Moroccan specie.

To identify the bioactive molecules of Moroccan *L. nobilis* extracts, an HPLC analysis was done. The results showed the presence of six components in the ethanolic extract and five in the aqueous extract. Many studies have shown the existence of other phenolic molecules. A study realized by Muchuweti et al identified ferulic, caffeic, and vanillic acids in Zimbabwean laurel extract (31). An HPLC analysis of Mexican laurel showed the presence of pyrogallol, resorcinol, gallic, and coumaric acids in ethanolic extract (32). The fact that quercetin is present in the ethanol extract could be related to the solvent and the extraction technique. One can explain its presence in the ethanolic extract by the fact of its solubility in ethanol (33).

Additionally, we were able to fully characterize the components of *L. nobilis* EO with the gas chromatography-mass spectrometry (GC-MS). Thirty-four compounds were identified with eucalyptol (44.14%), α-terpinyl acetate (11.11%), and β-phellandrene (6.74%) as major compounds. Our EO was characterized by a high content of eucalyptol, which was much higher than what was previously mentioned by Fiorini et al (34), who found an amount of 39% and lower than Yalçini et al (35) and Chericoni et al (36), who noticed the presence of eucalyptol at 58.59% and 50%, respectively. For α-terpinyl acetate, Yalçini et al and Chericoni et al reported a low content of 8.8% and 6%, respectively. Fiorini et al found 18% of α-terpinyl acetate, which is higher compared to our findings. Many factors can influence the composition of *L. nobilis* extracts and EO. Solvents, extraction techniques,

species, regions, and the climatic conditions of plant culture can play a role in this observed composition (37).

In this work, we also studied the immunomodulation effects of extracts and EO of *L. nobilis* compared to eucalyptol on human PMNs microbicidal activity. Our data revealed that all extracts, EO, and eucalyptol were significantly able to inhibit the microbicidal function of PMNs ($P<0.001$). According to these results, we can explain this effect with the presence of secondary metabolites that could exert an effect on the main PMNs functions, degranulation, and oxidative burst by interfering with their signaling pathways. During these twenty years, researchers have concentrated their attention on exploring the potential therapeutic and health-promoting effects of secondary metabolites (38). Numerous studies have demonstrated the biological properties of polyphenols as immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial agents (39). In the same context, a higher level of TPC was obtained in the aqueous extract than in the ethanolic, which could explain the difference in the observed immunomodulatory effects on PMNs. According to the literature, plant extracts can inhibit PMNs functions. Bedouhene et al studied the effect of Algerian olive mill wastewater polyphenol extract on PMNs. They reported that the polyphenols exert an inhibition of reactive oxygen species production by PMNs proposing their use as possible anti-inflammatory drugs (40). Denev et al studied the neutrophil-modulating effect of Bulgarian MP: raspberry (*Rubus idaeus*) leaves, meadowsweet (*Filipendula ulmaria*) aerial parts, chokeberry (*Aronia melanocarpa*) leaves, hawthorn (*Crataegus monogyna*) leaves, and blackberry (*Rubus fruticosus*) leaves and found that all six studied extracts blocked ROS production by neutrophils (41).

For EO, we could attribute the effect obtained to the presence of eucalyptol (44.14%). Regardless of using low concentrations of eucalyptol, our study showed an inhibitory effect slightly higher compared to EO which confirms that eucalyptol could be responsible for the suppressed microbicidal activity of PMNs. Eucalyptol is a natural monoterpene known as 1,8-cineole that possesses anti-inflammatory, analgesic, antimicrobial, and antioxidant effects (42). Eucalyptol can exert inhibition on human monocytes and lymphocytes cytokine production by decreasing tumour necrosis factor α and interleukin-1 beta release (43). In this regard, Kim et al found that eucalyptol inhibited the secretion of cytokines, in addition to nitric oxide along with the reduction of nuclear factor-kappa B to the cell number in the inflammatory site (44).

Conclusion

Phytochemical composition is crucial in determining extract effects. Indeed, the high presence of TPC in aqueous extract and eucalyptol in EO of *L. nobilis* showed significant inhibition of microbicidal PMNs activities, suggesting their immunomodulatory effects and their potential use as anti-inflammatory agents. This begs the question with regards to the signaling pathways involved in EO and eucalyptol effects on the main PMNs functions, which can be studied *in vitro*. In this context, more insightful studies are still required to evaluate the effect of EO and eucalyptol on degranulation and oxidative burst of N-formylmethionyl-leucyl-phenylalanine or phorbol 12-myristate 13-acetate (PMA)-stimulated PMNs.

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Author contributions

All authors contributed to the study design, experiments, data analysis, and interpretation. OEF: Conceptualization, writing original draft; SR: Conceptualization, validation; IEK: Conceptualization, formal analysis; AEA: Conceptualization, methodology, data analysis; MD: Conceptualization, methodology; YZ: Conceptualization, visualization; EMM: Conceptualization, supervision.

Conflict of interests

The authors have no conflicts of interest to declare.

Ethical considerations

All authors have inspected the ethical issues of plagiarism, misconduct, data fabrication, falsification, double publication, or redundancy related to the manuscript.

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