frontiers in Marine Science

TYPE Original Research
PUBLISHED 26 May 2026
DOI 10.3389/fmars.2026.1816745

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OPEN ACCESS

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RECEIVED 24 February 2026
REVISED 06 May 2026
ACCEPTED 07 May 2026
PUBLISHED 26 May 2026

CITATION
Molina-Roque L, Acosta F, Izquierdo M, Saromines CJ, Grifoll V, Docando J, Montero D and Torrecillas S (2026) Antioxidant, antibacterial, and immunostimulatory potentials of terrestrial and marine extracts from by-products and low-value biomass: an *ex vivo* study in gilthead seabream (*Sparus aurata*) head kidney leukocytes.
Front. Mar. Sci. 13:1816745.
doi: 10.3389/fmars.2026.1816745

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Antioxidant, antibacterial, and immunostimulatory potentials of terrestrial and marine extracts from by-products and low-value biomass: an *ex vivo* study in gilthead seabream (*Sparus aurata*) head kidney leukocytes

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The expansion of aquaculture and the drive toward more sustainable ingredients have promoted the incorporation of alternative and novel raw materials as alternatives to traditional marine raw materials, which can provide bioactive functions in addition to fulfill fish nutritional requirements. In this context, agro-industrial by-products and low-value marine biomass emerge as promising sources of antibacterial, immunomodulatory, and antioxidant bioactive compounds. Valorizing these raw materials within a circular economy framework offers the dual benefits of reducing waste and improving fish resilience. This study evaluated nine natural extracts of terrestrial and marine origin as potential functional ingredients for aquaculture. Terrestrial by-product extracts (TE) included pomegranate peel (rich in punicalagin or ellagic acid), citrus fruits, and grape seeds, whereas marine included marine macro- and micro-algal extracts (ME) (*Rhodomonas lens*, *Desmodesmus* sp., *Osmundea pinnatifida*, *Gracilaria* sp., and *Dictyota* sp.). Extracts were characterized by determining their total phenolic and flavonoid contents. Antioxidant activity was evaluated using two methods: 2, 2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and Ferric Reducing Antioxidant Power (FRAP) assays. Antibacterial activity was assessed against *Vibrio anguillarum, V. harveyi*, and *Photobacterium damselae* subsp. *piscicida*. To assess the effects of extracts at cellular level, *ex vivo* assays were performed on head kidney leukocytes from gilthead seabream (*Sparus aurata*), evaluating cytotoxicity, respiratory burst, phagocytic activity, and peroxidase activity. TE showed higher levels of both phenolic compounds and flavonoids than ME, which are usually related to higher antioxidant activity. In addition, TE showed stronger antibacterial effects against the three pathogenic bacteria tested. However, ME in general

terms, presented higher immunomodulatory potential, causing respiratory burst activation or higher peroxidase activity in leukocytes. These findings highlight distinct bioactivities depending on extract origin, suggesting that future *in vivo* studies evaluating the combined use of terrestrial and marine extracts may be of interest to explore potential complementary effects in aquaculture species such as *Sparus aurata*.

KEYWORDS

antioxidant activity, aquaculture sustainability, bioactive extracts, functional feed additives, immunomodulation, *Sparus aurata*, zero waste

1 Introduction

Aquaculture plays a fundamental role in food security and sustainable development as it is considered the main source of aquatic dietary protein (Tacon, 2023). Given the increase in global demand for fish due to population growth and increasing *per capita* consumption (FAO, 2024), the industry is moving towards intensifying production (Campanati et al., 2022). However, this intensification may entail significant challenges, such as disease outbreaks, antibiotic resistance, and environmental sustainability (Stentiford et al., 2020). In the pursuit of sustainability in the industry, global aquafeed production has largely shifted over the last two decades from using marine ingredients to plant-based raw materials, resulting in an increased carbon footprint (Glencross et al., 2025) and potential adverse effects on fish health due to fiber fractions, antinutritional factors and amino acid imbalance (Jannathulla et al., 2019). In this context, the use of functional feed additives (FFAs) emerges as a promising strategy, as they can be incorporated into diets and annul the adverse effects associated with antinutrients (Onomu and Okuthe, 2024). Therefore, the development of new ingredients in aquaculture must consider not only nutritional but also economic and environmental criteria (Olesen et al., 2023).

In this sense, the One Health concept, which recognizes the interconnectedness of human, animal, and environmental health, highlights the need for integrated and sustainable approaches, such as the reuse and recovery of terrestrial and marine by-products. These have emerged as a promising strategy, not only as a solution but also as an opportunity, since these natural matrices still contain considerable amounts of bioactive compounds (Schiener et al., 2016; Campos et al., 2020; Santos et al., 2023). This approach aligns with the four pillars defined by the European Animal Feed Federation (FEFAC, 2024) that underpin circular ingredient application: 1) minimizing the use of food-grade resources as feed, 2) minimizing dependence on land use, 3) maximizing the use of locally sourced ingredients, and 4) optimizing the nutritional characteristics of ingredients. Within this circular framework, plant by-products are particularly interesting as they have high nutritional value and low cost and are preferable to animal by-products as they have lower risk of zoonotic transmission (Dawood et al., 2022) and contain diverse bioactive compounds (Sagar et al., 2018) that can benefit the aquaculture industry. For example, antioxidant potential in fruit by-products, e.g. resulting from juice, jam, and canned food production (Dawood et al., 2022), is very significant (Campos et al., 2020; Saleh et al., 2021; Patra et al., 2022), due to the presence of phenolic compounds, flavonoids, anthocyanins, and carotenoids (Agourram et al., 2013; de Moraes Crizel et al., 2016). This antioxidant capacity is mediated by diverse bioactive compounds acting through different antioxidant pathways, including reactive oxygen species scavenging, peroxide degradation, metal ion chelation, and suppression of free radical chain reactions (van Gulcin, 2020). In addition, the benefits also include antitumor, antiviral, and antibacterial activities (Đilas et al., 2009; Reverter et al., 2014), as well as fish immunomodulation (Firmino et al., 2021; Galindo-Villegas et al., 2022). Consistent with these bioactivities, several studies have been conducted in this area, and important improvements have been demonstrated when applying these by-products to aquaculture species, such as enhanced antioxidant status and immune response through pomegranate (*Punica granatum*) peel supplementation in freshwater fish species (Avazeh et al., 2021; Yousefi et al., 2023); or an improvement of fish growth, antioxidant status and immune capacity in marine species when including dietary lemon (*Citrus limon)* (Garcia Beltran et al., 2019) or orange peel (*Citrus sinensis*) (Salem et al., 2019), highlighting the potential of sustainable phytogenic as feed additives to improve fish health and resilience.

Alongside plant by-products, marine by-products offer significant potential for sustainable valorization. The role of marine by-products has evolved over time from being considered a secondary material to an opportunity for value-added products in numerous industries, such as food, biotechnology, and pharmaceutical, due to the presence of compounds with significant bioactive potential, such as high-quality polyunsaturated fatty acids (PUFAs), proteins, peptides, polysaccharides, vitamins, and antioxidants (Mkadem and Kaanane, 2024). Marine seaweeds represent an abundant source of bioactive compounds, and in the case of invasive species, their use is also a way to mitigate the effects they have on the host habitat (Milledge et al., 2016; Vizcaíno et al., 2024). Bioremediation of wastewater using microalgae represents a viable and sustainable strategy due to their high efficiency in nutrient uptake (Lu et al., 2015). This approach not only reduces the environmental footprint of industrial activities but also generates a valuable biomass that can be valorized as a functional feed ingredient, supplying both essential nutrients and bioactive compounds (Han et al., 2019). Among these, fucoxanthin, a phycochemical present in both seaweed and microalgae, has attracted increasing interest due to its reported anti-diabetic, anti-obesity, anti-inflammatory, antioxidant, and anticarcinogenic properties (Peng et al., 2011; Mikami and Hosokawa, 2013; Afonso et al., 2019). The inclusion of *Gracilaria*

gracilis by-products has been shown to improve the inflammatory and innate humoral responses in *S. aurata* (Silva-Brito et al., 2022).

Despite the growing interest in both terrestrial and marine by-products, studies directly comparing their bioactivities within the same experimental framework remain limited. Therefore, a comparative evaluation of these two sources may provide valuable insights into their potential as functional ingredients in aquaculture.

In this context, the present study aimed to evaluate the potential of nine extracts from terrestrial by-products and marine biomass as functional ingredients in the diet of gilthead seabream (*Sparus aurata*). Due to their distinct chemical composition and origin, terrestrial and marine extracts are expected to exhibit different bioactive profiles, potentially offering complementary functional applications in aquaculture. In alignment with the 3Rs principle of replacing, reducing, and refining the use of animals in research, this study employed new approach methodologies (NAMs; EchA, 2016). Specifically, *in vitro* and *ex vivo* techniques were performed to evaluate their cytotoxic and immunomodulatory effects, as well as their antibacterial and antioxidant potential.

2 Material and methods

Extract selection was based on a combination of factors, including their reported bioactive compound content, documented functional properties (e.g., antioxidant, antibacterial, or immunomodulatory effects), and industrial relevance, such as availability as by-products and potential for large-scale production. A total of nine extracts were selected to represent a diverse set of candidates for preliminary screening. These included four extracts of terrestrial origin and five extracts of marine origin. Terrestrial extracts (TE) comprised two pomegranate (*Punica granatum*) peel, one rich in punicalagin (PG) and the other rich in ellagic acid (EA), a citrus flavonoid-rich extract (CF), and a grape seed extract (GS). Marine extracts (ME) were obtained from *Rhodomonas lens* (RH), *Osmundea pinnatifida* (OS), *Gracilaria* sp. (GR) two times and *Desmodesmus* sp. (DE) and *Dictyota* sp. (DI). Extracts/raw materials were kindly provided by DELACON (Engerwitzdorf, Austria), ANFACO (Vigo, Spain) and PTAQUA (Dublin, Ireland).

2.1 Extracts pressurized liquid extraction

The extraction procedures were provided by external partners and supplied as final products. The extraction of raw materials was performed using an accelerated solvent extractor (SpeedExtractor E-914; BÜCHI, Flawil, Switzerland). To ensure sample retention and prevent cross-contamination, cellulose acetate filters (BÜCHI, Flawil, Switzerland) were positioned at the top and bottom of each extraction cell. Extractions were conducted in triplicate using 120 mL cells at a constant pressure of 150 bar and a temperature of 60 °C. Two distinct solvent systems were evaluated: a hydroalcoholic mixture (ethanol:water, 50:50 v/v) and absolute ethanol (100%). The procedure consisted of three consecutive cycles, each comprising a 5-min heating/static holding phase, followed by an 8-min discharge. To ensure complete recovery, the system was flushed with the respective solvent and purged with nitrogen (N_2). The resulting extracts were collected and concentrated to dryness using a Syncore Plus Analyst (BÜCHI, Flawil, Switzerland). Evaporation was performed under reduced pressure at 40 °C with an oscillation speed of 250 rpm to prevent thermal degradation of the compounds. Finally, the dried extracts were stored at 4 °C until further analysis.

2.2 Characterization of extracts

Total phenolic (TPC) and flavonoid (TFC) contents were determined using commercial colorimetric kits (Abcam ab273293 and Novus NBP3-25916, respectively), according to the manufacturer's protocols. Absorbance was measured at 480 nm (TPC) and 510 nm (TFC) using a multiscan spectrophotometer (Multiskan FC, Thermo Fisher). Preliminary serial dilutions (20, 10, 5, and 1 mg/mL) were tested, and a concentration of 5 mg/mL was selected for analysis, as it fell within the linear range of the calibration curves for both assays. Results were normalized according to the amount of extract present in each reaction and expressed as moles of catechin equivalents per 100 mg of extract (TPC) and mg of flavonoids per 100 mg of extract (TFC).

2.3 Antioxidant capacity of extracts

Extract concentrations were selected following the same criteria described above. The antioxidant capacity of the extracts was determined using two complementary assays: ABTS and FRAP. The ABTS assay is based on radical scavenging activity and was performed using a Total Antioxidant Capacity (T-AOC) colorimetric Assay Kit (ABTS, Enzyme Method) (Ref. EEA023; Invitrogen™). The FRAP assay is based on the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) and was carried out using a Ferric Antioxidant Status Detection Kit (Ref. EIAFECL2, Invitrogen™). All assays were conducted according to the manufacturers' instructions. Absorbance was measured using a multiscan spectrophotometer (Multiskan FC, Thermo Fisher). Results were normalized according to the amount of extract present in each reaction and expressed as moles of Trolox equivalents per 100 mg of extract (ABTS) and moles of $FeCl_2$ equivalents per 100 mg of extract (FRAP).

2.4 Antibacterial capacity of extracts

2.4.1 Minimum inhibitory concentration assay

The MIC of the extracts against *Photobacterium damselae* subsp. *piscicida* (IRTA-23-66), *Vibrio anguillarum* (IRTA-22-11), and *V. harveyi* (IRTA-23-57) was determined using the broth microdilution method (EUCAST, 2003). Bacterial strains were obtained from the IRTA culture collection (Institute of Agrifood Research and Technology, La Ràpita, Spain), where they are routinely maintained, phenotypically characterized, and used in aquaculture pathogen research. Bacteria were recovered from stock cultures on tryptic soy agar (TSA; Scharlab S.L., Spain) and incubated for 24h at 23°C for *V. anguilarum* and *V. harveyi* and 48h at 26°C for *Photobacterium*. Subsequently, the inoculum was prepared in tryptic soy broth (TSB) with 1% NaCl (w/v) for 24h at the optimal temperature for each strain (23–26 °C) and adjusted to

10^5 CFU/mL. Extracts were prepared in Milli-Q water using a 1:2 serial dilution from 48 to 0.047 mg/mL, covering a wide concentration range, and were pre-incubated for 1 h at 45 °C when required. Bacterial suspensions with extracts were incubated in 96-well plates at the optimal temperature for each strain (23–26 ° C) under orbital agitation for 48 h. Growth was assessed by absorbance at 550 nm (INFINIT M200, TECAN Iberia, Spain), and the MIC was defined as the lowest concentration that completely inhibited growth. As MIC represents a threshold value, results are reported as single values. All assays were performed in triplicate to ensure reproducibility.

2.4.2 Minimum bactericidal concentration assay

The MBC was determined for bacteria that showed growth inhibition in the MIC assay. After 48h, 100 µl samples were collected from the MIC, plated on TSA agar plates supplemented with NaCl, and incubated at 23-26°C for 48h, depending on the bacterial strain. The MBC was defined as the lowest extract concentration at which no bacterial colonies were recovered.

2.5 Cell exposure

2.5.1 Isolation of head kidney leukocytes

Six gilthead seabreams (mean body weight: 100 g) were obtained from the University Institute ECOAQUA facilities (Canary Islands, Spain). Fish were maintained under standard rearing conditions in 500 L tanks under a flow-through seawater system with continuous aeration, a natural 12:12 h light:dark photoperiod, and fed *ad libitum* a commercial diet (Skretting, Burgos, Spain). Fish were randomly selected, slaughtered by overdose of anesthetic (clove oil, 2.5 mL/L) and bled from the caudal vein to avoid excessive tissue contamination with erythrocytes. Head–kidney leukocytes (HKLs) were isolated as previously described (Secombes, 1990; Román et al., 2015). Cell viability was determined by trypan blue staining, counted in a Neubauer chamber and adjusted to 10^6 viable cells/mL in L-15 medium supplemented with 1 penicillin/streptomycin (P/S; Ref. L0018, Biowest) and 5% Fetal Bovine Serum (FBS; Ref. S1810, Biowest). Head kidney leukocytes obtained from the six fish were pooled to obtain a sufficient number of cells for all assays. Pooling was performed to minimize inter-individual variability related to factors such as physiological status, stress, or feeding condition, ensuring a more homogeneous cell population.

2.5.2 Cell incubation

Serial dilutions of extracts were prepared from 96 mg/mL stock solutions in L-15 medium (1% P/S, 5% FBS) to final concentrations of 0 (control), 0.01, 0.1, 1, 10, and 100 µg/mL. This concentration range was selected based on previous studies using head kidney leukocytes from *Sparus aurata* and similar immunological assays (Campos-Sánchez et al., 2022), encompassing a broad range of concentrations to evaluate dose-dependent responses, including levels at which cytotoxic effects were observed. HKL were seeded (50 µL, 50, 000 cells/well) in sterile 96-wells white plates (Ref. 354620, Corning) and exposed to 50 µL of each dilution in triplicate wells (technical replicates) and then incubated for 24 h at 25 °C with agitation (100 rpm). This cell concentration was previously determined to obtain satisfactory luminescence and absorbance values.

2.5.3 Cytotoxicity assay

The cytotoxic effects of the extracts were assessed by ATP quantification using the Luminescent ATP Detection Assay Kit (Ref. ab113849; Abcam), according to the manufacturer's protocol. Luminescence levels were measured in a SpectraMax® Mini (Molecular Devices). The results are expressed as the means of relative light units (RLU) in triplicate wells.

2.5.4 Respiratory burst activity

Respiratory burst activity was assessed using the chemiluminescence protocol described by Campos-Sánchez et al. (2022), based on the method of Bayne and Levy (1991). After 24 h of incubation, 100 µL of HBSS (Hank's Balanced Salt Solution, Ref. 21-022-CV; Corning) with 1 µg/mL phorbol myristate acetate (PMA; Ref. 524400, Sigma-Aldrich) and 10^-4 M luminol (Ref. 123072, Sigma-Aldrich) were added to triplicate wells. In addition, for each extract and concentration, triplicate wells were performed without PMA to check the basal levels of reactive oxygen species (ROS). Plates were shaken (10 s), and luminescence kinetics recorded every 2 min for 40 min (SpectraMax Mini, Molecular Devices), expressed as mean maximum relative light units (RLU).

2.5.5 Phagocytic activity

In this assay, only macrophages were used. For this purpose, aliquots of the cell suspension (50 µL, 50, 000 cells/well) were incubated in sterile 96-wells transparent plates for two hours, as adherent cells have been previously characterized as macrophages (Mulero et al., 1998). After the fixation of macrophages, the wells were carefully washed with PBS and the 24 h incubation with the extracts was performed as explained above. The phagocytic activity was determined by a commercial kit, CytoSelect™ 96-Well Phagocytosis Assay (Cell Biolabs Inc.; CBA-224), following the manufacturer's protocol. Absorbance was read at 405 nm using a multiscan spectrophotometer (Multiskan FC, Thermo Fisher), and results are expressed as optical density (OD) at this wavelength.

2.5.6 Peroxidase activity

The HKLs peroxidase activity was measured according to the method of Quade and Roth (1997). Briefly, from 24h incubation plate, 5 µL was taken from each well (2, 500 cells) and pipetted into a transparent 96-well plate. The cells were lysed with 50 µL of 0.02% cetyltrimethylammonium bromide (CTAB, Ref. H9151; Sigma-Aldrich) and shaken for 10 min at 100 rpm in the dark. Next, 100 µL of TMB solution (Ref. 613548; Sigma-Aldrich) was added. The reaction was stopped after 2 min by adding 50 µL of 2M H_2SO_4 (Ref. 20685.295, VWR Chemicals). Absorbance was measured at 450 nm using a multiscan spectrophotometer (Multiskan FC, Thermo Fisher), and results are expressed as optical density (OD) at this wavelength.

2.6 Statistical analysis

Data are presented as means ± standard deviation (SD) from triplicates. Differences among treatments were analyzed by one-way ANOVA after confirming normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe); Tukey's *post-hoc* test was applied for parameters with significant effects ($p < 0.05$). Correlation analyses between extract concentration and peroxidase activity were evaluated using Spearman's rank correlation coefficient (ρ), since OS extract showed a non-normal distribution (assessed by the Shapiro–Wilk test), and applying the same correlation method across all extracts ensured methodological consistency. Statistical significance was set at $p < 0.05$, and exact p-values are reported where appropriate. GraphPad Prism 9.0 (GraphPad Software Inc.) was used for all statistical analyses.

3 Results

3.1 Characterization of extracts

The phenolic and flavonoid contents of terrestrial and marine extracts are presented in Table 1. Among terrestrial extracts, concentrations of phenolic compounds were higher in GS and CF than in PG and EA ($p < 0.05$). GS exhibited the highest total flavonoid content, while PG and EA showed comparable intermediate levels, and CF had the lowest.

In marine extracts, phenolic content varied significantly ($p < 0.05$), with DE and DI showing the highest values, whereas GR, OS, and RH presented comparatively lower levels. Total flavonoid content followed a similar trend, although RH exhibited higher values than OS and GR.

3.2 Antioxidant capacity

Antioxidant capacity determined by ABTS and FRAP assays is shown in Table 1, where exact p-values are also provided.

For TE, antioxidant capacity measured by the ABTS assay varied significantly ($p < 0.05$), with GS showing the highest values, followed by CF, while PG and EA exhibited lower and comparable levels. In contrast, the FRAP assay showed an opposite pattern, with PG and EA presenting higher values than CF and GS.

For ME, ABTS results showed significant differences ($p < 0.05$), with RH exhibiting the highest antioxidant capacity, followed by DE and DI, whereas GR and OS showed lower values. Similarly, FRAP results indicated that RH had the highest antioxidant capacity, followed by DE and DI, with OS and GR presenting the lowest values.

TABLE 1 Characterization, antioxidant and antibacterial capacity of 4 terrestrial extracts (PG, EA, CF and GS) and 5 marine extracts (RH, OS, GR, DE and DI).

Characterization of extracts	Terrestrial				*p*	Marine					*p*
	PG	EA	CF	GS		RH	OS	GR	DE	DI	
Phenolic content (mol Catechin Equivalents/100 mg extract)	0.263 ± 0.002^{b}	0.267 ± 0.004^{b}	0.352 ± 0.002^{a}	0.350 ± 0.005^{a}	<0.0001	0.018 ± 0.001^{d}	0.022 ± 0.001^{cd}	0.025 ± 0.001^{c}	0.052 ± 0.002^{a}	0.038 ± 0.001^{b}	<0.0001
Flavonoids content (mg flavonoids/100 mg extract)	17.42 ± 0.52^{b}	18.58 ± 0.69^{b}	9.69 ± 0.30^{c}	22.95 ± 0.25^{a}	<0.0001	0.468 ± 0.085^{c}	0.299 ± 0.006^{d}	0.219 ± 0.004^{d}	1.286 ± 0.051^{a}	1.061 ± 0.009^{b}	<0.0001
Antioxidant capacity											
ABTS (mol Trolox Equivalents/100 mg extract)	2.078 ± 0.003^{c}	2.077 ± 0.002^{c}	2.205 ± 0.007^{b}	2.250 ± 0.002^{a}	<0.0001	0.422 ± 0.018^{a}	0.149 ± 0.001^{c}	0.155 ± 0.009^{c}	0.343 ± 0.014^{b}	0.301 ± 0.007^{b}	<0.0001
FRAP (mol $FeCl_2$ Equivalents/100 mg extract)	1.782 ± 0.027^{a}	1.821 ± 0.046^{a}	1.514 ± 0.005^{b}	1.301 ± 0.041^{c}	0.0003	0.414 ± 0.001^{a}	0.130 ± 0.001^{d}	0.099 ± 0.001^{e}	0.292 ± 0.005^{b}	0.261 ± 0.002^{c}	<0.0001
Antibacterial capacity											
MIC (mg/mL)											
Vibrio anguillarum (IRTA-22-11)	0.094	0.188	1.5	3		>48	>24	>48	>24	>48	
Vibrio harveyi (IRTA-23-57)	6	0.375	6	12		>48	>24	>48	24	>48	
Photobacterium damselae (IRTA-23-66)	0.047	0.047	0.375	1.5		>48	>48	>48	12	48	
MBC (mg/mL)											
Vibrio anguillarum (IRTA-22-11)	0.188	0.188	3	3		>48	>48	>48	>48	>48	
Vibrio harveyi (IRTA-23-57)	12	6	6	12		>48	>48	>48	>48	>48	
Photobacterium damselae (IRTA-23-66)	0.094	0.047	0.375	6		>48	>48	>48	24	>48	

Pomegranate extract rich in punicalagin (PG), pomegranate extract rich in ellagic acid (EA), extract rich in citrus flavonoids (CF) and grape seed extract (GS). *Rhodomonas lens* extract (RH), *Osmundea pinnatifida* extract (OS), *Gracilaria* sp. extract (GR), *Desmodesmus* sp. extract (DE) and *Dictyota* sp. extract (DI). Data are the mean ± SD of triplicate wells. Different superscript letters in each row indicate significant differences after one-way ANOVA and Tukey test ($p < 0.05$).

3.3 Antibacterial capacity

MIC and MBC values against the tested bacterial strains are summarized in Table 1.

For terrestrial extracts, PG and EA consistently showed the strongest antibacterial activity, exhibiting the lowest MIC values across the three bacterial species. CF displayed intermediate effectiveness, whereas GS showed the lowest inhibitory activity. This pattern was generally consistent across *V. anguillarum*, *V. harveyi*, and *P. damselae* subsp. *piscicida*, although some similarities in MIC values among certain extracts were observed depending on the bacterial species (e.g., PG and CF in *V. harveyi*). MBC values generally followed the same trend, with PG and EA requiring lower concentrations to achieve bactericidal effects compared to CF and GS, although minor variations among bacterial species were observed.

For marine extracts, OS and DE showed comparatively higher antibacterial activity against *V. anguillarum* and *V. harveyi*, whereas RH, GR, and DI exhibited lower effectiveness. In the case of *P. damselae* subsp. *piscicida*, DE showed the strongest inhibitory activity among marine extracts. MBC values for marine extracts were largely similar across extracts and bacterial species, although DE showed slightly higher bactericidal effectiveness against *P. damselae* subsp. *piscicida*.

3.4 Cell exposure

3.4.1 Cytotoxicity assay

Cytotoxicity increased dose-dependently across extracts (Figures 1, 2). Regarding TE-induced cytotoxicity, significant effects were observed only at 100 µg/mL for PG and GS (both $p < 0.0001$; Figures 1A, D), from 10 µg/mL for CF ($p = 0.0006$; Figure 1C), and from as low as 1 µg/mL for EA ($p < 0.0001$; Figure 1B). Regarding ME-induced cytotoxicity, no significant effects were observed for RH or OS at any of the tested concentrations ($p = 0.1347$ and $p = 0.3716$, respectively; Figures 2A, B). In contrast, DE and DI induced significant cytotoxic effects at the highest concentration tested (100 µg/mL; $p < 0.0001$ and $p = 0.0297$, respectively; Figures 2D, E), whereas GR showed significant cytotoxicity from as low as 0.01 µg/mL ($p < 0.0001$; Figure 2C).

3.4.2 Respiratory burst activity

Respiratory burst results for terrestrial and marine extracts are shown in Figures 3, 4, respectively. TE tended to reduce respiratory burst activity in a dose-dependent manner, with significant effects observed at higher concentrations. Specifically, PG, CF, and GS

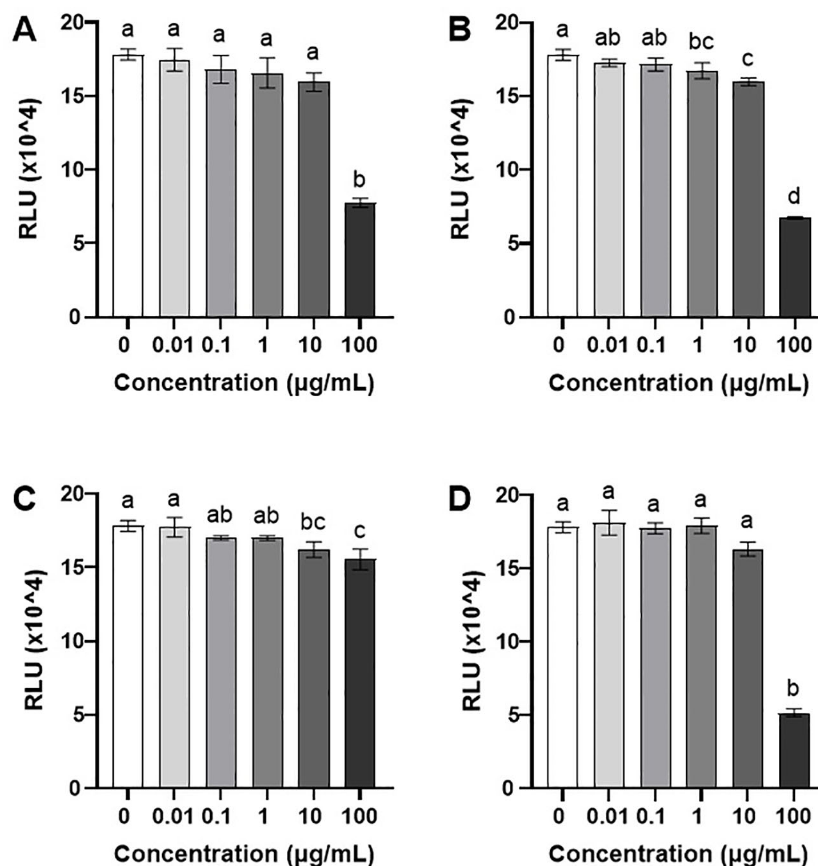

FIGURE 1
ATP levels (expressed as RLU, Relative Light Units) as a cytotoxicity indicator in *Sparus aurata* head kidney leukocytes exposed to 4 terrestrial origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. Pomegranate extract rich in punicalagin (PG, **A**), pomegranate extract rich in ellagic acid (EA, **B**), extract rich in citrus flavonoids (CF, **C**) and grape seed extract (GS, **D**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; p < 0.05).

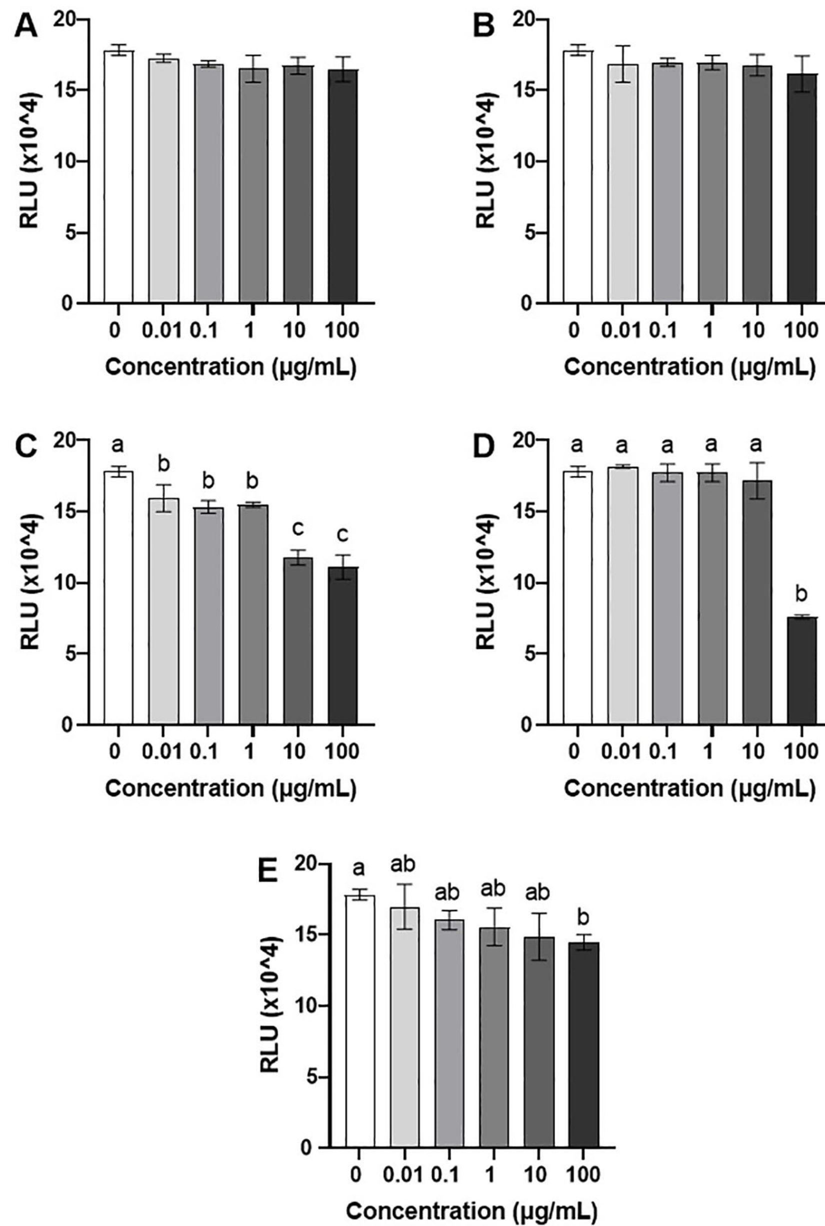

FIGURE 2
ATP levels (expressed as RLU, Relative Light Units) as a cytotoxicity indicator in *Sparus aurata* head kidney leukocytes exposed to 5 marine origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. *Rhodomonas lens* extract (RH, **A**), *Osmundea pinnatifida* extract (OS, **B**), *Gracilaria* sp. extract (GR, **C**), *Desmodesmus* sp. extract (DE, **D**) and *Dictyota* sp. extract (DI, **E**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; p < 0.05).

reduced respiratory burst activity from 10 µg/mL (all $p < 0.0001$; Figures 3A, C, D), whereas the EA extract induced a reduction from as low as 1 µg/mL ($p < 0.0001$; Figure 3B). For ME, respiratory burst activity decreased with OS, DE, and DI only at the highest dose of extract (all $p < 0.0001$; Figures 4B, D, E). In contrast, RH induced a significant decrease across all tested doses ($p = 0.0012$; Figure 4A), while GR showed a biphasic response, increasing from 0.1 µg/mL and decreasing at 100 µg/mL (both $p < 0.0001$; Figure 4C).

Basal ROS levels (without PMA stimulation) remained unaltered by TE (all $p > 0.05$; Figure 5). For ME (Figures 6), OS and GR dose-dependently increased basal ROS, reaching significance at 100 µg/mL ($p = 0.0070$ and $p = 0.0146$, respectively.

3.4.3 Phagocytic activity

Phagocytosis responses to TE and ME are shown in Figures 7, 8, respectively. For terrestrial extracts, PG and CF biphasically stimulated phagocytic activity up to 10 µg/mL and then returned to baseline (both $p < 0.0001$; Figures 7A, C), whereas EA and GS progressively inhibited phagocytic activity from 10 µg/mL (both $p < 0.0001$; Figures 7B, D). In the case of marine extracts, DI and GR did not affect the phagocytic capacity (Figures 8C, E), whereas OS and DE progressively inhibited phagocytic activity from 10 µg/mL (both $p < 0.0001$; Figures 8B, D). In contrast, RH dose-dependently stimulated phagocytosis, reaching a significant peak at 100 µg/mL ($p < 0.0001$; Figure 8A).

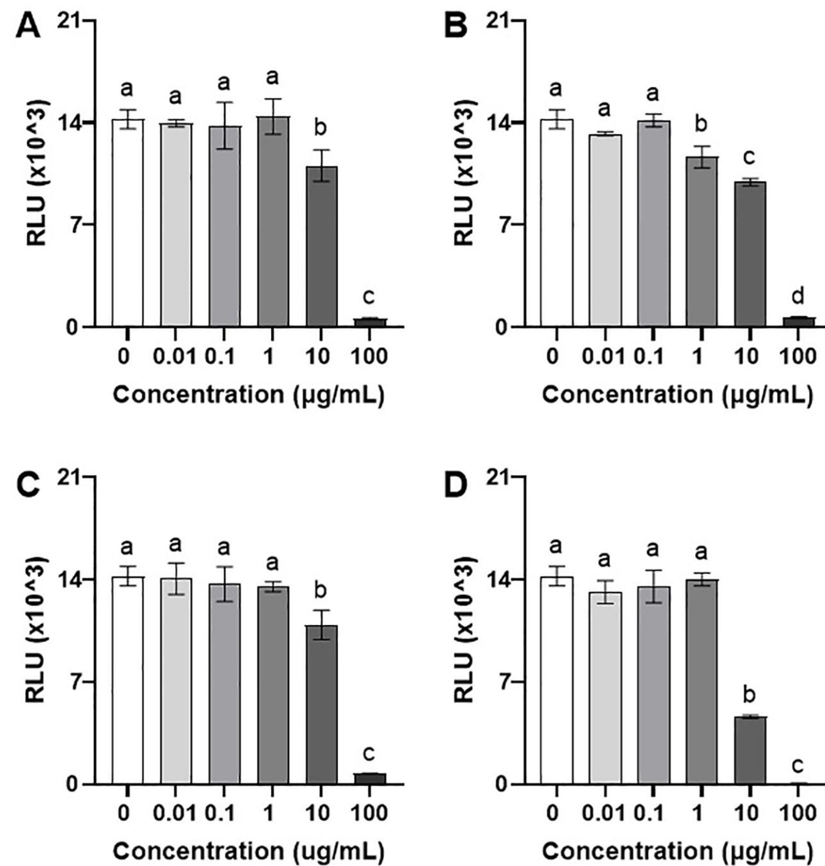

FIGURE 3
Respiratory burst (expressed as RLU, Relative Light Units) of *Sparus aurata* head kidney leukocytes exposed to 4 terrestrial origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. Pomegranate extract rich in punicalagin (PG, **A**), pomegranate extract rich in ellagic acid (EA, **B**), extract rich in citrus flavonoids (CF, **C**) and grape seed extract (GS, **D**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; p < 0.05).

3.4.4 Peroxidase activity

Peroxidase activity after exposure to TE and ME is shown in Figures 9, 10, respectively. All TE reduced peroxidase activity in a dose-dependent manner (PG, EA, and GS: $p < 0.0001$; CF: $p = 0.0014$; Figure 9). Significant reductions were observed from 0.01 µg/mL for PG and EA, 0.1 µg/mL for GS, and 10 µg/mL for CF. In contrast, most ME increased peroxidase activity proportionally with concentration (all $p < 0.0001$; Figure 10), with significant effects from 10 µg/mL for OS, DE, and DI, and from 1 µg/mL for GR. RH showed the opposite trend, reducing peroxidase activity from 10 µg/mL ($p = 0.0189$; Figure 10A). Consistently, Spearman correlation analysis revealed significant negative correlations between extract concentration and peroxidase activity for all terrestrial extracts ($\rho < 0$; $p < 0.05$), while marine extracts displayed significant positive correlations ($\rho > 0$; $p < 0.05$) except for RH, which showed a significant negative correlation ($\rho < 0$; $p = 0.03$). No correlation was detected for OS ($\rho > 0$; $p = 0.24$).

4 Discussion

Natural products from terrestrial and marine sources differ markedly in their chemical composition and structural features, as evidenced by cheminformatic analyses reported by Shang et al. (2018), which highlight the generally larger molecular size and lower solubility of marine-derived compounds. Moreover, marine natural products tend to be enriched in aliphatic chains rather than aromatic rings, a characteristic that may directly influence the prevalence of phenolic compounds and, consequently, their biological activity.

In line with these structural differences, in our study, extracts from terrestrial by-products showed a higher relative content of both phenols and flavonoids compared to those from marine origin. Among TE, a higher phenolic content was observed in both CF and GS. However, this does not necessarily correlate with a higher flavonoid content, in agreement with previous studies (Asem et al., 2020). Indeed, our results confirm a positive correlation between total phenolic content and ABTS antioxidant capacity, consistent with previous findings (Dudonne et al., 2009). Nevertheless, no positive correlation between total phenolic content and FRAP antioxidant capacity was observed, likely due to mechanistic differences: ABTS measures radical scavenging, whereas FRAP assesses ferric reducing power. For instance, flavonoid antioxidant activity is closely associated with their ability to chelate prooxidant metal ions (Cherrak et al., 2016; Kejík et al., 2021), suggesting that FRAP results may be directly affected by the relative flavonoid content rather than by the total phenolic content. Regarding ME, a general

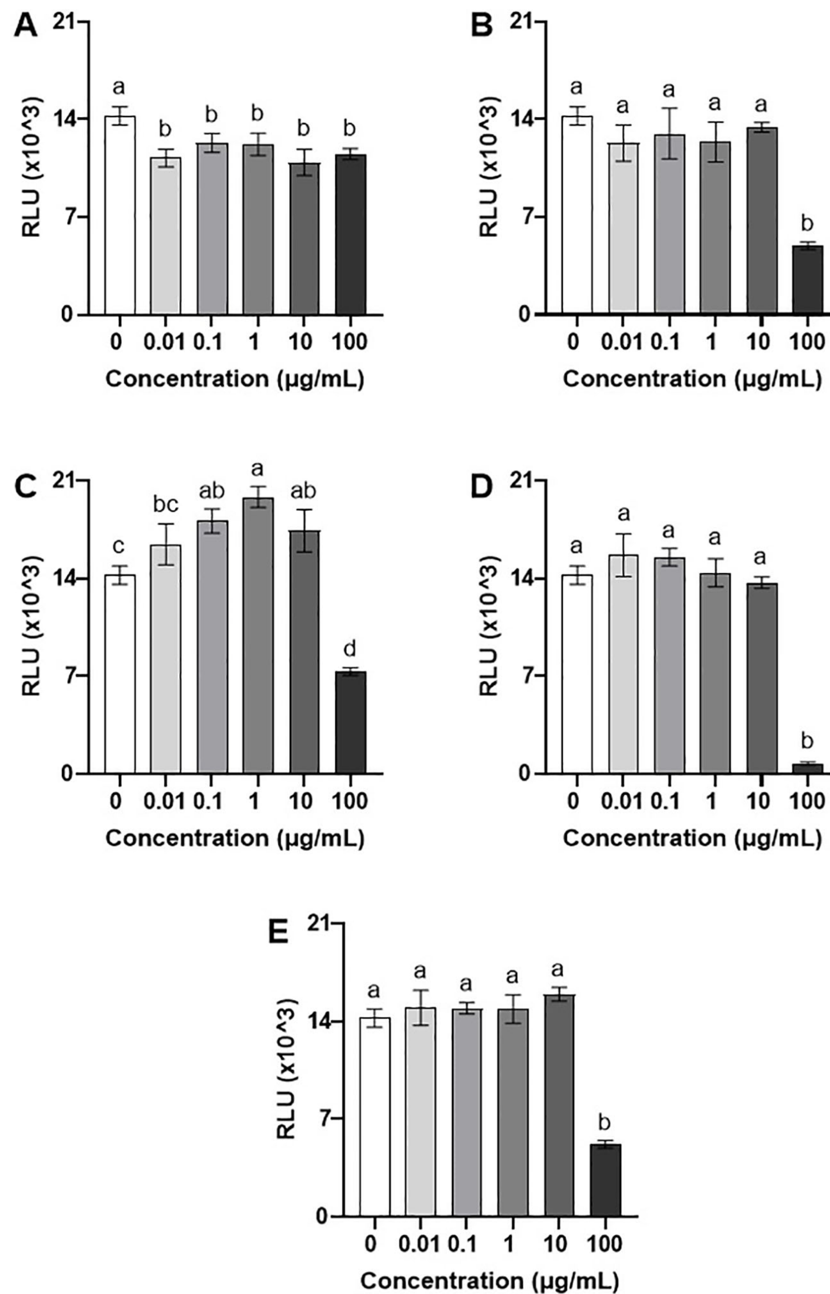

FIGURE 4
Respiratory burst (expressed as RLU, Relative Light Units) of *Sparus aurata* head kidney leukocytes exposed to 5 marine origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. *Rhodomonas lens* extract (RH, **A**), *Osmundea pinnatifida* extract (OS, **B**), *Gracilaria* sp. extract (GR, **C**), *Desmodesmus* sp. extract (DE, **D**) and *Dictyota* sp. extract (DI, **E**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

trend of association between TPC, TFC, and antioxidant capacity determined by both methodologies was observed. However, the *Rhodomonas* extract (RH) did not exhibit the highest TPC and TFC values but showed the greatest antioxidant activity, pointing to the importance of other compounds such as bioactive peptides and lipids with antioxidant potential (Suleria et al., 2016). Overall, the bioactivity of natural extracts reflects a multifactorial interplay between chemical structure and compound–compound interactions, emphasizing their intrinsic complexity and fundamental distinction from single, purified bioactive molecules (Seeram et al., 2005; Haminiuk et al., 2012; Caesar and Cech, 2019; Parcheta et al., 2021).

Although natural products often exhibit higher antibacterial activity against Gram-positive bacteria, likely due to their simpler cell wall structure and higher permeability (Dahham et al., 2010; Hafidh et al., 2011), the present study focused on Gram-negative pathogens of relevance in aquaculture, including *P. damselae* subsp. *piscicida*, *V. anguillarum*, and *V. harveyi*. In addition, these bacterial pathogens share common mechanisms such as the secretion of toxic extracellular products (ECPs) and the ability to sequester iron from

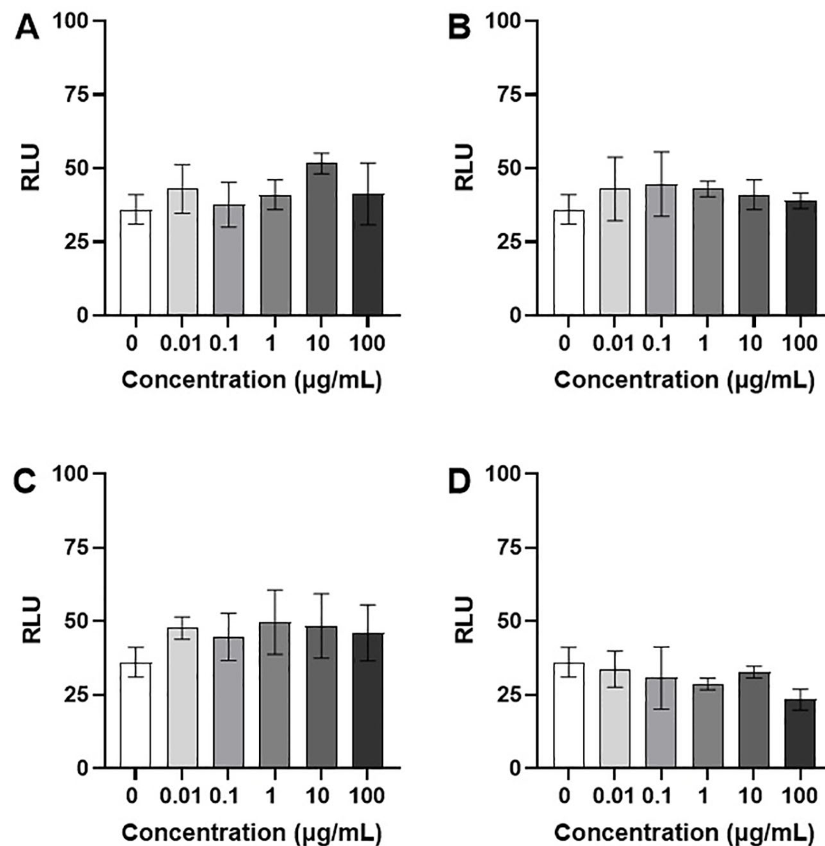

FIGURE 5
Reactive oxygen species (ROS) basal levels (without PMA stimulation) of *Sparus aurata* head kidney leukocytes exposed to 4 terrestrial origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. Pomegranate extract rich in punicalagin (PG, **A**), pomegranate extract rich in ellagic acid (EA, **B**), extract rich in citrus flavonoids (CF, **C**) and grape seed extract (GS, **D**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

the host (Miccoli et al., 2019). The extracts derived from pomegranate (PG and EA) showed a high effectiveness, with doses considerably lower than the other two extracts of terrestrial origin, CF and GS, in agreement with the results obtained by Rosas-Burgos et al. (2017) who observed that the highest antibacterial activity of pomegranate extracts were those with the highest concentration of punicalagin and ellagic acid, due to the involvement of these bioactives on the proliferation of pathogenic bacteria (Liu et al., 2023). On the other hand, marine compounds are not as effective in this regard, as relatively high concentrations are required to observe antibacterial effects compared to terrestrial compounds. In this sense, previous studies have reported antibacterial activity of macroalgae such as *Himanthalia elongata* at concentrations up to 60 mg/mL (Gupta et al., 2010), while microalgae including *Chlorella* sp. and *Spirulina* sp. typically exhibit MIC values in the range of 20–40 mg/mL against different bacterial species (Sraboni et al., 2024). These values are consistent with those obtained in the present study, indicating that the antibacterial activity of marine extracts falls within the expected range for natural bioactive compounds, particularly for extracts. Some of the compounds with antibacterial effects in algae are allelochemicals (Shannon and Abu-Ghannam, 2016; Karthikeyan et al., 2022) and phlorotannins, which are not found in terrestrial plants (Vatsos and Rebours, 2015), suggesting that a combination of terrestrial and marine extracts could provide complementary effects, acting through different pathways against pathogens.

A common bactericidal mechanism in natural products is membrane disruption, as compounds such as phenols or terpenes can compromise membrane integrity (García-Salinas et al., 2018). However, this mechanism may also affect host cells, highlighting the need to carefully evaluate the cytotoxic potential of natural extracts. In this study, increasing extract concentrations led to significant effects on cell viability, although the concentration at which cytotoxicity became evident differed among the tested extracts. Regarding terrestrial origin extracts, PG exhibited lower cytotoxicity than EA. Although PG and EA have similar components, as both come from pomegranate, punicalagin and ellagic acid differ in molecular size, polarity, solubility, and absorption progression (Sun et al., 2017), affecting the cytotoxicity of each compound. Previous studies have shown that EA exhibits cytotoxic effects at lower doses than PG, as it can form polyphenol–protein/lipid complexes that generate free radicals upon interaction with oxygen, potentially acting as a genotoxic agent (Labieniec et al., 2003; Kulkarni et al., 2007). In contrast, PG only exhibited cytotoxicity at the highest concentration, which may be attributed to the formation of macroaggregates. Given the higher solubility of PG compared to EA and its affinity to form bindings in protein-rich medium (Venusova et al., 2021), macroaggregates formation may

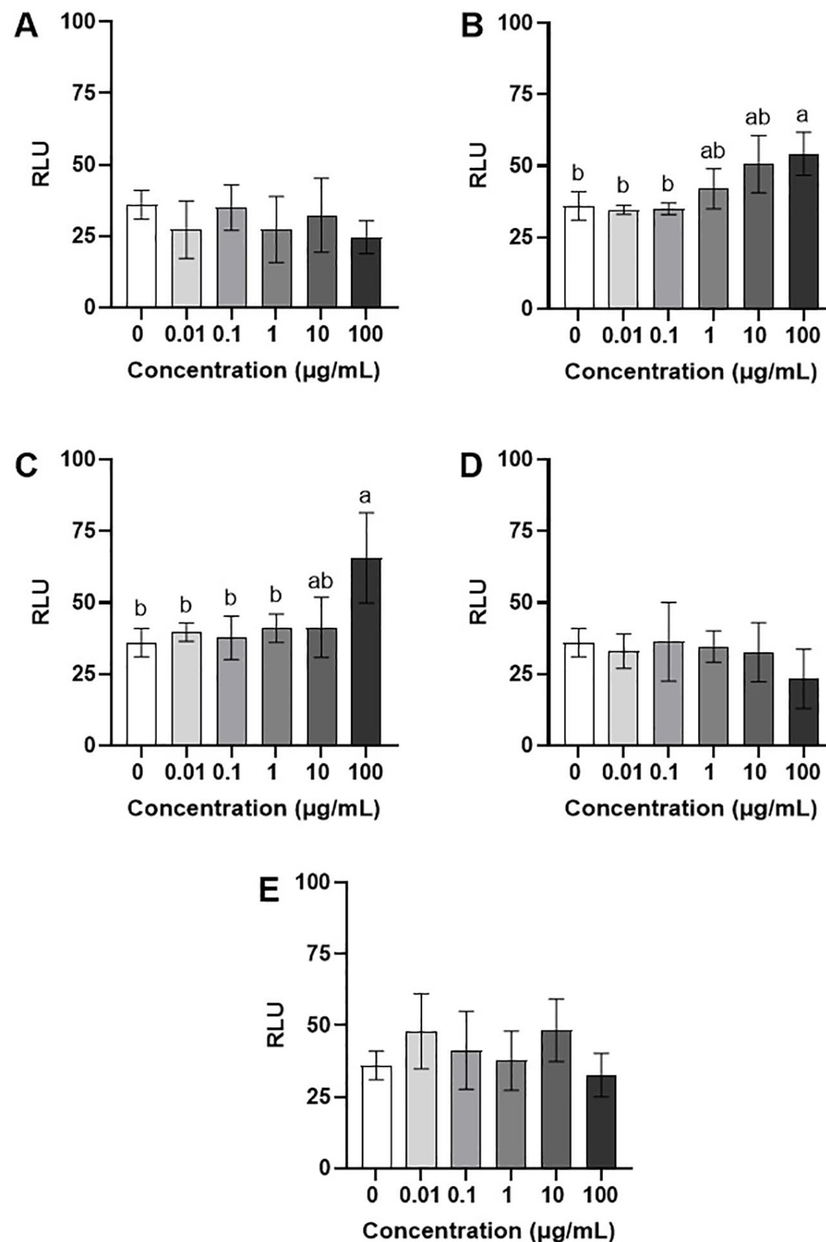

FIGURE 6
Reactive Oxygen Species (ROS) basal levels (without PMA stimulation) of *Sparus aurata* head kidney leukocytes exposed to 5 marine origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. *Rhodomonas lens* extract (RH, **A**), *Osmundea pinnatifida* extract (OS, **B**), *Gracilaria* sp. extract (GR, **C**), *Desmodesmus* sp. extract (DE, **D**) and *Dictyota* sp. extract (DI, **E**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

trigger frustrated phagocytosis, leading to an immunosuppressive effect (Campos-Sánchez et al., 2022). This hypothesis could be valid not only for the trend observed in PG, but also in GS, due to the presence of tannins in grape seeds (Mattivi et al., 2009) which can also cause the precipitation of proteins present in the medium, such as bovine serum albumin (BSA) (Yamauchi et al., 2023). However, this pattern is not observed in CF, where viability decreases progressively. Citrus fruits are rich in terpenoids, which likely contribute to cytotoxicity by compromising membrane integrity (Perestrelo et al., 2019; Saini et al., 2022). Therefore, as with their antioxidant activity, the biological effects of these extracts depend not only on total phenolic content, but also on the specific composition, structural characteristics, and their potential interactions. For marine extracts, both RH and OS showed no cytotoxic effects even at the highest doses. *Rhodomonas* is a source of phycoerythrin, a protein with antioxidant potential (Patel et al., 2018) that has been shown to regulate apoptosis pathways (Zhao et al., 2023). Interestingly, this pigment is also present in *Osmundea* sp (Schneider et al., 2020), suggesting that the presence of phycoerythrin could be related to the observed lack of cytotoxicity effects in both extracts. After exposure to DE, a trend similar to that observed with PG and GS was observed, with cell viability being drastically reduced only at the highest dose, which cannot rule out a potential contribution of macroaggregate formation due to its high

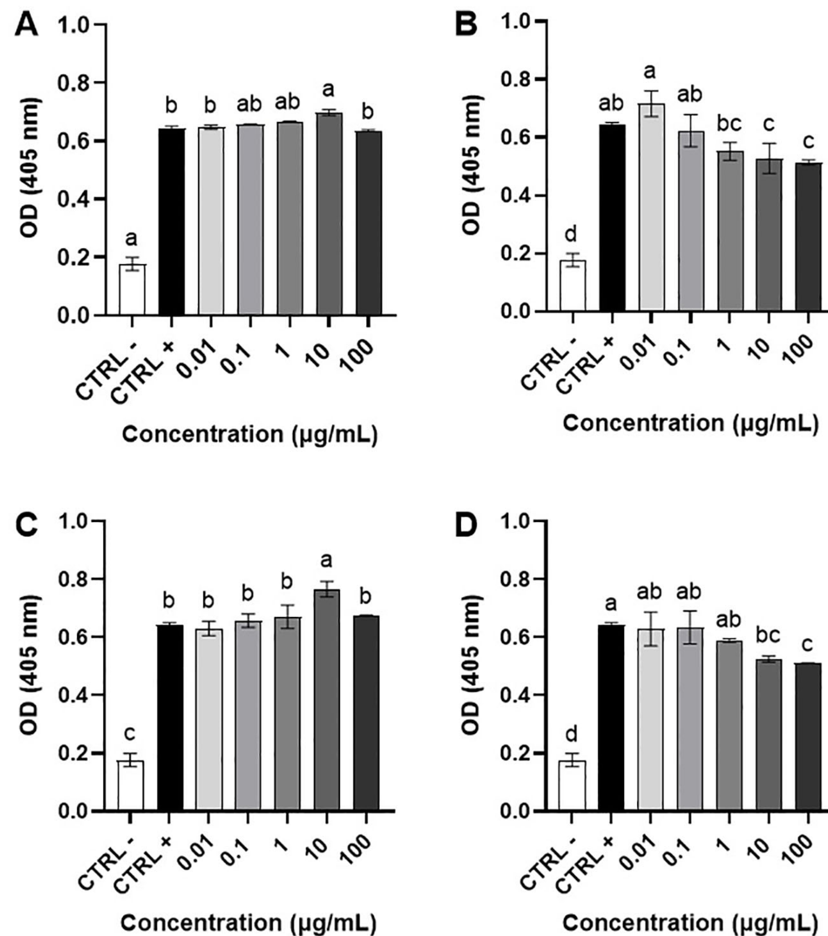

FIGURE 7
Phagocytic activity (OD) of *Sparus aurata* head kidney leukocytes exposed to 4 terrestrial origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 μg/mL). Data are the mean ± SD of triplicate wells. Pomegranate extract rich in punicalagin (PG, **A**), pomegranate extract rich in ellagic acid (EA, **B**), extract rich in citrus flavonoids (CF, **C**) and grape seed extract (GS, **D**). Positive control (CTRL+) corresponds to incubation with zymosan, whereas the negative control (CTRL-) was performed without zymosan. Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

phenolic and flavonoid contents among marine extracts. With the DI extract, a progressive decrease in cell viability was observed as the dose increased. *Dictyota* sp. is characterized by a relatively high and varied presence of terpenoids (Siamopoulou et al., 2004), which, as seen previously with CF, can be cytotoxic due to their membrane-disrupting effects. Finally, GR showed the strongest cytotoxic effect, reducing cell viability even at the lowest tested dose, which may be associated with apoptosis inducing effects of palmitic acid (Andriani et al., 2016; Park et al., 2014). Importantly, when comparing the antibacterial activity (MIC/MBC values) with the cytotoxicity results obtained in the present study, in several cases the concentrations required to inhibit growth or eliminate bacteria fall within or above the range that induces cytotoxic effects in head kidney leukocytes. This overlap highlights a key limitation for the direct application of some extracts, as their antibacterial efficacy may be constrained by host cell tolerance. These findings underscore the importance of considering dose-dependent effects when evaluating natural extracts as functional ingredients and emphasize the need to optimize dosage to achieve a balance between efficacy and safety. In this context, further *in vivo* studies are required to validate the biological relevance of these findings, determine safe and effective inclusion levels, and confirm whether the antibacterial effects observed *in vitro* can be achieved without compromising host cell viability under physiological conditions.

In addition to the effects on apoptotic pathways and considering the dose-dependent cytotoxic effects described above, certain compounds present in natural products may also modulate the immune system function, as some have been shown to inhibit pro-inflammatory pathways, acting as immunomodulatory agents (Beigoli and Boskabady, 2024). In this study, the respiratory burst was evaluated using two approaches: PMA-induced stimulation, which activates Protein Kinase C (PKC) and subsequently NADH oxidase (Iles and Forman, 2002), and direct stimulation by the extracts in the absence of PMA. Terrestrial extracts did not show immunomodulatory effects without stimulation of respiratory burst, potentially indicating a lack of recognition of Pathogen-Associated Molecular Patterns (PAMPs). However, upon stimulation with PMA, a dose-dependent decrease in respiratory burst activity was generally observed. At the highest dose tested this reduction was likely linked to the lower cell viability previously discussed. At lower doses, the reduced ROS levels may result from the inhibition of PKC-dependent NADPH oxidase activation by polyphenols (Pignatelli et al., 2006) or from

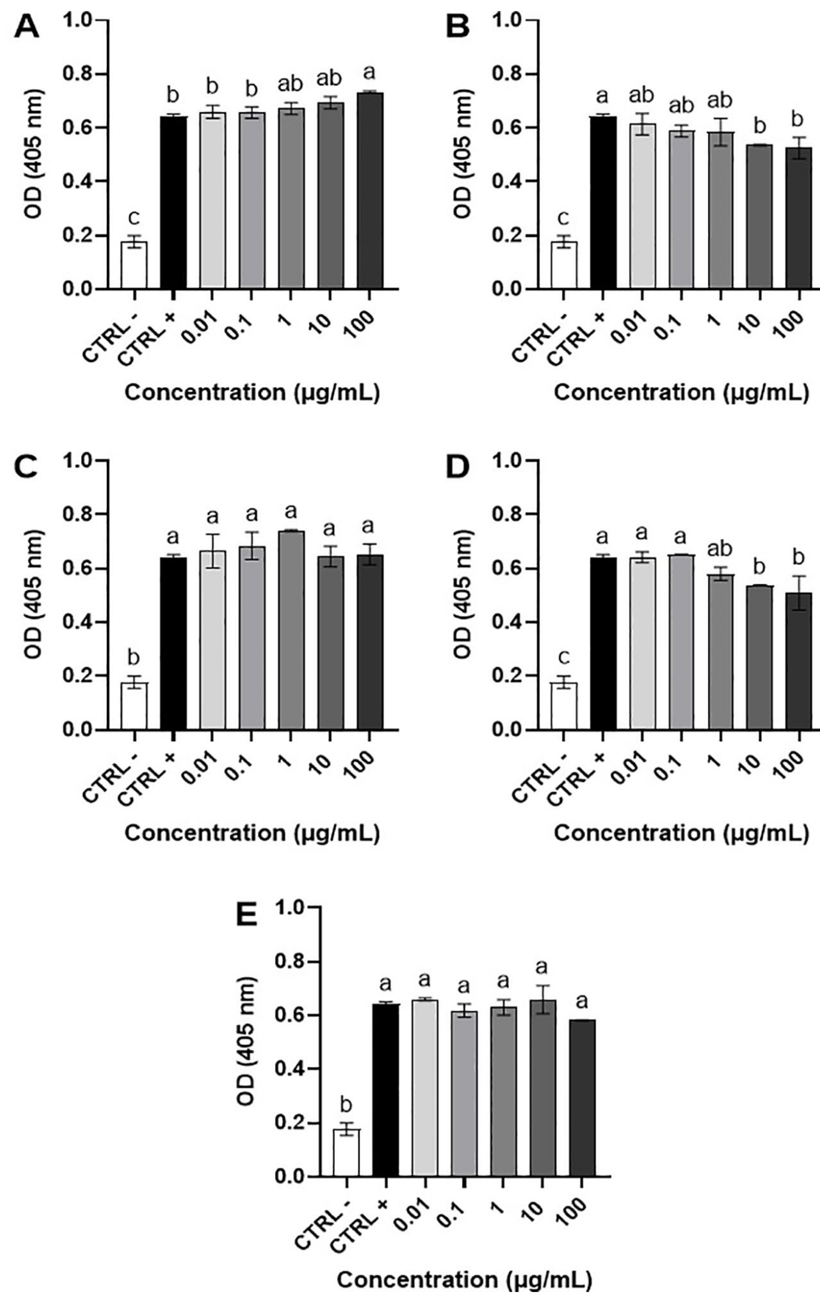

FIGURE 8
Phagocytic activity (OD) of *Sparus aurata* head kidney leukocytes exposed to 5 marine origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. *Rhodomonas lens* extract (RH, **A**), *Osmundea pinnatifida* extract (OS, **B**), *Gracilaria* sp. extract (GR, **C**), *Desmodesmus* sp. extract (DE, **D**) and *Dictyota* sp. extract (DI, **E**). Positive control (CTRL+) corresponds to incubation with zymosan, whereas the negative control (CTRL-) was performed without zymosan. Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

the antioxidant properties of the extracts themselves, which can scavenge the free radicals produced during the respiratory burst (Ren et al., 2014). However, a reduction in ROS production should be interpreted with caution, as it may not necessarily reflect a beneficial effect and could also be associated with a reduced microbicidal capacity of immune cells. In contrast, the responses to marine extracts varied across extracts, revealing distinct activity patterns. RH, DE, and DI did not activate the respiratory burst, whereas red algae OS and GR produced dose-dependent stimulation. A comparable effect was reported by Petit et al. (2024), who observed immunomodulatory activity in extracts rich in marine sulphated polysaccharides (MSPs) from the red algae *Solieria* sp. This response may be triggered by the stimulation of pattern recognition receptors (PRRs), since MSPs are considered as PAMP-like structures and MSP-rich extracts have been shown to activate immune cells via Toll-Like Receptor 4 (TLR4), initiating downstream signaling pathways (Berri et al., 2017). On the other hand, when cells were stimulated with PMA, three different patterns

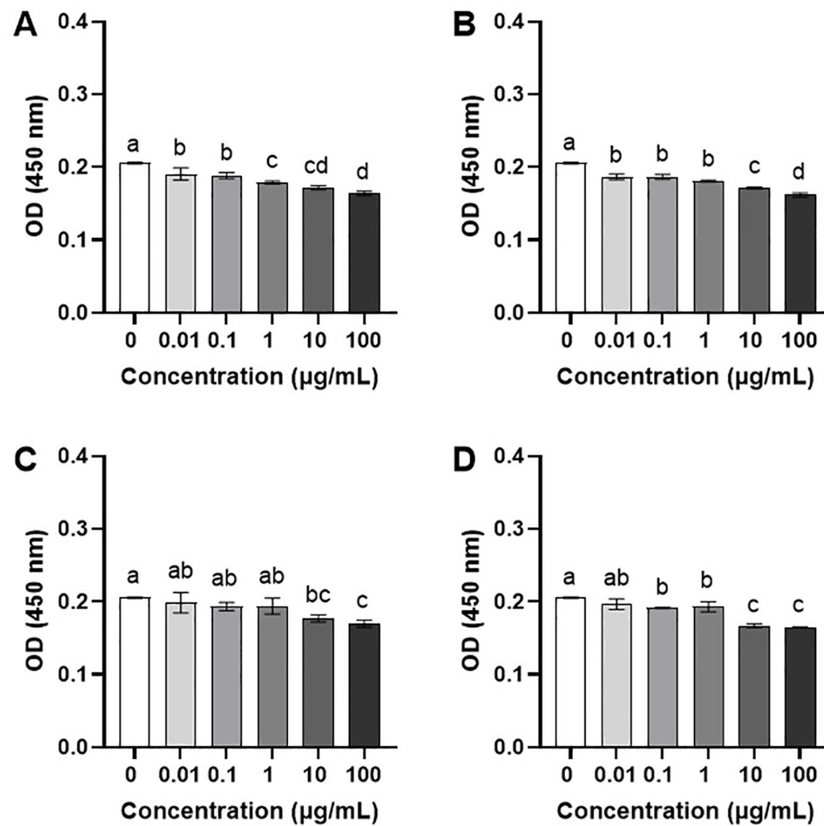

FIGURE 9
Peroxidase activity (OD) of *Sparus aurata* head kidney leukocytes exposed to 4 terrestrial-derived extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. Pomegranate extract rich in punicalagin (PG, **A**), pomegranate extract rich in ellagic acid (EA, **B**), extract rich in citrus flavonoids (CF, **C**) and grape seed extract (GS, **D**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

were observed. RH significantly reduced ROS production even at the lowest tested concentration, an effect that may be associated with its antioxidant capacity, potentially mediated by bioactive peptides through direct ROS neutralization and/or modulation of redox-related pathways (Derbel et al., 2023). Cells exposed to OS, DE, and DI showed an acute decrease in respiratory burst only at the highest dose, in relation to the cytotoxic effects detected. Finally, the pattern observed with GR followed a typical dose-response pattern, with stimulation of the respiratory burst at intermediate doses. *Gracilaria* species are major sources of agar (Lee et al., 2022), and polysaccharides are closely linked to the stimulatory capacity of macroalgae (Castro et al., 2004), especially when they contain sulphate groups (Petit et al., 2024). Taken together, these results emphasize that the immunomodulatory potential of the extracts is strongly dependent on their chemical composition and applied concentration, underscoring the importance of carefully selecting and dosing bioactive ingredients when considering their use in functional feeds.

Phagocytosis, which plays a crucial role in innate immunity, but also in tissue homeostasis and remodeling (Esteban et al., 2015) was differentially modulated by the extracts evaluated. From terrestrial origin, PG and CF moderately enhanced phagocytic activity, although the highest doses may have partially masked this effect due to reduced cell viability. Nevertheless, the overall phagocytic activity remained relatively high, possibly reflecting an increased particle uptake per macrophage rather than changes in macrophage abundance. Similar responses have been described following supplementation with vitamins C and E, which enhanced the phagocytic capacity but not the proportion of phagocytic cells in *S. aurata* phagocytes (Mulero et al., 1998). The bioactive compounds present in these extracts could be enhancing the recognition and uptake of foreign particles by modulating complement (CRs) and Fcγ receptors (FcγRs), either by activating the complement pathway or upregulating receptor expression (Narayanaperumal et al., 2022). On the other hand, EA and GS caused the opposite trend, a decrease in phagocytic activity as the extract concentration increased. This effect may be attributed to their anti-inflammatory properties, as compounds such as resveratrol, a well-studied phenolic compound, have been reported to downregulate macrophage microbicidal activity by reducing pro-inflammatory cytokine production (Martínez et al., 2010; Rahimifard et al., 2017; Martínez et al., 2019). Regarding marine extracts, RH dose-dependently enhanced phagocytic activity, reaching maximum effect at the highest concentration tested. This stimulatory effect could be related to cytoskeletal remodeling induced by marine bioactive compounds, as previously reported in *Megalobrama amblycephala* macrophages (Li et al., 2023). In contrast, a dose-dependent decrease in phagocytic capacity was observed when using DE and OS extracts. This

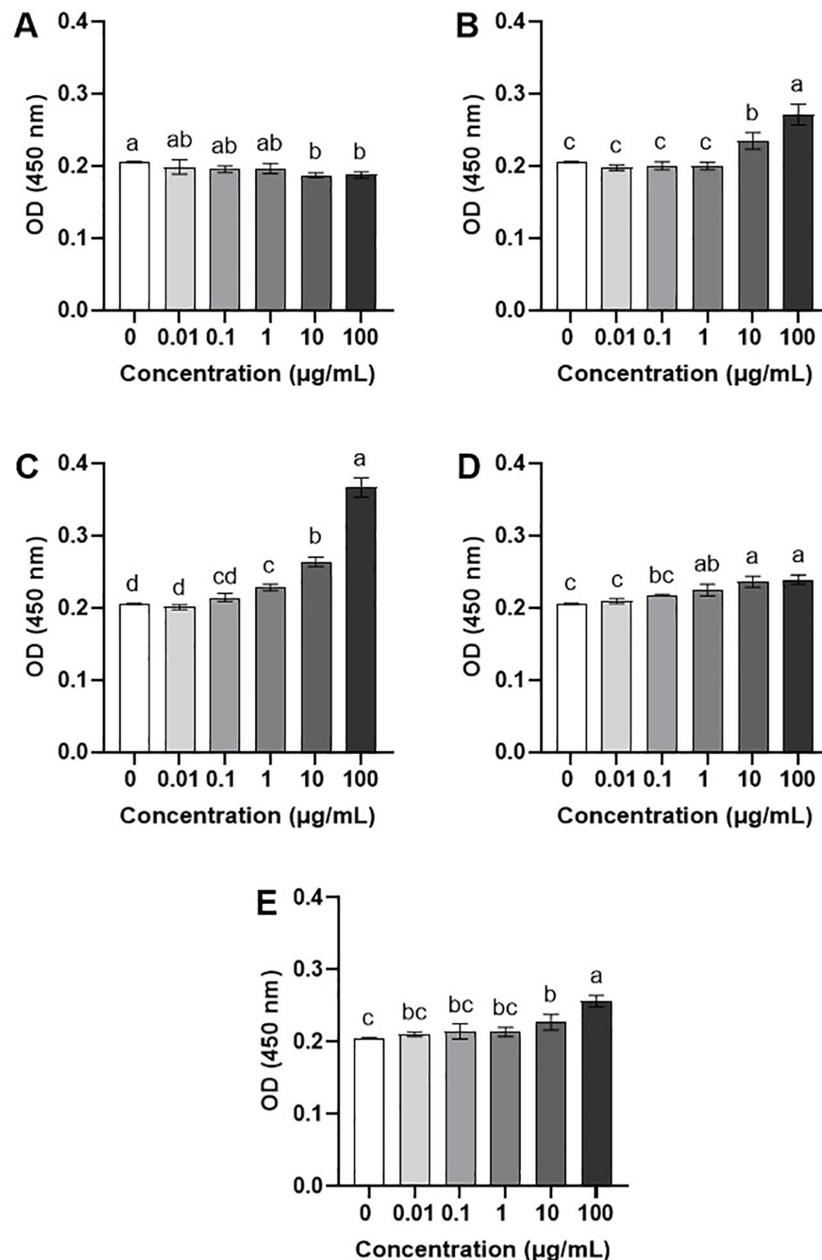

FIGURE 10
Peroxidase activity (OD) of *Sparus aurata* head kidney leukocytes exposed to 5 marine origin extracts at 5 different doses (0, 0.01, 0.1, 1, 10 and 100 µg/mL). Data are the mean ± SD of triplicate wells. *Rhodomonas lens* extract (RH, **A**), *Osmundea pinnatifida* extract (OS, **B**), *Gracilaria* sp. extract (GR, **C**), *Desmodesmus* sp. extract (DE, **D**) and *Dictyota* sp. extract (DI, **E**). Different letters within each graph indicate statistically significant differences among treatments (one-way ANOVA followed by Tukey's *post hoc* test; $p < 0.05$).

inhibitory effect may result from anti-inflammatory compounds, as observed for the terrestrial extracts EA and GS, but in this case, it could be mediated by PUFAs or chlorophyll A, which have been shown to reduce pro-inflammatory cytokine production (Robertson et al., 2015). Finally, neither GR nor DI affected phagocytic capacity at any of the concentrations tested. Similar observations have been reported for certain macroalgal compounds, such as fucoidans, which do not alter the production of key cytokines involved in phagocytosis (Maruyama et al., 2015). Overall, responses observed at lower extract concentrations are more likely to reflect true immunomodulatory effects, whereas those detected at higher concentrations should be interpreted with caution, as they may be influenced by cytotoxicity.

Two differentiated trends were identified in the peroxidase activity depending on the origin of the extracts. Terrestrial extracts demonstrated a dose-dependent reduction in peroxidase activity, whereas marine extracts showed an increase. Interestingly, Castro et al. (2008) found that phenolic compounds such as resveratrol can act as powerful inhibitors of myeloperoxidase (MPO), an abundant enzyme in fish neutrophils. This inhibition occurs not only because resveratrol is a competing substrate but also by blocking MPO expression. In this context, such mechanisms may help explain the

decreasing peroxidase trend observed in terrestrial extracts, which generally have a higher phenolic content than marine extracts. In contrast, marine compounds appear to stimulate peroxidase activity, which could be indicative of immune activation of leukocytes. However, this increase may also reflect changes in cellular redox status or oxidative processes and should therefore be interpreted in the context of both antioxidant activity and immune modulation. Notably, RH exhibited a response pattern comparable to that of terrestrial extracts despite not having the highest total phenolic content among marine samples, highlighting that the functional effects of such ingredients are likely driven by their overall chemical composition and combined interactions among multiple bioactive compounds. However, part of the response observed at higher concentrations may be influenced by cytotoxic effects and should therefore be interpreted in the context of reduced cell viability.

A limitation of the present study is the lack of detailed chemical profiling of the evaluated extracts. While basic compositional parameters such as total phenolic and flavonoid contents were assessed, a more comprehensive characterization of individual bioactive compounds would provide deeper insight into the mechanisms underlying the observed biological effects. However, the objective of this work was to perform a functional screening of a diverse set of extracts with potential applicability in aquaculture. In this context, the approach adopted here prioritizes the identification of promising candidates for further development and application in the sector, while future studies integrating detailed chemical characterization and *in vivo* validation will help to elucidate the mechanisms of action and optimize their practical use.

Natural terrestrial and marine extracts have different but complementary bioactivities *in vitro*, that are strongly influenced by their chemical composition and origin. Terrestrial by-products were characterized by higher phenolic content, antioxidant capacity, and antibacterial activity, whereas marine extracts showed a greater capacity to modulate key immune-related parameters, including respiratory burst, phagocytosis, and peroxidase activity. This variability highlights the inherent complexity of interpreting bioactivity in natural products and underscores the importance of considering both source and chemical composition when assessing their functional potential. Importantly, the distinct bioactive profiles observed according to extract origin suggest that future *in vivo* studies evaluating the combined use of terrestrial and marine extracts may help explore potential complementary effects between both sources. Overall, these findings provide a scientific basis for the development of natural product-based functional ingredients in aquaculture, supporting fish health through preventive and sustainable alternatives to antibiotic use.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: AccedaCRIS: https://accedacris.ulpgc.es/.

Ethics statement

The animal study was approved by Animal Experimentation Ethics Committee (C.E.E.A.) of the University of Las Palmas De Gran Canaria (ULPGC) (CEEA_ULPGC 33-2023). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LM-R: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. FA: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. MI: Conceptualization, Writing – review & editing. CS: Formal analysis, Writing – review & editing. VG: Resources, Writing – review & editing. JD: Resources, Writing – review & editing. DM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing. ST: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declared that financial support was received for this work and/or its publication. This work was conducted within the LARVOOST project (PID2021-122474OB-I00) funded by MCIN/AEI/10.13039/501100011033 and by ERDF/EU. LM-R was supported by a predoctoral grant (PRE2022-103656) funded by MICIU/AEI/10.13039/501100011033 and by ESF+. ST is financed by a Ramón y Cajal fellowship (RYC2021-031414-I) funded by MICIU/AEI/10.13039/501100011033 and by European Union NextGenerationEU/PRTR.

Acknowledgments

The authors gratefully acknowledge Martiña Ferreira and Paula Frade for kindly providing the *Rhodomonas lens* and *Osmundea pinnatifida* samples used in this study.

Conflict of interest

JD was employed by company PTAqua.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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