Ultrasound- and enzyme-assisted extraction of *Moringa oleifera* polysaccharides: bioactivity evaluation

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Abstract

Polysaccharides from *Moringa oleifera* (PMS) leaves were extracted using neutrase (PMS-N) or savinase (PMS-S) enzymes combined with ultrasonic (US) treatment, and their biological activities were investigated. Both US time (10 min) and enzyme type positively influenced PMS yield, regardless of the enzyme used. Among the detected neutral sugars, xylose was the major monosaccharide in US-pretreated PMS, reaching 72% and 69% in PMS-S-US-10 and PMS-N-US-10, respectively. In addition, US-pretreated PMS exhibited the highest antioxidant capacities and significantly prolonged blood clotting period and reduced fibrinogen levels, accomplishing 0.7 g/L and 1.5 g/L, by the addition of PMS-S-US-10 and PMS-N-US-10, respectively. Antiproliferative assays revealed dose-dependent inhibition effect of PMS against two types of cancer cells, where PMS-S-US-10 displayed the highest inhibitory rates, reaching 96% and 99% (at 200 µg/mL) against K562 and Caco-2 cells, respectively.

Keywords: anticoagulant activity; antimicrobial activity; antioxidant activities; antiproliferative effect; *M. oleifera*; polysaccharides

Introduction

Moringa oleifera Lam. (*M. oleifera* Lam.) is abundantly found in numerous tropical and subtropical countries. Renowned as the "miracle tree," it is recognized for its substantial nutritional content, rapid growth, and remarkable resilience to drought conditions (Horn *et al.* 2022). Over the last decades, *M. oleifera* has been investigated by many researchers for its beneficial properties. This tree has been used for its therapeutic and nutritional value. It is well known that the various components of *M. oleifera* exhibit diverse properties that contribute to health and nutrition. Besides providing edible food and medicinal properties, this plant is also

used as an ornamental plant, as it has wide applications in medicine for treating many diseases, such as diabetes, blood pressure, headaches, constipation, and bronchitis (Muhammad *et al.*, 2016; Yang *et al.*, 2022).

Numerous scientific studies have corroborated the extensive biological benefits of *M. oleifera* in enhancing human health. These benefits include its anti-inflammatory, anticancer, antimicrobial, antitumor, hypotensive, hypoglycemic, and wound-healing properties (Fernandes *et al.*, 2021). The various organs of the Moringa tree are acknowledged as a potential reservoir of numerous natural metabolites, such as natural antioxidants (Padayachee *et al.*, 2020). Previous studies have indicated that polysaccharides (Chen *et al.*, 2017; He *et al.*, 2018; Yang *et al.*, 2022), phenolics (Singh *et al.*, 2020), and flavonoids are the primary active components found in *M. oleifera*.

Polysaccharides represent a group of macromolecules that have numerous functional properties (He *et al.*, 2018; Sharma *et al.*, 2022). These are a significant class of natural biopolymers with diverse biological applications and provide significant promises to resolve various health problems. In fact, these bioactive compounds provide numerous benefits, such as immunomodulatory, antioxidant, antimicrobial, antiviral, anticancer, anticoagulant, anti-diabetes, and hypoglycemic activities, as well as protection against ultraviolet (UV) rays (Benalaya *et al.*, 2024; Salehi and Rashidinejad, 2024). The extraction of polysaccharides from *M. oleifera* has garnered significant attention from researchers across various fields, and they have demonstrated intriguing activities, making it imperative to conduct studies aiming to characterize and assess their medicinal and nutritional benefits (Wang *et al.*, 2022).

The isolation of biopolymers from natural sources has sparked considerable interest across multiple disciplines because of their advantageous properties. Extensive investigations have predominantly centered on the purification, separation, characterization of the chemical structure, physical properties, and biological functionalities of polysaccharides derived from *M. oleifera* (Fernandes *et al.*, 2021; He *et al.*, 2018; Zhang *et al.*, 2022). As a result, the necessity to investigate these natural antioxidants, such as polysaccharides from *M. oleifera*, has grown for their potential use in food additives and in health-promoting products. It is also important to highlight that the bioactivities of *M. oleifera* polysaccharides have a direct correlation with factors such as their molecular weight, arrangement of glycosidic linkages, chemical modifications and their extraction and purification techniques, and their structural diversity (Yang *et al.*, 2022).

There are various methodologies that are applied for the recovery of polysaccharides from *M. oleifera*, encompassing conventional techniques, such as hot water extraction (Wang *et al.*, 2018) and ethanol extraction (Zheng *et al.*, 2021), as well as advanced approaches, such as microwave (Chen *et al.*, 2017) and ultrasonic (US)-assisted cellulase extraction (Yang *et al.*, 2020). Compared to other processes (subcritical water extraction and microwave-, enzyme-, pulsed electric field-assisted extraction), US extraction enhanced the molecular movement rate and penetration capacity by applying mechanical cavitation, increasing the enzyme capacity and extraction efficiency of polysaccharides (Leong *et al.*, 2021).

Enzymes are highly efficient biocatalysts that have gained increasing attention in the extraction of polysaccharides from plants because of their effect on promoting cell permeability as well as their green and environment-friendly properties (Yang *et al.*, 2022). It has been reported that enzyme-assisted extraction has several benefits, such as improving extraction efficiency and promoting the bioactivity of extracted polysaccharides (Yang *et al.*, 2022). To the best of our knowledge, the use of proteases in US-assisted polysaccharides extraction process from Moringa leaves is not studied.

Therefore, in this investigation, two distinct proteases are utilized to extract polysaccharides from *M. oleifera* leaves cultivated in Tunisia. This is achieved through the implementation of two types of enzymes in conjunction with US treatment. Subsequently, the effects of extraction method on the biological activities of extracted polysaccharides are investigated.

Materials and Methods

Plant material

M. oleifera Lam. (Moringaceae) leaves were collected from the southeastern region of Tunisia, specifically Medenine, which experiences an arid climate with an average annual rainfall of 150 mm. The leaves were carefully collected and subjected to air-drying in a shaded area until a consistent mass was achieved (over a period of 20 days). Subsequently, the dried leaves were crunched into a fine powder and stored in a dry and dark place at room temperature until they were ready to use.

Extraction of polysaccharides from *M. oleifera* (PMS) leaves

Polysaccharides were extracted using two enzymes, neutrase (PMS-N) and savinase (PMS-S), using a modified extraction procedure as described by Abdelhedi *et al.* (2024). Initially, 100 g of *M. oleifera* powder

was suspended in 100 mL of distilled water. For savinase extraction, the optimized conditions included an extraction time of 18 h, pH of 9, temperature of 50°C, and an enzyme dosage of approximately 300 mg. In the case of neutrase extraction, the optimized conditions comprised 4.4 mL of Neutrase, an extraction time of 18 h, 50°C temperature, and pH of 7.

After incubation, ethanol was added to each mixture to precipitate polysaccharides. Then the resulting solution was separated through filtration. The pretreated sample underwent centrifugation at 6,000 rpm for 15 min. In the subsequent step, the precipitate was washed twice with distilled water and centrifuged again at 6,000 rpm for 15 min. Finally, two groups of polysaccharides, PMS-S and PMS-N, were obtained. Each group was dried and subsequently crushed for further analysis.

Ultrasound-assisted extraction of *M. oleifera* polysaccharides

The ultrasound-assisted extraction was conducted according to the protocol described by Liao *et al.* (2015) and Lin *et al.* (2023) with minor adjustments. Approximately 100 g of *M. oleifera* leaf powder was measured and placed in a beaker. Subsequently, 250 mL of buffer solution was added, and the mixture had extraction by ultrasound treatment (500 W) for 10 min (US-10) at a controlled temperature. Thereafter, enzymes (savinase or neutrase) were introduced to initiate enzymatic digestion process.

The subsequent steps involved in the extraction were the same as described in the previous extraction methods. The resulting crude polysaccharide obtained through ultrasound-assisted extraction and enzymatic treatment was designated as PMS-S-US-10 and PMS-N-US-10, referring to the use of savinase (S) and neutrase (N), respectively.

Chemical composition

Chemical composition of *M. oleifera* polysaccharides was assessed using two distinct methodologies. First, the protein content was evaluated by Bradford assay, which is widely used for protein quantification. Different concentrations of each PMS sample were combined with Coomassie blue dyes, vigorously mixed, and incubated for a period of 10 min. Subsequently, the absorbance was measured at 595 nm by following the protocol outlined in Ben Othman *et al.* (2024).

The phenol-sulfuric acid method was used to determine total carbohydrate content as described by Ben Yakoub *et al.* (2020). This method is commonly utilized for the accurate measurement of total carbohydrates in samples. Additionally, the yield of PMS-S and PMS-N was calculated to determine the polysaccharides content obtained from respective extractions.

Evaluation of antioxidants activities

Iron (Fe^{2+})-chelating activity

The chelating effect of various PMS samples was assessed using the methodology outlined by Salem *et al.* (2021). In this experiment, approximately 100 µL of each PMS, prepared at different concentrations, was combined with 50 µL of 2-mM $FeCl_2$ and 450 µL of water. Following a 3-min incubation period at room temperature, 200 µL of 5-mM ferrozine solution was added to each mixture. The resulting mixtures were vigorously shaken and left at room temperature for 10 min to allow complete chelation of iron ions. Control tubes were also prepared to serve as a reference, where water was substituted for PMS samples. This allowed the comparison of the chelating effect of PMS samples to the control group.

Finally, the absorbance was measured at 562 nm, and the chelating activity (%) was calculated as follows:

$$\text{Metal chelating activity } (\%) = \frac{OD_C + OD_B - OD_S}{OD_C} \times 100, \quad (1)$$

where OD_C, OD_B, and OD_S are absorbance values of the sample, the control, and the blank (the blank was made in the same way by replacing ferrozine with distilled water), respectively. The test was administered in triplicate.

Ferric-reducing antioxidant power (FRAP)

M. oleifera polysaccharides were tested for their ability to reduce iron using the technique outlined by Thimmaraju *et al.* (2023) with a few minor adjustments. For every test sample, three replicates were run to guarantee the precision and dependability of findings.

β-carotene bleaching method

The ability of PMS samples to inhibit β-carotene bleaching was determined following the method described by Ben Yakoub *et al.* (2020). First, 0.5 mg of β-carotene, 25 µL of linoleic acid, and 200 µL of Tween 40 were dissolved in 1 mL of chloroform to create a new emulsion of β-carotene–linoleic acid. A rotary evaporator operating at 50°C was used to evaporate chloroform under vacuum. After that, 100 mL of distilled water was added, and the mixture was swirled. In all, 2.5 mL of the newly made emulsion was put into test tubes holding 0.5 mL of each PMS at various concentrations. Control tubes were

prepared in the same manner as test tubes, except water was introduced in place of PMS sample.

Before and after the tubes were incubated for 1–2 h at 50°C, the absorbance of each tube was measured at 470 nm.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The antiradical activity of PMS was assessed using the DPPH method, following the protocol outlined by Sankarapandian *et al.* (2022). In this analysis, 1 mL of DPPH solution (0.02%) was added to 1 mL of PMS at various concentrations dissolved in water (ranging from 0.5 to 4 mg/mL). The resulting mixture was vigorously shaken, allowed to stand at room temperature for 20 min, and the absorbance was measured at 517 nm. Afterward, the DPPH scavenging effect was calculated according to the following equation:

$$\text{Scavenging ability} = \frac{C + B - R}{C} \times 100, \quad (2)$$

where C is the absorbance of the control, B is the absorbance of the blank, and R is the absorbance of polysaccharide samples.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed by following the methodology reported by Zhang *et al.* (2024) with minor adjustments.

Total antioxidant capacity (TAC)

The TAC of PMS was determined using a modified version of the method described by Asril *et al.* (2025). Initially, a reagent solution consisting of 4-mM ammonium molybdate in 28-mM sodium phosphate with 0.6-M sulfuric acid was prepared. Next, 0.1-mL aliquots of each sample solution at various concentrations were mixed with 1 mL of reagent solution. The resultant mixtures were incubated at 90°C for 90 min. Once the incubation period was completed, the tubes were set aside to cool down to room temperature, and the liquids' absorbance was determined at 695 nm. The presence of electron-donating species initiated the reduction of Mo (VI) to Mo (V), which was marked by the development of a green hue. In fact, the color shift happened when a phosphate–Mo (V) complex was formed in an acidic environment. The result of TAC assay was presented as α-tocopherol equivalent (μmol/mL), which referred to antioxidant ability in relation to the reference component α-tocopherol.

Antibacterial activity

Bacterial strains

Antibacterial activities of PMS were evaluated against different Gram+ strains: *Micrococcus luteus* (ATCC 4698), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), and *Listeria*, and five Gram- strains: *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 43972), *Enterobacter spp.*, and *Pseudomonas bacteria*.

Agar diffusion method

The antibacterial activity of each PMS was assessed following the method outlined by Ben Yakoub *et al.* (2020). Culture suspensions, each containing 200 μL of microorganisms (with a concentration of 106 colony-forming units/mL [CFU/mL] determined by absorbance at 600 nm), were evenly spread on Luria–Bertani (LB) agar plates.

Then, 50 μL of *M. oleifera* polysaccharide at a concentration of 10 mg/mL was carefully placed in 6-mm diameter wells, which were punctured in the agar layer. Gentamycin at a concentration of 30 μg/well was used as a positive control for the tested bacteria. In order to enable the diffusion of PMS, petri dishes were kept at 4°C for 1 h. Following this, the plates were incubated for 24 h at 37°C. The effectiveness of antibacterial activity was determined by measuring the diameter of inhibition zones in milliliter, which indicated the extent of growth inhibition caused by PMS or the positive control (gentamycin).

Anticoagulant activity

Sodium citrate buffer-containing siliconized Vacutainer TM tubes (Becton Dickinson, Le Pont de Claix, France) were used to draw blood from healthy subjects. To prepare platelet poor plasma (PPP), to use it in coagulation tests, the obtained blood was processed according to the protocol described by de Araujo *et al.* (2021).

Coagulation parameters

Activated partial coagulation parameters

A precise volume of 5 μL from each sample was combined with 45 μL of PPP, and the mixture was incubated for 3 min at 37°C. Subsequently, 50 μL of activated partial thromboplastin time (aPTT) reagent (CK-PREST®; Diagnostica Stago S.A.S, France) was added to the mixture, followed by another incubation period of 3 min at 37°C. Afterward, 100 μL of 25-mM solution of calcium chloride ($CaCl_2$) was added to the mixture, and time (in seconds) taken for blood clotting was immediately recorded. It is important to note that in aPTT test, measurements exceeding 120 s were considered insignificant.

Prothrombin time (PT)

From each PMS solution, 5 μL was mixed with 45-mL of PPP, and the mixture was incubated at 37°C for 3 min. Post-incubation, 100 μL of Neoplastin® reagent was

added, and the clotting time was noted. The recorded clotting time was used to determine prothrombin time expressed in seconds.

Thrombin time (TT)

Thrombin time was assessed by mixing 10 µL of each PMS solution with 90 µL of PPP, followed by an incubation period of 3 min at a temperature of 37°C. Subsequently, 100 µL of thrombin (80 NIH) was incorporated into the prepared solution, and the clotting time was measured and recorded in seconds.

Fibrinogen level (FL)

To determine the fibrinogen concentration of each PMS, 5 µL of each sample was mixed with 45 µL of PPP, which was diluted 20-fold with Owren–Koller solution. The resulting mixture was incubated at 37°C for 3 min. Subsequently, 100 µL of Multifibren® U reagent was added to the mixture, and the fibrinogen levels were directly quantified in grams per liter (g/L).

Antiproliferative effect of PMS using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The antiproliferative effect of PMS was investigated against K562 human myelogenous leukemia and Caco-2 colon cancer cell lines, following the methodology described by Secme *et al.* (2023). The results were presented as the mean values obtained from three independent experiments, reflecting the antiproliferative activity of PMS on the tested cell lines.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) from at least three independent replications. Results were statistically analyzed using SPSS ver. 17.0, professional edition (SPSS Inc., Chicago, USA). A one-way analysis of variance (ANOVA) was then performed to estimate significance among mean values at 5% probability level.

Results and Discussion

Yield and chemical composition of *M. oleifera* polysaccharides

The yields of polysaccharides extracted from the leaves of *M. oleifera* and their chemical composition were determined. Carbohydrates present in the leaves were recovered after hydrolysis using two types of enzymes to produce PMS, followed by ethanol precipitation. The results revealed that the yields of PMS-S-US-0 and PMS-S-US-10 were approximately 11.21% and 15.96%, respectively, while the yields of PMS-N-US-0 and PMS-N-US-10 were approximately 8.12% and 10.35%, respectively. Based on these findings, it was concluded that both US time and enzyme type used had a significant impact on the yield of polysaccharides. These results emphasized the importance of optimizing these parameters to achieve high polysaccharide yields in future applications. In the same context, Yang *et al.* (2023) proved that ultrasound treatment (495 W for 10 min) contributed more to increase the yield of polysaccharides from *Zingiber mioga* leaves, compared to microwave-assisted extraction, reaching 6.22% when combining both extraction processes.

The chemical composition of PMS-S-US-0, PMS-S-US-10, PMS-N-US-0, and PMS-N-US-10, regarding total carbohydrate, protein, and monosaccharide contents, was analyzed (Table 1). The findings revealed that the US treatment applied to PMS led to an increase in chemical constituents. These results aligned with the findings reported by Otu *et al.* (2020), demonstrating the effectiveness of US method and incorporation of enzymes in enhancing the rate of molecular movement. Additionally, these factors improved the solvent's ability to diffuse, thereby increasing the efficiency of polysaccharide extraction (Abdelhedi *et al.*, 2024; Gu *et al.*, 2022; Yang *et al.*, 2022).

As shown in Table 1, five neutral sugars (rhamnose [Rha], xylose [Xyl], mannose [Man], Glc [glucose], and Gal [galactose]) were found. The molar ratios of monosaccharides seem to vary, suggesting that different extraction methods influenced the composition of monosaccharides (Yang *et al.*, 2020). Xylose and glucose were the major monosaccharides in both PMS-S-US-10 and PMS-N-US-10 ($P < 0.05$). The contents of xylose in PMS-S-US-10 (72.4%) and PMS-N-US-10 (69%) were higher than those in other samples. In contrast, findings of Chen *et al.* (2017) suggested that *PMS* obtained through microwave-assisted extraction, predominantly consisted of Xyl, Glc, and Man. The study further indicated that the extraction method had a significant influence on extraction efficiency, monosaccharide composition, and glycosidic bond structure of the resulting polysaccharides (Abdelhedi *et al.*, 2024).

Polysaccharides' antioxidant activities

The antioxidant activity of PMS was assessed through various complementary tests, including the FRAP assay, iron (Fe^{2+})-chelating activity test, β-carotene bleaching inhibition capacity assay, DPPH• radical scavenging activity assay, ORAC assay, and TAC assay. These tests collectively provided insights into the antioxidant potential of PMS.

Iron (Fe^{2+})-chelating activity

The Fe^{2+}-chelating activity serves as an important antioxidant mechanism because it helps to reduce the

Table 1. Yield and protein, carbohydrate, and monosaccharide composition of polysaccharides extracted from *M. oleifera* (PMS) leaves.

Sample	PMS-S-US-0	PMS-S-US-10	PMS-N-US-0	PMS-N-US-10
Extraction yield (%)	11.21 ± 0.71^{b}	15.96 ± 0.28^{a}	8.12 ± 0.58^{c}	10.35 ± 0.03^{b}
Protein	5.20 ± 0.05^{b}	2.30 ± 0.06^{c}	6.30 ± 0.03^{a}	2.60 ± 0.05^{c}
Carbohydrate	23.00 ± 0.07^{c}	32.00 ± 0.05^{a}	21.00 ± 0.05^{d}	29.00 ± 0.04^{b}
Monosaccharide composition (molar ratio)				
Rha (%)	12.30 ± 0.05^{b}	4.50 ± 0.04^{c}	19.80 ± 0.03^{a}	3.70 ± 0.02^{d}
Xyl (%)	61.60 ± 1.06^{c}	72.40 ± 0.90^{a}	56.30 ± 1.10^{d}	69.00 ± 0.99^{b}
Man (%)	8.20 ± 0.33^{a}	7.80 ± 0.09^{b}	7.90 ± 0.07^{a,b}	6.30 ± 0.08^{c}
Glc (%)	14.50 ± 0.78^{b}	13.30 ± 0.10^{c}	12.70 ± 0.20^{d}	17.00 ± 0.10^{a}
Gal (%)	3.40 ± 0.43^{b}	2.00 ± 0.07^{c}	3.30 ± 0.09^{b}	4.00 ± 0.05^{a}

Different superscript letters ($^{a, b, c, d}$) within the same row indicate significant differences between samples at $P < 0.05$ based on Tukey's *post hoc* test. Xyl: xylose; Glc: glucose; Rha: rhamnose; Man: mannose; Gal: galactose.

concentration of catalyzing transition metals involved in lipid peroxidation. In this context, the chelating capacity of the reactive substance is assessed by its ability to disrupt the formation of Fe^{2+}–ferrozine complex. A reduction in the intensity of the purple-colored complex indicates disruption and reveals the chelating potential of the substance being tested. In the present study, the chelating capacity of PMS was tested. Results showed that obtained polysaccharides, PMS-S and PMS-N, exhibited significant dose-dependent chelating activities. PMS displayed significant chelating activity, which increased in a dose-dependent manner (Abdelhedi *et al.*, 2024). Notably, at a low concentration (1.5 mg/mL), both PMS-S-US-10 and PMS-N-US-10 were more effective than the untreated PMS (86.78% and 79.50%, respectively). The combined application of US treatment and enzyme extraction resulted in a noteworthy enhancement of antioxidant properties observed in PMS-S-US-10 and PMS-N-US-10, compared to untreated samples (PMS-S-US-0 and PMS-N-US-0). The utilization of ultrasonication in conjunction with enzyme extraction holds promising potential in the production of polysaccharides as well as the enhancement of their antioxidant properties (Noman *et al.*, 2020) (Figure 1A).

Ferric-reducing antioxidant power

FRAP assay, which relies on electron transfer reactions, is a widely employed method for assessing the antioxidant capacity of samples. Antioxidants in the sample can reduce Fe^{3+}–ferricyanide complex to its Fe^{2+} form. This reduction process signifies the presence of antioxidants and their potential to donate electrons, thus exhibiting antioxidant activity. The FRAP values of different polysaccharides increased significantly in a concentration range of 0.5–3 mg/mL (Figure 1B). The reducing power of PMS, obtained through various pretreatments, exhibited a significant increase with higher sample concentrations. This indicated that PMS samples could neutralize free radicals by donating electrons, thereby converting them into more stable products.

While the reducing ability of PMS-S-US-0 and PMS-N-US-0 did not differ significantly at an approximate concentration of 3 mg/mL, PMS-S-US-10 demonstrated significantly higher reducing power compared to PMS-N-US-10 at the same concentration (3 mg/mL), with respective values of 1.87 and 1.67. This implied that both PMS samples, after undergoing pretreatment, were capable of effectively donating electrons and engaging in interactions with free radicals. As a result, free radicals were converted into more stable compounds, leading to a higher absorbance value in FRAP assay, indicating an enhanced reducing power. Similar results were obtained by Ben Jeddou *et al.* (2016) and Ben Yakoub *et al.* (2020), who reported that polysaccharides from potato peels and Tossajute leaves, respectively, showed significant reducing power in a dose-dependent manner. Furthermore, Hu *et al.* (2021) discovered that mulberry leaf polysaccharides with lower molecular weight, resulting from enzymatic digestion, exhibited a greater iron scavenging ability, compared to those without enzymatic digestion. In addition, Noman *et al.* (2020) revealed that extraction methods (US and microwave) led to the generation of polysaccharides with distinct sequences, compositions, and sizes, thereby influencing their antioxidant activities.

β-carotene bleaching method

The ability of polysaccharides to neutralize linoleic hydroperoxyl radicals is depicted in Figure 1(C). Both PMS-S samples exhibited a higher capacity for bleaching β-carotene in a dose-dependent manner, with the highest effect observed in PMS-S-US-10. Particularly,

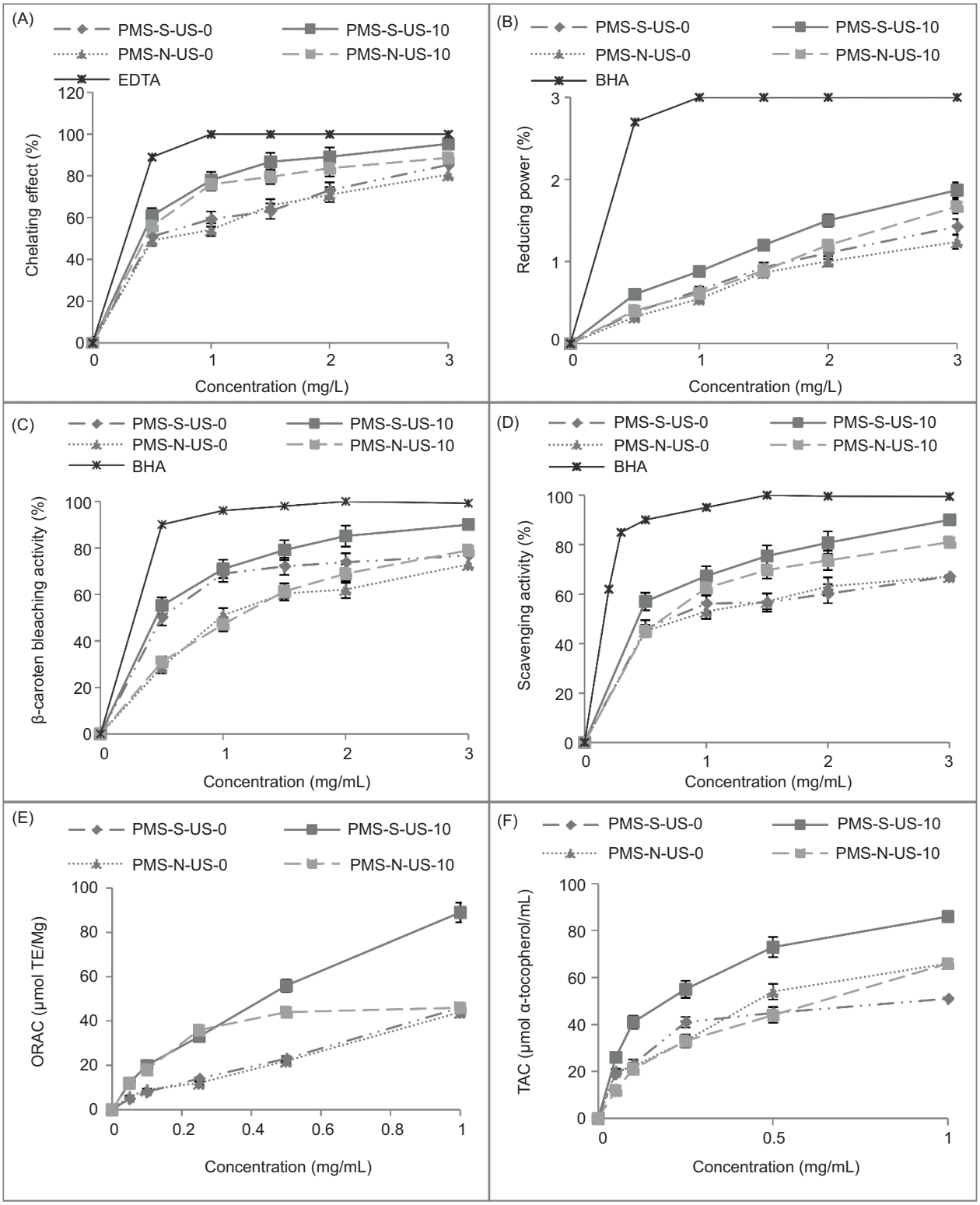

Figure 1. Antioxidant activities of polysaccharides extracted from *M. oleifera* leaves: (A) Fe^{2+}-chelating activity; (B) ferric-reducing antioxidant power (FRAP); (C) β-carotene bleaching method; (D) DPPH assay; (E) oxygen radical absorbance capacity (ORAC); and (F) total antioxidant capacity (TAC).

PMS-S-US-10 demonstrated significant activity (approximately 79.1%) at a concentration of 1.5 mg/mL whereas PMS-N-US-10 exhibited lower activity at the same concentration (approximately 61.32%). These findings indicated that PMS-S-US-10 displayed superior inhibition of β-carotene bleaching compared to the other PMS samples. This could be attributed to the combined treatment's ability to enhance antioxidant power. Similar results were reported by Ben Yakoub *et al.* (2020), who described that PMS derived from Tossa jute leaves exhibited significant antioxidant activity at a concentration of 1.5 mg.

DPPH assay

Antioxidants interact with DPPH• radicals, leading to their neutralization and subsequent decrease in absorbance, indicating their scavenging activity. The DPPH scavenging activity of obtained polysaccharides at different concentrations (0.5–4 mg/mL) is depicted in Figure 1(D). The US treatment combined with enzymatic extraction of PMS exhibited a clear quantitative relationship with the scavenging effects of DPPH radicals. The DPPH• radical scavenging activity was around 67% for both PMS-S-US-0 and PMS-N-US-0. However, PMS-S-US-10 and PMS-N-US-10 demonstrated higher inhibition of the DPPH• free radicals, indicating their high hydrogen donating capacity. Specifically, at a concentration of 3 mg/mL, PMS-S-US-10 exhibited higher scavenging activity (90.01%), compared to PMS-N-US-10 (80.98%). These results were also compared to standard antioxidant butylated hydroxyanisole (BHA), suggesting that PMS-S-US-10 showed promising scavenging activity, slightly lower than BHA antioxidant. Similarly, Yang *et al.* (2023) found that the highest antioxidant values of Microwave–US-assisted extraction of polysaccharides from *Z. mioga* leaves waste reached at 3 mg/mL, estimated at about 95.3%, for radical scavenging and an absorbance of 0.75 ± 0.04 for reducing power.

Moreover, He *et al.* (2018) reported that PMS extracted from *M. oleifera* leaves presented higher activity in a concentration-dependent manner at the same range of concentration, while Ben Jeddou *et al.* (2016) suggested that potato peels exhibited scavenging activity at higher concentration. In another study, the antioxidant activity of *M. oleifera* gum polysaccharide (arabinogalactan) was investigated using water extraction. The scavenging activity of DPPH free radicals was dose-dependent up to a concentration of 200 μg/mL. At a concentration of 300 μg/mL, the radical scavenging efficacy was 86%. The findings suggested that *M. oleifera* polysaccharides could potentially be used as a natural antioxidant.

Oxygen radical absorbance capacity

ORAC test determines the protection level of a substrate on fluorescein (target molecule) oxidized by peroxyl radicals of (2,2'-azobis(2-amidinopropane) dihydrochloride) (AAPH). The method used in the study involved a hydrogen atom transfer-based approach to evaluate the ability of polysaccharides to quench peroxyl radicals through hydrogen donation. Peroxyl radicals were generated using AAPH, while fluorescein was utilized as a fluorescent probe (Avilés-Gaxiola *et al.*, 2021).

Change in the area under curve (AUC) of kinetic profiles of the fluorescence brought on by fluorescein consumption is the basis for calculating ORAC. To determine Trolox equivalent (measurement used to express the antioxidant capacity of a substance relative to Trolox, a water-soluble vitamin E analog) of the sample's ORAC, the integrated AUC of the ORAC fluorescence quenched by PMS-S-US, PMS-N-US, and Trolox was utilized. Figure 1(E) displays ORAC values for each polysaccharide. The effectiveness of PMS-S-US-10 in preventing fluorescein from oxidation was demonstrated by the results, which were obtained at various tested concentrations. According to ORAC values, at doses < 1 mg/mL, PMS-S-US-10 had a significantly greater effect than other samples.

The ORAC value of PMS-S-US-10 at 0.1 mg/mL was around 20 μmol TE/mg of sample; in contrast, for PMS-S-US-0, PMS-N-US-0, and PMS-N-US-10 at the same dosage, the ORAC values were 8 μmol TE/mg, 9 μmol TE/mg, and 18 μmol TE/mg, respectively. These findings showed that PMS-S-US-10 could neutralize free radicals and prevent changes in fluorescence, with an increased capacity to do so with increased concentration. The biological activity of PMS is affected by the duration of ultrasonication and the composition of monosaccharides (Yang *et al.*, 2022). Moreover, structural characteristics strongly affected the antioxidant capacity of polysaccharides. Indeed, Zhang *et al.* (2023) reported that sulfated derivatives from *Sagittaria trifolia* polysaccharides showed different antioxidant potential depending on the degree of substitution, where higher degree of sulfation reduced antioxidant capacity, but a moderate substitution and the smallest molecular weight (MW) exerted better antioxidant activity, particularly of scavenging nitrogen-containing radicals.

Total antioxidant capacity

Figure 1(F) displays TAC of PMS in phosphormolybdenum test findings. It demonstrated that each polysaccharide displayed varying levels of activity, which increased with increase in concentration. In case of all tested concentrations, PMS-S-US-10 outperformed other PMS in terms of effectiveness. In contrast to PMS-S-US-0, PMS-N-US-0, and PMS-N-US-10, which had the same concentration, PMS-S-US-10 was able to decrease Mo (VI) to Mo (V) at 86 μmol/mL α-tocopherol equivalents (eq.), 51 μmol/mL α-tocopherol eq., and 66 μmol/mL α-tocopherol eq., respectively. This effect could be related to monosaccharide's composition and the effect

of US to enhance antioxidant activity. As observed, TAC increased with increase in polysaccharides concentration.

This was in line with the findings of other studies, reporting that the TAC efficiency of *Kappa phycus spp.* and *Opuntia stricta Haw.* fruit peels increased with increasing concentration of samples, thereby enhancing scavenging activity (Koubaa *et al.*, 2015; Palpperumal *et al.*, 2020).

Antibacterial activity

The agar diffusion method was used to assess the antibacterial activity of extracted polysaccharides. The extracted polysaccharides were tested against a panel of bacterial strains, including four Gram+ strains and five Gram– strains. The inhibition zones, measured in millimeter, indicated the extent of bacterial growth inhibition by extracted polysaccharides. Data revealed that PMS-S-US-10 and PMS-US-10 presented an interesting antibacterial potential against all tested microorganisms. Indeed, the inhibition zone of tested strains showed varying degrees of inhibition. It was more sensitive against *K. pneumonia* (Gram–) and *S. aureus* (Gram+), which varied from 20.5 mm to 27.5 mm and from 13.5 to 15.5 mm, respectively. PMS-S was more effective for all tested strains than PMS-N, especially PMS-S-US-10 after US treatment. Samples without US treatment presented lower antibacterial activity than other PMS. Arora and Kaur (2019) conducted research and discovered that *M. oleifera* polysaccharides possessed notable antibacterial properties that contributed to wound healing. In another study conducted by Rubio-Elizalde *et al.* (2019), the antimicrobial efficacy of *M. oleifera* polysaccharides was specifically evaluated against *S. aureus* and *E. coli* bacterial strains. In addition, polysaccharides extracted from two algal species exerted an inhibitory effect against several pathogenic microorganisms, including *E. coli*, *B. cereus*, and *S. aureus* (Ismail and Amer, 2021).

The findings of the present study revealed that the application of US treatment enhanced the antibacterial properties of PMS. Notably, the PMS-S fraction exhibited the highest level of antibacterial activity. This could be due to various factors, including the higher content of total sugars present in PMS-S, compared to PMS-N, as well as the monosaccharide composition of PMS-S. The increased total sugars and specific monosaccharides in PMS-S potentially contributed to its superior antibacterial efficacy, compared to PMS-N (Table 2).

Anticoagulant activity of PMS

The objective of the present study was to assess the impact of various polysaccharides derived from *M. oleifera* leaves on coagulation cascade. To achieve this, two specific coagulation tests: aPTT and prothrombin time (PT), were used. The aPTT test measured the time needed for fibrin formation, starting from the initiation of intrinsic pathway of coagulation cascade. On the other hand, the PT test measured the time required for fibrin production after the activation of factor VII, representing the extrinsic coagulation pathway (Abdallah *et al.*, 2022). Applying both tests enabled the evaluation of anticoagulant activity of polysaccharides.

The aPTT test was utilized to examine impact on intrinsic pathway, while the PT test focused on extrinsic coagulation pathway. Both tests provided insights into the influence of polysaccharide's anticoagulant activity on

Table 2. Antibacterial activities of different PMS extracted from *M. oleifera* leaves.

	PMS-S-US-0	PMS-S-US-10	PMS-N-US-0	PMS-N-US-10
Gram–				
E. coli	15.0 ± 0.14^{b}	18.0 ± 0.14^{a}	15.0 ± 0.14^{b}	15.5 ± 0.07^{b}
K. pneumonia	20.5 ± 0.14^{b}	27.5 ± 0.07^{a}	15.5 ± 0.14^{c}	17.5 ± 0.07^{c}
S. enterica	16.0 ± 0.14^{c}	17.5 ± 0.07^{b}	17.0 ± 0.14^{b}	18.5 ± 0.07^{a}
Pseudomonas	13.0 ± 0.14^{c}	16.5 ± 0.21^{a}	14.5 ± 0.21^{b}	16.0 ± 0.14^{a}
Enterobacter spp.	12.5 ± 0.07^{d}	13.5 ± 0.07^{c}	16.0 ± 0.14^{b}	17.0 ± 0.14^{a}
Gram+				
M. luteus	14.0 ± 0.0^{b}	15.5 ± 0.0^{a}	14.0 ± 0.0^{b}	15.0 ± 0.0^{a}
S. aureus	13.5 ± 0.14^{c}	15.5 ± 0.21^{a}	14.0 ± 0.07^{b}	14.5 ± 0.07^{b}
B. cereus	13.0 ± 0.14^{c}	14.5 ± 0.07^{b}	13.5 ± 0.07^{c}	15.0 ± 0.14^{a}
Listeria	14.0 ± 0.14^{a}	14.5 ± 0.35^{a}	13.0 ± 0.14^{b}	14.0 ± 0.14^{a}

Different superscript letters within the same row indicate significant differences between samples at $P < 0.05$ based on Tukey's *post hoc* test.

different stages of coagulation cascade. Then, thrombin time (TT) and fibrinogen level (FL) assays for coactivity were performed.

Activated partial thromboplastin and prothrombin period

Both PT and aPTT assays are frequently used to evaluate anticoagulant activity.

aPTT measures the time it takes for clot formation through intrinsic and/or common pathways. Prolonged aPTT values indicated inhibition or impairment of these pathways. On the other hand, PT measures the clotting time of extrinsic pathway. Any prolongation in PT suggests a delay in clot formation through this pathway. It is important to note that blood coagulation is a tightly regulated process. Imbalances in this system, whether due to genetic or environmental factors, can disrupt normal coagulation, leading to the formation of abnormal blood clots within blood vessels and posing a risk to overall health (Abdallah *et al.*, 2022). Understanding the mechanism and regulation of coagulation cascade, as well as evaluating its functional status through tests such as aPTT and PT, could provide a better understanding of potential anticoagulant effects of substances or the presence of coagulation disorders. That's why, the effect of PMS on human blood clotting was studied (Figures 2A and 2B).

When compared together, PMS-S-US-10 had a more pronounced effect on prolonging PT blood clotting time, reaching its maximum of 70 s at 1 mg/mL. However, in aPTT test, all PMS prolonged blood coagulation time in a similar manner irrespective of the concentration used ($P \geq 0.05$). According to these results, neither US nor enzyme treatments influenced the effectiveness of PMS on extending aPTT. These findings showed that, albeit to varying degrees, an isolated PMS could block both intrinsic and extrinsic blood coagulation pathways. In fact, the polysaccharides isolated from blackberry seeds in a study conducted by Wang *et al.* (2017) prolonged both clotting time and aPTT. Furthermore, Wang *et al.* (2017) studied the effect of *Angelica dahurice* roots' polysaccharides on blood coagulation and confirmed its ability to shorten PT, suggesting its exclusive action on the extrinsic pathways of blood coagulation. Moreover, Souza *et al.* (2015)

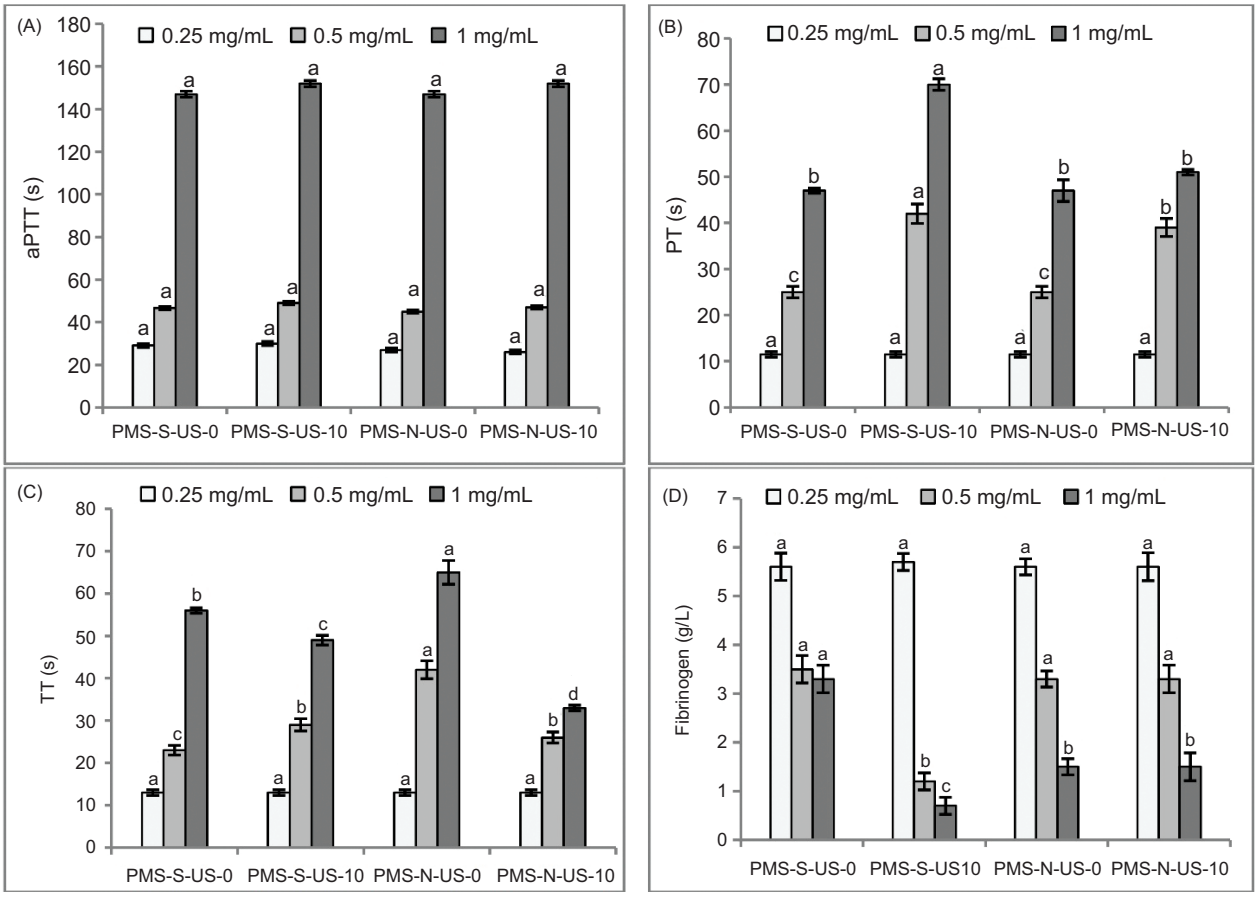

Figure 2. Anticoagulant activity of PMS at different concentrations: (A) Activated partial thromboplastin time (aPTT); (B) prothrombin time (PT); (C) thrombin time (TT); and (D) fibrinogen level. Different letters assigned to different samples within the same concentration indicate significant differences at $P < 0.05$ based on Tukey's *post hoc* test.

used purified polysaccharides from *Geoffroea spinosa* barks and reported that clotting Factor III (or thromboplastin/FIII) (0.066 mg/mL) increased aPTT clotting to 121 s. In their study, the authors observed that the discovered anticoagulant activity of polysaccharides could be attributed to a delicate balance between inhibiting coagulation proteases, leading to an anticoagulant effect, and activating factor XII, which has a pro-coagulant activity. However, further investigations are needed to delve deeper into this aspect, specifically in relation to plant polysaccharides.

In aPTT test, the application of crude polysaccharides derived from red algae *Corallina officinalis* and *Pterocladia capillacea* resulted in the prolongation of both aPTT and PT results (Ismail and Amer 2021). Specifically, at a concentration of 0.1 mg/mL, the red algae polysaccharides extended aPTT to 46 and 51 s for *Corallina officinalis* and *Pterocladia capillacea*, respectively. Additionally, PT values were prolonged to 20 s for *Corallina officinalis* and 23 s for *Pterocladia capillacea* at the same concentration. These findings indicated that crude polysaccharides extracted from both algal species possessed anticoagulant properties, as evidenced by prolonged aPTT and PT values in aPTT test. The authors suggested that these red algae polysaccharides might have an inhibitory effect on coagulation cascade, potentially affecting both intrinsic and extrinsic pathways.

Similarly, de Araujo *et al.* (2021) found a 3.5-fold clotting aPTT increase in the presence of 0.3 mg/mL *Caesalpinia ferreastem* barks' polysaccharides, but with a minimal effect on PT coagulation time. On the contrary, polysaccharide extracted from *Pinus halepensis* using ethanol precipitation exhibited a PT value of 82 s at 0.1 mg/mL (Abbou *et al.*, 2019). In a more recent study, Wu *et al.* (2024) extracted four water-soluble polysaccharides (ethanol precipitation and enzymatic hydrolysis) from *Clausena lansium* fruit pulp, showing anticoagulant activity by prolonging aPTT and disturbing intrinsic coagulation pathway. The most active polysaccharide fraction had a MW of 510.1 kDa and was mainly composed of Gal, Ara and GalA. However, Cao *et al.* (2019) stated the hypothesis that the anticoagulant activity of polysaccharides is determined from the specific sites of sulfation within their structure. Additionally, the distribution and proportion of sulfation pattern in polysaccharide (SP) structure played a crucial role in influencing its anticoagulant properties. According to Cao *et al.*'s (2019) suggested explanation, the presence of sulfate groups at specific positions within polysaccharide chain is essential for exhibiting anticoagulant activity. The arrangement and density of these sulfate groups, as well as the overall sulfation pattern, contribute to the structural basis of polysaccharide's anticoagulant effects. In other words, the anticoagulant activity of polysaccharides is intricately linked to the sites of sulfation within their structure as well as the distribution and proportion of sulfate groups along the polysaccharide chain. Understanding these structural aspects is crucial for elucidating the underlying mechanisms of their anticoagulant properties.

Thrombin time and fibrinogen level

Following their respective activations, both intrinsic and extrinsic pathways of the blood coagulation cascade converge into a common pathway leading to the activation of factor Xa. Once activated, factor Xa plays a crucial role in the subsequent steps of coagulation process. Factor Xa acts to cleave prothrombin, an inactive precursor, into its active form known as thrombin. Thrombin, in turn, plays a central role in the clotting process. One of its key functions is the conversion of fibrinogen, a soluble plasma protein, into fibrin—a fibrous protein that forms a mesh-like structure, essential for clot formation and stabilization. In other words, after the activation of intrinsic and extrinsic pathways, the common pathway is initiated with the formation of factor Xa, which then facilitates the conversion of prothrombin to thrombin. Thrombin subsequently leads to the conversion of fibrinogen into fibrin. This fibrin network serves as the foundation for blood clot formation (Abdallah *et al.*, 2022). In this test, both thrombin time (TT) and fibrinogen level (FL) were evaluated (Figures 2C and 2D). In the TT test, all the extracted polysaccharides were able to extend blood clotting time. At high concentration (1 mg/mL), PMS-S-US-0 and PMS-N-US-0 prolonged blood coagulation time by 56 s and 65 s, respectively, while PMS-S-US-10 and PMS-N-US-10 did not exceed 49 s and 33 s, respectively. According to obtained results, the US effect seems to inhibit thrombosis phenomenon. In FL test, results were enhanced for treated samples with an important decrease of fibrinogen concentration, reaching 0.7 g/L and 1.5 g/L, by the addition of PMS-S-US-10 and PMS-N-US-10, respectively. These findings confirmed the ability of PMS-S-US-10 and PMS-N-US-10 to reduce the level of fibrin that inhibits the formation of a dense blood clot in common coagulation pathway.

In a related study, Wang et al. (2017) investigated the anticoagulant properties of polysaccharides derived from blackberry seeds. The researchers specifically focused on three polysaccharides designated as BSP-1b, BSP-2, and BSP-3. Their findings revealed that these polysaccharides exhibited notable anticoagulant activity. The authors further noted that the anticoagulant effect was influenced by several factors. First, a lower MW of polysaccharides was found to be associated with enhanced anticoagulant activity. Additionally, the monosaccharide composition of polysaccharides played a significant role in determining their anticoagulant properties. Specific arrangement and distribution of monosaccharides within the polysaccharide backbone structure influenced the

observed anticoagulant activity. To sum up, the anticoagulant activity of polysaccharides derived from blackberry seeds, namely BSP-1b, BSP-2, and BSP-3, was dependent on factors such as lower MW, monosaccharide composition, and their positioning within the polysaccharide structure. Understanding these structural characteristics provides insights into the mechanisms underlying the observed anticoagulant effects of these polysaccharides.

Oxidative stress is known to contribute to endothelial damage and the activation of cascade coagulation pathways, leading to thrombus formation (Li *et al.*, 2024). *M. oleifera* polysaccharides, with their strong free radical scavenging, can help to reduce oxidative damage, thereby maintaining vascular homeostasis and inhibiting the aggregation of platelets. Consequently, the combined antioxidant and anticoagulant activities of *M. oleifera* polysaccharides could be considered as a promising solution for managing oxidative stress and thrombotic disorders, and therefore the prevention of cardiovascular matters. In the same context, Yang *et al.* (2023) stated an excellent antioxidant capacity and a promising anticoagulant property of *Z. mioga* leaves' polysaccharides by acting as a DPPH-radical scavenging agent and by prolonging aPTT, TT, PT, and lowering FL, at a concentration of 5 mg/mL. Based on these activities, the authors qualified the extracted polysaccharide as a novel cardiovascular-protective candidate.

Antiproliferative activity of PMS

The MTT assay was used to investigate the antiproliferative effects of PMS on K562 human myelogenous leukemia cells and Caco-2 colon cancer cells at concentrations of 100 µg/mL and 200 µg/mL (Figures 3A and 3B).

All PMS demonstrated potential inhibitory effects against the tested cancer cells, where the activity significantly increased with PMS concentration ($P < 0.05$). Data showed that PMS-S-US-10 displayed the highest inhibitory cells' proliferation levels of 96% and 99% for K562 and Caco-2 cells, respectively, while PMS-N-US-0 exhibited the lowest inhibitory activity against the tested cells at 200 µg/mL. The results showed that PMS demonstrated antiproliferative potential, as it significantly decreased the viability of K562 cells at both concentrations. The potent antiproliferative activity of PMS-S-US-10 is potentially attributed to its notable antioxidant effect, as previous research indicated that compounds capable of augmenting anti-oxidation levels and eliminating reactive oxygen species in cells could hinder the growth of cancer cells (Abdelhedi *et al.*, 2024).

Other studies have also revealed that PMS from *M. oleifera* possessed hepatoprotective and anticancer properties. For instance, Sun *et al.* (2019) isolated phenolic glycoside 1-O-(4-hydroxymethylphenyl)-α-L-rhamnopyranoside (MPG) from *M. oleifera* Lam seeds, and it was found to mitigate the hepatotoxicity of CCl4-induced L02 cells and ICR mouse models. MPG led to a substantial increase in cell viability and superoxide dismutase (SOD) activity, regulated levels of inflammatory cytokines (TNF-α, IL-1β, and MCP-1), and reduced hepatotoxicity in CCl4-treated mice. The abundance of

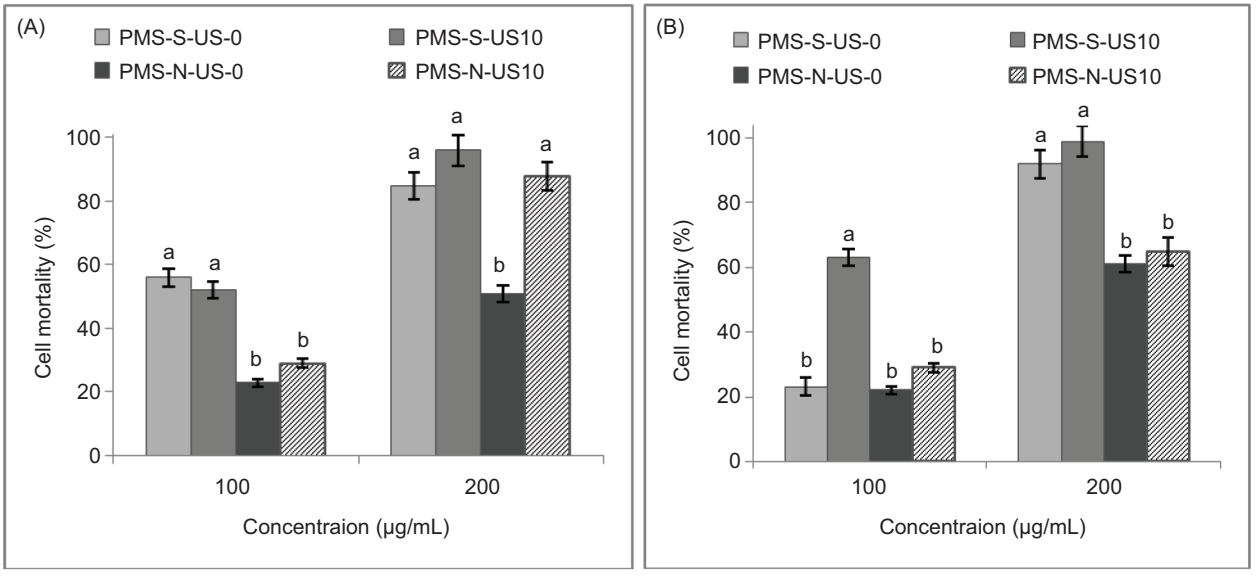

Figure 3. Antiproliferative activity of PMS against (A) K562 human myelogenous leukemia cells, and (B) Caco-2 colon cancer cells at different concentrations. Different letters assigned to different samples within the same concentration indicate significant differences at $P < 0.05$ based on Tukey's *post hoc* test.

antioxidants in *M. oleifera* allowed it to alleviate oxidative stress, consequently aiding in the prevention of cancer (Singh *et al.*, 2020).

Prior reports have also highlighted the anticancer properties of the *M. oleifera* tree (Al-Asmari *et al.*, 2015; Jung *et al.*, 2014). Furthermore, other natural compounds derived from *M. oleifera* have potential for inhibiting cancer proliferation. Jung *et al.* (2014) demonstrated that cold water extracts of *M. oleifera* exhibited higher anticancer activities, compared to hot water extracts, possibly due to the inactivation of certain heat-sensitive bioactive molecules present in the leaves of hot water extracts.

Conclusion

Based on the findings of this study, it was concluded that PMS biological activities strongly depended on extraction methods, where the alkali (savinase)-US-assisted extraction provided the most active PMS. In fact, PMS-S-US10 displayed the strongest antioxidant activity, potentially decreased the production of fibrin clots, and inhibited the extrinsic (PT) coagulation pathways, as well as limited the proliferation of colon cancer and human myelogenous leukemia cells. In summary, these findings underline the advancements in extracting and applying polysaccharides from *M. oleifera* leaves as health-promoting agent. The relationship between the structural characteristics of PMS and their biological functioning is worthy to be conducted to enhance the understanding of their structure–activity correlations.

To bridge the gap between *in vitro* efficacy and practical application, conducting comprehensive *in vivo* studies is essential for assessing pharmacokinetics, bioavailability, and safety profiles. Finally, clinical trials are necessary to confirm health benefits and therapeutic relevance of PMS in humans.

Declaration of Competing Interest

The authors confirmed the absence of any conflict of interest, and disclosed no significant financial support that may have biased study findings.

Data Availability

Data is made available on request.

Author Contributions

MM: Investigation, Writing – Original Draft. **OA**: Investigation, Data Curation, Writing – Original Draft, Visualization. **WE**: Data Curation, Writing – Original Draft, Visualization. **HB**: Methodology, Statistics, Writing – Review & Editing, **NF**: Conceptualization, Writing – Review & Editing. **NZ**: Conceptualization, Writing – Review & Editing, Supervision. **MJ**: Methodology, Writing – Review & Editing, Supervision. All authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declared no conflict of interest.

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