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LC-MS/MS-Based Phenolic Profiling of Flaxseed (*Linum usitatissimum* L.) and Safflower (*Carthamus tinctorius* L.) Seed Extracts and Their Antibacterial Activity Against Selected Fish Pathogenic Bacteria

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ABSTRACT

The growing demand for sustainable disease-control strategies in aquaculture has increased interest in plant-derived compounds with potential antibacterial activity against fish pathogens. This study investigated the LC-MS/MS-based phenolic profile and *in vitro* antibacterial activity of aqueous methanolic flaxseed (*Linum usitatissimum* L.) and safflower (*Carthamus tinctorius* L.) seed extracts against selected fish pathogenic bacteria. The extracts were obtained using 40% methanol, concentrated, and prepared as aqueous stock solutions. The stock concentrations were 0.148 g mL^{-1} for flaxseed and 0.101 g mL^{-1} for safflower. The phenolic profiles of the final aqueous stock solutions were then determined by LC-MS/MS and expressed as μg L^{-1} (ppb). LC-MS/MS analysis showed that the flaxseed extract was mainly characterized by a tannic acid-rich profile, together with trans-ferulic acid, caffeic acid, gallic acid, quercetin, rutin trihydrate, cinnamic acid, and 2,5-dihydroxybenzoic acid. In contrast, the safflower seed extract contained trans-ferulic acid, cinnamic acid, 2,5-dihydroxybenzoic acid, ellagic acid, caffeic acid, and quercetin as the detected phenolic constituents. Antibacterial activity was tested by the broth microdilution method against *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Pseudomonas putida*, *Yersinia ruckeri*, and *Vibrio anguillarum*. The strongest antibacterial activity was observed for flaxseed extract against *A. hydrophila* and *A. salmonicida*, with MIC values of 25 and 50 μg mL^{-1}, respectively. Safflower seed extract showed its highest inhibitory activity against *A. salmonicida*, with an MIC value of 50 μg mL^{-1}, while higher MIC values were recorded against *A. hydrophila*, *Y. ruckeri*, and *V. anguillarum*. No inhibitory activity was detected against *P. putida* for either extract within the tested concentration range. These results indicate that aqueous methanolic flaxseed and safflower seed extracts, particularly flaxseed, have species-dependent antibacterial activity against fish pathogens. Although these findings suggest that the tested extracts may be considered as preliminary natural antibacterial candidates for aquaculture-related research, further studies are needed to evaluate their safety, stability, and *in vivo* applicability.

INTRODUCTION

Bacterial infections remain one of the persistent constraints in intensive aquaculture, where both opportunistic and primary pathogens can cause mortality, reduced growth, treatment costs, and economic losses in farmed fish. Species belonging to *Aeromonas*, *Yersinia*, *Vibrio*, and *Pseudomonas* are frequently associated with bacterial diseases in freshwater, marine, and brackish-water fish culture (Tobback et al., 2007; Öztürk & Altınok, 2014; Terzi et al., 2023). Among these bacteria, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia ruckeri*, *Vibrio anguillarum*, and *Pseudomonas putida* are particularly relevant to fish health studies because of their association with disease outbreaks and opportunistic infections under culture conditions. Antibiotics are still used for the treatment and control of bacterial diseases in aquaculture. However, their repeated or inappropriate application may contribute to antimicrobial resistance, environmental contamination, and residue-related concerns (Ramesh & Souissi, 2018; Terzi & Isler, 2019). These limitations have encouraged the evaluation of non-antibiotic strategies, including vaccines, probiotics, bacteriophages, organic acids, and plant-derived bioactive products that may support disease control in aquaculture (Van Hai, 2015; Tadese et al., 2022; Li et al., 2022). Medicinal and aromatic plants are important sources of phenolic and other bioactive compounds, including phenolic acids, flavonoids, tannins, terpenoids, and alkaloids. These compounds have been associated with antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities, although their effects may vary according to compound type, concentration, extraction method, and target organism (Van Hai, 2015; Bouarab-Chibane et al., 2019; Tadese et al., 2022). In antibacterial screening studies, however, crude plant extracts need to be interpreted with caution, since their activity is rarely explained by a single constituent. Rather, the observed response may result from combined interactions, including additive, synergistic, or antagonistic effects among several compounds in the extract (Liu, 2003; Terzi et al., 2023). Despite the growing body of research on plant-derived antibacterials in aquaculture, the antibacterial potential of oilseed extracts, particularly flaxseed (*Linum usitatissimum* L.) and safflower (*Carthamus tinctorius* L.), against multiple fish pathogens remains insufficiently explored. Flaxseed is known for its nutritional value and phenolic constituents, including phenolic acids, lignans, flavonoids, and tannin-related compounds, whereas safflower is valued for its seed oil and phytochemical composition. Evaluating the phenolic composition and strain-specific antibacterial activity of these seed extracts may therefore contribute to the identification of candidate plant-derived products for aquaculture-related research.

Although a number of studies have evaluated plant extracts against fish pathogens, comparative investigations that combine targeted phenolic profiling with antibacterial screening remain relatively scarce and provide a stronger basis for selecting candidate extracts for aquaculture-related research (Bilen et al., 2016; Karga et al., 2020; Metin et al., 2021; Pires et al., 2021; Li et al., 2022). Chemical characterization is particularly important because it helps relate the observed antibacterial activity to the phytochemical composition of the extract, rather than interpreting inhibition data alone. The present study aimed to determine the phenolic composition of aqueous methanolic flaxseed and safflower seed extracts using LC-MS/MS and to evaluate their *in vitro* antibacterial activity against selected fish pathogenic bacteria using the broth microdilution method.

MATERIAL AND METHODS

Seed Materials and Extract Preparation

Seeds of safflower and flaxseed were purchased from a commercial herbal supplier in Kastamonu, Türkiye. The seed materials were transported to the laboratory under dry conditions and stored at room temperature until extraction. Before extraction, the seeds were cleaned manually to remove foreign materials and then ground into a fine powder using a laboratory grinder.

For each plant, 50 g of dried seed powder was extracted with 1 L of 40% methanol (v/v) for 72 h at room temperature. During extraction, the mixtures were inverted twice daily. After extraction, the

mixtures were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated to dryness under reduced pressure using a rotary evaporator. The remaining dry extract residue was dissolved in 30 mL of distilled water. The final aqueous stock extract concentrations were determined gravimetrically as 0.101 g mL^{-1} for safflower seed and 0.148 g mL^{-1} for flaxseed. Based on these gravimetrically determined stock concentrations, the amount of dry extract obtained from 50 g of seed powder was calculated as 3.03 g for safflower seed and 4.44 g for flaxseed, corresponding to extraction yields of 6.06% and 8.88%, respectively.

LC-MS/MS Analysis of Phenolic Compounds

The phenolic composition of the extracts was determined at Kastamonu University Central Research Laboratory using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer system. The injection volume was 10 μL. Chromatographic separation was performed on an Inertsil ODS-4 column (3 μm, 2.1 × 50 mm). Mobile phase A consisted of water containing 0.1% formic acid, and mobile phase B consisted of methanol containing 0.1% formic acid. The flow rate was 0.4 mL min^{-1}, and the column temperature was maintained at 40°C. The gradient program was applied as follows: 5% A and 95% B from 4.00 to 7.00 min, followed by a shift to 95% A and 5% B at 7.01 min, which was maintained until the end of the run at 12.00 min.

The monitored phenolic compounds included catechin, tannic acid, myricetin, naringenin, ellagic acid, quercetin, luteolin, chrysin, cinnamic acid, caffeic acid, trans-ferulic acid, 2,5-dihydroxybenzoic acid, rutin trihydrate, and gallic acid. The corresponding precursor/product ion transitions are presented in Table 1. Results were expressed as μg L^{-1} (ppb) for the final aqueous extract solutions. The LC-MS/MS analysis was performed as a targeted phenolic profiling analysis using the available laboratory method parameters, including chromatographic conditions, ionization modes, and precursor/product ion transitions. A full validation dataset including LOD, LOQ, recovery, and precision values was not available for all monitored phenolic compounds. Therefore, the LC-MS/MS results were interpreted as targeted chemical profiling data for the final aqueous stock extract solutions rather than as fully validated absolute quantification.

Table 1. LC-MS/MS ion transitions used for the determination of phenolic compounds in the extracts

Compound	Ionization mode	Precursor > product ions (m/z)
Catechin	Positive	291.1 > 138.90, 122.90
Tannic acid	Negative	182.90 > 123.60, 78.20
Myricetin	Negative	316.80 > 178.90, 151, 137
Naringenin	Negative	270.80 > 150.90, 118.90, 92.90
Ellagic acid	Negative	300.80 > 229.10, 257.10
Quercetin	Negative	300.80 > 150.80, 121.10, 106.90
Luteolin	Negative	284.80 > 217, 198.80, 174.90
Chrysin	Negative	252.80 > 142.90, 119, 209.10, 106.90
Cinnamic acid	Positive	149.10 > 130.90, 103.20
Caffeic acid	Negative	178.90 > 135, 134, 89
trans-Ferulic acid	Negative	192.80 > 132.90, 178.00
2,5-Dihydroxybenzoic acid	Negative	153.10 > 107.90, 109.00
Rutin trihydrate	Negative	609 > 300, 271, 301
Gallic acid	Negative	168.80 > 124.90, 78.90, 81.00

Bacterial Strains

The antibacterial activity of the extracts was tested against five fish pathogenic bacteria: *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Pseudomonas putida*, *Yersinia ruckeri*, and *Vibrio anguillarum*. The bacterial strains had been previously isolated from diseased rainbow trout (*Oncorhynchus mykiss* Walbaum) and identified using conventional morphological and biochemical analyses, together with 16S rRNA gene sequencing (Terzi et al., 2023). For the present antibacterial assays, the isolates were revived from stock cultures and used at the first passage after subculture on Mueller Hinton Agar. The plates were incubated at 25°C for 24–48 h until colonies developed. Bacterial suspensions were prepared in sterile Mueller Hinton Broth and first adjusted to the turbidity of a 0.5 McFarland standard. The suspensions were then diluted in sterile Mueller Hinton Broth to obtain a final inoculum concentration of approximately 10^6 CFU mL^{-1} for use in the microdilution assay.

Minimum Inhibitory Concentration Determination

The minimum inhibitory concentration (MIC) values of the extracts were determined using the broth microdilution method, in accordance with the general principles of antimicrobial susceptibility testing outlined by the Clinical and Laboratory Standards Institute (CLSI, 2018), with minor adaptations for plant extracts. Serial dilutions of safflower seed and flaxseed extracts were prepared in Mueller Hinton Broth in sterile 96-well microplates to obtain final extract concentrations ranging from 25 to 1600 μg mL^{-1}. Each well contained the appropriate extract dilution and the standardized bacterial suspension. Wells containing bacterial suspension without extract served as growth controls, whereas wells containing broth without bacterial inoculum served as sterility controls. The plates were incubated at 25°C for 48 h. The MIC was defined as the lowest extract concentration that visibly inhibited bacterial growth after incubation (CLSI, 2018; Hajji et al., 2010; Terzi et al., 2023). All assays were performed in triplicate, and MIC values were expressed as μg mL^{-1}.

Data Reporting

LC-MS/MS results were expressed as μg L^{-1} (ppb) for the final aqueous stock extract solutions. The LC-MS/MS analysis of each final aqueous stock extract solution was performed as a single analytical measurement; therefore, standard deviation values could not be calculated. The results were presented descriptively as targeted phenolic profiling data. Compounds not detected were reported as "nd" (not detected) (Table 2). MIC values were presented descriptively, as MIC represents an endpoint dilution value obtained from serial dilution assays and corresponds to discrete intervals; therefore, no inferential statistical analysis was applied to the MIC endpoints.

RESULTS

Phenolic Composition of the Extracts

LC-MS/MS analysis showed that the two extracts had distinct phenolic profiles (Table 2). In the safflower seed extract, trans-ferulic acid was detected at the highest concentration (590.54 ppb), followed by cinnamic acid (183.04 ppb), 2,5-dihydroxybenzoic acid (154.22 ppb), ellagic acid (63.45 ppb), caffeic acid (19.38 ppb), and quercetin (4.73 ppb). Rutin trihydrate, gallic acid, and tannic acid were not detected in this extract. The flaxseed extract showed a broader phenolic profile. Tannic acid was the dominant compound, with a concentration of 25596.31 ppb. Considering the gravimetrically determined flaxseed stock extract concentration of 0.148 g mL^{-1}, the detected tannic acid concentration corresponds to approximately 0.017% of the total dry extract in the stock solution. Other detected compounds included trans-ferulic acid (761.81 ppb), caffeic acid (183.60 ppb), gallic acid (142.52 ppb), quercetin (79.26 ppb), 2,5-dihydroxybenzoic acid (63.75 ppb), rutin trihydrate (33.79 ppb), and cinnamic acid (12.44 ppb). Ellagic acid was not detected in the flaxseed extract. Catechin, myricetin, naringenin, luteolin, and chrysin were not detected in either extract. Overall, the flaxseed extract contained a greater number of detected phenolic compounds, and tannic acid was the dominant compound within the monitored phenolic panel.

Table 2. Comparative phenolic composition μg L^{-1} (ppb) of aqueous methanolic seed extracts from flaxseed and safflower determined by LC-MS/MS.

Compound	Safflower	Flaxseed
Cinnamic acid	183.04	12.44
Caffeic acid	19.38	183.60
trans-Ferulic acid	590.54	761.81
Rutin trihydrate	nd	33.79
Quercetin	4.73	79.26
Ellagic acid	63.45	nd
Gallic acid	nd	142.52
Tannic acid	nd	25596.31
2,5-Dihydroxybenzoic acid	154.22	63.75

**Note:* Values are expressed as μg L^{-1} (ppb) and represent single LC-MS/MS measurements of the final aqueous stock extract solutions; therefore, standard deviation values are not presented. nd: not detected. Compounds monitored but not detected in either extract: catechin, myricetin, naringenin, luteolin, and chrysin.

Antibacterial Activity of the Extracts

The extracts showed species-dependent inhibitory activity against the tested fish pathogenic bacteria (Table 3). Flaxseed extract exhibited the lowest MIC values recorded in the study, with MICs of 25 μg mL^{-1} against *A. hydrophila*, 50 μg mL^{-1} against *A. salmonicida*, and 400 μg mL^{-1} against *Y. ruckeri*. No inhibitory activity was detected against *P. putida* or *V. anguillarum* within the tested concentration range. Safflower seed extract showed an MIC value of 50 μg mL^{-1} against *A. salmonicida*. Higher MIC values were recorded against *A. hydrophila* (400 μg mL^{-1}), *Y. ruckeri* (800 μg mL^{-1}), and *V. anguillarum* (1600 μg mL^{-1}), whereas no inhibition was observed against *P. putida*. Among the tested pathogens, *A. hydrophila* was the most susceptible bacterium to flaxseed extract, while *A. salmonicida* was inhibited by both extracts at 50 μg mL^{-1}. In contrast, *P. putida* showed no detectable susceptibility to either extract under the tested conditions.

Table 3. MIC values of aqueous methanolic safflower and flaxseed seed extracts against selected fish pathogenic bacteria

Bacteria	Safflower	Flaxseed
A. hydrophila	400	25
A. salmonicida	50	50
P. putida	NI	NI
Y. ruckeri	800	400
V. anguillarum	1600	NI

Note: Values are expressed as μg mL^{-1}. MIC: minimum inhibitory concentration; NI: no inhibition detected within the tested concentration range.

DISCUSSION

The present study demonstrated that aqueous methanolic flaxseed and safflower seed extracts have detectable *in vitro* antibacterial activity against selected fish pathogenic bacteria. The activity was not uniform across bacterial species, indicating that the antibacterial response was influenced by both the extract type and the target microorganism. This pattern is consistent with the behavior of crude plant extracts, whose effects may vary depending on their phenolic composition, extraction characteristics, and the susceptibility of the tested bacterium (Bouarab-Chibane et al., 2019; Li et al., 2022).

A key finding was the strong activity of flaxseed extract against *A. hydrophila* and *A. salmonicida*, with MIC values of 25 and 50 μg mL^{-1}, respectively. These bacteria are important in aquaculture because *Aeromonas* species are associated with economically relevant bacterial diseases in cultured fish. In particular, *A. hydrophila* has been linked to motile aeromonad septicemia, hemorrhagic septicemia, and ulcerative or red-sore disease, whereas *A. salmonicida* is recognized as the causative agent of furunculosis and septicemic infections, especially in salmonid fish (Öztürk & Altınok, 2014; Dallaire-Dufresne et al., 2014; Semwal et al., 2023). In addition, *A. hydrophila* has been widely used in experimental challenge models to evaluate the protective potential of plant-derived products in rainbow trout (Bilen et al., 2016).

Therefore, the low MIC values recorded for flaxseed extract are particularly relevant as preliminary *in vitro* antibacterial evidence.

The more pronounced antibacterial activity of flaxseed extract may be partly associated with its broader targeted phenolic profile. LC-MS/MS analysis showed that tannic acid was the dominant compound within the monitored phenolic panel of this extract, together with trans-ferulic acid, caffeic acid, gallic acid, quercetin, rutin trihydrate, cinnamic acid, and 2,5-dihydroxybenzoic acid. Phenolic compounds can interact with bacterial cell surfaces and may affect microbial growth through various mechanisms, including membrane disruption and enzyme inhibition (Bouarab-Chibane et al., 2019). Tannins, in particular, have been reported to inhibit bacterial growth through iron chelation, interference with cell wall synthesis, disruption of membrane integrity, and inhibition of certain metabolic pathways (Farha et al., 2020). The predominance of tannic acid among the monitored phenolic compounds may therefore be associated with the distinct chemical profile of flaxseed extract; however, this relationship should not be interpreted as evidence that tannic acid alone was responsible for the observed antibacterial activity. In addition, the apparently high tannic acid value should be interpreted in relation to the concentrated final stock extract solution and the total dry extract concentration, rather than as a direct concentration in the original seed material.

However, the antibacterial effect should not be attributed to tannic acid alone. Although the high tannic acid concentration in flaxseed extract may have contributed to the lower MIC values, crude plant extracts represent complex chemical mixtures rather than single-compound preparations. Moreover, the LC-MS/MS values reported in the present study represent the concentrations of selected phenolic compounds in the final aqueous stock solutions, whereas the MIC values are expressed as total dry extract concentrations. Therefore, the quantified phenolics should be interpreted primarily as chemical markers of the extract profile, rather than as direct evidence that the measured individual compounds reached antibacterial concentrations in the MIC wells. The observed inhibition may also involve unmonitored constituents of the crude extract and additive or synergistic interactions among multiple compounds. Liu (2003) emphasized that the biological effects of plant-derived products are often associated with combined phytochemical interactions rather than isolated compounds alone. Similarly, Bouarab-Chibane et al. (2019) reported that the antibacterial activity of polyphenols depends on multiple physicochemical properties and interactions with bacterial cell surfaces. In the present study, flaxseed extract contained not only tannic acid but also trans-ferulic acid, caffeic acid, gallic acid, quercetin, rutin trihydrate, cinnamic acid, and 2,5-dihydroxybenzoic acid. These phenolic acids, flavonoids, and tannin-related compounds may have contributed to the observed antibacterial effect through complementary mechanisms, including membrane disturbance, enzyme inhibition, metal chelation, and interference with bacterial metabolic processes (Cushnie & Lamb, 2005; Bouarab-Chibane et al., 2019; Farha et al., 2020). Flavonoids such as quercetin and rutin have also been associated with antimicrobial activity in previous reviews (Cushnie & Lamb, 2005). Therefore, the stronger activity of flaxseed extract against *A. hydrophila* and *A. salmonicida* should be interpreted as the possible result of its overall phenolic profile, rather than as a direct effect of tannic acid alone.

The phenolic composition of the extracts was also compared with previous reports on flaxseed and safflower. Flaxseed has been reported to contain several phenolic acids and flavonoids, including ferulic acid, cinnamic acid derivatives, rutin, quercetin, and related phenolic constituents (Oomah et al., 1995; Koçak, 2024). In agreement with these reports, the present flaxseed extract contained trans-ferulic acid, caffeic acid, gallic acid, quercetin, rutin trihydrate, cinnamic acid, and 2,5-dihydroxybenzoic acid. Notably, tannic acid was detected as the dominant compound within the monitored phenolic panel, distinguishing flaxseed extract from safflower seed extract. For safflower seed, previous studies have reported phenolic acids and flavonoids such as trans-ferulic acid, quercetin derivatives, rutin, luteolin, naringin, and catechin-type compounds (Yu et al., 2013). In the present study, safflower seed extract was mainly characterized by trans-ferulic acid, cinnamic

acid, 2,5-dihydroxybenzoic acid, ellagic acid, caffeic acid, and quercetin. The detection of trans-ferulic acid as the main compound in the present safflower seed extract is in line with previous reports showing that safflower seeds contain ferulic acid and related hydroxycinnamic acid-derived phenolic constituents, including N-feruloylserotonin and N-(p-coumaroyl)serotonin (Kim et al., 2007; Katsuda et al., 2009; Yu et al., 2013). Nevertheless, direct numerical comparison with the literature should be made cautiously because the present LC-MS/MS results were expressed as μg L^{-1} (ppb) in the final aqueous stock solutions, whereas many previous studies report total phenolics as mg GAE g^{-1} or individual compounds as mg g^{-1} dry material or extract. Therefore, differences among studies may reflect plant material and variety, extraction solvent, sample preparation, target analyte panel, analytical method, and unit expression. For this reason, the present results are more appropriately compared with previous studies in terms of detected compound groups and relative compositional trends rather than direct absolute concentrations. Within this context, the tannic acid-rich and broader targeted phenolic profile of flaxseed extract may be associated with its lower MIC values compared with safflower seed extract; however, this association should not be interpreted as direct evidence of a single-compound effect.

The safflower seed extract showed a different inhibitory pattern. Its lowest MIC value was observed against *A. salmonicida* (50 μg mL^{-1}), which was comparable to the value recorded for flaxseed extract against the same pathogen. Higher concentrations were required to inhibit *A. hydrophila*, *Y. ruckeri*, and *V. anguillarum*. The absence of tannic acid and the generally lower detected levels of several phenolic compounds compared with flaxseed extract may partly account for the higher MIC values observed for safflower seed extract. However, this interpretation should also be considered associative, since purified compounds and compound combinations were not tested in the present study.

Both extracts inhibited *Y. ruckeri*, although the MIC values were higher than those recorded for the more susceptible *Aeromonas* species. *Y. ruckeri*, the causative agent of enteric redmouth disease, remains an important pathogen in salmonid aquaculture (Tobback et al., 2007). The MIC values of 400 μg mL^{-1} for flaxseed and 800 μg mL^{-1} for safflower seed indicate modest but detectable inhibitory activity against this pathogen. These results justify further screening against *Y. ruckeri*, but they also suggest that extract concentration, formulation, and exposure conditions would need to be optimized before any aquaculture-related application is considered.

No inhibitory activity was detected against *P. putida* within the tested concentration range. Members of the genus *Pseudomonas* are known for variable antimicrobial susceptibility, and resistance-related traits in this group may involve mechanisms such as reduced membrane permeability and efflux-mediated tolerance (Pang et al., 2019). Therefore, the lack of inhibition against *P. putida* should be interpreted as a species- or isolate-dependent response. Together with the MIC differences observed among the other bacteria, this finding supports the view that the antibacterial activity of the extracts was selective rather than uniformly broad-spectrum.

The activity against *V. anguillarum* was weak compared with the activity observed against the *Aeromonas* species. Only the safflower seed extract inhibited this pathogen, and this inhibition was recorded at a relatively high MIC value of 1600 μg mL^{-1}. *V. anguillarum* is an important fish pathogen and the causative agent of vibriosis, particularly in marine and brackish-water aquaculture (Frans et al., 2011). The lower susceptibility of *V. anguillarum* in the present study may reflect a species-dependent response to the tested extracts. Safflower seed extract should not be considered a strong candidate against *V. anguillarum* at this stage, although further work on extract concentration, formulation, and exposure conditions may clarify its potential.

When compared with previous plant-derived antibacterial studies against fish pathogens, the MIC values obtained in the present study fall within a broad activity range, but clear extract- and pathogen-dependent differences are evident. Karga et al. (2020) reported a lower MIC value for *Laurus nobilis*

aqueous methanolic extract against *A. hydrophila* (3.125 μg mL^{-1}) than those observed in the present study for flaxseed and safflower extracts against the same pathogen (25 and 400 μg mL^{-1}, respectively). In the same study, *Brassica nigra* extract inhibited *V. anguillarum* at 100 μg mL^{-1}, which was lower than the MIC value recorded for safflower seed extract against *V. anguillarum* in the present study (1600 μg mL^{-1}). In contrast, Terzi et al. (2023) reported considerably higher MIC values for *Vaccinium arctostaphylos* aqueous methanolic extract against *A. hydrophila, A. salmonicida, Y. ruckeri,* and *P. putida* (8.75 mg mL^{-1}; equivalent to 8750 μg mL^{-1}), indicating that the present flaxseed extract showed stronger inhibitory activity against *A. hydrophila, A. salmonicida,* and *Y. ruckeri.* Similarly, Pires et al. (2021) reported MIC values ranging from 400 to 1600 μg mL^{-1} for Maclura tinctoria heartwood extract against Aeromonas strains, with an MIC of 400 μg mL^{-1} against one *A. hydrophila* strain. Therefore, the flaxseed extract in the present study, with an MIC of 25 μg mL^{-1} against *A. hydrophila*, appeared more active than several previously reported plant extracts, whereas safflower seed extract showed moderate or weaker activity depending on the target bacterium. Overall, these comparisons indicate that flaxseed extract showed relatively strong activity against *Aeromonas* species within the context of plant-derived antibacterial studies, whereas safflower seed extract showed moderate to weak activity, particularly against *Y. ruckeri* and *V. anguillarum*. However, direct comparison among studies should be made cautiously because extraction solvent, extract type, bacterial strain, assay conditions, and reporting units may differ.

A methodological point worth noting is that the extract composition was evaluated using LC-MS/MS-based targeted phenolic analysis, which provided ppb values for selected phenolic compounds in the final aqueous extract solutions. Unlike relative peak-area percentages commonly reported in GC-MS based plant extract studies, these quantitative data directly represent compound concentrations rather than chromatographic abundance. This distinction is important because the results reflect the targeted phenolic profile within the analyzed compound panel, rather than relative abundance based solely on detector response. Studies combining quantitative chemical profiling with antimicrobial screening have used comparable data to support the biological interpretation of plant extracts (Hajji et al., 2010; Terzi et al., 2023).

Since the present study was limited to *in vitro* antibacterial screening, the findings should be regarded as preliminary evidence. Another limitation of the present study is that full LC-MS/MS validation parameters, including LOD, LOQ, recovery, and precision values, were not available for all monitored phenolic compounds. Therefore, the LC-MS/MS results should be considered as targeted phenolic profiling data supporting comparison between the extracts, rather than as fully validated absolute quantification. Further *in vivo* studies are required to evaluate the safe concentration range, formulation suitability, and biological effects of the extracts in fish. Additional bactericidal and compound-level assays would also help clarify the mechanisms underlying the observed inhibitory activity. Despite these limitations, the findings provide relevant preliminary evidence that aqueous methanolic seed extracts of flaxseed and safflower seed have antibacterial potential against selected fish pathogens. The notable inhibitory activity of flaxseed extract against *A. hydrophila* and *A. salmonicida*, together with its broader targeted phenolic profile, supports its further evaluation as a natural antibacterial candidate for aquaculture-related research.

CONCLUSION

Aqueous methanolic seed extracts of flaxseed and safflower showed species-dependent antibacterial activity against selected fish pathogenic bacteria. Flaxseed extract exhibited the strongest inhibitory activity, particularly against *Aeromonas hydrophila* and *Aeromonas salmonicida,* with MIC values of 25 and 50 μg mL^{-1}, respectively. Safflower seed extract also inhibited *A. salmonicida* at 50 μg mL^{-1}, although higher MIC values were required against the other susceptible pathogens. LC-MS/MS analysis showed that flaxseed extract had a broader detected phenolic profile and was distinguished by the presence of tannic acid as the dominant monitored phenolic

compound, whereas safflower seed extract was mainly characterized by trans-ferulic acid, cinnamic acid, 2,5-dihydroxybenzoic acid, and ellagic acid. The broader targeted phenolic profile of flaxseed extract was associated with lower MIC values against some pathogens, particularly *A. hydrophila* and *Y. ruckeri*. However, the observed antibacterial activity should be interpreted as the response of the whole crude extract rather than as the direct effect of a single quantified phenolic compound. Further *in vivo* studies are needed to evaluate the safety, effective concentration range, formulation suitability, and practical potential of these extracts in fish health.

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Compliance with Ethical Standards

Authors' Contributions

MARS: Conceptualization, Investigation

MK: Methodology, Writing original draft

ONK: Writing original draft, Formal analysis

SB: Supervision, Writing – review & editing

All authors critically reviewed and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

Ethical approval was not required for this study because no live animals were used. The study was conducted using bacterial isolates and plant extracts under in vitro laboratory conditions.

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Not applicable.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI Disclosure

ChatGPT (GPT-5.5 Thinking, OpenAI) was used for language editing and structural improvement of the manuscript. The authors reviewed and validated all outputs and take full responsibility for the final content.

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