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Preliminary study of phenolic content in farmed *Mytilus galloprovincialis* from the Black Sea coast

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Marine bivalves constitute a rich source of nutrients and antioxidants, essential for providing a balanced diet. There are numerous studies devoted to the nutritional quality of farmed black mussels (*Mytilus galloprovincialis*) which reported the presence of health-beneficial components such as polyunsaturated fatty acids, fat-soluble vitamins and carotenoids. However, data about the phenolic content of mussels from the Bulgarian Black Sea waters is limited. The aim of this study was to determine and compare the total phenolic contents and phenolic composition of farmed black mussels (*M. galloprovincialis*) cultured in the Black Sea. Mussel tissue was extracted with five solvent systems: methanol, acetone:water, ethanol:water, hot water and ethyl acetate. Total phenolic content (TPC) of each extract was determined by Folin-Ciocalteu method. All extracts were further subjected to RP-HPLC/UV to analyze individual phenolic acids (4-hydroxybenzoic, gallic, caffeic, p-coumaric and cinnamic acid) and quercetin. The highest TPC of *M. galloprovincialis* was shown in methanol (84.5±7.1 μgGAE.g^{-1} ww) and ethanol:water (66.7±4.8 μgGAE.g^{-1} ww). The chromatographic analysis confirmed the presence of phenolic compounds in all mussel extracts, revealing that farmed black mussels (*M. galloprovincialis*) from the Black Sea could be a good source of phenolic compounds. Further studies are needed to explore the antioxidant potential of this commercially important species.

Keywords: Black mussels (*Mytilus galloprovincialis),* phenolic acids, quercetin, HPLC/UV, total phenolic content

INTRODUCTION

Over the past decades, there has been a growing interest in marine bivalves as inexpensive and easily accessible source of high-quality proteins, lipids and secondary metabolites. The black mussel (*Mytilus galloprovincialis*) is the main species in marine aquaculture and the most consumed shellfish in Bulgaria. A number of studies devoted to the nutritional quality of *M. galloprovincialis* farmed along the Bulgarian Black Sea coast reported fatty acid composition, vitamin and protein content of mussels meat [1-3]. Moreover, health-beneficial potential of Black Sea mussels is being complemented by the functional properties of their tissue extracts [4-7].

Marine organisms are exposed to a variety of exogenous and endogenous oxidants, consequently they produce a number of secondary metabolites with antioxidant activity – peptides, polysaccharides, carotenoids, etc. In addition, marine shellfish could be a good source of other natural antioxidants, such as flavonoids and phenolic acids. The main sources of phenolic compounds are plants and plant-derived foods, but polyphenols and their metabolites are also found in animal tissues and fluids [8]. The data about the phenolic content and antioxidant activity of marine bivalves is limited. Few studies investigated the total phenolic content (TPC) of green mussel (*Perna veridis*) [9, 10] and Moncheva *et al*. suggested that polyphenols in Black Sea *M. galloprovincialis* extracts play an important role for their antioxidant capacities [5]. However, data about the phenolic content and individual phenolic components in mussels from the Bulgarian Black Sea waters is scarce. Therefore, the aim of this study was to determine and compare the total phenolic contents and individual phenolic compounds in different extracts (methanol, acetone:water, ethanol:water, hot water and ethyl acetate) from farmed *M. galloprovincialis* from the Black Sea.

MATERIALS AND METHODS

Chemicals

All solvents and standards were of HPLC grade. The five phenolic acids: 4-hydroxybenzoic acid (4HBA), gallic acid monohydrate (GA), 3,4-dihydroxycinnamic acid (CA) and *trans*-cinnamic acid (CiA) were purchased from Acros Organics, New Jersey, USA; *trans*-4-hydroxycinnamic acid (p-coumaric acid, pCoA) – from Alfa Aesar, Thermo Fisher, Germany; and quercetin (Q) – from Fluorochem, Hadfield, UK. The solvents (water (W), methanol (Me), ethanol (E), acetone (Ac) and ethyl acetate (EAc)) were purchased from Fisher Chemicals, Thermo Fisher, Germany.

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Mussels sampling and pre-treatment

Live mussels (*M. galloprovincialis*) were purchased in the summer of 2019 from a mussel farm near Sozopol, Bulgaria. Samples were transported to the laboratory in iceboxes. Individual shell length of one hundred mussels was measured using a digital calliper and only mussels of a mean size (45.0±5.9 mm) were chosen for analysis. Mussels shells were thoroughly brushed, washed with distilled water and steamed for 6 min at 90±5 ºC.

Extraction

Three pools (n=20) of steamed mussels were homogenized using a blender. A three-step extraction procedure was applied for each extractant [11, 12]. The method includes triple liquid extraction of homogenized tissue with a portion of the following solvents – 100% methanol (Me), 70% acetone (AcW), 50% ethanol (EW), 100% ethyl acetate (EAc) and hot water (80 ºC). Combined extracts were centrifuged and filtered through 0.45 µm PTFE filter.

Total polyphenols determination

Total phenolic content of each mussel extract was determined by Folin-Ciocalteu method [13, 14]. The spectrophotometric analysis was performed using UV-Vis spectrometer Evolution 220 (Termo Fisher Scientific, USA). The absorbance was measured at 746 nm. Gallic acid was used as calibration standard and results were expressed in microgram gallic acid equivalents per gram wet weight (µgGAE.g^{-1} ww) as mean values (n=3) ± standard deviation.

HPLC analysis of phenolic acids and quercetin

The five phenolic acids – 4HBA, GA, CA, pCoA, CiA and Q in mussel extracts were analyzed by an HPLC/UV/FL system (Termo Fisher Scientific, USA), coupled with reverse phase column Acclaim™ Phenyl-1 Dionex Bonded Silica (C18, 120Å, 3µm, 250×3.0 mm, Termo Fisher Scientific, Waltham, MA, USA). The chromatographic elution of analytes was performed using a gradient program by the method of Özturk *et al*. [15]. Solvent A consisted of methanol:water:formic acid = 10:88:2 (v/v) and solvent B – methanol:water:formic acid = 45:53:2 (v/v). The chromatographic system used the following gradient program: from 0 to 27 min – 100% A, from 28 to 65 min – 100% B, then returned to 100% A. The flow rate of the mobile phase was 0.4 ml/min from 0 to 27 min and 0.5 ml/min from 28 to 65 min and the column temperature was 40 ºC. Gallic acid, CA, pCoA, CiA and Q were detected at 280 nm, and 4HBA – at 255 nm. Results were expressed in micrograms per gram wet weight (µg.g^{-1} ww) as mean values (n=3) ± standard deviation.

RESULTS AND DISCUSSION

Total phenolic content

It is widely accepted that polyphenols are most abundant in plants and plant-derived foods. However, the filter-feeding nature of marine shellfish suggests that the primary sources of polyphenols in mussels are algae and phytoplankton, comprising a major part in their diet. In the cited literature, most of the proposed methods for antioxidant determination of mussels as potential biomarkers are based on the water-soluble enzymes. The approach of extracted polyphenols as the main antioxidants has not been used for these purposes. The results for TPC in *M. galloprovincialis*, presented on Fig. 1 show that all prepared extracts could exhibit antioxidant potential. Methanolic extracts showed the highest TPC (84.5 µg GAE.g^{-1} ww), followed by EW and HW extracts. Lowest TPC was measured for AcW extract (36.3 µg GAE.g^{-1} ww).

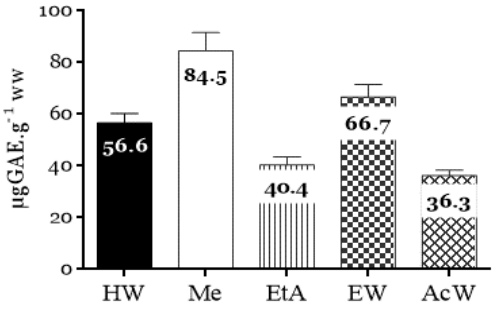

Fig. 1. Total phenolic content in *M. galloprovincialis* extracts

Gorinstein *et al*. [4] reported significant differences in TPC (varying from 391.8±35.8 to 892.7±76.9 µg GAE.100 g^{-1} DW) of methanolic extracts of boiled *M. galloprovincialis* collected from two regions of the Black Sea coast – Cape Galata and the area of Port Varna. Mussels from ecologically clean regions (Cape Galata) presented lower TPC than samples from the more polluted region of Port Varna [4]. Since *M. galloprovincialis* is a filter-feeding species the total phenolic content found in their tissues could be self-defense reaction against various pathogens or pollutants in their habitats. Our results for the methanolic extracts of farmed *M. galloprovincialis* from Sozopol were significantly higher than those reported by Gorinstein *et al*. [4] and Moncheva *et al*. [5]. On the other hand, aqueous extracts of Indian fresh-water pearl mussel (*Lamellidens marginalis*) showed higher TPC (82.81±0.75 µg GAE.mg^{-1} DW)

compared to our results [16]. Microwave assisted extraction and the use of protease inhibitors have been applied to facilitate the extraction of total polyphenols from the green mussel (*Perna viridis*) with water, methanol and ethanol. Ethanolic extracts showed the highest TPC (13.5±5.8 mg GAE.g^{-1}) compared to methanol and water extracts [9]. The discrepancies in the results published by other authors are most probably species-specific, related to environmental (geographical distribution, food availability) and extraction conditions (solvents, temperature and duration). The diverse nature of mussel species, extraction methods and results representation makes the comparison of data rather complicated.

Phenolic acids and quercetin content

Qualitative and quantitative results for the major individual phenolic acids and quercetin in *M. galloprovincialis* extracts are presented in Table 1.

Table 1. Individual phenolic acids and quercetin content in *M. galloprovincialis* extracts

	HW	Me	EtA	EW	AcW
4HBA	40.8±5.1	25.9±1.9	1.6±0.2	2.7±0.3	2.8±0.3
GA	10.5±0.9	5.5±0.6	nd	6.6±0.5	2.2±0.3
CA	3.1±0.4	1.7±0.2	nd	1.6±0.1	1.8±0.2
pCoA	2.3±0.3	0.34±0.02	0.35±0.02	0.41±0.02	1.0±0.09
CiA	1.1±0.08	0.4±0.01	<LOQ	0.08±0.01	1.1±0.08
Q	nd	0.32±0.01	0.3±0.01	0.47±0.02	<LOQ

LOQ – limit of quantification; nd – not detected

Five phenolic acids and quercetin were detected in *M. galloprovincialis* extracts. Several previous studies only examined the TPC of bivalves [4, 5, 9, 10, 16] but did not progress to further phenolic profiling. This study identified 4HBA and GA as the major phenolic acids, regardless of the extraction solvent used. Ethyl acetate and acetone:water extracts yielded the lowest TPC and phenolic compounds, while methanol and hot water extracts of *M. galloprovincialis* gave the highest phenolic content. Not surprisingly, quercetin was detected only in Me, EtA and EW extracts, since it has a low-polarity structure and is commonly extracted from plants with ethanol or aqueous-based ethanol and methanol solutions [17].

CONCLUSIONS

This preliminary study reveals that farmed black mussels (*M. galloprovincialis*) from the Black Sea could be an interesting source of phenolic compounds. Further qualitative and quantitative analyses explaining the relationship between total phenolic contents and total antioxidant capacity could be helpful to explore the antioxidant potential of this commercially important species.

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