Effect of Three-spot Seahorse Petroleum Ether Extract on Lipopolysaccharide Induced Macrophage RAW264.7 Inflammatory Cytokine Nitric Oxide and Composition Analysis

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Abstract: Three-Spot seahorse is a traditional medicine in Asian countries. However, the alcohol extract is largely unknown for its anti-inflammatory activity. This study aimed at elucidating fraction of potent anti-inflammatory activity of seahorse. A systematic solvent extraction method of liquid–liquid fractionation of ethanol crude extract gave four fractions petroleum ether (PE), and ethyl acetate (EtOAc), water saturated butanol (n-BuOH), water (H₂O). In this study, PE extract was selected for further study after preliminary screening test, and was connected to silica column chromatography and eluted with different polarity of mobile phases, and obtained four active fractions (Fr I, Fr II, Fr III, Fr IV). Effect of separated fractions on inflammation was investigated in lipopolysaccharide (LPS) stimulated murine RAW264.7 cells in vitro. The result shows that seahorse extract was capable of inhibiting the production of nitric oxide (NO) significantly in a dose dependent manner and exhibited no notable cytotoxicity on cell viability. IC₅₀ of fraction IV was 36.31 μg/mL, indicating that separated fraction possessed potent NO inhibitory activity against LPS-induced inflammatory response, thus, demonstrated its in vitro anti-inflammatory potentiality, it may be at least partially explained by the presence of anti-inflammation active substances, phenolic compounds, phospholipids and polyunsaturated fatty acids, especially phospholipids and polyunsaturated fatty acids. It could be suggested that seahorse lipid-soluble components could be used in functional food and anti-inflammatory drug preparations.

Key words: three-spot seahorse, anti-inflammation, nitric oxide, lipids

1 INTRODUCTION

Inflammation is a defense reaction of the body to penetration of an infectious agent, entrance of antigen, or cell damage. It is a fundamental biological process and the most frequent indicator of disease¹. In most cases, the inflammatory response is resolved by the release of endogenous anti-inflammatory mediators (anti-inflammatory cytokines) as well as the accumulation of intracellular negative regulatory factors. Thus, the inflammatory cells are eliminated at an appropriate time⁵. However, the continuous accumulation and activation of leukocytes are a hallmark of chronic resulting in dysfunction of these negative regulatory mechanisms⁵. Therefore, it is crucial to develop drugs capable of strengthening and accelerating the limitation and resolution of inflammation, it will be beneficial to be used as therapeutic targets to control the transition from acute to chronic inflammation.

Macrophages are major cells that are involved in innate immune system⁴. The activation of macrophages plays a pivotal role in inflammatory responses when infected with pathogens. Macrophages can kill pathogens directly by phagocytosis and indirectly through secretion of various pro-inflammatory mediators. Excessive production of the inflammatory mediators by activated macrophages has been implicated in the pathophysiology of many inflammatory diseases, including rheumatoid arthritis, pulmonary fi-
brosis. Lipopolysaccharide (LPS)-induced macrophage activation stimulated the production of pro-inflammatory cytokines, nitric oxide (NO) by inducible nitric oxide synthase (iNOS), which is the main cytotoxic and pro-apoptotic mechanisms participating in the innate response in many mammals. Therefore, LPS-stimulated macrophages could be validly used as a model to investigate inflammation and potential anti-inflammatory mediators.

Hippocampus trimaculatus Leach belongs to Syngnathidae of Syngnathiformes in Steichthyes of vertebrate phylum. Most animals in Syngnathidae can be used as traditional Chinese medicine material and were well documented by all versions of China ancient pharmacopoeia. Recent pharmacological studies suggested that seahorse not only had hormone-like activities, but also exhibited activities of anti-tumor, anti-aging, Ca²⁺ channel blocking, anti-microbial and anti-oxidative. And these biological activities had close correlation with water-soluble components being in aqueous extract, for instance, hydrolyzed amino acids, macro and trace elements, and lipid-soluble components, such as phospholipids and steroids. The limit of these studies was neglect of its anti-inflammation of lipid-soluble components, such as fatty acids, phospholipids and steroids. Meanwhile, according to the efficacy described in the literature, seahorse has the effect of dispersing swelling and dissipating mucus, nevertheless, there were almost no relevant reports conducted on exploring the anti-inflammatory activities that were published. Therefore, the present article had focused on isolating components of small polarity that solved in organic solvent and evaluating the biological effects of solvent fractions derived from seahorse on the production of inflammatory mediator NO in vitro experiment.

2 EXPERIMENTAL

2.1 Materials and methods

Three-spot seahorse was purchased from Chinese marine products market of GuangZhou. Mouse mono macrophage RAW264.7 cells were obtained from the Beijing Xiehe cell resource center (Beijing, China). Cell culture basic media DMEM was purchased from Gibco, life technologies (USA), penicillin/streptomycin (10000 U/mL), fetal bovine serum (FBS) were purchased from Gibco, life technologies (Australia). Griess reagent and LPS of Escherichia coli O111:B4 and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals, The MTT reagent ([3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide]) were purchased from Invitrogen Molecular Probes (Eugene, Oregon, USA), thin-layer chromatography (TLC) plates (Silica pH = 5, MF254, Agela Technologies) were used in analytical TLC. Methanol used was of liquid chromatography grade, other chemicals and reagents used were of analytical grade available commercially.

2.2 Preparation of PE extract

Three-spot seahorse were washed with tap water to remove organs and sediment, then put in the constant temperature drying box and allowed to be dried for 24 h
under 60°C and through 20 mesh sieve after crushing. Then carried out in the following Fig. 1. Analytical 95% ethanol was used for reflux extraction at 1:10 (m/v) of solid-liquid ratio, the temperature was 80°C and lasted for 1.5h, repeated the procedure three times. The obtained crude ethanol extract was filtrated through the vacuum Buchner funnel, the solvent was evaporated in vacuum to obtain the extract. Suspended the extract at 1:1 (m/v) of raw material and deionized water. Extracted successively using PE and EtOAc, n-BuOH on the basis of systematic solvent extraction method\textsuperscript{19}. The supernatant and sediment were separated through decompress filtration, the residue was re-extracted. The polarity of extracts obtained was variable from small to large (PE, EtOAc, n-BuOH, H\textsubscript{2}O). Combined the obtained extracted solution and concentrated by rotary evaporator at 60°C, then were freeze-dried. The yield rate of each fraction was 1.70%, 1.28%, 0.96%, 3.36%, respectively. The extracts were stored in refrigerator under −20°C before used (Fig. 1).

2.3 Fractionation of PE extract

UV scanning spectrum of PE extract from 400 to 200 nm showed that 205 nm was the maximal absorption wavelength, thus, 205 nm was selected as detection wavelength. PE extract was further separated using medium voltage preparation and purification system (CHETATMP MP 100/200) through silica gel column (40-60 μm, 2.1 cm*9.0 cm, Agela) chromatography with linear gradient of solvents (A phase PE and B phase ethanol) (B5%	extendash B5%, 0-5 min, B30%	extendash B55%, 5-18.5 min) at a flow rate of 10 mL/min, volume of the dissolved sample was 1 mL. Elution peaks were detected at 205 nm and monitored at 210 nm, each ultraviolet absorption peak was collected and concentrated using a rotary evaporator, and the samples were then lyophilized and stored under −20°C until used.

2.4 TLC analysis

TLC plates (Silica pH = 5, MP254, Agela Technologies) were used in analytical TLC, TLC on silica gel was performed eluting with PE/Dichloromethane/Acetic acid (7:3:0.2, v/v/v) as mobile phase, TLC spots were visualized under UV light (λ = 254 nm) and were stained with 1% vanillin in sulfuric acid solution as used for cholesterol detection\textsuperscript{19}, followed by heating under 105°C for around 5 min. For each sample, 10 μL of around 10 mg/mL \textsuperscript{2} solutions in the appropriate solvent were spotted and allowed to migrate over 8 cm. Cholesterol (RF = 0.16) was used as references at a concentration of 1 mg/mL \textsuperscript{2}.

2.5 Analysis of total phenolic content

Total phenolic content was estimated as epigallocatechin gallate (EGCG) equivalents as described by published Folin–Ciocalteau’s method\textsuperscript{20} with slight modifications. All four fractions at 0.1 mL were added into 24 well plate, and mixed with 0.5 mL Folin–Ciocalteau reagent (diluted doubling before use) followed by addition of 0.6 mL of 20% (m/v) sodium carbonate solution after vortex mixing for 3 min, the mixtures were shielded from the light and incubated for 90 min at room temperature. After the incubation the absorbance were measured at 750 nm on an ELISA microplate reader (Synergy HT, Gene Company Limited). The results were compared with the EGCG standard curve (3.12-100 μg/mL) and expressed as EGCG equivalents (μg EGCG/g of added extract). Analysis was performed in triplicate.

2.6 Determination of total phospholipids content

Analysis of total phospholipid content was carried out as method described by C. Arnaiz\textsuperscript{21}. Accurately weighed 5-20 mg of the four fractions that separated from column chromatography of silica gel, added 0.25 mL digestant (70% perchloric acid) in the hard glass test tubes, placed them in the laboratory electric furnace, slowly digested with heating until the color of the solution changed from brown black to transparent colorless which dedicating the termination of digestion, after cooling, added deionized water 1mL and phosphorus chromogenic reagent (3 mol/L sulfuric acid/water/2.5% ammonium molybdate phosphate/10% ascorbic acid (1:5:1:1, v/v/v/v) rinsed and set the volume of volumetric flasks to 10 mL, and then placed in the thermostatic water bath at 60°C for 10 min, adjusted the absorbance of blank pipe solution to zero, the absorbance was measured at 700 nm by spectrophotometry. Total phospholipids content was calculated according to the standard curve generated by known concentrations of potassium dihydrogen phosphate (y = 0.0346x-0.007, R² = 0.9959, liner range (2-16 μg/mL)).

2.7 Analysis of fatty acid

Analysis of fatty acid of four fractions was carried out according to Raffaella Boggia\textsuperscript{22} with slight modification. Weighed 15-40 mg fractions, added 15 mL mixing solution of chloroform/methanol/water (8:4:3 (v/v/v)), centrifuged 10 min at 4000 rpm under 25°C after vortex mixing 5 min, and then let it stand 1 min, collected the lower organic phase to clean centrifuge tubes. The tubes were placed on the ventilation cabinet to remove solvents. Added 3 mL 2% potassium hydroxide methanol solution (m/v), put in the thermostatic water bath under 65°C for 1 h after mixing, added 3 mL 14% boron trifluoride methanol solution, put in the in the thermostatic water bath under 65°C for 1 h after mixing, after cooling, 200 μL saturated sodium chloride solution and 2 mL cyclohexane were added, centrifuged 5 min at 4000 rpm after the mixture was shaken vigorously and left to stand for 1min, the upper hexane layer was collected for further analysis.

GC-MS analyses were performed by an HP 6890 GC equipped with an Agilent 5975C (EI) MS detector (Agilent
Technologies, USA), using the MSD Chemstation Software E.02.01 for data acquisition and processing. A 30 mm × 0.25 mm × 0.25 μm film thickness ZB-5MSI 5% Phenyl-95% DiMethylpolysiloxane fused-silica capillary column was used for the gas chromatographic analysis at a constant Helium flow rate. The column temperature was 50°C (retain 1 min), and was heated to 280°C at the speed of 5°C/min. The temperature of vaporization chamber was kept at 50°C. Pre column pressure was 7.62 psi, carrier gas flow was 1.0 mL/min, ratio of diversion was 40:1, solvent delay time was 5 min. EI was adopted as ion source, temperature of ion source was 230°C, the quadrupole temperature was 150°C, electron energy was 70 eV, emission current of 34.6 μA, the voltage of multiplier was 1526 V, interface temperature was 280°C, the quality range was 20-450 amu. The total ion current of each peak in the graph by search the standard mass spectrogram in Nist2005 and check computer data system of Wiley 275 MS, relative quality fraction for each chemical constituent were determined by peak area normalization method.

2.8 Determination of cholesterol content

Analysis of cholesterol content was carried out as method described by A. Daneshfar23. Quantification of cholesterol was performed by HPLC. Prior to injection, fractions were filtered through 0.22 μm nylon syringe filters and were injected in an Agilent HPLC system consisting of binary pumps, thermostat and auto sampler connected to an Agilent 1260 Infinity variable wavelength detector (Agilent, Milford, USA). The separation of cholesterol was performed on a reverse phase AA C18 (5 μm, 2.1 mm×200 mm, Hypersil) column used as a stationary phase at ambient temperature. Mobile phase was composed of methanol solely and the flow rate was kept at 1 mL/min, the analysis time was 25 min, the injection volume was 10 μL and peaks were monitored at 205 nm. Column temperature was kept at 35°C. The flow rate was kept at 1.0 mL/min. The gradient programme was as follows: 0% A/100%B 0–25 min. Peaks were identified by congruent retention times compared with standards. Analyses were performed in triplicate. Amount of detected compounds were estimated from calibration curves obtained by injecting mixtures of high purity of cholesterol (Yuanye Bio-Technology Co., Ltd, Shanghai, China) as reference standard (y = 4125.3x + 127.32, R² = 0.9972, linear range (0.20-1.20 mg/mL)). Identified peaks were confirmed and quantified by data acquisition, spectral evaluation and manual integration using chemistry workstation chromatographic software (Agilent).

2.9 Cell culture and cell viability

Murine macrophage cell line RAW264.7 were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL streptomycin-penicillin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. RAW264.7 cells were maintained via two to three times passage per week and cells were utilized for experimentation at 70-80% confluency. Cell viability was determined by MTT reduction assay as described by Hansen et al.24. MTT is used as an indicator of cell viability via its mitochondrial reduction to formazan. Briefly, adjusted cell density to 1×10⁵ per milliliter, seeded 100 μL per well to 96 plate, incubated in an atmosphere of 5% CO₂ and 95% air at 37°C overnight. Cells were treated with various concentrations (10, 50, 100 μg/mL) of four fractions (Fr I, II, III, IV) for 24 h, each carried out in triplicate. 20 μL MTT solution (5 mg/mL final concentration, dissolved in phosphate-buffered saline (PBS, pH 7.4)) was added and incubated at 37°C for 4h. After the removal of culture supernatant, the resulting dark blue crystals were added 100 μL DMSO, allowed to shake 15 min on a mini shaker, determined the absorbance values of 570 nm in a microplate reader. Cell viability of control group was taken as 100%, relative cell viability of treated groups was calculated compared with untreated control group.

2.10 NO production assay

NO levels in the culture supernatants were measured by Griess reagent as described previously25. In brief, RAW264.7 cells were pre-incubated for 24 h in 96-well plates using DMEM without phenol red at a density of 1×10⁷ cells per well, followed by the treatment of four fractions (10, 50 and 100 μg/mL). NO production was stimulated by incubating with LPS (1 μg/mL final concentration) for 48 h. Each was carried out in quadruplicate. Fifty microliter of culture supernatants from each sample was collected and mixed with a same volume of Griess reagent and allowed to incubate for 15 min at room temperature. Absorbance values were read at 540 nm on an ELISA microplate reader (Synergy HT, Gene Company Limited). The nitrite (stable oxidation product of NO) levels of the media were calculated from regression analysis using different concentrations of sodium nitrite to deliver a standard curve (y = 0.0124x + 0.0104, R² = 0.995, linear range (3.12-100 μM)).

2.11 Statistical analysis

One-way analysis of variance was used for all statistical analysis using independent experiments and data are presented as Means ± S.D. The mean values were calculated based on the data taken from at least three independent experiments conducted on using freshly prepared reagents. Set up the analysis of variance (one way ANOVA, Duncan test) for trials. Differences with p<0.05 were considered significant.
3 RESULTS

3.1 Fractionation of silica gel column chromatography

UV scanning spectrum of PE extract from 200 to 400 nm showed that 205 nm was the maximal absorption wavelength. In the following separation, 205 nm was selected as detection wavelength. Four fractions were obtained via silica gel column chromatography, the retention time of four fractions were 1.6, 4.5, 9.9, 14.5 min, respectively. Among these fractions, the yielding rate of Fr II was 31.1%, which was the highest, and Fr IV was the lowest, 8.4% (Fig. 2).

3.2 TLC analysis

Blue purple spots were observed in Fr I - III and that the Rf values of them and standard cholesterol were equal indicated that Fr I - III contained cholesterol, while it was not detected in Fr IV (Fig. 3).

3.3 Chemical components analysis of four fractions of PE extract

Table 1 shows contents of lipid-soluble composition. Lipid-soluble contents are calculated by averaging three independent experiments, and taken as X ± S.D. (n = 3). Total phospholipids content should be composed of total phosphorus content multiplying a coefficient. The phenolic compound contents of four fractions increased along with the polarity, the content of total phospholipids of Fr IV was the highest. The cholesterol of Fr IV was not observes and detected, while Fr III has the highest content of cholesterol.

3.4 Fatty acid analysis

The gas chromatogram for analysis of fatty acid of four fractions showed that the composition and distribution were different along with the polar divergence. Combined analysis of mass spectrometric, obtained the composition and percentage of fatty acid (Table 2). With the increase of polarity, the contents of polyunsaturated fatty acids increased. Fr IV has the highest content of polyunsaturated fatty acids, and the content of omega-3 polyunsaturated fatty acids was likewise the highest.

Fig. 2  TLC spectrogram of cholesterol.
The first sampling point was standard cholesterol, from the second to the fifth were Fr I - IV. Cholesterol analysis by TLC revealed that Fr I - III have cholesterol that showed purple spots after spraying chromogenic agent and heating (Rf=0.16) while cholesterol was not detected in Fr IV.

Fig. 3  Silica gel column chromatography spectrogram of medium pressure system for preparation.
3.5 Cell viability

As depicted in Fig. 4, all four fractions showed no significant \((p < 0.05)\) cytotoxicity on RAW264.7 cells in all tested concentrations after 24 h treatment compared with the normal group. Non-toxic concentrations ranging from 10 to 100 \(\mu g/mL\) were selected from all fractions for further analysis (Fig. 4).

3.6 Nitrite analysis

NO amount increased with the increase of concentration of LPS concentration varying from 0-2 \(\mu g/mL\), while the concentrations were above 1 \(\mu g/mL\), nitrite production increased gradually, meanwhile, cell viability decreased slightly. Hence, this experiment selected 1 \(\mu g/mL\) LPS to be used to stimulate inflammatory response (Fig. 5).

The present study found that all fractions treatment could inhibit NO amount at all the tested concentrations. Inhibitory activity of NO of fractions was not strong as positive control group regarding the result. The NO IC\(_{50}\) values of Fr \(\text{I} \), \(\text{II} \), \(\text{III} \), \(\text{IV} \) were 277.85, 230.56, 61.90, 36.31 \(\mu g/mL\) respectively, while dexamethasone was 12.68 \(\mu g/mL\). As shown in Fig. 6, among the tested four fractions, Fr \(\text{IV} \) showed the highest NO inhibitory potentiality and the inhibitions were dose dependent compared with the stimulated control group \((p < 0.05)\). At the highest concentration of Fr \(\text{IV} \) (100 \(\mu g/mL\)) NO production was more or less similar to the non-stimulated normal group (Fig. 6).

### Table 1 Contents of lipid-soluble composition.

| Fraction | Yield(%) | Total phenolic content/(mg/g)\(^1\) | Total phospholipid content/(mg/g)\(^2\) | Cholesterol content/(mg/g)\(^3\) |
|----------|----------|-------------------------------------|--------------------------------------|---------------------------------|
| \(\text{I} \) | 10.5     | 1.223 ± 0.145                       | 3.198 ± 0.123                       | 43.402 ± 0.191                  |
| \(\text{II} \) | 31.1     | 1.913 ± 0.105                       | 1.060 ± 0.138                       | 55.970 ± 1.481                  |
| \(\text{III} \) | 26.3     | 3.702 ± 0.158                       | 10.700 ± 0.179                      | 116.737 ± 5.195                 |
| \(\text{IV} \) | 8.4      | 15.346 ± 0.458                      | 75.612 ± 0.899                      | –                               |

### Table 2 Fatty acid composition and percentage content of four fractions.

| Compound                                | Fr \(\text{I} \) | Fr \(\text{II} \) | Fr \(\text{III} \) | Fr \(\text{IV} \) |
|-----------------------------------------|-----------------|-----------------|-----------------|-----------------|
| cyristic acid                           | –               | 0.155           | 0.122           |                 |
| 13-methyl myristic acid                 | 0.280           | 0.138           | 0.204           | 0.273           |
| pentadecanoic acid                      | 1.614           | 1.001           | 1.320           | 1.729           |
| 14-pentadecanoic acid                   | 0.261           | 0.275           | 0.277           | 0.337           |
| hexadecanoic acid                       | 38.537          | 30.168          | 27.66           | 27.355          |
| methylpalmitic acid                     | 11.659          | 7.877           | 9.994           | 11.390          |
| margaric acid                           | 1.416           | 1.860           | 1.893           | 1.857           |
| 6-hexadecenoic acid                     | 11.659          | 7.877           | 9.994           | 11.390          |
| 6,9,12- calendic acid                   | 0.180           | –               | 0.163           | 0.261           |
| cis-9,12,15-linolenic acid              | 1.337           | 4.630           | 1.711           | 1.479           |
| 8,11-octadecadienoic acid              | 1.337           | 4.630           | 1.711           | 1.479           |
| 9-octadecenoic acid                     | 24.403          | 33.823          | 33.580          | 26.193          |
| docosahexenoic acid                     | 3.437           | 2.891           | 4.305           | 6.149           |
| 5,8,11,14-arachidonic acid              | 2.225           | 2.637           | 2.940           | 3.356           |
| eicosatrienoic acid                     | 0.451           | 0.230           | 0.659           | 0.680           |
| cis-11-eicosenoic acid                  | 0.688           | 1.136           | 1.231           | 1.077           |
| 4,7,10,13,16,19-eicosapentaenoic acid   | 3.526           | 1.840           | 3.348           | 5.068           |
| unsaturated fatty acid                  | 56.649          | 48.423          | 59.734          | 57.632          |
| n-3 fatty acid                          | 5.546           | 7.702           | 8.865           | 12.434          |
| n-6 fatty acid                          | 7.267           | 3.742           | 4.814           | 5.069           |
| n-9 fatty acid                          | 33.823          | 24.403          | 33.580          | 26.283          |
| Fatty acid content/(mg/g fraction)       | 93              | 108             | 312             | 547             |

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4 DISCUSSIONS

NO has been identified as important mediators of inflammation and are produced by iNOS\(^26\) in many biological systems. Inducible nitric oxide synthase (iNOS) is responsible for generating high levels of NO in tissues. The product of L-arginine, NO, has been demonstrated to modulate COX-2 expression in degenerative and inflammatory diseases\(^{27,28}\). Hence, the inhibition of iNOS expression is consistent with the impediment of NO production, and thus reduction of inflammation.

Phenolic compounds retain redox properties which enable them to act as reducing agents, hydrogen donors, single oxygen quenchers and metal chelating agents\(^{29}\). Furthermore, phenolic compounds have been reported to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of NO. These phenolic compounds are capable to quench the generated NO radicals during inflammation. And also Surh et al.\(^{30}\) reviewed anti-inflammatory action of some phenolic compounds and showed their ability in inhibiting the expression of iNOS by down regulating their transcriptional factor NF-κB. Therefore, it can be suggested that the high content of phenolic compounds in seahorse may mediate the anti-inflammatory actions.

Phospholipids has significant anti-inflammatory action on acute exudation of inflammatory animal model\(^{31}\), it may due to the enhancement of phagocytic activity of peritoneal macrophage. In addition, it may also be the outcome of affecting the metabolism of arachidonic acid. Arjun H. Banskota et al.\(^{32}\) found that macroalga palmaria palmate ex-

**Fig. 4** Effects of Three-spot hippocampus PE extract fraction. Cyto-compatibility of four fractions (Fr I, II, III, IV) were tested at three concentrations (10, 50 and 100 µg/mL) using the MTT cell viability assay in RAW264.7 cells, results of three independent experiments were averaged and are shown as percentage cell viability compared with the viability of untreated control cells.

**Fig. 5** Cell viability and NO generated by RAW 264.7 induced by LPS.
tracts could suppress LPS-induced NO production in RAW264.7 macrophage cells, and amongst the polar lipids, phospholipids were responsible for the NO inhibitory effect. Also, a research from Monika Vicenova demonstrated that biological active phospholipids extracted from hen egg yolk exerted anti-inflammatory activity in vitro under controlled conditions of monocyte-derived macrophages stimulated with LPS.

The anti-inflammatory activity of extracts could not only be explained on the basis of their contents of phenolic compounds and phospholipids. Numerous effects of omega-3 fatty acids EPA and DHA on functional responses of cells involved in inflammation have been investigated. Valerie Boutar et al. found that fish oil supplementation and essential fatty acid deficiency reduce NO synthesis by rat macrophages. Fatty acid-derived modifications in membrane sequence and in the availability of substrates for eicosanoid synthesis are enduring mechanisms that are deemed important in explicating the effects observed, and the differential effects of fractions upon iNOS indicates that iNOS may be regulated by unsaturated fatty acids or compounds derived from them.

Pre experiment showed that seahorse PE extract could inhibit NO in RAW264.7 induced by LPS effectively than other bigger polar constituents may indicate its potent anti-inflammatory activity may attributed by the presence of lipid soluble components. After separated by silica gel column chromatography on the basis of polarity difference, FrIV showed the highest NO inhibitory activity beyond that it was superior to crude extract in inhibiting NO production, indicating that the separation was effective. The differentiated effects observed in this study indicated that each fraction was composed of different types or proportions of the active compounds. FrIV has higher amount of phospholipids and phenolic compounds, however, it does not contain cholesterol, its impelling anti-inflammatory activity may stem from the high contents of phospholipids and phenolic compounds, besides the high levels of the unsaturated fatty acids, especially, the high content of omega-3 fatty acids, could bring the inhibitory action of the production of NO effectively. The contents of unsaturated fatty acids, phospholipids and polyphenols of FrIII were lower than FrIV, however, FrIII has higher proportion of cholesterol. Although there is no significant inhibition of inflammation at the cellular level in comparison with FrIV, it may emerge an increase in the inhibition of inflammation of the metabolism of the organism. Thus, considering all the obtained results, it could be suggested that the anti-inflammatory activity of seahorse may due to the existence of unsaturated fatty acids, phospholipids and polyphenolics, furthermore, polyunsaturated fatty acids, omega-3 in particular are an essential part of active anti-inflammation components. Besides, it may be explained by the synergistic effects of different components in consideration of complexity of mechanisms of Chinese medicine. The presence of these components in seahorse suggests that consumption of seahorse as a functional food may help to reduce inflammation associated with various diseases.

![Graph](image-url)

**Fig. 6** Effects of seahorse separated fractions of PE extract on LPS-induced NO production in RAW264.7 cells. Percentage inhibition was calculated based on the ability of extracts to inhibit NO formation by cells compared with the control (cells in media without samples containing stimuli and DMSO), which was considered as 0% inhibition. Values correspond to mean ± S.D., *p < 0.05, #p < 0.01, compared with LPS alone treated control group.
5 CONCLUSIONS

FyLV separated from PE extracts of seahorse could suppress the production of NO induced by LPS in RAW264.7 significantly. Its anti-inflammatory actions may due to the existence and high levels of phospholipids and unsaturated fatty acids, phenolic compounds. Among these, polyunsaturated fatty acids and phospholipids may play a vital role in restraining inflammatory activity. The results obtained in this study validate the use of organic solvent extracts of seahorse against inflammation. Extracts of seahorse might be of value in the management of various diseases emerging from chronic inflammation. The bioactive compounds should be isolated and its safety should be determined to investigate potential use. The composition of phospholipids should be analyzed and the forms they are combined with other components should be detected. Animal studies are also demanded to ascertain if in vitro activity of extracts equates to in vivo activity.

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