

HPLC Profile of Medicinal Plant Extracts and its Application in Aquaculture

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Abstract

High Performance Liquid Chromatography technique based screening of medicinal proteins as fish feed were prepared from the selected medicinal plants such as *Leucas aspera*, *Achyranthes splendens*, and *Swertia chirayita*. The present study aims at arrival of quality profile of protein in the medicinal plants which are main ingredients in the fish feed. Antibacterial activity of the medicinal plant extracts was assessed and based on that the self-formulated fish feed was formulated. The medicinal feed surpassed the quality of commercial fish feed by having higher protein and fibre along with medicinal properties. In the present study fish pathogens were isolated from the infected fishes and cultured on a suitable medium. Control of pathogens by 80%, 92% and 87% *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* respectively revealed the efficiency of medicinal plants than the control. This study enabled that the medicated fish feed curtails the high cost of fish diet and can be used as potential high quality alternative source in the aquaculture industry.

Keywords: Medicated fish feed; Protein; HPLC; Fish pathogens; Antimicrobial activity

Introduction

HPLC analytical technique is used for the isolation of various natural products. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture [1]. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants [2]. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize its properties. The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale. Several authors describe the use of HPLC for characterization and quantification of secondary metabolites in plant extracts [3-6].

In the present investigation HPLC technique was used to study the secondary metabolites as medicated proteins from the selected medicinal plants. Medicated proteins were used along with the fishery wastes to prepare the pellets. The self-formulated fish feed made from the extracts of *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* leaves and fishery wastes. The medicinal feed surpassed the quality of commercial fish feed by having higher protein and fibre along with medicinal properties. This enabled the fish to combat commonly found fish pathogens in water thereby increasing the survival rate and health of fishes.

Materials and Methods

Preparation of phyto extracts

Aqueous extracts of selected plants such as *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* were prepared at 1:1 w/v.

Protein extraction using HPLC

1 g of sample was weighed and macerated in pestle and mortar with 5 ml of phosphate buffer, and the contents were transferred to a centrifuge tube. The tube was centrifuged at 8000 rpm for 20 minutes. Supernatant was collected and the extraction was repeated 4-5 times. Supernatant was combined and the volume was made to 50 ml with phosphate buffer. To 5 ml of supernatant, 5 ml of 20% TCA was added

and incubated for half an hour, and centrifuged at 8000 rpm for 20 minutes. Supernatant was discarded, and the pellet was washed twice with acetone, 5 ml of 0.1 N NaOH was added to the pellet and mixed well to dissolve the pellet [7,8].

Ion exchange separation: Ion exchange separation is based on the binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix. Substances are bound to ion exchangers when they carry a net charge opposite to that of ion exchanger. This binding is electrostatic and reversible.

Selection of ion exchanger: The pH value at which a bio-molecule carries no net charge is called the iso-electric point (pI). When exposed to a pH below its pI, the bio-molecule will carry a net positive charge and will bind to a cationic exchanger. At pH above its pI, the bio-molecule will carry a net negative charge and will bind to an anionic exchanger.

Selection of buffer pH and ionic strength: Buffer pH and ionic strength are critical for binding and elution of material in ion exchange chromatography. Selection of appropriate pH and ionic strength for the start and elution buffers allows the use of 2 possible separation strategies. In the first strategy, binding is achieved by choosing a start buffer with a low pH for CM Sepharose and high pH for Q Sepharose fast flow. The ionic strength should be kept as low as possible to allow all components to bind to the ion exchanger. This results in concentration of the target substance and a complete picture of the total sample. In the second strategy, enrichment of target protein is achieved by choosing a start buffer with a pH optimized to allow maximum binding of target protein, and as high possible as ionic strength to suppress binding of sample contaminants. This strategy results in a concentration of the target substances [9].

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Received January 23, 2017; **Accepted** June 22, 2017; **Published** June 24, 2017

Citation: Thirumal Y, Laavu S (2017) HPLC Profile of Medicinal Plant Extracts and its Application in Aquaculture. J Aquac Res Development 8: 484. doi: [10.4172/2155-9546.1000484](https://doi.org/10.4172/2155-9546.1000484)

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Choice of gradient: Continuous salt gradients are the most frequently used type of elution. Two buffers of different ionic strength, the start and the elution buffer are mixed together and if the volume ratio is changed linearly, the ionic strength changes linearly.

Sample protein purification: Buffers used:

(a) **Start buffer:** Tris-HCl (pH-9.0): 100 ml of 0.2 M Tris+10 ml of 0.2 M HCl diluted to 400 ml of distilled water gives Tris buffer of pH 9.0.

(b) **Elution buffer:** Tris-NaCl (pH-8.5): 150 ml of Tris-HCl (start buffer)+150 ml of 2M NaCl gives Elution buffer.

Samples: Crude protein extracts of medicinal plants *L. aspera*, *A. splendens*, *S. chirayita*.

Initially the column was washed off the preservatives with 5 ml of 20% ethanol. The column was washed again with 5 ml of elution buffer. It was equilibrated with 5 ml to 10 ml of start buffer. Sample was applied at a flow rate of 1 ml/min using a syringe. The column was washed again with 5 ml of start buffer until no material appears in the effluent. The sample was eluted with 5 ml to 10 ml of elution buffer. Continuous gradient elution was performed and the pure protein fractions were collected. After the completed elution, the column was regenerated by washing with 5 ml of elution buffer followed by 5 ml to 10 ml of start buffer.

Protein estimation

It is the most commonly used method for the determination of proteins in cell free extracts because of its high sensitivity, and quantities as low as 20 µg of protein can be measured. Suitable aliquots (1 ml) of the extracts were taken and to them, 4.5 ml of freshly prepared alkaline copper sulphate reagent was added. Folin's reagent was added and the contents were mixed instantaneously. The color was allowed to develop for 10 minutes. The absorbance was recorded at 660 nm after setting the spectrometer with reagent blank containing 1 ml of 0.1 N NaOH, instead of sample aliquot. In another set of test tubes, suitable aliquots of BSA solution (0 µg to 100 µg range) were taken. The total volume was made to 2 ml with 0.1 N NaOH and the color was developed as before. A standard graph of absorbance at 660 nm versus µg of BSA was plotted. From the standard graph, the amount of the protein present in the samples was determined [10].

Preparation of medicated feed pellets

Fresh leaves of *Leucas aspera*, *Achyranthes splendens*, and *Swertia chirayita* were collected from the college campus, air dried for 2 to 3 days and powdered. The dried leaves were ground to a fine powder in a mixer. The dried powder was taken in the following order and proportion (W/W).

Leucas aspera: *Achyranthes splendens*: *Swertia chirayita* (LAS)

- 1:1:1 (LAS)
- 0.5:1:1 (LAS)
- 1:0.5: 1 (LAS)
- 1:1:0.5 (LAS)

Along with 50% fishery wastes the medicated mix was bound into a thick dough using the gel of Aloe vera as binding agent. The dough was then made into fine uniform pellets which were eventually air dried.

Test on fish pathogens

The test was conducted with the triplicates of medicinal plant

extracts and the commercial fish food (as control) in completely randomized block design. To assess the antimicrobial activity, the samples were subjected to Agar diffusion test and modified Hohenstein test [11]. The fish pathogens were isolated and used in both the tests were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas species*. These species belong to the family of bacteria and they cause most of the diseases in aquaculture. The evaluation of agar diffusion test was made on the basis of zone of inhibition of bacteria around the test sample and the evaluation of modified Hohenstein test was made on the basis of the percentage reduction of bacteria by the sample. Percentage reduction was calculated using the following formula.

$$R = (B-A)/B$$

Where R is percentage reduction, A is the number of bacteria recovered from the inoculated treated test samples in the jar incubated over the desired contact period (18 hours) and B is the number of bacteria recovered from the inoculated treated test sample swatches in the jar immediately after inoculation i.e., at zero contact time.

Results and Discussion

HPLC study of medicinal plant materials

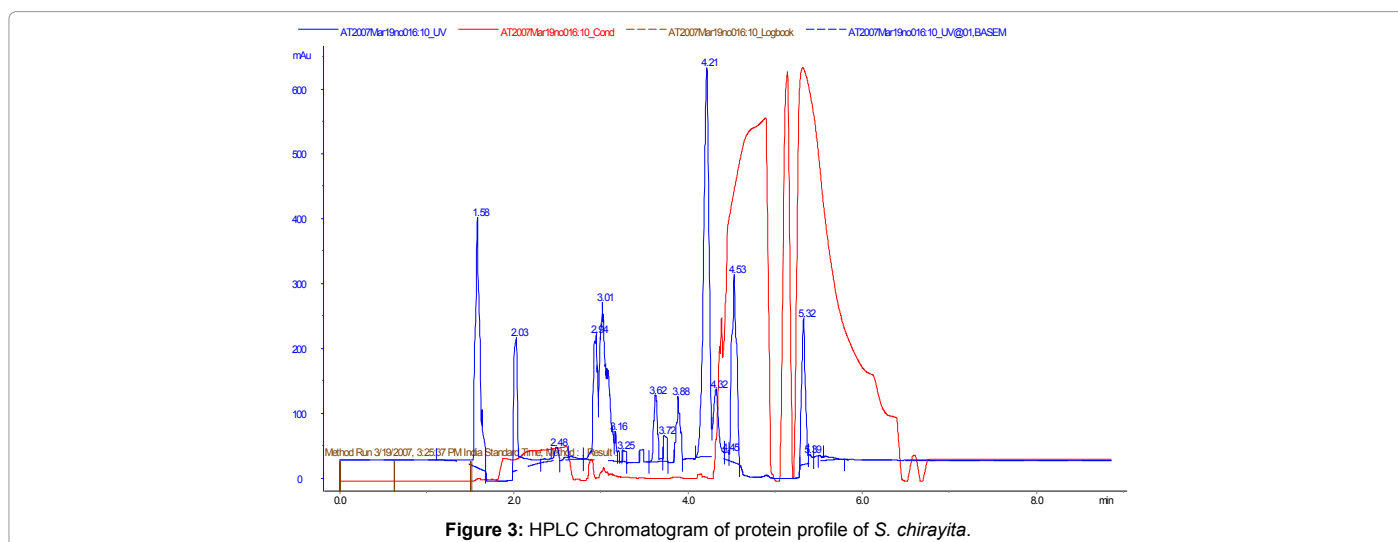
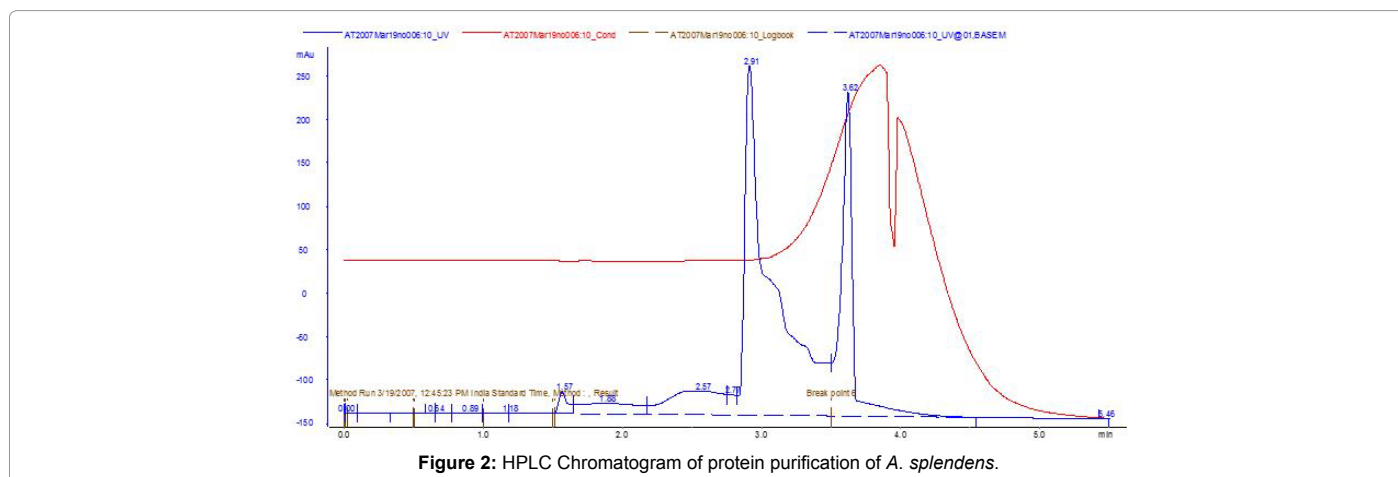
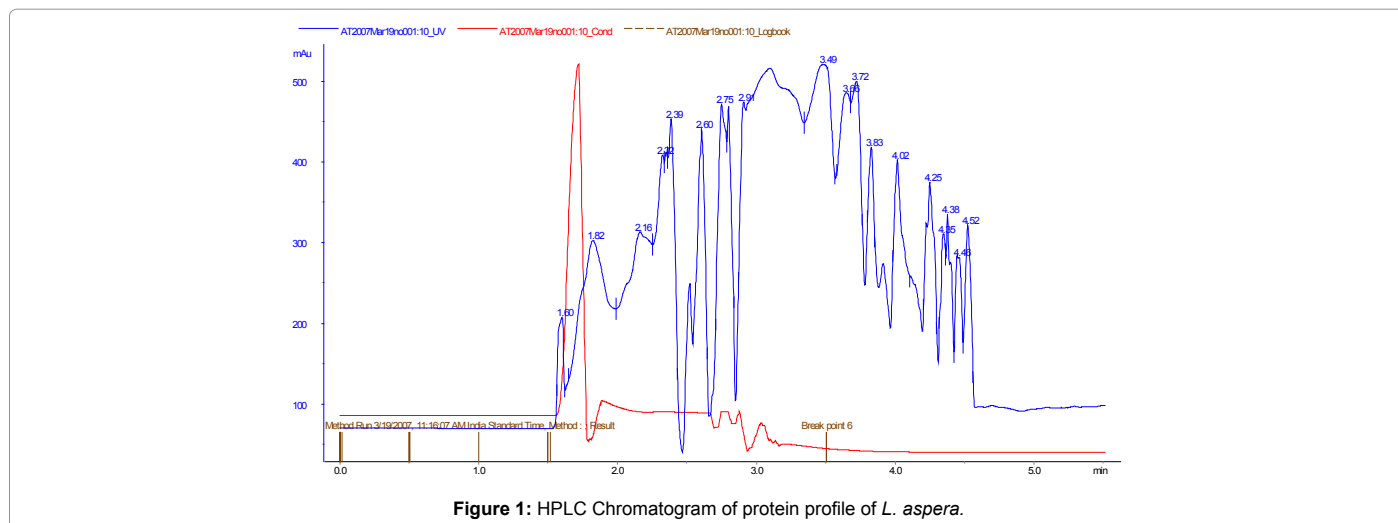
The results pertaining to the HPLC profile of the leaves of *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* revealed their highest peaks for the secondary metabolites as protein source. Similar kind of earlier experiments demonstrates the processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation [3]. The source material, e.g., dried powdered plant, will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extracting and following a period of maceration, solid material is then removed by decanting off the extract by filtration [12,13]. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract.

The qualitative study of the proteins of the selected phyto extracts was performed by High Performance Liquid Chromatography. The resulting chromatograms were recorded and presented in the Figures 1-3.

Protein profile of different sources of fish feed

The qualitative analysis was supportive to the quantitative analysis. The protein content of the medicinal plants was purified and taken as pellets along with the fishery wastes. The self-formulated fish feed made from the extracts of *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* leaves and fishery wastes contains 97,46.5,84.5 and 69 µg of crude protein Hence the present study confirms the quality and quantity of protein in the fish feed (Table 1). This kind of quality profile is essential in aquaculture industry and many fish farming practices concern about the quality of protein. Commercial protein Jet-X was used to compare the protein profile of fish feed.

Similar studies of Webster et al. [14]; Bai and Gatlin [15] revealed that protein is used for fish growth if adequate levels of fats and carbohydrates are present in the diet. If not, protein may be used for energy and life support rather than growth. Proteins are composed of carbon (50%), nitrogen (16%), oxygen (21.5%), and hydrogen (6.5%). Fish are capable of using a high protein diet [16,17], but as much as 65% of the protein may be lost to the environment. Most nitrogen is



excreted as ammonia (NH₃) by the gills of fish, and only 10% is lost as solid wastes.

Antimicrobial activity

From the results, it was found that the antibacterial effect in terms

of percentage reduction for antimicrobial agents treated for different pathogens in the following order:

L. aspera < *A. splendens* < *S. chirayita* < commercial fish food (control)

The investigation had been revealed that the minimum quantity of

S. No	Source	Crude protein content (µg/g)
1	Jet-X fish food	49.6
2	<i>L. aspera</i>	97.0
3	<i>A. splendens</i>	46.5
4	<i>S. chirayita</i>	84.5
5	Fishery wastes	69.4

Table 1: Crude protein content of commercial fish feed, fishery wastes and the phyto extracts.

Pathogenic microbes	<i>Leucas aspera</i> µg/ml	<i>Achyranthes splendens</i> µg/ml	<i>Swertia chirayita</i> µg/ml	Jet-X fish food (control) µg/ml
<i>Staphylococcus aureus</i>	25 ± 0.03	20 ± 0.01	20 ± 0.01	35 ± 0.01
<i>Staphylococcus epidermidis</i>	25 ± 0.05	25 ± 0.03	20 ± 0.01	39 ± 0.01
<i>Escherichia coli</i>	20 ± 0.03	20 ± 0.04	20 ± 0.02	38 ± 0.02
<i>Pseudomonas species</i>	20 ± 0.02	25 ± 0.05	20 ± 0.01	35 ± 0.01

*Mean of triplicates; ± Standard deviation

Table 2: Minimum Inhibitory Concentration (MIC) values of medicinal plant extracts.

Pathogenic microbes	<i>Leucas aspera</i> (%)	<i>Achyranthes splendens</i> (%)	<i>Swertia chirayita</i> (%)	Jet-X fish food (control) (%)
<i>Staphylococcus aureus</i>	81.0 ± 0.05	93.5 ± 0.05	87.0 ± 0.03	54 ± 0.05
<i>Staphylococcus epidermidis</i>	79.5 ± 0.02	92.5 ± 0.02	88.2 ± 0.01	49 ± 0.01
<i>Escherichia coli</i>	81.0 ± 0.02	92.7 ± 0.01	87.5 ± 0.04	46 ± 0.02
<i>Pseudomonas species</i>	80.5 ± 0.03	93.5 ± 0.02	87.1 ± 0.03	59 ± 0.01

*Mean of triplicates; ± Standard deviation

Table 3: Confirmation of antibacterial activity by modified Hohenstein test method-Challenge test-Reduction Percentage.

20 µg/ml to 25 µg/ml was found to increase the antimicrobial activity of the medicinal plants to target the fish pathogens. Whereas the quantity of the commercial fish food (control) was found to be more 35 µg/ml to 39 µg/ml to control the fish pathogens. Hence the results for the antimicrobial test (Table 2) and modified Hohenstein test (Table 3) were confirmed the viability of the antimicrobial treatment when it is used as fish feed.

Similar experiments were conducted using different plant materials as protein sources [18-20] and to control different pathogenic bacteria [21].

Conclusion

To isolate and purify the plant based products like secondary metabolites and proteins HPLC is highly useful for researchers and industrialists those who focus on quality. Hence from the present experiments it is concluded that the protein content of *Leucas aspera*, *Achyranthes splendens* and *Swertia chirayita* and fishery wastes could be used as high protein alternative sources of fish feed. Medicated fish feed curtails the high cost of fish diet and can be used as potential high quality alternative source in the aquaculture industry.

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