



DETERMINATION AND QUANTIFICATION OF AVAROL IN *DYSIDIA AVARA* BY HPLC-DAD

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ABSTRACT

Avarol is major compound of *Dysidia avara*. In this study avarol has been quantified by using reverse-phase High Performance Liquid Chromatography (HPLC) with diode array detector (DAD). The identification of avarol has been based on retention time and UV spectra by comparison with standard. The experimental results have shown high amount of avarol in *D. avara* from Ayvalık Turkey. The precision, accuracy, and reproducibility of the method have been apparent from good linearity.

KEYWORDS: Avarol, *Dysidia avara*, HPLC-DAD, Quantification.

INTRODUCTION

Sponges are spineless animals that belong to phylum^[1] Marine sponges are a rich source of potentially pharmaceutically bioactive compounds.^[2] Most of these compounds show antiviral activity^[3], antibacterial activity^[4], cytotoxicity activity^[5], antioxidant activity^[6] antiplatelet and antiprotozoal activity.^[7] Thus isolation, identification and determination of these compounds are very important steps in drug discovery.^[2] Spongothymidine and spongouridine are the first compounds that were isolated from sponges in 1950s.^[8] *Dysidea avara* is a common Mediterranean sponge. The sesquiterpene hydroquinone (avarol) is the major secondary metabolite in this sponge species. The structure of avarol is shown in Figure 1. Minale, in 1974 for the first time, isolated avarol and a minor amount of its oxidised derivative avarone from *Disidea avara*.^[9] Avarol is known as an antiviral agent. Studies have shown antiviral activity at concentration of 0.1 µg/mL against replication of the etiologic agent of acquired immune deficiency syndrome (AIDS) and human T-lymphotropic retrovirus (HTLV III)/lymphadenopathy-associated virus in human H9 cells.^[10] Avarol and avarone also have many bioactivity properties like, cytotoxicity activity^[11], antiinflammatory activity^[12], antimicrobial activity^[13], TNF-α generation and NF-κB activation inhibitory.^[14] Furthermore, natural and synthetic derivatives of avarol are biologically active. For example, avarol-3'-thiosalicylate, avarol-3'-thiolactate, avarol-4'-thiolactate, avarol-3'-thiobenzoate, avarol-4'-thioglycolate and avarol-3'-thioglycolate that show anti-acetylcholinesterase activities^[15] and avarol-3'-thiosalicylate shows antipsoriatic activity.^[16] In our

previous study, we isolated avarol and avarone from *D. avara*.^[17] In this study, we have studied the determination of avarol amount in *D. avara* by HPLC-DAD.

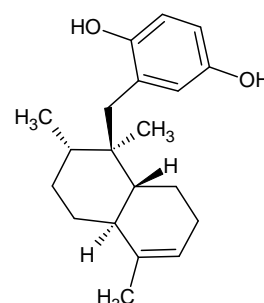


Figure 1: Avarol structure.

MATERIAL AND METHODS

Sponge Material

D. avara was collected from Ayvalık Turkey in August 2016, through 10 meter scuba diving and transferred immediately to the laboratory in Ankara and it was kept in 70% ethanol during the transfer and later on put in a deepfreeze until the experimental process. The sample was identified by Dr. Bülent Gözcelioğlu based on conventional macroscopic and microscopic marine sponge identification procedures. A voucher specimen was deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University.

Sponge Extraction

The sample was cut into small pieces and then extracted by methanol and was dried under vacuum. The crude

extract was kept in 4°C for further use. The extract was redissolved in HPLC grade methanol (10mg/ml) and filtered through 0.45 µm membranes.

Avarol used as standard in this experiment has been isolated by our group.^[17] A stock solution of avarol (100µg/mL) was prepared and filtered through 0.45 µm membranes. To make calibration, curve was used 100, 50, 25, 10 and 5 ppm of stock solution. HPLC analysis of avarol was performed by a reverse phase column (150 × 4.6 mm i.d, 5 µm) (Agilent, Eclips XDB-C18, USA), on a gradient program with two solvents system[A: 2 % phosphoric acid in water; B: Methanol] at a constant solvent flow rate of 1 mL/min on a HPLC system (Agilent Technologies 1260 infinity). Mobile phase consisting of solution A and solution B is as follows: gradient system from 0-2 min,70% A; 4 min, 80% A; 8 min 90% A, 8-12 min, 90 A. Injection volume was 20 µL. The signals were detected at 270 nm by DAD detection with column temperature at 25°C. The identification of avarol was executed by comparing retention times and spectral data with standard. Calibration curve of avarol was used for quantification.

Validation of HPLC Method

The method validation was evaluated in terms of linearity, detection limits, quantification limits, recovery, precision and selectivity according to ICH guidelines.^[18]

Determination Linearity

Five different concentrations of avarol in the range of 5-100 ppm were analyzed in three replicates. The peak areas plotted against each concentration of avarol solutions to obtain the calibration graph and establish a linear regression equation and to identify value of correlation coefficient.

Determination of Limits of Detection and Quantification

The values of limit of detection (LOD) and limit of quantification (LOQ) were estimated by a signal-to-noise ratio (S/N), For LOD threefold of S/N and for LOQ tenfold of S/N is accepted.

Determination of Recovery

For accuracy assay (recovery) of the method the extract of *D.avara* was spiked with known amounts of avarol standard solutions at 5, 25 and 50 µg/ml concentrations. Accuracy was evaluated as the mean concentration that recovered and were represented as recovery percentage.

Determination of Precision

Precision of method was determined in two steps: the intra-day and inter-day. In the intra-day precision, solutions were prepared at different concentrations and analyzed in triplicate in a day. In the inter-day precision, solutions were prepared at different concentrations and analyzed in triplicate during three different days. The results were calculated as the relative standard deviation (% RSD) of retention times and % peak areas.

$$\%RSD = (SD / \text{Mean}) \times 100.$$

Determination of Selectivity

The method selectivity was appraised by the resolution study between the standard peaks.

Quantitative Analysis of *Dysidea avara*

The determination of avarol in the *D.avara* was carried out by the HPLC-DAD (HPLC running condition was the same as avarol analysis). Avarol peak in methanolic extract of *D.avara* was found by comparing avarol standard solution peak. The HPLC spectrum of avarol is shown in Figure 2. The methanolic extract of *D.avara* was injected three times in order to verify the method. The HPLC spectrum of *D.avara* is shown in Figure 3. Retention time for avarol was 8.98 min.^[19,20]

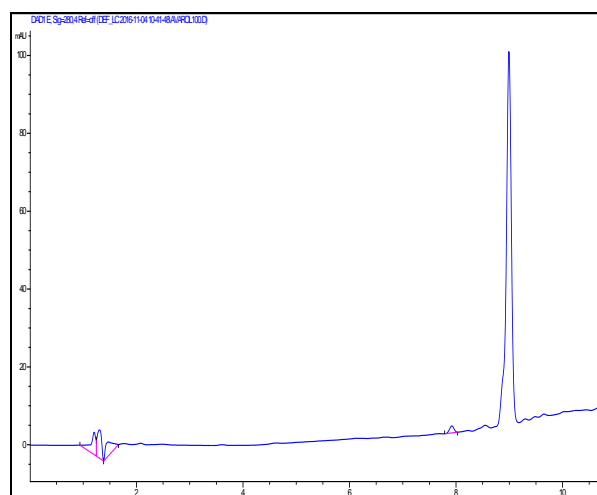


Figure 2: HPLC spectrum of avarol.

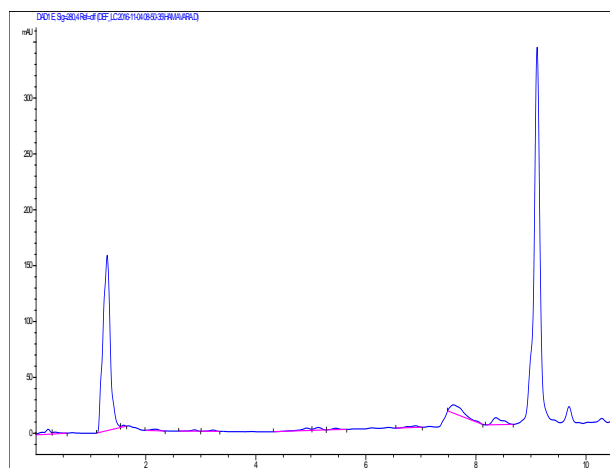


Figure 3: HPLC spectrum of methanolic extract of *D.avara*.

RESULTS

In this study we investigated and quantified amount of avarol by HPLC-DAD in Turkish *D.avara*. The calibration curve of standard (avarol) was established and the equation was determined as $y = 2.5329x - 9034.2$ and correlation coefficient was calculated as $R^2 = 0.999$. The calibration curve of avarol is shown in Figure 4. The

precision, accuracy, and reproducibility of the method were apparent from good linearity. LOD and LOQ values were 0.35 µg/mL and 1.18 µg/mL, respectively. According to the results, %RSD values for intra-day (repeatability) and inter-day (intermediate precision) variations were 0.03% and 0.23%, respectively. The recovery values were in the range of 97.1% and 101.9%. Furthermore, results demonstrated that the quantitative method was precise, accurate, and sensitive for the determination of avarol in methanolic extract of *D. avara*. Consequently, the avarol amount of *D. avara* was calculated as 3.39 mg/g dry weight of methanolic extract.

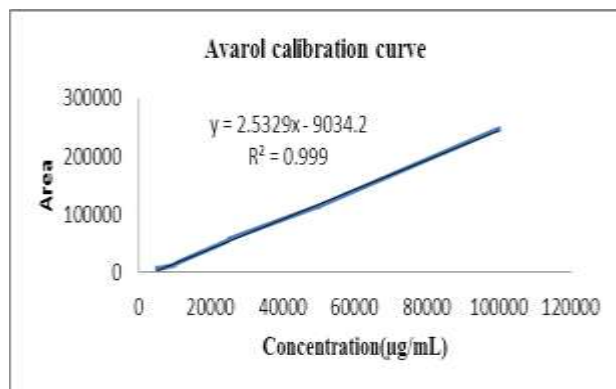


Figure 4: Calibration curve of avarol.

DISCUSSION

According to the published literatures we found only one study about temporal variability of avarol yields in *D. avara* from NW Mediterranean Sea. It showed 2.09% to 4.83% of avarol (relative to sponge dry weight), also established the peripheral zones that were close to other invertebrates of sponge significantly had more avarol than the center of sponge.^[21] In this study, we investigated avarol amount in *D. avara* that was collected from the Aegean Sea. Several analytical methods and chromatographic techniques are used for the chemical standardization of bioactive compounds in plant and marine species. For this purpose, HPLC with different types of detectors as UV/Vis, MS and MS/MS are preferred.^[22] HPLC-DAD yields rapid, accurate, sensitive and reproducible results.^[23]

CONCLUSION

The aim of this study was to study production of avarol in *D. avara* that was collected from the Turkish coast. Avarol, which was isolated previously from *D. avara*, was chosen as a standard compound, and standardization of the methanol extract of *D. avara* was established in this study. Avarol is the major compound of *D. avara* that has many bioactive properties.

Avarol, because of its various and vital bioactive effects, is an essential raw material for pharmaceutical industry. Total chemical synthesis of avarol has been achieved by a different group. Although its synthesis has been successful, the debate on whether it is a synthetic compound or natural one is still going on and several

groups prefer natural raw materials. And this has brought forward obtaining avarol in a natural way.

CONCLUSION

The combination of a number of expensive substrates, a low yield, and more than 20 reaction steps contributed to our decision to not assess the feasibility of chemical synthesis of avarol for this article. The enormous biomedical potential of avarol has made it an interesting compound for pharmaceutical industry. There is a provision problem of avarol for research and possible commercialization of the product. Unfortunately the amount of avarol in sponge is not sufficient for economical purposes of pharmaceutical industry. Because of this supply problem, different culture techniques, such as mariculture, ex-situ culture (in tanks) cell culture and in situ experimental culture have been assayed. Further studies based on present results will help to develop marine culture for large scale of avarol for economic purposes.

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