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by

BIODIVERSITAS
Volume 23, Number 3, March 2022
Pages: 1196-1203

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230302

Nutritional potential of *Semele* sp. shellfish on estrogen levels and follicle stimulating hormone receptor genes in perimenopausal women

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Manuscript received: 23 October 2021. Revision accepted: 11 February 2022

Abstract. *Sjafaraenan, Umar MR, Herwin, Islamiyati A, Tuwo M, Zulkifli A. 2022. Nutritional potential of* Semele *sp. shellfish on estrogen levels and follicle stimulating hormone receptor genes in perimenopausal women. Biodiversitas 23: 1196-1203.* The Bonea village community that often consume shellfish usually have a high libido and experience menopause above the age of 50 years. Therefore, this study aims to determine the nutritional potential of *Semele* sp. on the Follicle-Stimulating Hormone Receptor (FSH-R) gene in perimenopausal women as well as the levels of estrogen and polymorphisms. The experimental method with a one-group pre and post-test design was used by measuring 30 perimenopausal women respondents aged 40-45 before and after consuming *Semele* sp. 2 times a week for 2 months. Furthermore, blood samples were collected through the median cubital vein 2-3 mL before and after the treatment. The nutritional measurement of *Semele* sp. including protein was carried out using the Kjeldhal method, carbohydrates using the Anthrone method, and fat using the gravimetry. Also, the mineral content was determined using Atomic Absorption Spectrophotometry (AAS), while the estrogen levels were assessed with the ELISA technique. The FSH gene was examined using DNA extraction and amplification, electrophoresis, as well as Restriction Fragment Length Polymorphism (RFLP) with the Bsrl enzyme, while the t-test was conducted to examine the effect of shellfish consumption. The results showed that the consumption of *Semele* sp. increased estrogen levels significantly, as the Asn680Ser, Asn680Asn, and Ser680Se genotypes were found in each respondent with the FSH-R gene. Based on the results, it was concluded that the regular consumption of *Semele* sp. increases estrogen levels and is applicable as an alternative treatment to delay the incidence of menopause.

Keywords: FSHR, hormonal disorders, local ghiwo shellfish, Muna District, RFLP

INTRODUCTION

Shellfish have long been known and used as a food source by the community, specifically in coastal areas as they contain nutrients that are essential for the reproductive process including fats, vitamins, and minerals (Gopalsamy et al. 2014). The fat content functions to dissolve vitamins A, D, E and K, while vitamin C or ascorbic acid in the form of dehydroascorbic acid is more stable to heat and water soluble vitamins. Furthermore, fish and shellfish are the best sources of vitamin B12 or cobalamin which is also found in the liver and meat. Shellfish contain minerals such as Zn and Ca which play a role in the reproductive system (Vickram et al. 2021). Calcium levels contained in these invertebrates are higher compared to fish and meat (Furkon 2012; Venugopal and Kumarapanicker 2017). Shellfish Low-calorie is also rich in Fe, Cu, protein and fatty acid omega-3 (Irkin 2021) Hence, it is widely used by people in coastal areas as a traditional medicine to treat jaundice, anemia, internal disease, diabetes, and colds, while some species of bivalves namely *Semele* sp. are commonly used to increase libido (Zaman 2018). On average, women who often consume shellfish experience longer menopause compared to women who do not eat shellfish. Based on the empirical community experience, shellfish consumption tends to delay the menopause period beyond 50 years (Sjafaraenan 2014).

Menopause or perimenopause occurs at an age range of 48-53 years and is characterized by a decrease in the production of the estrogen hormone, thereby affecting the formation of collagen, bone health, reproductive system (Schubert et al. 2019), as well as the function of high-density lipoprotein (Ko and Hyun 2020). These hormonal changes interfere with the activities of a woman's life (Gold 2011) and the symptom is called menopausal syndrome (Sullivan 2017). One of the preventive measures to delay menopause is Hormone Replacement Therapy (HRT) which has great potential to overcome the accompanying complaints during perimenopause such as hot flushes, sleep disturbances, and night sweats (Bliuc et al. 2014; El Khoudary et al. 2020).

In developing countries, the rising elderly population is accompanied by an increase in the incidence of osteoporosis, which is estimated to be found in approximately 200 million people worldwide and 54 million in the United States. Although osteoporosis occurs

in all genders, women are 4 times more likely to develop this condition than men, after age 50, 1 in 2 women and 1 in 4 men reportedly experience an osteoporosis-related fracture, while approximately 30% have a low bone density which increases the risk for osteoporosis and this condition is called osteopenia (Tian et al. 2017). Meanwhile, Estrogen and Follicle-Stimulating Hormone Receptor (FSHR) hormone play an important role in reproductive physiology, as polymorphism in exon 10 of the FSHR gene affect ovarian dysfunction by identifying variations in the alleles that influence ovarian response to gonadotropin stimulation (Livshyts et al. 2009; Sjafaraenan et al. 2018; Laven 2019) showed that regular consumption of shellfish affects the menopause period. Therefore, it is expected that osteoporosis-related bone health and activity, as well as hormonal disorders in women, are overcome with increasing age.

This is a cross-sectional and experimental study that examines the effect of *Semele* sp. on the levels of estrogen and Follicle-Stimulating Hormone Receptors (FSHR). The result is expected to obtain a solution for increasing the estrogen and FSH content in women approaching menopause by utilizing minerals and vitamins derived from natural marine ingredients to maintain bone health and hormonal disorders.

MATERIALS AND METHODS

Study area

The materials used include *Semele* sp. and blood serum of perimenopausal women from Bonea Village, Lasalepa Subdistrict, Muna District Southeast Sulawesi Province, Indonesia. To avoid harmful substances including bacteria, these shellfish were initially left for 24 hours in an open container, then they were consumed. The sampling locations are as shown in Figure 1, while sampling and treatment were carried out purposively.

Data collection

The samples used were 10 women and the inclusion criteria included age between 40-45 years, had not experienced a tubectomy nor entered menopause, not on medication and not participated in the Family Planning (KB) program, nor consumed *Semele* sp. Before the study. Recruitment of respondents by visiting a family welfare empowerment group at the village hall. Then an explanation about the research and a consent form was given after the explanation. Meanwhile, shellfish are widely known by the local name Ghiwo in the Muna community.

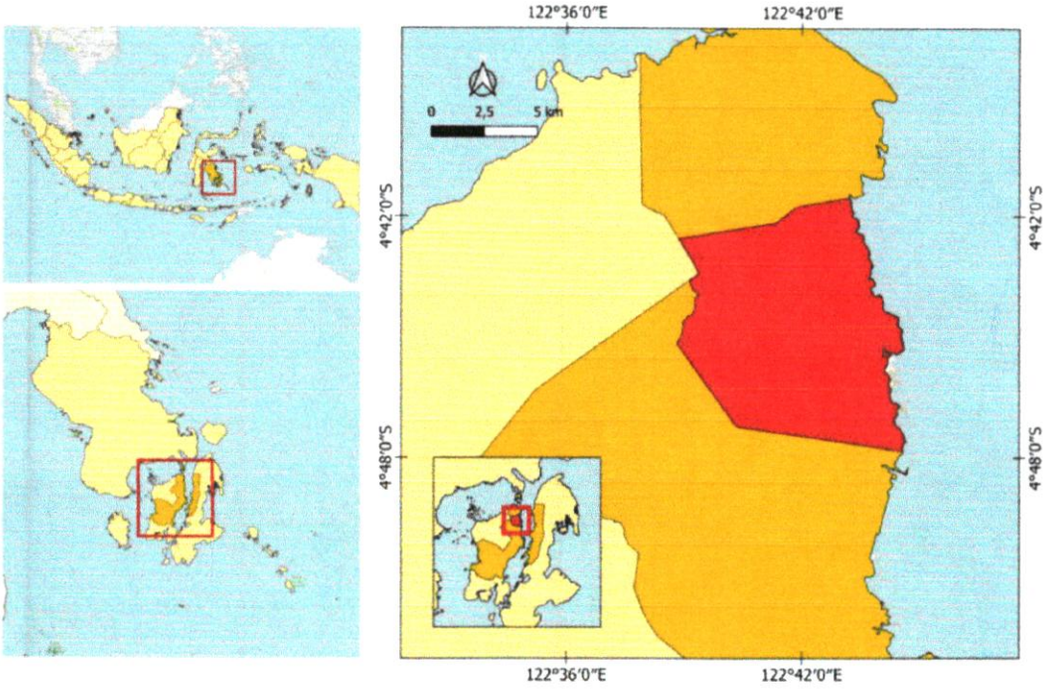


Figure 1. Locations for sampling *Semele* sp. and treatment of respondents in Lasalepa Subdistrict, Muna District, Southeast Sulawesi Province, Indonesia

The study design was an experimental one-group pre and post-test with a cross-sectional approach, while the blood sample was collected using a Project Disposable Syringe of 2-3 mL through the median cubital vein to determine the levels of Estrogen and Follicle-Stimulating Hormone Receptor (FSH-R) as a pre-test. The treatment was carried out by consuming 40-50 g of *Semele* sp. 2 times a week for 2 months, based on a study which used 39 g of shellfish per consumption (Guéguen et al. 2011), then after 4 and 8 weeks, blood was drawn again for the second and third time as a post-test. Furthermore, the protein, carbohydrate, and fat levels content of *Semele* sp. were tested using the Kjeldahl, Anthrone, and Gravimetry methods respectively, while the minerals content was determined with Atomic Absorption Spectrophotometry (AAS). The estrogen level was tested based on a procedure by DRG International USA (DRG CE 2010), while the FSHR polymorphism examination was carried out with the ELISA technique by extracting DNA with Geneaid PrestoTM Mini gDNA Blood Kit. Also, FSHR gene amplification was performed with Forward 5' TTT GTG GTC ATC TGT GGC TGC 3' primer and Reverse 5' TCAAAGGCAAGACTGAATTATCATT 3' (Livshyts et al. 2009), while DNA amplification, Electrophoresis, and Restriction Fragment Length Polymorphism (RFLP) were assessed using BsrI Enzyme (restriction enzyme). This study received ethical approval from the Health Research Ethics Committee, Faculty of Medicine, Hasanuddin University with letter number: 85/H4.8.4.5.31/PP36-KOMETIK/2018, January 30, 2019.

Mineral content test

5 g of *Semele* sp. powder was placed into a furnace at a temperature of 100°C for 2 hours until it became ash. The sample was then cooled, while 3 drops of HNO_3 were added and placed back into the furnace at 500°C for 1 hour. Furthermore, 1 mL of HNO_3 was added and diluted with distilled water to a volume of 100 mL and the mixture was then filtered using a Buchner. The result was determined/read using the AAS (Atomic absorption solution) device. The total calculation was the concentration multiplied by the sample volume per weight.

Proximate content test

The carbohydrate content was tested using the Anthrone method, 1 g of dry *Semele* sp. meat was placed into a test tube and dissolved with 50 mL of distilled water. The mixture was boiled on a hot plate for 20 minutes, cooled to room temperature, and filtered, then the filtrate obtained was collected in a volumetric flask and aquadest was added until the volume reached 50 mL. Furthermore, 4 mL of Anthrone solution was added and the results were obtained through readings on a UV visible spectrophotometer at a wavelength of 546 nm. The glucose standard series was used as a comparison. The total Carbohydrates is the concentration of the sample multiplied by the volume per weight.

The protein content was tested using the Kjeldahl method, 1 g of the sample was placed into a Kjeldahl flask, then 3 mL of HNO3 was added and boiled for 1½ hours. Water was added slowly to maintain a stable volume, then the solution was transferred to a distillation flask. The 125 mL Erlenmeyer was filled with 5 mL of H_2BO_3 solution, 2 drops of indicator, and 8-10 mL of $NaOH$-$Na_2S_2O_3$ solution, while the end of the condenser tube was submerged under the H_2BO_3 solution. Moreover, distillation was carried out until 15 mL volume was reached and diluted to 50 mL, then the solution was titrated using 0.02 N HCl until the color changed from white to purple. Calculation: % N= mL HCL minus mL blank multiplied by Normality multiplied by 14,007 per sample weight multiplied by 100%.

The fat levels were assessed by the gravimetry method, 2 g of the sample was dissolved in chloroform and filtered using filter paper, then the filtrate obtained was evaporated in an oven at a temperature of 105-110°C for 2 hours and the treatment was repeated 3 times. The crucible was cooled and weighed to a constant weight, while the fat content was obtained by subtracting the weight of the cup containing the fat from the weight of the empty cup.

Furthermore, the HDL content was tested with the precipitation dilution method by mixing the HDL cholesterol reagent 400 µL adds 100 µL aqua dest, and 200 µL *Semele* sp. Extract, and then centrifuged for 5 minutes at a speed of 3,000 rpm. 100 µL of the supernatant was mixed with 1000 µL of cholesterol reagent until the mixture turned pink, then the solution was placed into the 5010 Spectrophotometer at a wavelength of 546 nm, by first measuring the blank. The value listed on the tool indicates the HDL value. To test the cholesterol content, the indirect method was used by first making a solution containing 1,000 µL of cholesterol reagent, then the standard solution was made with a mixture of 1 µL of cholesterol and 10 µL of standard. The samples were provided and contained a mixture of 1000 µL cholesterol reagent and 10 µL of the extract. Each blank, standard, and sample were incubated in a water bath at 37°C for 5 minutes and were mixed, then the readings were made on a 5010 spectrophotometer with a wavelength of 546 nm. The value listed on the tool indicates the cholesterol content value.

Estrogen level test

Estradiol

25 µL of the serum was placed into an Eppendorf tube for standard and control with 200 µL of enzyme conjugate, the mixture was then stirred and incubated for 120 minutes at room temperature without covering the plate, while washing was carried out three times with 400 µL of washing solution and added with another 100 µL of the substrate. The mixture was reincubated at room temperature for 15 minutes and added with 50 µL of the stock solution. Furthermore, the readings were performed on a spectrophotometer at a wavelength of 450±10 nm with a maximum of 10 minutes after the addition of the stock solution.

Estron

50 µL of the standard solution, control, and serum were placed in a tube with 100 µL of enzyme conjugate added to

each, then the mixture was shaken for 10 seconds at a speed of 3000 rpm, and incubated for 60 minutes at room temperature with the tube open. It was washed 4 times with the washing solution, reincubated for 30 minutes at room temperature, while 50 μL of substrate solution was added. The calculations were carried out on a spectrophotometer at a wavelength of 450±10 nm for a maximum of 10 minutes.

Estriol

20 μL of standard solution, sample, and control were placed into an Eppendorf tube, while 200 μL of diluted enzyme conjugate was added. The mixture was incubated at 37°C for 1 hour, then washing was carried out using distilled water, and a 300 μL fraction was reincubated at room temperature between 22-28°C for 15 minutes. Moreover, 100 μL of the stock solution was added and the readings were carried out with a spectrophotometer at 450±10 nm wavelength with a maximum of 10 minutes.

FSH receptor test

DNA extraction

The DNA extraction was carried out according to the protocol in the DNA Blood Kit. Blood samples taken from the freezer were heated in a water bath at 60°C for 1 minute, then 300 µL in the EDTA tube was transferred to a sterile microcentrifuge tube marked 1-1. After adding 100 µL of RBC Buffer, the sample was incubated at room temperature for 10 minutes and centrifuged for 5 minutes at 3000 RCF, then the supernatant was discarded. Furthermore, 200µL of GB Buffer was added and incubated at 60°C for 10 minutes until the sample was lysed, during incubation, it was inverted every 3 minutes. Meanwhile, the Elution Buffer was heated to 60°C before use. In the DNA binding lysate stage, 200µL of Absolute Vortex Ethanol solution was added for 10 seconds, then the GD Column was transferred and centrifuged at 13,000 RCF for 2 minutes, while the collection tube was replaced with a new one. Washing was carried out by adding 400 µL of the W1 Buffer to the GD Column and centrifuge for 30 seconds at 14,000 RCF, then the liquid was removed from the collection tube, and placed in the GD Column. It was added with 600 µL of the Wash Buffer mixed with ethanol, centrifuged for 30 seconds at 14,000 RCF. The liquid in the collection tube was discarded, placed into the GD Column, and centrifuged for 3 minutes to dry the column matrix. In the DNA Elution stage, the heated 100 µL Elution Buffer solution was added to the center of the GD Column matrix which had been placed in a new microcentrifuge tube. It was then incubated for 3 minutes and centrifuged for 30 seconds at 14,000 RCF to elute pure DNA.

PCR mix preparation

Each 10 µL Primer FSHR Forward and Reverse was taken and placed into a sterile effendorf tube, the Forward was 5' TTT GTG GTC ATC TGT GGC TGC 3', while the Reverse was: 5' TCA AAG GCA AGA CTG AAT TAT CAT 3' (Ganna et al. 2009). Furthermore, 100 µL of Go Taq Master Mix Green was placed into a sterile effendorf tube and mixed with primer, while 90 µL of H_2O was added. The 20 µL Mix Primer and Go Taq Mastermix were placed into the PCR tube, and then 5 µL of DNA was added to each.

Detection of DNA amplification by PCR method

The tube containing PCR (polymerase chain reaction) Mix and DNA was inserted into the Veriti-Applied Biosystem Thermal Cycler machine, then, it was run under PCR conditions of 5°C Predenaturation 2 minutes for 1 cycle, Denaturation 94°C for 1 minute, Annealing 58°C for 45 seconds, Extension 72°C for 45 seconds, and post-extension 72°C for 5 minutes in 34 cycles. Furthermore, the FSHR gene PCR results were electrophoresed to observe the amplified bands.

Detection of PCR products by electrophoresis

2% Agarose gel was placed into an Erlenmeyer containing 100 mL of 0.5x TAE buffer heated in the microwave for 2 minutes and added with 8 µL of Ethidium Bromide. The gel liquid was poured into an electrophoresis gel mold using a gel comb of 17 wells and then cooled at room temperature. Moreover, 2 µL of loading dye was taken and mixed into 5 µL of PCR product for each sample, then the mixture was placed into the wells. A 5 µL ladder of 100 bp was taken and placed in the far end of the agarose gel, then the power supply was turned on with 100 volt setting for 50 minutes. Observation was carried out under UV light and recorded in the Gel Documentation to determine the 520 bp FSHR gene amplification band.

FSHR gene RFLP with BsrI

The RFLP technique was used with the PCR product of the FSHR gene along 520 bp added with the restriction enzyme BsrI (5'-ACTG_Gn 3'), then incubated at 65°C for 20 minutes and inactivated at 80°C for 15 minutes. The PCR product was further prepared for electrophoresis to determine the allele of the FSHR gene.

Data analysis

The primary data obtained were analyzed descriptively with the univariate analysis, while the Kolmogorov Smirnov test obtained a p-value >0.05 indicating that the data were normally distributed. Furthermore, the Paired T-test was performed with an alpha value of 0.05 to determine the average comparison between the variables.

RESULTS AND DISCUSSION

The descriptive test results showed the nutrient content of *Semele* sp., estrogen levels, and PCR as presented below:

Nutrient content of *Semele* sp.

Nutrient content of Semele sp. can be seen in Table 1.

Estrogen levels (estradiol, estron, and estriol) of respondents

The average estrogen levels in the respondents before and after receiving treatment for 4 and 8 weeks are shown

in Table 2. The estrogen levels obtained after further testing with the Paired T-test are shown in Table 3.

The paired T-test results showed an average difference in the levels of estradiol, estrone, and estriol before, as well as 4 and 8 weeks after the treatment. This is indicated by the smaller P-value in each test compared to the alpha value of 0.05. Furthermore, the mean differences indicate that the treatment has significant effects on the levels of estradiol, estrone, and estriol.

PCR results of FSHr and RFLP genes

Before treatment, 2-3 cc of venous blood was taken, while the PCR results of FSHr (Follicle-Stimulating Hormone receptor) and RFLP (Restriction Fragment Length Polymorphism) Genes are shown in Figures 2 and 3. The respondents were given treatment with consumption of *Semele* sp. 2 times a week, then after 4 and 8 weeks, 2 ccs of blood was taken through the median cubital vein. The results of amplification and RFLP are shown in Figures 2 and 3 (A and B).

Table 1. Content of nutrients and minerals in dry *Semele* sp.

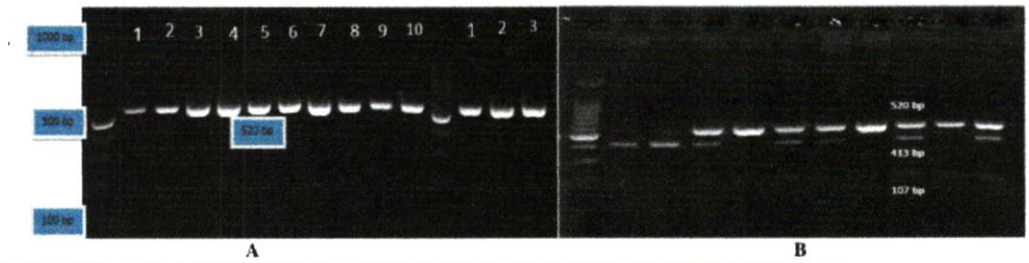
Component	Value	Unit
Nutrient		
Protein	7.157	%
Carbohydrate	67.678	%
Fat	6.620	%
Cholesterol	10.000	Mg/dL
HDL	7.000	Mg/dL
Minerals		
Ca	266.375	Ppm
Mg	29.467	Ppm
Fe	1.759	Ppm
Zn	1.258	Ppm

Table 2. Average estrogen levels (pg/mL) before and after consumption of *Semele* sp.

Estrogen	Before consumption	After consumption	
		4 weeks	8 weeks
Estradiol	27.862	32.559	38.406
Estron	78.274	84.094	88.815
Estriol	4.173	4.516	5.085

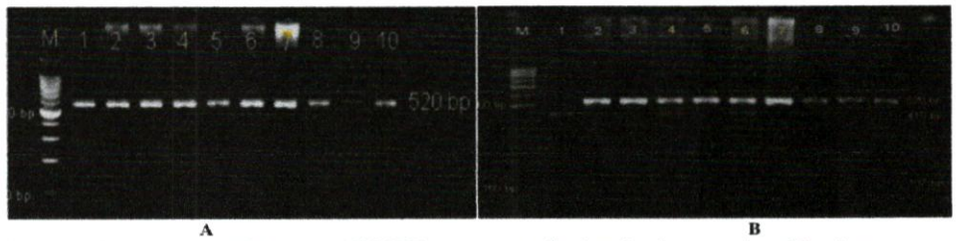
Table 3. Combined characteristics of paired T-test analysis of estrogen levels (pg/mL) before and after consumption of *Semele* sp.

Treatment (before and after consumption)	Estradiol		Estron		Estriol	
	T-count	P-value	T-count	P-value	T-count	P-value
Before vs 4 weeks	-4.686	0.001	-5.909	<0.001	-4.033	0.003
Before vs 8 weeks	-7.607	0.000	-7.577	0.000	-5.898	0.005
4 and 8 weeks	-5.527	<0.001	-6.054	0.000	-3.955	0.003



A B

Figure 1. PCR results. A. DNA amplification, B. RFLP FSH receptor gene before consumption of *Semele* sp.



A B

Figure 2. PCR results. A. DNA amplification, B. RFLP FSH receptor gene after 4 weeks of consumption of *Semele* sp.

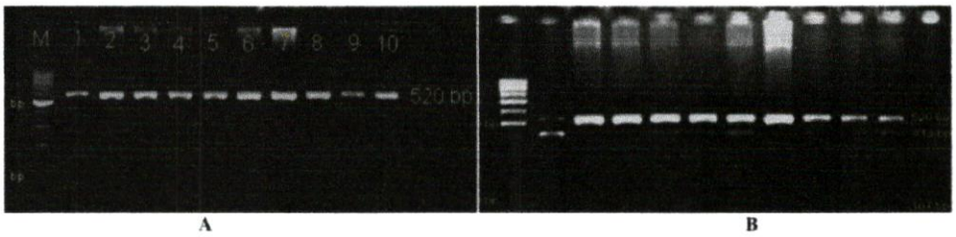


Figure 3. PCR results. A. DNA amplification, B. RFLP FSH receptor gene after 8 weeks of consumption of *Semele* sp.

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BioEdit version 7.1.3.0 (11/4/2011) Restriction Mapping Utility
(c)1998,  Tom Hall

Homo sapiens chromosome 2, GRCh37.p10 Primary Assembly Restriction Map
18/07/2013 14:09:01
520 base pairs
Translations:  none
Restriction Enzyme Map:

1       TTTGTGGTCATCTGTGGCTGCTATATCCACATCTACCTCACAGTGCGGAACCCCAACATCGTGTCCTCCTCTAGTGACAC   80
1       AAACACCAGTAGACACCGACGATATAGGTGTAGATGGAGTGTCACGCCTTGGGGTTGTAGCACAGGAGGAGATCACTGTG   80

81      CAGGATGGCCAAGCGCATGGCCATGCTCATCTTCACTGACTTCGTCTGCATGGCACCCATTCTTTCTTTGCCATTTCTG   160
81      GTCCTAGCGGTTCGCGTACCGGTACGAGTAGAAGTGACTGAAGGAGACGTACCGTGGGTAAAGAAAGAAACGGTAAAGAC   160

161     CCTCCCTCAAGGTGCCCCTCATCACTGTGTCCAAAGCAAAGATTCTGCTGGTTCTGTTTCACCCCATCAACTCCTGTGCC   240
161     GGAGGGAGTTCCACGGGGAGTAGTGACACAGGTTTCGTTTCTAAGACGACCAAGACAAAGTGGGGTAGTTGAGGACACGG   240

241     AACCCCTTCCTCTATGCCATCTTTACCAAAAACTTTCGCAGAGATTTCTTCATTCTGCTGAGCAAGTGTGGCTGCTATGA   320
241     TTGGGGAAGGAGATACGGTAGAAATGGTTTTTGAAAGCGTCTCTAAAGAAGTAAGACGACTCGTTCACACCGACGATACT   320

321     AATGCAAGCCCAAATTTATAGGACAGAAACTTCATCCACTGTCCACAACACCCATCCAAGGAATGGCCACTGCTCTTCAG   400
321     TTACGTTCGGGTTTAAATATCCTGTCTTTGAAGTAGGTGACAGGTGTGTGGGTAGGTTCCTTACCGGTGACGAGAAGTC   400

401     CTCCCAGAGTCACCAGTGTTCCACTTACATACTTGTCCCTCTAAGTCATTTAGCCCAAAACTAAAACACAATGTGAAAA   480
401     GAGGGTCTCAGTGGTCACAAGGTGAATGTATGAACAGGGAGATTCAGTAAATCCGGGTTTTGATTTTGTGTTACACTTTT   480
                  BsrI

481     TGTATCTGAGTATTGAATGATAATTCAGTCTTGCCTTTGA   520
481     ACATAGACTCATAACTTACTATTAAGTCAGAACGGAAACT   520

Restriction table:
Enzyme     Recognition                  frequency  Positions
-------------------------------------------------------------
BsrI       ACTG_Gn'                     1          414
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Figure 4. Restriction mapping utility

Discussion

Nutrient contents of Semele sp.

The nutrient composition in Table 1 shows that *Semele* sp. contains 7.157% protein, 67.678% carbohydrates, as well as 6.620 mg/dl fat and cholesterol. The fat level is relatively high compared to the result obtained by Nurjannah et al. (2005) on blood clams (*Anadara granosa*) of 0-2% proximate content with a fat value of 2.60%. Also, it was higher than the shellfish content of Pokea or *Batissa violeacea celebensis* (Yenni et al. 2011) which showed 29.13% carbohydrates and 4.6% fat.

The minerals Cu, Fe, Zn, and Ca were tested for their roles as antioxidants in the body's defense system against free radicals. Specifically, Ca (calcium) is a mineral for bone formation and is incorporated into enzymes that play a role in protecting cell membranes as well as components in the cytosol. Additionally, it is easily oxidized, while Zn (zinc) is an important mineral in enzyme and hormone systems. Based on the results, the mineral content of *Semele* sp. is dominated by Ca with 266.375 ppm which plays an important role in maintaining healthy bones, teeth, and is needed by women in maintaining the balance of estrogen (Henderson 2008) as well as to prevent the risk of osteoporosis.

The influence of Semele sp. *on estrogen levels*

The examination of Estrogen levels including Estradiol, Estron, and Estriol after consuming *Semele* sp. for 2 and 8 weeks showed a significant increase, indicated by the P-value in each test which was smaller than the alpha value of 0.05 as shown in Table 3. This implies that the treatment has significant effects on estradiol, estrone, and estriol levels, therefore, it was concluded that the consumption of Semele sp. increases estrogen levels. Furthermore, the nutritional content of the shellfish in Table 1 shows the presence of fat and cholesterol. Cholesterol is a structural

component of cell membranes and functions as a building block for the synthesis of various steroid hormones, vitamin D, and bile acids. Also, it provides stability and fluidity, as well as plays an important role in regulating cell function (Heffner 2010; Ahmadabad et al. 2019). Meanwhile, fat hydrolyzed in metabolism produces fatty acids, which are then broken down into acetic acid and forms acetoacetyl coenzyme. This molecule further produces mevalonic acid in a biogenetic pathway to form lanosterol and then through a series of changes, synthesize cholesterol which is the precursor for the biosynthesis of steroid hormones such as progestins, androgens, and estrogens (Hu et al. 2010). In addition, the dominant mineral in shellfish namely calcium plays an important role in maintaining the balance of the estrogen hormone.

Relationship of Semele sp. *and follicle-stimulating hormone receptor genes*

The paired T-test results showed that there was a significant effect before and after consumption for 4 and 8 weeks, while the nutritional content examination of *Semele* sp. found fat and cholesterol. Meanwhile, cholesterol is a precursor of steroid hormones through the biogenetic pathway of mevalonic acid using aromatase enzyme to produce an aromatic compound namely 17β-estradiol (E2) which is an estrogen with 18 carbons and 2 OH functional groups (Hinson. et al. 2010; Aquirre et al. 2018). Perimenopausal women experience decreased levels of estrogen (Hestiantoro et al. 2019), due to reduced ovarian function which leads to an increase in the numbers of free estrogen receptors. However, the nutrient content in *Semele* sp. tends to replace the role of estrogen, hence, regular shellfish consumption enables free estrogen receptors to bind with cholesterol, leading to an increase in estrogen levels including estradiol, estrone, and estriol (Hu et al. 2010).

This process affects the Follicle Stimulating Hormone Receptor (FSHR) gene encoded on chromosome 2 of the P-arm at locus P21-P16 (Schubert et al. 2019). This gene consists of 10 exons and 9 introns, where the first 9 exons function to code for the receptor domain on the cell surface and the 10th exon encodes the C-terminal of the extracellular, transmembrane, and intracellular groups of FSHR. Presently, 1,800 SNPs have been reported in the FSHR gene in NCBI, specifically in exon 10, which has 7 SNP sites located at codons 307, 329, 449, 524, 567, 665, and 680. However, only 2 SNP sites were found in this exon which when mutated leads to the failure of follicular cell development, such as SNP (Single nucleotide polymorphisms) in the FSHR Asn680Ser (Laven 2019). The FSHR gene in exon 10 consists of 520 base pairs in line with the blast results from NCBI. Using 2 specific primers that bind to the 5' end of each side of the DNA, the amplification of the FSHR gene was 520 bp. Moreover, PCR amplification through the DNA extraction process used the Geneaid PrestoTM Mini gDNA Blood Kit. The extraction results were then processed with DNA amplification using specific primers for the FSHR gene. The amplification process used the Abi Veriti PCR machine with optimal PCR conditions, while an electrophoresis process was carried out using 2% Agarose to observe the luminescence of the DNA from the amplified FSHR gene in the form of a band with a size of 520 base pairs (bp). Figures 1, 2, and 3 show that each subject has the FSHR gene, indicating the presence of a gene encoding the FSH receptor in the various cells (Livshyts et al. 2009). These genes play a role in structural FSH receptor formation including the membrane receptor superfamily G-protein-coupled receptor (GPCR) (Alfredo et al. 2018) which are expressed on granulosa cells and convey signal transduction by activating adenylate cyclase and increasing intracellular cAMP (Sheika et al. 2011).

All subjects had the FSHR gene in the cells, but RFLP (Restriction Fragment Lenght Polymorphism) was performed to determine the presence of Asn680Ser, Asn680Asn, and Ser680Ser genotypes in the subjects. This FSHR gene polymorphism shows the conversion of Adenine to Guanine at codon 680 in exon 10, causing a cleavage site for the restriction enzyme BsrI (5'-ACTG_Gn 3').

Figure 4 above shows the sequence of the *Homo sapiens* FSHR gene chromosome 2, GRCh37.p10 Primary Assembly from the NCBI gene bank. The sequences show the Ser680Ser allele, where the cleavage site by the BsrI enzyme is at base 413, when there is a change in C to T, then the cleavage site is lost, leading to the formation of the Asn codon (TTG) to produce the Ser680Asn or Asn680Asn allele.

Subjects 1 and 2 have Ser680Ser genotype with 2 restriction bands of 413 bp and 107 bp, while 3, 5, 6, 8, and 10 had Asn680Ser genotype with 3 restriction bands of 520 bp, 413 bp, and 107 bp. Furthermore, subjects 4, 7, and 9 had the Asn680Asn genotype with 1 band of 520 bp, but without a cleavage area for the BsrI enzyme in DNA. Figure 3 shows the RFLP results of the FSHR gene with subject 1 having Ser680Ser, 4, 6, 8, and 10 with Asn680Ser, and subjects 2, 3, 5, 7, and 9 with Asn680Asn genotype. Also, Figure 3B shows the RFLP results of the FSHR gene 8 weeks from the first blood collection, with subject 1 having Ser680Ser, 4, 6, 8, and 10 with Asn680Ser, and 1, 2, 3, 5, 7, and 9 with Asn680Asn. Subjects that had the FSHR phenotype experienced a change compared to the first RFLP, for example, subject 2 initially with the Ser680Ser genotype after intervention with *Semele* sp. Changes were maintained until the third blood draw, this is presumably due to the content of nutrients and minerals in the shellfish. Besides, the dominance of calcium, fat, and cholesterol tends to affect the estrogen levels in the blood (Balkan 2010). According to Kennneth et al. (2020), polymorphisms of receptor and estrogen genes are associated with cholesterol, HDL, and calcium levels (Ahmadabad et al. 2019). Further research is needed to find bioactive compounds in *Semele* sp. that affect the hormone estrogen.

A study also reported that respondents treated with regular consumption of *Semele* sp. experienced fluctuations in the lipoprotein concentrations as well as the calcium, fat, and cholesterol which greatly affect the estrogen levels (Balkan et al. 2010). Furthermore, Koga (2020) stated that the Asn680Ser gene polymorphism led to a decrease in

plasma FSH levels, but there were variations in the proportion of genotypes due to the observed ethnic differences of the subjects. According to Gold (2011) and Bansal et al. (2013), ethnic and racial differences affect menopause. The subjects observed in this study were women in coastal areas from different ethnicities.

Based on the discussion and analysis, it was concluded that the consumption of *Semele* sp. significantly increased estrogen levels because the genotypes Asn680Ser, Asn680Asn, and Ser680Se were found in each respondent with the FSH-R gene, hence, *Semele* sp. can be used as an alternative treatment to delay the incidence of menopause.

ACKNOWLEDGEMENTS

The authors are grateful to Hasanuddin University, Makasar, Indonesia for funding this study under the Specific Indonesian Maritime Continent (BMIS) scheme with Number 12287/UN4.21/PL.00.00/2018 June 22, 2018. As well as to all respondents who participated in this study.

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