



# Effect of *Batissa violacea celebensis* Martens, 1897, Extract on $\beta$ -Catenin Gene in Mice (*Mus musculus*) Induced by Azoxymethane and Dextran Sulfate Sodium using Polymerase Chain Reaction-restriction Fragment Length Polymorphism Method

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## Abstract

**Edited by:** Slavica Hristomanova-Mitkovska  
**Citation:** Rasyid SA, Sanatang S, Syarif S, Sugireng S, Purnama T, Suwarny S, Saputra YK. Effect of *Batissa violacea celebensis* Martens, 1897, Extract on  $\beta$ -Catenin Gene in Mice (*Mus musculus*) Induced by Azoxymethane and Dextran Sulfate Sodium using Polymerase Chain Reaction-restriction Fragment Length Polymorphism Method. Open-Access Maced J Med Sci. 2021 Sep 24; 9(A):964-970. https://doi.org/10.3889/oamjms.2021.6987  
**Keywords:**  $\beta$ -catenin gene; Colorectal cancer; Mutation; Polymerase chain reaction-restriction fragment length polymorphism

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**Received:** 03-Aug-2021

**Revised:** 20-Aug-2021

**Accepted:** 14-Sep-2021

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**Funding:** This research did not receive any financial support

**Competing Interest:** The authors have declared that no competing interest exists

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**BACKGROUND:** At present, the  $\beta$ -catenin gene is a genetic change considered as the most important factor in the initiation and the progress of cancer. Bivalve extract contains steroid compounds that are thought to have an aphrodisiac and anti-inflammatory effect. The triterpenoid component detected in the crude bivalves extract is thought to have antitumor activity.

**AIM:** This study aimed at knowing the effect of ethanol (EtOH) extract, ethyl acetate fraction, and the n-hexane fraction of poka clam on  $\beta$ -catenin gene mutation in mice induced by azoxymethane (AOM) and dextran sulfate sodium (DSS) using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

**METHODS:** The testing of the effect of poka clam extract was conducted on several treatment groups, namely, AOM/DSS-infected mice treated with EtOH extract, ethyl acetate fraction, and N-hexane fraction. The positive control and the negative control of the three treatment groups were done by giving the extract with a dosage of 0.25 mg/bb to mice for 14 days.

**RESULTS:** Based on the result of  $\beta$ -catenin gene amplification using the PCR method and RFLP analysis, the EtOH extract and ethyl acetate fraction groups had the same band, namely, 56 bp and 12 bp, consecutively; meanwhile, the n-hexane fraction was marked by the band formation of 191 bp, 82 bp and 48 bp, 69 bp, 116 bp, 151 bp, 180 bp, and 234 bp. From the types of band and the band size that have been formed, it shows that they have the same formation and similarities in the positive and negative treatment characterized by the amplification that occurred in the band size of 227 bp, and the result of amplification from RFLP analysis showed the same band with the same band size, namely, 89 and 138 bp.

**CONCLUSION:** Based on this, it can be concluded that N-hexane fraction affects  $\beta$ -catenin gene mutation at the first or second base at codon position 32 or 33, and the third base, namely, the bands of 82 and 145 bp that also characterize the effect of N-hexane on  $\beta$ -catenin gene mutation at the second or third base. It is supported by the sequencing method obtaining the homological percentage of 99.13%.

## Introduction

$\beta$ -catenin gene plays an important role in the cell adhesion system mediated by cadherins and in signal transduction at the Wnt signaling pathway binding T-cell factor in the nucleus and regulates gene transcription related to cell development and differentiation. The regulation derivative of the  $\beta$ -catenin gene binds adenomatous polyposis coli (APC) protein and phosphorylation by glycogen synthase kinase-3 beta (GSK3 $\beta$ )/serine-threonine kinase causing the  $\beta$ -catenin gene stabilization in the cytoplasm and can induce the activation of the constitutive transcription of Tcf derivation from DNA-binding proteins.  $\beta$ -catenin gene mutation has been found in the 50% of human colon tumors in APC. This indicates that the activation of  $\beta$ -transcriptional pathways mediated by catenin/Tcf is

caused by the mutation of the APC or  $\beta$ -catenin gene that is strongly important for colorectal neoplasia [1].

$\beta$ -catenin-CTNNB1 gene is the central component of adherens junctions (AJs) having the capability to bind E-cadherin in the epithelial cells and stabilize the cytoskeleton in preventing abnormal cell growth. AJ is crucial in the growth regulation and adhesion between epithelial cells. At the cell level,  $\beta$ -catenin gene mutation can transmit the signal that causes cell lyses, especially during the growth plate of epithelial cells [2].

The factors involved in colorectal cancer development can be categorized into three groups, namely, genetic factor, epigenetic factor, and environmental factor. These factors result in the variation of growth pathways and cell proliferation. The genetic factor is one of the most significant factors where the gene changes and signaling pathways cause

a failure of normal gene functions. The cell process of the  $\beta$ -catenin gene involves many genes that interact with each other and regulates cellular activities, such as cell proliferation, transformation, cell growth, and cell invasion [3].

Azoxymethane (AOM) is a pro-carcinogen that is structurally similar to cycasin, a compound that can induce colorectal tumors. AOM is used in biological research and is strongly effective for inducing colorectal carcinoma or cancer that grows from the skin tissues or the tissues structuring the organ walls. In the carcinogenesis of the colon in mice induced by AOM, K-ras gene mutations often happen as colorectal tumors happened in humans, yet, in APC, gene changes are rarely observed [3].

The bivalve extract has steroid compounds that are assumed to increase stamina (aphrodisiacs) and anti-inflammation. The component of triterpenoids detected in the crude extract of bivalves is believed to have antitumor activities. Natural triterpenoids have antitumor activities since they can inhibit the performance of type II topoisomerase by binding the active site of the enzyme that will bind DNA and cleaving it, thus, the enzyme is locked and it cannot bind DNA [4]. Pokea extract can be collected by the extraction process. Extraction is the process of separating a substance from a mixture using a suitable solvent. The extraction process is stopped when the balance between the concentration of compounds in the solvent and the concentration in the plant cells or animal cells is achieved. The solvents used in this study were non-polar N-hexane, ethanol (EtOH) as a polar solvent, and semi-polar ethyl acetate [5].

Based on the statement above, it is important to conduct a study on  $\beta$ -catenin gene mutation in mice that have been given pokea (*B. violacea celebensis* Martens, 1897) extract available in Southeast Sulawesi. The researcher was interested in conducting a study entitled "Effect of Pokea (*Batissa violacea Celebensis* Martens 1897) Extract on  $\beta$ -Catenin Gene Mutation in Mice Induced by AOM and Dextran Sulfate Sodium (DSS)."

## Methods

### **Maceration process and filtering the *B. violacea celebensis* Martens, 1897, extract (Rasyid et al., 2017) [5]**

Pure EtOH solvent used 2 L of distillation results. Soaking was done with 2 L of pure EtOH, after that, it was allowed to stand for 3 × 24 h. For 1 × 24 h, the concentration process is carried out by evaporation, then the filtrate is filtered (Rasyid et al., 2017) [5].

### **Evaporation stage**

Two liters of macerates from the filtering process are evaporated. The evaporation process was conducted using a rotary vacuum evaporator. This process was conducted until the thick extract was formed. Then, it was moved and scaled (the extraction product of 1 kg pokea clam produced 6 g of extract).

### **Fractionation process of EtOH extract using ethyl acetate and n-hexane solvents [6]**

- a. The ethyl acetate fraction was obtained by taking 24 g of EtOH extract, dissolved again with EtOH until dissolved. Then, the extract filtrate was put into a separating funnel and 200 ml of ethyl acetate was added to form a layer. Added 50 ml of distilled water. Then, the fractionation process is carried out with the solvent indicator becoming clear. A filtrate was formed with an aqueous phase of EtOH and an organic filtrate of ethyl acetate. The ethyl acetate filtrate was concentrated to become ethyl acetate extract
- b. The n-hexane fraction was obtained by taking 24 g of EtOH extract and diluted with EtOH until dissolved. The extract filtrate was put into a separating funnel and 300 ml of N-hexane was added to form a layer. Added 60 ml of distilled water. Then, the fractionation process is carried out with the solvent indicator becoming clear. A filtrate is formed with the water phase of EtOH and organic N-hexane filtrate.

### **Preparation of experimental mice**

This study had obtained ethical approval from the ethical committee of the Faculty of Medicine, Universitas Hasanuddin, with the following registration number: UH20110627. The colon sample was taken after injecting AOM/DSS as a treatment and ensuring that the mice experience dysplasia, except for the negative control, the AOM/DSS treatment was not given [7]. Next, EtOH extract, ethyl acetate fraction, and n-hexane fraction were given with a dosage of 0.25 mg/ml to each mouse group for 14 days.

### **DNA extraction**

#### **Protein removal**

Put 15 mg of fresh animal tissue (colon) into a 1.5 ml microcentrifugation tube. Added 25 l of GST buffer and 20 l of proteinase K. Divortex, then incubated at 60°C until the sample looks clear.

#### **Cell lysis**

The insoluble material was centrifuged for 2 min at 14–16,000 × g, the supernatant was transferred

to a new 1.5 ml microcentrifuge tube. Added 200 L of GSB buffer and vortexed for 10 s.

#### *DNA binding*

Added 200 l of absolute EtOH to the sample lysate and shaken vigorously for 10 s. The GS column was placed in a 2 ml collection tube, then centrifuged at 14–16,000 × g 1 min. Discard the 2 ml collection tube containing the filtrate and transfer the GS column to a new 2 ml collection tube.

#### *Washing*

Add 400 L of buffer W1 to the GS column tube at 14–16,000 × g for 30 s and discard the filtrate. Place the GS column back into the 2 ml collection tube. Add 600 L of wash buffer (make sure absolute EtOH is added) to the GS column at a centrifuge of 14–16,000 × g 30 s then discard the filtrate. Place the GS column back into the 2 ml collection tube. Centrifuge again for 3 min at 14–16,000 × g. The dry GS column was transferred to a 1.5 ml microcentrifuge tube. Add 100 L of preheated elution buffer. Let stand for 3 min, then centrifuge at 14–16,000 × g 30 s.

#### ***Polymerase chain reaction (PCR) process***

Added DNA template 200 mg. Each primer was taken 1 L each. Then, the MyTaq HS Red Mix reagent kit, 2× as much as 25 L was added. Water was added until the sample volume became 50 µl, then homogenized. The amplification process was carried out in a PCR machine with pre-denatured conditions of 95°C for 1 min and followed by denaturation of 95°C for 15 s, annealing at 57°C, and extension at 72°C for 10 s.

#### ***Restriction fragment length polymorphism (RFLP) analysis (restriction digestion)***

The results of PCR amplification were digested using the Hinf1 enzyme which consisted of mixing 10 µL PCR reaction (~0.1–0.5 g DNA), 18 µL nuclease-free water, 10× buffer R 2 µL, and 1–2 L Hinf1, then stir gently and turn it down for a few seconds. It was incubated at 37°C for 1 h and the incubation temperature was raised to 65°C for 20 min.

#### ***Electrophoresis process***

##### *PCR results in the electrophoresis process*

The tray position was adjusted and 1XTAE buffer pH 8.0 500 ml was poured into the

electrophoresis tank. Then, 5 L of the DNA sample was mixed and inserted into the agarose gel well, the current-voltage and running time were set at 100 V, 35 A, and 30 min.

#### ***Analysis of Hinf1 digestion products using electrophoresis***

To know the result of DNA amplification, electrophoresis was applied to the Hinf1 digestion products in agarose gel of 2–3%. Around 2–3% was made by dissolving 2–3 g of agarose in 100 ml buffer 1×TAE with pH 8 and was heated until boiled. After being homogenized and the agar was cool (approximately 60°C), DNA staining was added (1 µL/30 ml). The agarose solution was poured into an agarose gel container, and the electrophoresis comb was installed at one of the tips of the agarose gel container. After the gel was solid, the comb was removed carefully.

The container position was set and 500 mL of buffer TAE 1× pH 8.0 was poured into the electrophoresis tank. Furthermore, 10 µL of DNA sample mixture was poured into the agarose gel well; the voltage, electric current, and running time were set at 100 V, 1 A, and 90 min, consecutively. After the electrophoresis finished, the gel was placed in the ultraviolet (UV) transilluminator; then, the visualized DNA bands were observed.

#### ***DNA visualization of PCR and Hinf1 digestion products***

DNA visualization of PCR results and Hinf1 digestion had the same procedure, namely, placing the gel into the UV transilluminator device by removing the gel from the mold. Then, the gel is exposed to UV light. The image of the gel formed under UV light is then documented. The DNA bound to the red gel will be exposed under UV light.

#### ***DNA sequences analysis (PT *genetica* sains)***

DNA bands of the Beta-catenin gene obtained from the sample the results of electrophoresis carried out DNA sequencing through PT. Genetics Science Indonesia to see changes in the sequence of the Beta-catenin gene. DNA sequencing, the PCR method is used as the basis. The DNA for which the ACGT base sequence will be determined is used as a template and then amplified using enzymes and materials similar to PCR reactions, but with the addition of certain reagents. This process is called cycle sequencing.

While the analysis of sequencing result, Blast can be used to identify a questionable nucleotide sequence (query sequence) that we have with the nucleotide database so that the output obtained is in the form of the identity of the nucleotide, including the

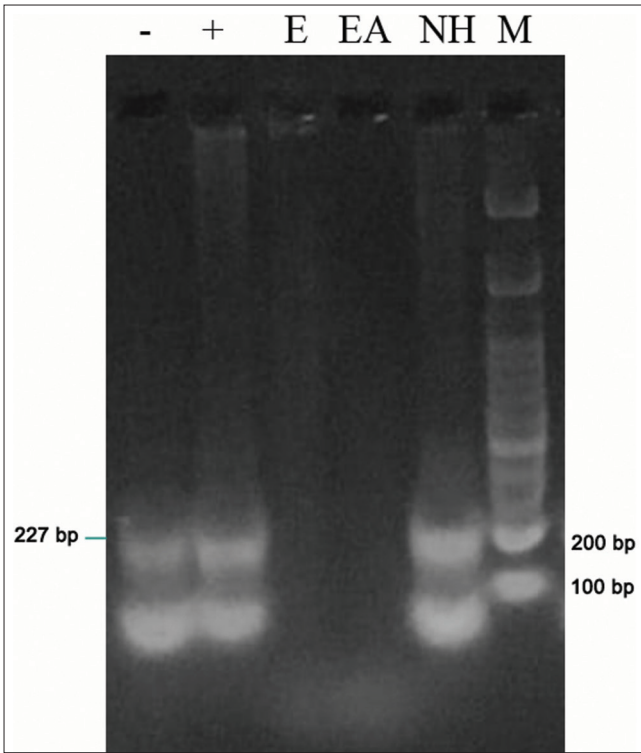


Figure 1: The result of  $\beta$ -catenin gene amplification using the PCR method, (Source: Personal Documentation, 2021). Desc: - = Negative, + = (Positive), E = (EtOH), EA = (Ethyl acetate), NH = (n-hexane), and M = (Marker)

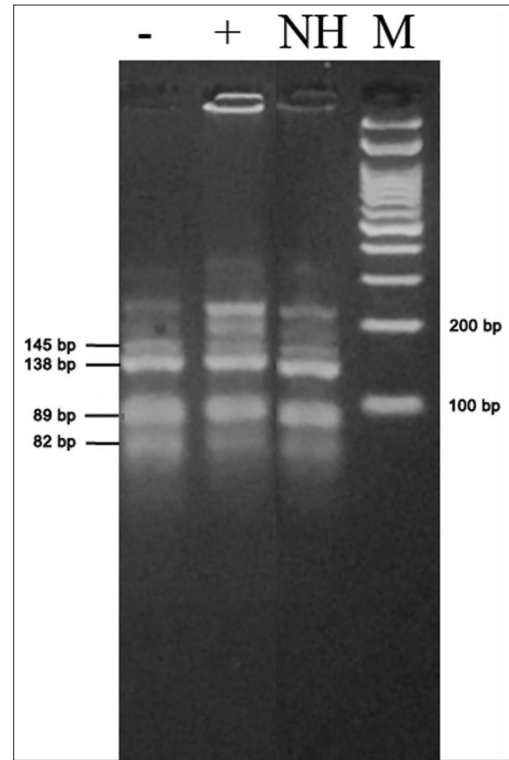


Figure 2: The result of  $\beta$ -catenin gene amplification using the RFLP analysis (restriction digestion). Desc: - = Negative, + = (Positive), NH = (n-Hexane), and M = (Marker)

name of the gene and the producing species of the complete sequence.

## Results

This study investigated the effect of the EtOH extract, ethyl acetate fraction, and N-hexane fraction of pokea extract on  $\beta$ -catenin gene mutation in mice using the PCR method and RFLP analysis (restriction digestion). The result of the  $\beta$ -catenin gene examination using the PCR method and the result of the  $\beta$ -catenin gene examination using the RFLP analysis (restriction digestion) can be seen below:

### Result of the examination of $\beta$ -catenin gene using the PCR method

The result of  $\beta$ -catenin gene amplification using the PCR method is presented in Figure 1.

Figure 1 shows that  $\beta$ -catenin gene amplification using the PCR method in the positive control, negative, and n-hexane obtains the same base pair, namely, 191 bp and 82 bp. Meanwhile, EtOH and ethyl acetate treatments obtain the same result, namely, 56 bp. In this treatment, the total migration rate was 440 bp. The target band in this result was, if it was amplified, the band with a size of 227 bp was formed [8].

### Result of the examination of $\beta$ -catenin gene using the RFLP analysis (restriction digestion)

The result of  $\beta$ -catenin gene amplification using the RFLP analysis (restriction digestion) is presented in Figure 2.

Based on Figure 2, to determine the sizes of the bands formed from the amplification of the  $\beta$ -catenin gene with RFLP Digestion, a standard curve of the  $\beta$ -catenin gene DNA was calculated based on the DNA marker size, migration rate, and total migration rate, from the PCR yield curve. DNA standard obtained the equation  $y = -2.4932x + 3.4276$ ; with a value of  $R^2 = 0.9587$ , the formula is used to calculate the length of the sample DNA so that the length of the DNA sample in each well is obtained as follows: The negative control obtained 48 bp, 70 bp, 155 bp, 180 bp, 249 bp, and 362 bp. The positive control obtained 53 bp, 69 bp, 127 bp, 151 bp, 191 bp, 234 bp, and 394 bp and in the n-hexane treatment obtained 48 bp, 69 bp, 116 bp, 151 bp, 180 bp, and 234 bp.

In this treatment, the total migration rate was 660 bp. The band with the objective criteria of the RFLP analysis (restriction digestion) in this study was, first, if the bands were 82.7 bp and 138 bp, it indicated no  $\beta$ -catenin gene mutation (wild type). Second, if the bands were 89 bp and 138 bp, the mutation happened in the first or second base at codon positions 32 or 33. Finally, if the bands were 82 bp and 145 bp, the

**Table 1: Consensus and refseq alignment results (NM.001165902.1)**

Consensus	Refseq
P3 TTT GCT GAC CTG ATG GAG TTG GAC ATG GCC ATG GAG CCG GAC AGA AAA GCT GCT GTC AGC CAC TGG CAG CAG CAG TCT TAC TTG GAT TCT GTGCA ATC CAT TCT GGT GCC ACC ACC ACA GCT CCT TCC CTG AGT GGC AAG GGC AAC CCT GAG GAA GAA GAT GTT GAC ACC TCC CAA GTC CTT TAT GAA TGG GAG CAA GGC TTT TCC CAG TCC TTC ACG CAA GAG CAA GTA GCA AA	NM.00116590 2.1 1 CGT CAG TGC AGG AGG CCG AGG CCG AGC GGG CCG CCG CGA GGT ACC TGA AGC TCA GCG CAC 61 AGC TGC TGT GAC ACC GCT GCG TGG ACA ATG GCT ACT CAA GCT GAC CTG ATG GAG TTG GAC 121 ATG GCC ATG GAG CCG GAC AGA AAA GCT GCT GTC AGC CAC TGG CAG CAG CAG TCT TAC TTG 181 GAT TCT GGA ATC CAT TCT GGT GCC ACC ACC ACA GCT CCT TCC CTG AGT GGC AAG GGC AAC 241 CCT GAG GAA GAA GAT GTT GAC ACC TCC CAA GTC CTT TAT GAA TGG GAG CAA GGC TTT TCC 301 CAG TCC TTC ACG CAA GAG CAA GTA GCT GAT ATT GAC GGG CAG TAT GCA ATG ACT AGG GCT

mutation happened in the second or third base at codon positions 34 and 35 [1].

The result of identifying the bad size obtained in Figures 1 and 2 in the n-hexane fraction was continued by conducting the sequencing test to know the gene types that were considered experiencing the repair mechanism of mutation.

**Table 2: Result of the BLAST for  $\beta$ -catenin gene in the n-hexane fraction**

No.	Name of species	Homological percentage	Accession
1.	<i>Mus musculus</i> targeted KO-first, conditional ready, lacZ-tagged mutant allele Ctnnb1:tm1a(EUCOMM)Hmgu; transgenic	99.13	JN956086.1
2.	<i>Mus musculus</i> targeted non-conditional, lacZ-tagged mutant allele Ctnnb1:tm1e(EUCOMM)Hmgu; transgenic	99.13	JN952341.1
3.	<i>Mus musculus</i> catenin (cadherin-associated protein), beta 1 (Ctnnb1), transcript variant 2, mRNA	99.13	NM_001165592.1
4.	<i>Mus musculus</i> catenin (cadherin-associated protein), beta 1 (Ctnnb1), transcript variant 1, mRNA	99.13	NM_007614.3

The sequencing results in the form of a chromatogram, shown in Figure 3, were then edited using the BioEdit program to remove unnecessary bases and combine the two sequence results from the forward and reverse primers, namely, contig analysis. The nucleotide arrangement of the isolates obtained from the results of the contig analysis was then identified by comparing the sample DNA sequences with the  $\beta$ -catenin gene reference (NM.001165902.1), shown in Table 1, at the National Center for Biotechnology Information in the BioEdit software to see changes in the sequence that occurred. After the comparison, it can be seen that there has been a change in the GGA  $\rightarrow$  GTGC sequence at codon 30 bases to 188 which is a transversion mutation where the base G becomes T, and insertion is identified, namely, the addition of a nitrogen base, namely, base GC.

## Discussion

Colorectal cancer is cancer leading to the malignant tumor in the colon (the longest part of the large intestine/colon) and the rectum (the longest part of the large intestine/colon before the anus). Gene mutation is a change occurred in the genetic materials in the cells. The material changes in colorectal cancer can cause characteristic changes, both at the cell level and in the living creatures.  $\beta$ -catenin gene mutation, APC will decrease the  $\beta$ -catenin improvement. The result of this depletion of the  $\beta$ -catenin gene will translocate the  $\beta$ -catenin gene in the nucleus; with this change, the genetic change will activate the mutation in an oncogene that will allow the neoplastic transformation into cancer [2].

PCR-RFLP is one of the DNA characterizations based on the different cutting sites. The cutting was done by an endonuclease restriction enzyme that can digest DNA and cut the DNA in a certain restriction site into fragments. The PCR-RFLP utilization in this identification is knowing the effect of Poke extract treatment (n-hexane, EtOH, and ethyl acetate) in mice infected with pada AOM/DSS) [9].

One of the factors causing cancer is the result of mutations in chromosomal DNA of normal cells that can be triggered by external factors (tobacco, alcohol, chemical substances, infection agents, and radiation); meanwhile, the internal factors are hormone, immunity, congenital mutation, and mutation happened in the metabolism. It can be characterized by the clinical utilization in the pharmacology of drugs from marine organisms. The anti-cancer effect is marked by an evaluation of the apoptosis effect of clam extract, showing that it has an inhibitory effect in human cancer cell proliferation [10].

Figure 1 shows the result of the PCR in this study indicates the success in the  $\beta$ -catenin gene amplification in the positive control, negative, and n-hexane. It is characterized by the occurrence of a target band of 227 bp, while the EtOH and ethyl acetate treatments do not show any amplification with a size of 227 bp. It shows that the  $\beta$ -catenin gene is not formed. The occurrence of a band in the negative treatment was caused by the characteristic of  $\beta$ -catenin gene mutation in the normal DNA of animals and humans in the normal condition. Therefore, in the negative treatment, several bands nearly similar to those of positive control occur [11].

Figure 2 shows the PCR-RFLP analysis (restriction digestion) with the bands of 89 bp and 138 bp, indicating that the mutation occurs in the first or second base at codon position 32 or 33; besides, the bands of 82 bp and 145 bp show a mutation in the second or third base at codon position 34 and 35 in the n-hexane fraction. It is also supported in the sequencing test in the n-hexane showing the repair mechanism of mutation.

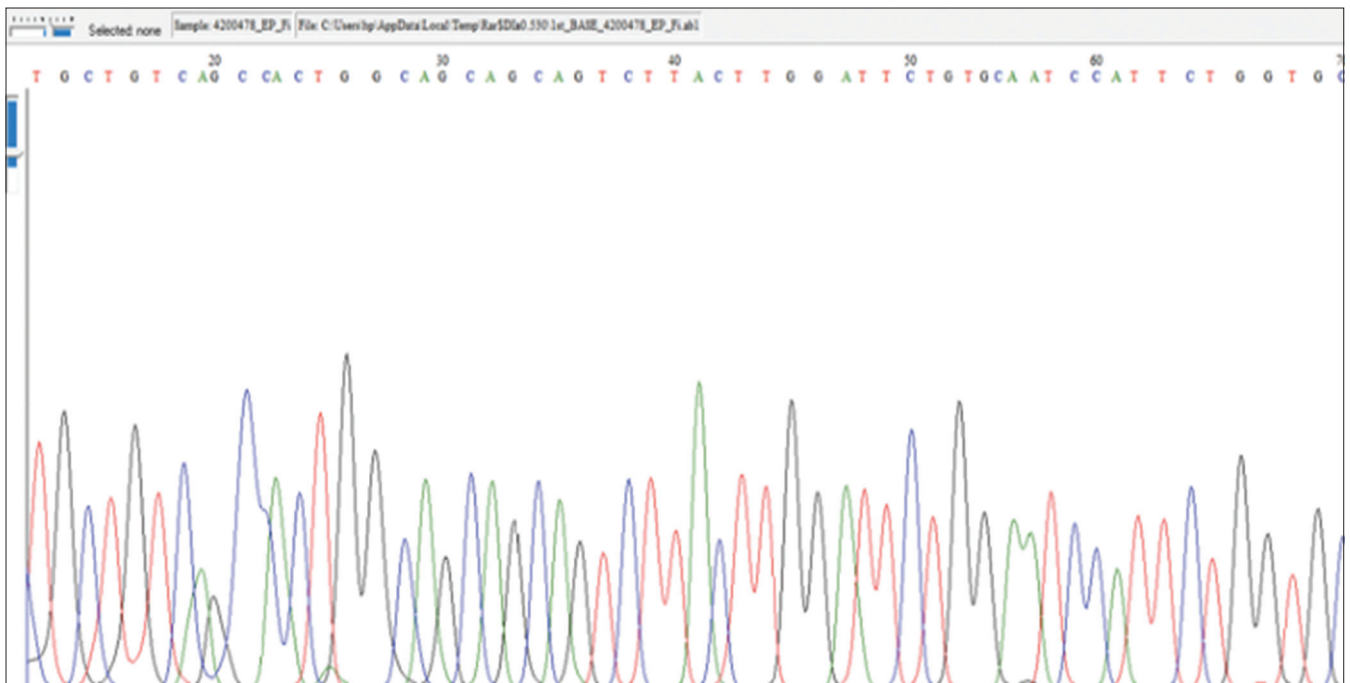


Figure 3: Sequencing results of mouse isolates after the treatment of *Batissa violacea* extract with *N*-hexane fraction as a solvent used

The occurrence of non-specific bands in this study is affected by the optimization of the annealing temperature of DNA in the PCR machine. The optimization of annealing temperature becomes the most important part of the amplification process. Too low and too high annealing temperatures make the primer unable to recombine on the specific place, improper primary annealing, the unstable optimal temperature during incubation with a constant temperature of 65°C for 20 min leading to primary annealing that does not fulfill the target, inaccurate process of pipetting or contamination when performing the work [12]. It is also revealed in the relevant study that the success of amplification is based on the suitability of primer and the efficiency and optimization of the PCR process [13].

Molecular identification in Table 2 shows that *n*-hexane obtains 99.13% of the  $\beta$ -catenin gene. The result of the  $\beta$ -catenin gene sequencing from the sample treated with *n*-hexane was traced its homology in the similar type of sample that existed in the GenBank through the BLAST program. If the homological presentation of a species is above 97%, the species is denoted as the same species [14].

The  $\beta$ -catenin gene is frequently mutated at codons 33, 41, and 45 of the GSK-3 $\beta$  phosphorylation motif in human colon cancer. In addition, mutations often occur in rat colon tumors induced by AOM at codons 32 and 34. The second G nitrogenous base of the CTGGA sequence is generally mutated to the nitrogenous base A in codons 32 and 34 of the mouse  $\beta$ -catenin gene and this pathway is considered a hotspot mutation with AOM (Takahashi *et al.*, 1998). In the study of Mami *et al.* (2000), the mouse  $\beta$ -catenin gene was found to be mutated at codons 34, 33, 41, and 37, nine out of 10 mutations were G: C  $\rightarrow$  A: T transition, but not at

codon 32. Thus, the hotspot in codon 34 may be similar, further supporting the present study's hypothesis that the second G of the CTGGA sequence is critical.

Based on the results obtained in this study, it can be assumed that the DNA of mice colon isolate samples that had been treated with pokea extract experienced changes in the beta-catenin gene sequence. Hence, it can be concluded that the treatment of pokea extract in the colon of mice has not been able to fully improve the mutations that occur in the  $\beta$ -catenin gene sequence. Mutations occurring in the  $\beta$ -catenin gene in AOM-induced colorectal cancer of mice suggest that the consequent changes in protein stability and localization may play an important role in this model of colon carcinogenesis.

The current laboratory examination applies the molecular diagnostic-based method more since it is considered more sensitive, time saving, and the result is more optimal than the conventional method. One of the ways in performing the treatment using the molecular technique is collection the new and safe raw material of drugs from food materials using bioactive components from mollusks.

## Conclusion

Based on the result of the study on using EtOH extract, ethyl acetate fraction, and *n*-hexane fraction for testing the effect of  $\beta$ -catenin gene mutation in mice induced by AOM and DSS, it shows that only the *n*-hexane fraction of pokea clam displaying the bands relevant to the target, namely, in the PCR process and

restriction digestion with HinfI. The result is reinforced by the identification using the sequencing method by obtaining the homological presentation of  $\beta$ -catenin of 99.13%.

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