Original Research Article

Inhibitory Power Effect of Star Fruit Extract (*Averrhoa bilimbi* Linn) Against Vibrio cholera of Tuna

ABSTRACT

Aims : . This study aims to determine the effect of ethanol concentration and extraction temperature on the characteristics of star fruit extract,That content of bioactive compounds (polyphenols, flavonoids, alkaloids, saponins, and tannins of star fruit) can inhibit bacterial activity as a preservative for tuna.

Study design: This research was carried out in 2 stages, namely: (1) determine the ethanol concentration and temperature to produce the best extract and (2) determine the antibacterial power of the extract against Vibrio cholera - BSIL Randomized block design (RBD) with two factors, namely 1 (ethanol concentration 96 and 70%) and 2 (temperature extraction 30, 40 and 50^0 C. Variance analusis was used for analyzing data and further tests were carried out with Honest Significant Difference.

Place and Duration of Study: This research was conducted at Food Technology laboratorium ,Faculty of Agriculture Technology, Udayana University on November – April 2022

Results: the best quality (P<0,05) of star fruit extract by 96% ethaol at 30^0C , such as: yield (77%), total flavonoids (7.10%), total tannins (0.914 mg QE /g) , total acids (34.24%). and with a concentration of 80% the extract was able to inhibit the growth of Vibrio cholera (VC-BSIL) with very strong criteria (inhibition zone diameter . 20.78 mm). .

Keywords: *Star fruit Extrak, Tuna, Vibrio cholera*

1.INTRODUCTION

Fish is a food commodity that supplies animal protein rich in saturated and unsaturated fatty acids. Unconducive marketing of fish, lack of sanitation, causes quality to decline, consumer health problems and financial losses increase. This poisoning is caused by contamination of

pathogenic bacteria such as *Vibrio cholerae, Escherichia coli, Salmonella* and *Enterobacteriacea.*

Prolonged use of antibiotics will increase the resistance of contaminant bacteria which will endanger consumer health. Therefore, it is necessary to apply an alternative solution by soaking fish in star fruit juice (*Averrhoa bilimbi* L) before being marketed. This effort can inhibit the activity of contaminant bacteria, especially *Eschericia coli, Staphylococcus aureus* (Sugitha, et al, 2021).

Wuluh star fruit (Figure 1) is the result of the plants that are often found in people's yards, maximum height of 10-15 m, grows naturally and is rarely commercialized. Flavonoid and saponin compounds are antimicrobial compounds that are very effective in inhibiting the growth of viruses, bacteria and fungi as well as easily soluble in polar solvents such as ethanol, butanol, and acetone. Likewise, alkaloids have the ability as an antibacterial by interfering with the constituent components of peptidoglycan in bacterial cells, while tannins are polyphenolic compounds (C6-C3-C6) that precipitate proteins and form complexes with polysaccharides, resulting in microbial cell walls lysis .

Figure 1. Star fruit

This study aims to determine the effect of ethanol solvent concentration and extraction temperature on the characteristics of star fruit extract, to determine the best ethanol concentration and extraction temperature to produce star fruit extract, and to determine the extract concentration that has the best antibacterial activity against Vibrio cholerae.

RESEARCH METHODS

Materials and tools

The material used in the study consisted of star fruit (green color, size 3-5cm), with the criteria of green fruit skin, obtained from Jimbaran Village, Badung Regency. The chemical used as a solvent is ethanol (Merck bratachem) with a concentration of 96% and 70%. Aquades, NaNO2, AlCl3, NaOH. Vibrio cholerae isolate was obtained from the Bali Seafood Inspection Laboratory (BSFIL VC Isolate) as a result of identification of Bali tuna poisoning in Tabanan, TCBS (Thiosulfate Citrate Bile Salts Sucrose), LB (Lactose Broth), NA (Nutrient Agar), PW (Peptone Water), 95% alcohol.

The equipment used is: for the extraction process blender (Phillips), extraction flask, hot plate, magnetic stirrer, stainless steel knife, oven (Blue M, USA), rotary evaporator (Janke & Kunkel RV 06 – ML, Switzerland), UV-spectrophotometer Vis (Thermo scientific, USA), colorimeter (PCE-CSM4, Spain), analytical balance (Mattler Toledo AB 204 and Shimadzu, Japan), 60 mesh sieve (Retsch, Germany), pH meter (Hanna HI98107) and glassware (Pyrex).

Research design

This research was done in 2 stages, . Stage 1 aims to determine the best ethanol solvent concentration and extraction temperature to produce star fruit extract, using a Randomized Block Design (RBD). Factorial pattern: the first factor is solvent concentration (P) which consists of 2 levels, namely: P1 (Ethanol 96 %), P2 (70% ethanol); and the second factor is the extraction temperature (T) which consists of 3 levels, namely T1(30^oC), T2(40^oC), T3(50^oC) with 3 repetitions. The data were analyzed using analysis of variance and a follow-up test was carried out for Honest Significant Differences.

Stage 2, aims to test the antibacterial activity using the best percent extract in the Phase I study (20%, 40%, 60%, 80% and 100%) on VC SFIL isolates, with tetracycline as a positive control and distilled water as a negative control. Completely randomized design (CRD) with 3 times and observed variables: yield (AOAC International, 2005), total flavonoids (Rahman et al., 2006), total tannins ((Muaja et al., 2013), and total acids ((Hadiwiyoto et al. , 2012) followed by the best treatment effectiveness test (Dynes et al. (1984); while for the best extract antibacterial activity test against Vibrio cholerae ((Buldani et al., 2017); data were analyzed using analysis of variance and follow-up tests with significant difference tests honest and determine the best treatment (effectiveness index test, Dynes et al., 1984

Making Star Fruit Extract

Star fruit (*Averrhoa bilimbi* L) is cleaned using running water, then sliced thinly and then dried in an oven at 50^oC until it is easily broken, then blended and then sieved with a 60 mesh sieve to obtain dried star fruit powder. Star fruit extract is made by maceration process. A sample of 10 g was put into a 1000 ml erlemeyer then added 200 ml of solvent, then macerated for 24 hours. During the extraction, stirring was carried out using a magnetic stirrer at a speed of 300 rpm, then filtered using coarse filter paper. Filtrate I is collected while the dregs are added with solvent to a volume of 100 ml, shaken and filtered again with coarse filter paper, and filtrate II combined with filtrate I is filtered with Whatman filter paper No. 1. Filtrate III was evaporated with a rotary vacuum evaporator at a temperature of 40^oC, a pressure of 110 mbar at a speed of 100 rpm until all the solvent had evaporated and a viscous extract was obtained. This thick extract was analyzed according to the research variables: yield, total flavonoids, total tannins, total acids) and its datas were analyzed using analysis of variance and carried out a follow-up test of Honest Significant Differences.

Confirmatory Vibrio cholerae test

In this confirmation test by observing the shape of the colony, gram staining and cell observation. Bacterial isolates that had been refreshed were then cultured on TCBS media to obtain single colonies and incubated for 48 hours at 37^{o}C. Then the shape and color of the bacterial colonies were observed.

Preparation of Vibrio cholerae culture stock

For the preparation of bacterial culture stock, a colony of Vibrio cholerae (VC-SFIL) bacteria was taken using an ose needle, then instilled in a slanted Nutrien Agar medium by scraping, after which it was incubated in an incubator at 37°C for 24 hours.

Antibacterial activity test of the extract against Vibrio cholerae

The sterile disc paper with a diameter of 6 mm is dripped with the best extract which has been diluted with a concentration of 20, 40, 60, 80 and 100% until completely wetted, let stand until the extract is completely absorbed. The control solution used in this experiment was distilled water as a negative control and tetracycline as a positive control. Tetracycline was chosen as the positive control in this study because this antibiotic is a selective antibiotic for the test bacteria. The disc paper was then placed on the surface of the media according to the specified position, each treatment was repeated 3 times. The media was incubated at 37^{o}C for 1x24 hours. Furthermore, the inhibition zone formed (clear zone around the disc) was observed which showed no bacterial growth (Buldani et al., 2017). This shows that star fruit extract has bioactive compounds that act as antibacterial inhibiting the growth of Vibrio cholerae (VC-SFIL).

RESULT AND DISCUSSION

Yield

The results of the analysis of variance showed that the treatment of ethanol concentration and extraction temperature had a highly significant effect ($P<0.01$), while the interaction between treatments had a significant effect ($P<0.05$) on the yield of starfruit extract. (Table 1.)

Table 1. The average yield (%) of starfruit extract in the treatment of ethanol concentration and extraction temperature.

Ethanol Concentration	Ectraction temperature		
	30^{o}C	40^{o}C	50^{o}C
96%	$77 \pm$ 0,025a	$73 \pm 0{,}00$a	$61 \pm 0{,}015$b
70%	$65 \pm 0{,}036$b	$52 \pm 0{,}036$c	$47 \pm 0{,}064$d

Description: Different letters behind the mean value indicate real difference Tukey's BNJ test with 95% confidence interval

An decrease in the concentration of ethanol (96-70%) resulted in a lower yield (P<0.05), as well as an increase in the extraction temperature(Table 1) , but at temperatures of 30^0 C and 40^0C with an ethanol concentration of 96 % had no significant effect (P>0.05). This is do to the compounds in star fruit have the polarity most similar to that of 96% ethanol. . The more compound components dissolved in the solvent will produce a higher yield.(Sabila Pratiwi, 2016)

Total Flavonoids

Ethanol concentration and extraction temperature had a highly significant effect (P<0.01) and the interaction of the two treatments had a significant effect (P<0.05) on the total flavonoids of star fruit extract (Table 2).

Table 2. The average value of total flavonoids (%) of star fruit extract in the treatment of ethanol concentration and extraction temperature.

Ethanol Concentration	Ectraction temperature		
	30°C	40°C	50°C
96%	7,10 ±0,65a	4,96±0,29c	4,38±0,38d
70%	6,6 ± 0,24b	6,48 ±0,40b	6,47±1,35b

Description: Different letters behind the mean value indicate significant difference in the BNJ Tukey test with a 95% confidence interval

The highest average value of total flavonoids (7.10%) was obtained from the combination treatment of 96% ethanol concentration at 30°C and the lowest average value of total flavonoids (4.38%) was obtained from the combination treatment of 96% at 50°C. Based on Table 2, the total flavonoids treated at 70% at 30°C, 40°C and 50^0C were no significant lower (P>0.05), but at 96% ethanol concentration the different value were significant (P<0.05). This is due to the difference caused by the concentration of ethanol can result in a change in the polarity of the solvent which affects the solubility of the flavonoids. According to (Harborne, 1987) each type of flavonoid has a different polarity depending on the number and position of the hydroxyl groups of each type of flavonoid.

The polarity of the ethanol solvent decreases at higher temperatures, so that the binding of flavonoid compounds in starfruit extract is not optimal. (Sa'adah and Nurhasnawati, 2017).

Total Tannins

The results of the analysis of variance showed that the extraction temperature treatment had a very significant effect (P<0.01), but the solvent concentration and the interaction of the two treatments had a significant effect (P<0.05) on the total tannins of starfruit extract (Table 3).

Table 3. The average value of total tannins (mg QE/g) of star fruit extract wuluh in the treatment of ethanol concentration and extraction temperature.

Ethanol Concentration	Ectraction temperature		
	30°C	40°C	50°C
96%	0,914 ±0,022a	0,646 ± 0,092d	0,434 ± 0,088e
70%	0,802 ± 0,134b	0,794 ± 0,128c	0,683 ± 0,080d

Note: Different letters behind the mean value indicate a significant difference in the BNJ Tukey test with a 95% confidence interval

The highest average total tannin value (0.914 mg QE/g) was obtained from the combination treatment with 96% ethanol concentration at 30oC and the lowest average total tannin value (0.434 mg QE/g) was obtained from the combination treatment with 96% ethanol concentration at room temperature. 50°C. Increasing solvent concentration and extraction temperature caused a significant decrease in total tannins ($P<0.05$) due to decreasing solvent polarity. The polarity of the ethanol solvent for each concentration also affects the level of solubility of tannin compounds so that the total tannins produced are reduced (Andriani et al. 2019).

Total Acid

The results of the analysis of variance showed that the effect of solvent concentration and extraction temperature on the total acid of the belimbing wuluh extract and its interaction was significantly different ($P<0.05$), (Table 4). The treatment of ethanol solvent concentration and extraction temperature had a significant effect ($P<0.05$) on total acid, the highest (34.24%) was produced in the process with 96% ethanol. and the lowest total tannin (%) was produced with 70% ethanol at 30oC. Table 4 shows that increasing the concentration of ethanol solvent and extraction temperature will increase the total acid in general. This is due to the increase in extraction temperature triggering the evaporation of intracellular water which will produce pressure inside the cell. This pressure will cause the cell wall to rupture so that it can increase the compound diffused into the solvent. However, the extraction temperature that exceeds the optimal time results in the degradation of the active compound by heat (Silaban et al., 2013). The total acid in starfruit extract tends to be extracted more effectively in solvents with lower polarity (Krisanta et al. (2021).

Table 4. Average total acid value (%) of starfruit extract at ethanol concentration and extraction temperature.

Ethanol Concentration	Extraction Temperature.		
	30°C	40°C	50°C

96%	34,24 ±0,554a	30,08 ± 1,109b	32,96 ± 0,554a
70%	10,88 ± 1,466d	13,44 ± 0,960c	14,40 ± 0,000c

Note :Different letters behind the average value indicate a significant difference in the BNJ Tukey test

Best Treatment

In this study, 96% ethanol concentration at 30oC obtained the highest value of all treatments (used for Phase 2 research). This treatment is the best treatment to produce star fruit extract which has the potential for antibacterial Vibrio cholerae.

Vibrio cholerae Confirmation Test

Confirmatory tests on Vibrio cholerae include color and colony shape as well as gram staining and cell shape. Prior to testing the antibacterial activity, the colony count was first performed using the TPC method. Vibrio cholerae grows well on TCBS agar media. The color of Vibrio cholerae colonies will change the green selective TCBS media to yellow. Large colony morphology with diameter <3 mm, smooth surface, rather flat, opaque center and bright edges, yellow in color (Figure 2.)

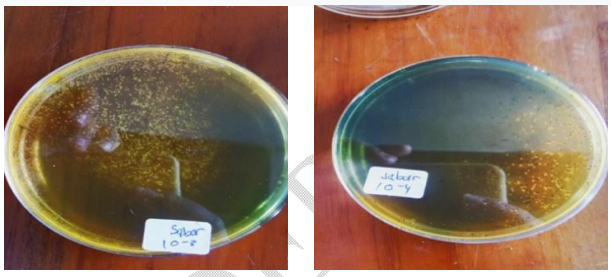

Figure 2. Form and color of Vibrio cholerae colonies

The shape of the cell in Vibrio cholerae shows the form of bacilli, gram negative, . with a length of 2-4 µm and a crooked rod-shaped like a comma (. Figure 3.)

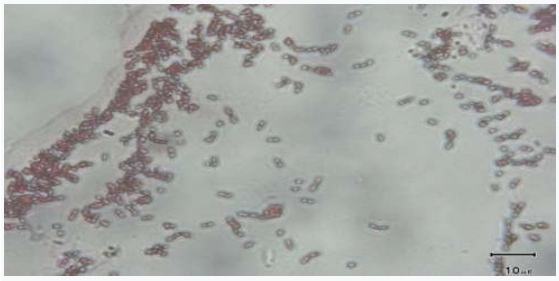

Figure 3. Morphology of Vibrio cholerae under a microscope at 1000x magnification.

Antibacterial Activity

The results of the analysis of variance showed that the concentration treatment had a very significant effect (P<0.01) on the antibacterial activity of starfruit extract. (Table 5). The highest average diameter of inhibition (20.78mm) was obtained from the 80% concentration treatment which was not significantly different (P<0.05) with 100% concentration treatment of mm . Antibacterial activity test against Vibrio cholerae was carried out using the disc diffusion method. Based on Table 5, star fruit extract was able to inhibit the growth of Vibrio cholerae

Table 5. The average diameter of the inhibition of star fruit extract on the growth of Vibrio cholerae

Concentration Treatment)	The Inhibition Diameter (mm)	Inhibition Category
20%	$12{,}63 \pm 0{,}11c$	Strong
40%	$13{,}43 \pm 0{,}23b$	Strong
60%	$14{,}21 \pm 0{,}28b$	Strong
80%	$20{,}78 \pm 0{,}36a$	Very strong
100%	$19{,}01 \pm 0{,}69a$	Strong

The total Vibrio cholerae used in this study was 2.24 x 107CFU/ml. The inhibition zone on TCBS media appeared after 24 hours of storage. The formation of an inhibition zone indicates that star fruit extract has bioactive compounds that act as antibacterial compounds , so that they can inhibit the growth of Vibrio cholerae (Figure 4).

Figure 4. Zone of inhibition of star fruit extract against Vibrio cholerae with a concentration of 80%

The wider the diameter of the inhibition zone formed, the stronger the bioactive compound inhibits the growth of bacteria which are categorized as very strong.

Table 5 shows the diameter of the barriers continued to increase from up to 20.78 mm at concentrations of 20% - 80%. but decreased at 100% concentration. The decrease in inhibition diameter at 100% concentration was due to the fact that the extract was unable to diffuse properly because the concentration of the extract was too concentrated so that the active component of the star fruit had difficulty interacting with VCSIL.

The content of secondary metabolite compounds (flavonoids, tannins) in starfruit extract is able to inhibit the growth of VCSFIL by inhibiting cytochrome c-reductase, so that the metabolism of microbial cells is disrupted (Buldani et al., 2017). The inhibition of this metabolic activity will result in the death of bacterial cells. Flavonoids also play a role in reducing energy metabolism by inhibiting the use of oxygen by bacteria, thus preventing the formation of energy in the cytoplasmic membrane and inhibiting bacterial motility which plays a role in antimicrobial activity.

Tannins work by inhibiting the enzymes reverse transcriptase and DNA tropoisomerase, so that bacterial cells are not formed (Buldani et al., 2017). Tannin activity as an antibacterial with its ability to inactivate microbial cell adhesion (molecules attached to host cells) found on the surface of the cell which will cause damage to the cell wall, so that the membrane will leak (disturbing permeability) resulting in liquid metabolites coming out and bacterial metabolism disrupted so that growth inhibited bacteria can even experience death.

The cell wall plays a role in the survival of bacteria, regulates the entry and exit of secondary metabolites such as flavonoids and tannins. into the bacterial cell, thereby disrupting the permeability of the cell membrane. Apart from the secondary metabolites of starfruit extract, acids play a role in inhibiting bacterial growth. (Dibner and Buttin, 2002) explained that almost all types of acids have strong antibacterial activity, which work by dissociating and producing hydrogen ions. If the amount of acid that is not dissociated is large, it results in a large number of hydrogen ions so that in the cytoplasm acidification of the cell will occur, which will damage the membrane and the bacteria will die.

CONCLUSION

Based on the results of the study, several things were concluded as follows:

1. The best characteristics of starfruit extract made with 96% ethanol solvent at 30^0 C consists of: yield 77%, total flavonoids 7.10%, total tannins 0.914 mg QE/g, and total acids 34.24%.
2. Star fruit extract is able to inhibit the maximum growth of Vibrio cholerae (20.78 mm) in the very strong category. at a concentration of 80%

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