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RESEARCH ARTICLE

IDENTIFICATION OF INULIN PROFILE FROM RED FRUIT (PANDANUS CONOIDEUS L) PEDICEL EXTRACT USING LC-MS AND ITS IN VITRO PREBIOTIC ACTIVITY TEST.

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Abstract

Pedicel is a by-product of red fruit oil production. Identify the profile of inulin using LCMS (liquid chromatography-mass spectrometry) extracted from red fruit pedicels with conventional methods and hydrodynamic cavitation and testing their prebiotic activity. The results showed that the filtrate extracted from red fruit pedicels contained inulin compounds, evidenced by the presence of glucopyranoside and fructofuranosyl monomers associated with β bonds as markers. The chromatogram peak of the filtrate by conventional methods at retention time of 9.77 minutes had a base peak m/z of 686.740 g/mol with the alleged molecular formula $C_{30}H_{54}O_{17}$, while the hydrodynamic cavitation method at 7.42 minutes retention time had a base peak m/z of 668.597 g/mol with the alleged molecular formula $C_{30}H_{36}O_{17}$. The polymerization degree of inulin with both extraction methods was 4. In vitro test showed that the inulin in the red fruit pedicel extract supported the growth of the *Lactobacillus casei* colony. The number of colonies in the extract of the hydrodynamic cavitation method was 9.09 log CFU/ml higher than the conventional method, which was 8.89 log CFU/mL at 48 hours incubation time. The highest prebiotic activity value in the filtrate media of the hydrodynamic cavitation method was 0.9 at 48 hours incubation.

Introduction:-

Consumer awareness on the correlation between diet, intestinal microflora, and health provides the view that the food consumed must be beneficial for the health of the digestive tract. To achieve this condition, the composition of the intestinal microflora is adjusted so that the number of beneficial bacteria such as Bifidobacterium and Lactobacillus increases, while harmful bacteria such as Escherichia coli and Streptococcus decreases. But in certain conditions, the group of beneficial bacteria can decrease. According to Collins and Gibson (1999), factors that can affect the number of beneficial bacteria are age, susceptibility to infection, nutritional needs, the immunological

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status of the host, pH, transit time, interaction between flora and the availability of fermentable material in the intestine.

The number of beneficial bacteria in the digestive tract can be maintained by the consuming probiotics, prebiotics, or a combination of probiotics and prebiotics called symbiotics. Probiotics are live bacteria that are beneficial for health, especially the digestive system, while prebiotics is substances from undigested food and selectively increase the culture and activity of beneficial bacteria in the large intestine (Collins & Gibson, 1999; Aureli et al., 2011; Al-Sheraji et al., 2013). Consumption of a combination of probiotics and prebiotics or synbiotics can have a synergistic effect on the probiotics because relevant or specific substrates are available (Collins & Gibson, 1999).

Compared with probiotics, prebiotics is more efficient in modifying the composition of the intestine because it has several advantages that can stimulate the growth of beneficial bacteria, activation of bacterial metabolism, and its physiological effects (Gibson & Roberfroid, 1995). Another conclusion reported by Al-Sheraji et al., (2013) that the utilization of prebiotics is preferred because, in addition to being able to stimulate the growth of probiotic bacteria, it is proven to be able to increase the immune system, increase the absorption of calcium and magnesium, affect blood glucose levels, and increase plasma lipids. Another reason is convenience. Prebiotics can be incorporated into a variety of foods such as milk and yogurt.

Common prebiotic types isolated from natural sources are undigested oligosaccharides including fructooligosaccharides (FOS), galactooligosaccharides, lactulose, and inulin (Collins & Gibson, 1999; Ranadheera et al., 2010). Many studies have shown that inulin is proven as a prebiotic that promotes the growth of Bifidobacterium and Lactobacillus (Lopez-Molina et al., 2005; Huebner et al., 2007; Pompei et al., 2008; Winarti et al., 2013). Inulin is a fructan with β (2-1) bonds between monomers in their poly or oligomer. The β configuration of C2 anomeric makes inulin indigestible by digestive enzymes so that it can act as a prebiotic (Niness, 1999; Roberfroid, 2005). Inulin is not digested in the large intestine but undergoes fermentation to produce a substrate that is useful for beneficial bacteria. On the contrary, the fermentation results can inhibit the development of harmful bacteria.

Research by Djayani (2016) has proven that red fruit pedicels (Pandanus conoideus L) which are by-products from the red fruit oil industry have the potential to be a source of raw materials with 3.11% (d.w) inulin content. Pedicel is a by-product of red fruit oil production with the highest percentage of weight (51-61%) compared to drupa (39-49%) and seeds (27-36%) (Sarungallo et al., 2019). At present, pedicel has not been used only as animal feed and only disposed of. In addition, until now there has never been a study of the profile and activity of inulin prebiotics from red fruit pedicels. Therefore, this study aimed to identify the inulin profile, including the alleged relative molecular mass and the molecular formula of the filtrate obtained by conventional methods and hydrodynamic cavitation using LCMS. Also, this study aimed to determine prebiotic activity in media added by filtrate from conventional extraction methods and hydrodynamic cavitation.

Materials And Methods:-

Materials and tools

The raw material was red fruit obtained from the Papua University (UNIPA) Manokwari experimental garden. The chemicals used were Merck standard inulin, standard glucose, and technical ethanol. Bacteria for testing were L. casei FNCC-90 from the Food and Nutrition Culture Collection, Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta and E.coli ATCC 25922. Bacterial growth media was MRS (Man Rogosa Soy Protein) liquid from Oxoid for the growth of L. casei. MRS media was formulated by replacing glucose with inulin. TSA (Trypticase Soy Agar) and TSB (Trypticase Soy Broth) for the growth of E. coli. M9 media, for testing E. coli using glucose and inulin as carbon sources.

The equipment used was an incubator shaker, a series of hydrodynamic cavitation extractors equipped with a 0.5 hp Showfou centrifugal pump, stopwatch, analytical balance, refrigerator, and some glassware for preparation and analysis. LCMS analysis used UPLC-MS which was equipped with a binary pump.

Preparation of red fruit pedicel powder

The pedicels were taken from ripe red fruit. The pedicels were obtained by separating the drum from the red fruit cephalium then washed with running water to remove any dirt. Furthermore, the red fruit pedicels were chopped with a thickness of ± 5 mm. Drying was done using sunlight until the moisture content reached ± 5%. After drying, the size was reduced to ± 80 mesh powder using a hammer mill.

Inulin extraction

Extraction with conventional methods was done by maceration of 200 mL liquid (a mixture of distilled water and pedicel powder) in an Erlenmeyer baffle flask using an orbital shaker. The acidity (pH) level of the liquid was 9. The extraction process was carried out at 300 rpm, the particle size of 80 mesh, the ratio of material and solvent of 1:50 (w/v) for 60 minutes at 50 ^{o}C. The extract was filtered using filter paper to separate the filtrate from the pulp. The filtrate was further processed by precipitation using ethanol.

Inulin extraction by the hydrodynamic cavitation method was carried out using a hydrodynamic cavitation device equipped with a venturi with a 4 mm nozzle and a throat length of 100 mm. The flow in the hydrodynamic cavitation device consisted of two pathways, namely the main and the bypass. The bypass line was intended to control the flow rate of liquid through the mainline, because the flow rate of the pump was fixed, the bypass path was not used. The liquid flow rate was generated by a centrifugal pump and was fixed, while the gas flow rate was fixed at 4.5 LPM. The gas used was UHP (Ultra High Purity) nitrogen.

Analysis using liquid chromatography-mass spectrometry (LCMS)

A total of 5 µL of sample filtrate was injected into the LC-ESI-QTOF instrument system. LCMS analysis used UPLC-MS, which was equipped with a binary pump. The liquid chromatography was connected with a QTOF (Quadrupole-Time of Flight) mass spectrometer equipped with an ESI (Electrospray Ionization) ionization source. Xevo G2-S QTOF system mass spectrometer with a positive ionization method was used in this study. ESI parameters included a capillary temperature of 150 ^{o}C and 50 l/hour of fogging gas, the voltage source of +3.0 kV. Full scan mode from m/z 50-1500 with a source temperature of 50 ^{o}C. The UPLC column used was Acquity UPLC BEH C18 1.7 µm 2.1 x 150 mm. The effluent used was methanol : water that was regulated at a total flow rate of 0.3 ml/min. Isocratic elution system was carried out at 0-0.5 minutes with a rasio of 90:10, minutes 0.5-8 elution linear gradient elution from 90% to 10%, minutes 8-9 isocratic elution 10:90, minutes 9-10 gradient elution linear solvent from 90% to 10%.

LCMS data were analyzed using the MassLynx V4.1 SCN884 2012 program from Waters Laboratory Informatics. The inulin profile was obtained from reading the results of LCMS through a comparison of peak mass spectra with databases contained in the Chemspider online library.

Effect of Inulin on the Growth of L. casei and E. coli

Lactic acid bacteria growth was observed in MRS broth growth media (without carbon sources). The basic media was then autoclaved and supplemented with 2% of filtered red fruit pedicel inulin extract. MRS base media added with glucose in the same amount as inulin extract and used as a control. Each was duplicated in each observation, e.g. 0 hours, 12 hours, 24 hours, and 48 hours to avoid contamination. Incubation was carried out in an incubator at 37 ^{o}C.

Observation of E. coli growth was incubated in TSB media, and about 1% (v/v) was added to a separate tube containing M9. One liter of M9 media was made with a composition of $Na_2HPO_4.7H_2O$ 64 g, KH_2PO_4 15 g, NaCl 25 g, then sterilized. For E.coli testing/growth, 200 ml of solution was added with 700 ml sterile distilled water, 2 ml of 1M sterile $MgSO_4$, 20 ml of 20% sterile glucose (another carbon source), 100 µl of sterile $CaCl_2$ 1 M, and added sterile distilled water up to 1000 ml.

Calculation of the number of L casei and E. coli colonies

Calculation of the number of colonies was done by a suspension of 1 ml sample in a 0.85% NaCl physiological solution (10^{-1} dilution) and put in 9 ml of 0.85% NaCl physiological solution so that a 10^{-2} dilution was obtained, then the same dilution was made 10^{-3}, 10^{-4} and so on to the desired degree of dilution (it is expected that the platting results will be between 25-250 colonies). Calculation of the colonies number was carried out by the pouring method, 1 ml of suspension from the appropriate dilution level pipetted and fertilized into a sterile petridish then poured on MRS media agar for L. casei and TSA media for E. coli, it was shaken well until flat and incubated at 37 ^{o}C for 24 hours.

Prebiotic activity score

Prebiotic activity score was a comparison between the difference in the number of cells (log CFU/ml) of probiotic bacteria in a certain time/24 hour prebiotic media and the number of 0 hour cells with the difference in the number of probiotic bacterial cells in a given glucose time/24 hour and number of probiotic bacterial cells in the 0^{th} hour

glucose media minus the rasio between the difference in the number of enteric bacterial cells in the prebiotic media for a certain time/24 hours and the number of enteric bacterial cells in the 0th hour by the difference in the number of enteric bacterial cells in the glucose time media/24 hours and 0-hour enteric bacterial cell count (Moongngarm et al., 2011).

Results:-

Inulin profile from the extraction of red fruit pedicels

The amount of the alleged composition of compounds in a sample can be determined by looking at the percent relative area, which is the area of the peak to the total area of the peak detected. Figure 1 shows the chromatogram produced by LCMS from samples of conventional extraction methods (A) and hydrodynamic cavitation (B). Based on these peaks, the filtrate from the conventional method has 6 (six) highest peaks with the area percentage of 24.61%, 15.70%, 14.46%, 13.69%, 5.98%, and 4.17%, respectively and retention times of 7.35, 21.29, 1.85, 9.77, 13.71, and 4.82 minutes, respectively. The filtrate from the hydrodynamic cavitation method has 7 highest peaks with the percentage of successive areas of 17.87%, 16.64%, 13.60%, 7.56%, 5.46%, 5.11%, and 4.73%, respectively and retention times of 7.42, 6.92, 9.39, 21.29, 13.59, 11.92, and 3.97 minutes, respectively.

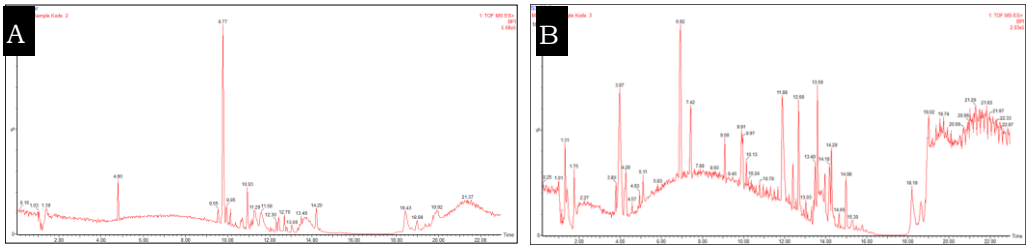


Figure 1:-Chromatogram results of LCMS samples from (A) conventional methods and (B) hydrodynamic cavitation methods

The growth of L. casei and E. coli colonies

The in vitro testing showed that L. casei bacteria were able to ferment the media supplemented with red fruit pedicel extract. These results indicate that the inulin in the red pedicel extract has the potential as a prebiotic, characterized by the growth of bacterial colonies (Figure 2).

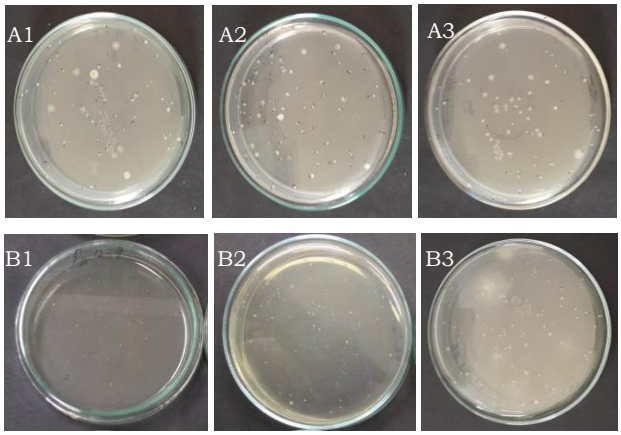


Figure 2:-Growth of L casei bacterial colonies on the red fruit pedicel extract substrate by conventional methods (A) and hydrodynamic cavitation (B) at incubation times of 12 hours (1), 24 hours (2), and 48 hours (3).

The growth of L.casei bacteria on glucose (control) media and filtrate-containing media from conventional methods and hydrodynamic cavitation are presented in Figure 3A. The highest growth of L. casei colonies on inulin-containing media from the highest conventional method filtrate was obtained at an incubation period of 24 hours, which was 8.93 log CFU/ml and then decreased at 48 hours incubation period to 8.89 log CFU/ml. Conversely, the growth of L. casei colonies on inulin-containing media from the hydrodynamic cavitation filtrate method continued to increase during the incubation period of 12 hours, 24 hours and 48 hours, which were 8.89, 8.93, and 9.09 log CFU/ml, respectively. Colony growth in the filtrate from conventional extraction and hydrodynamic cavitation was

lower than the media added with glucose as a control. Growth of L. casei colonies in glucose media decreased to 9.67, 961, and 9.48 log CFU/ml, respectively, in the incubation period of 12, 24, and 48 hours.

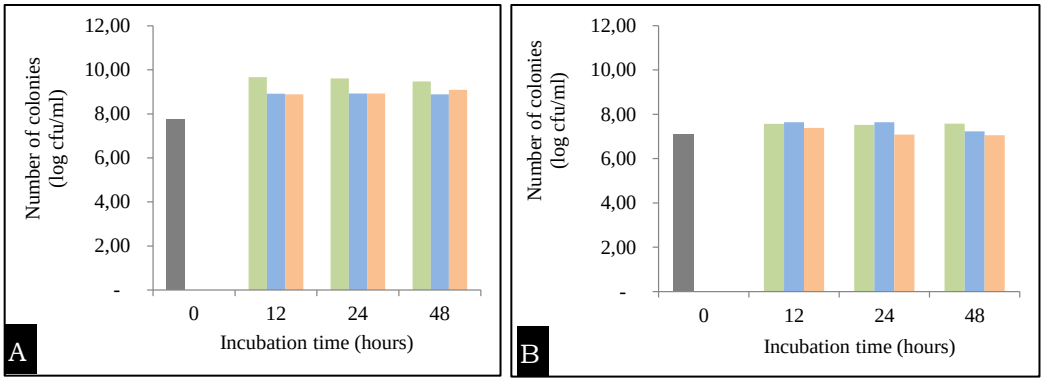
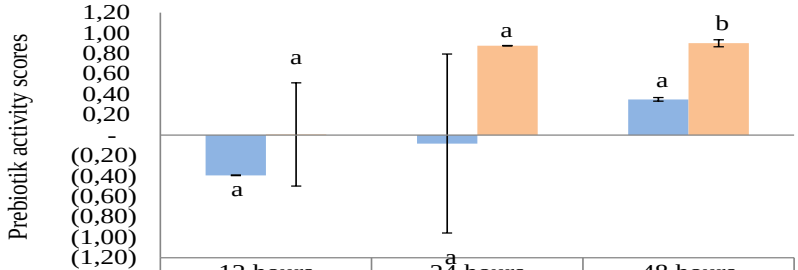


Figure 3:-Number of L. casei (A) and E. coli (B) colonies on control , glucose media , conventional method filtrate , and method hydrodynamic cavitation filtrate

Prebiotics activity scores

Prebiotics activity scores can be determined based on the total number of L. casei and E.coli (Figure 4). This value shows how far inulin can support prebiotic growth and inhibit enteric (Huebner et al., 2007). Prebiotics activity scores on the media added with filtrate from conventional methods and hydrodynamic cavitation during the incubation periods of 12, 24 and 48 hours are presented in Figure 4.



	12 hours	24 hours	48 hours
Conventional	(0,39)	(0,08)	0,35
Hydrodynamic cavitation	0,01	0,88	0,90

Figure 4:-Prebiotic activity score

Discussion:-

LCMS chromatogram data were analyzed with MassLynx software (Version 4.1), then used to identify the structure of chemical compounds with the Chemspider online database. Estimation of the relative mass and molecular formula of inulin compounds was addressed by examining the main peak of the mass spectrum. The identification results show that the group of inulin compounds in the filtrate by conventional extraction methods and hydrodynamic cavitation are found at retention times of 9.77 and 7.42 minutes. Red fruit pedicel inulin filtrate from conventional methods at a retention time of 9.77 minutes had a base peak m/z of 686.740 g/mol with the alleged molecular formula $C_{30}H_{54}O_{17}$. In the hydrodynamic cavitation method, a retention time of 7.42 minutes has a base peak m/z of 668.597 g/mol with the alleged molecular formula of the $C_{30}H_{36}O_{17}$ compound. According to Chemspider, the molecular name for the filtrate from the conventional method is β-D-Fructofuranosyl 6-O-dodecanoyl-α-D-galactopyranosyl- (1-> 6)-α-D-glucopyranoside, whereas from the hydrodynamic cavitation method, 3-O-[(2E)-3-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propenoyl]-beta-D-fructofuranosyl6-O- (4-hydroxybenzoyl)-alpha-D glucopyranoside. The alleged structural formula for red fruit pedicel filtrate using conventional methods and hydrodynamic cavitation is presented in Figure 5.

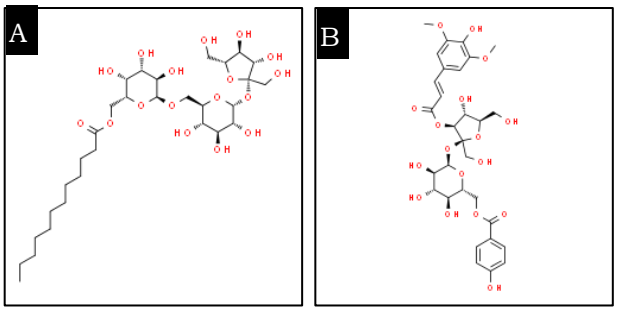


Figure 5:-Structural formula for red fruit pedicle extract from (A) the conventional method, and(B) the hydrodynamic cavitation method

Alleged names of inulin molecules in extracts by conventional methods and hydrodynamic cavitation have the composition of glucopyranoside and fructofuranosil monomers associated with β bonds. This result is in accordance with several literature which mention the chemical name of inulin. Roberfroid (1999) stated that inulin-type fructans have a composition of β-D-fructofuranose which are interconnected with β (2-1) bonds with the first monomer of the chain is β-D-glucopyranosyl or β-D-fructopyranosyl bonds. Identification by Li et al.. (2013) on inulin-type FOS using NMR found that the 1H NMR spectrum of oligosaccharides has two main regions. First, the anomeric proton in the glucopyranose residue to H-C (1) is between δ 5.20 and 5.40 ppm. Second, the region between δ 3.10 and 4.40 ppm with anomeric proton signal in the glucopyranose residue becomes H-C (2) and H-C (4). The characteristics of the 13C NMR spectrum of FOS are included with the chemical shift C-2 from the β-Fruc residue at 102.7-103.8 ppm and methylene (-CH2) from the α-glucopyranose residue at 92.0 and 92.5 ppm.

Analysis of the estimated inulin relative molecular mass based on peak mass m/z (g/mol) using LCMS was used as the basis for calculating the degree of polymerization (DP). Campa et al. (2004) explained that DP is determined using the following equation:

$$DPn = \frac{\sum_{i=1}^{\infty} ni\ DPi}{\sum_{i=1}^{\infty} ni}$$

Where DP_n is the polymerization degree, ni is the number of mol of oligomer with residue i, and DP_i is the suitable DP. A clearer DP calculation is described in Winarti et al. (2013), namely:

MW of inulin = $(C_6H_{10}O_5)n + H_2O$

= (162)n + 18

Based on the DP calculation theory, the number of inulin polymers from conventional methods and hydrodynamic cavitation were 4.13 and 4.02, respectively. From the estimated inulin relative molecular masses, each has m/z values of 686.740 g/mol and 668.597 g/mol. The amount of DP polymer between conventional extraction and hydrodynamic cavitation do not differ. It shows that depolymerization due to cavitation phenomena in the inulin extraction process does not occur.

The DP value of the red fruit pedicel filtrate was 4, indicating that the prebiotic types contained in the filtrate can be classified as inulin compounds. Greg Kelly (2008) described that inulin as a prebiotic is bifidogenic, classified into inulin, oligofructose, and fructooligosaccharides (FOS). The difference in terms of the three compounds is based on DP and the extraction method. Inulin as a general term for hot water extraction with DP between 3-60, oligofructose is the result of inulin hydrolysis by endoglycosidase enzymes with DP between 2-20, and FOS is a compound produced by transfructosylation of sucrose with DP between 2-4 (Van de Wiele et al., 2004; Greg Kelly, 2008). Although the DP values are different, the three compounds have the same bond, namely β (2-1).

The growth of L. casei colonies in the media with the addition of red fruit pedicel extract confirmed that the filtrate contained inulin, which was potential as a prebiotic source. The growth of probiotic bacterial colonies was tested using the L. casei culture. According to Daud et al. (2009), from four lactic acid bacteria (B. bifidum, B. animalis, L. casei Rhamnosus, and L. bulgaricus) grown on MRS media containing oligosaccharide sugar purified from purified extracts of rumbia fruit flour, L.casei Rhamnosus from the genus Lactobacillus has the higher growth. Another

opinion by Huebner et al. (2007) stated that the highest value of prebiotic activity was found in L. paracasei 1195, which grew on the inulin substrate.

Prebiotic compounds cannot be digested by the small intestine and will reach the large intestine, which will then be degraded or fermented by intestinal bacteria to stimulate the growth of beneficial bacteria (Gibson & Roberfroid 1995). Fermentation of oligosaccharides by intestinal bacteria will produce metabolic energy and short-chain fatty acids (mainly acetic acid and lactic acid) and change the composition of the intestinal microflora (Al-Sheraji et al., 2013). The production of these acids will decrease intestinal pH so that the percentage of beneficial bacteria such as Bifidobacterium and Lactobacillus increases, while the percentage of spoilage bacteria such as E. coli and Streptococcus faecalis will decrease. The growth of pathogenic bacteria such as Salmonella and E. coli will be inhibited in the presence of acids and antibacterial substances.

The number of L. casei colonies in the media using the hydrodynamic cavitation method was higher than the media with conventional methods. It means that the addition of filtrate from the hydrodynamic cavitation method is more effective than conventional methods. The effectiveness of prebiotics inulin-type fructan depends on the concentration and DP value (Van Loo 2004). In the study, the same concentration was added to the growth media, which was 2%. Likewise, the results of this study indicate that DP inulin from conventional methods and hydrodynamic cavitation has the same value of 4. The number of L. casei colonies on the media added to the filtrate from the hydrodynamic cavitation method was higher. It is presumably due to the higher inulin concentration in the filtrate from the hydrodynamic cavitation method.

The number of E. coli colonies was lower than the growth of L. casei colonies for all media both control and extracted filtrate (Figure 3B). The number of E. coli colonies in inulin-containing media from both conventional and hydrodynamic cavitation filtrate methods decreased with an increase in the incubation period. The lowest number of colonies was found in conventional filtrate media and hydrodynamic cavitation at 48 hours incubation period, respectively 7.23 log CFU/ml and 7.06 log CFU/ml. E. coli growth is low because the inulin in the red fruit pedicel extract can create a more acidic environment and inhibit the growth of enteric bacteria.

In general, the score probiotics activity of inulin with the addition of filtrate from the hydrodynamic cavitation method at incubation times 12, 24, and 48 hours was higher than the filtrate from conventional methods (Figure 4). The filtrate extracted from the two methods did not have a significant effect ($p < 0.05$) on the value of prebiotic activity at the incubation times of 12 and 24 hours. However, at the incubation time of 48 hours, different extraction methods had a significant effect ($p < 0.05$) on the value of the prebiotic activity. This result is related to the growth phase of lactic acid bacteria. Li et al. (2015) explained that the growth phase of lactic acid bacteria in inulin media entered a lag period of 12 hours, and the right time to measure the prebiotic effect was after 32 hours.

Figure 4 also shows that at the incubation times of 12 and 24 hours, there is low or negative prebiotic activity. Huebner et al. (2007) explain that the low or negative prebiotic activity value is caused by poor growth of lactic acid bacteria in prebiotic media (based on cell density) compared to prebiotic growth in the control media and is less than the growth of enteric bacteria in prebiotic media. Incubation for 48 hours on filtrate media from the hydrodynamic cavitation method produced the highest prebiotic activity value, 0.90. These results indicate that the filtrate from the hydrodynamic cavitation method is easier and has the potential as a growth medium for L. casei.

Conclusion:-

Filtrate extracted from red fruit pedicels contains inulin compounds confirmed by the presence of glucopyranoside and fructofuranosil monomers associated with β bonds as markers. The peak of chromatogram filtrate by conventional method at retention time of 9.77 minutes had a base peak m/z of 686,740 g/mol with the alleged formula $C_{30}H_{54}O_{17}$, whereas the hydrodynamic cavitation method at 7.42 minutes retention time had a base peak m/z of 668,597 g/mol with the alleged formula molecule compound $C_{30}H_{36}O_{17}$. The polymerization degree of inulin with both extraction methods was 4. In vitro test results showed that the inulin in the red pedicel extract could support the growth of L. casei colonies. The highest prebiotic activity value in the filtrate media hydrodynamic cavitation method was obtained at 48 hours incubation time, which was 0.9.

Conflict Of Interest

The authors declared of no conflict of interest.

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