

Formulation of handsanitizer with antibacterials substance from n-hexane extract of soursop leaves (*Annona Muricata* Linn)

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Graphical abstract



Abstract

One of the plants which are efficacious as antibacterial is the soursop leaves. Soursop leaves were extracted by maceration using n-hexane. The extract was evaporated using rotary evaporator. Soursop leaves extract was then formulated in a gel dosage form. This study aims to formulate hand sanitizer from soursop leaves extract based on Growing Minimum Inhibitory Concentrations (MIC) of n-hexane extract of soursop leaves, and to know the evaluation result of gel dosage with the active substance of soursop leaves extract. Testing of physical properties of the gel includes organoleptic test, dispersive power test, homogeneity, pH, and consistency test. Antibacterial activity was tested by using a diffusion method. The evaluation of the gel showed SNI standards which were able for topical use. The organoleptic test resulted that the dosage is odorless, transparent and gel. Homogeneity test resulted that all gel dosage concentrations are homogenous. The pH tests at concentrations of 1, 5 and 10 ppm respectively are 5.38 to 6.22; 5.48 to 6.28; and from 5.29 to 5.90. The dispersive power test resulted for 6.47 to 7 cm; 6.20 to 6.87 cm; and 6.09 to 6.59 cm. The consistency test resulted that all gel dosages are consistent in gel form. Gel dosage with extract concentrations of 1, 5 and 10 ppm can inhibit the growth of bacteria *P. acne* with antibacterial activity of 3.53; 3.26 and 2.20 mm.

Keywords: antibacterial, soursop leaves, hand sanitizer, *P. acne*

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INTRODUCTION

Health is a very important aspect of life. Maintaining hand hygiene is one of the most important things in maintaining a body health (Kumesan, *et al.*, 2013). However, the Indonesian public awareness to the importance of hand hygiene is lacking. Society does not realize that hands are often contaminated with microbes while doing an activity.

Washing hands is a simple matter to eliminate and minimize germs on the hands with water and by adding certain ingredients. One of the simplest and common way to maintain the cleanliness of hands is by washing hands with soap. But along with increasing people's activities, especially in urban areas, and many instant products, then comes to the innovative waterless hand cleaners known as antiseptic hand sanitizer. The gel is one of the dosage form popularly used as hand sanitizer (Shu, 2013).

Antiseptic materials used for gel dosage formulation typically are from alcohol (ethanol, propanol, isopropanol) at a concentration of \pm 50% to 70% and other types of disinfectant such as kloeksidin, triclosan (Cahyani, 2014). Alcohol as a disinfectant has bactericidal activity by damaging proteins. Alcohol is as an organic solvent that can dissolve the fatty layers and sebum on the skin, which serves as a protective layer against infectious microorganisms. Alcohol is also flammable and repeated use of alcohol can cause dryness and irritation to the skin. While it is well known that antiseptic hand gel is

always necessary at any time, in this case used in repeated use (Kurniawan, *et al.*, 2012).

The risk posed by an alcohol need to be addressed in particular by seeking an alternative which is a natural and safe antibacterial ingredients without side effects. One of the plants that is potential as an antibacterial is soursop plant (*Annona muricata* Linn). Soursop plant is can be used as a natural medicine including bark, leaves, roots, fruits and seeds. The ethanol extract of soursop leaves contain flavanoid compound (Cushnie and Lamb, 2005). The n-hexane extract, chloroform and methanol of soursop leaves are evidently able to inhibit the growth of *E. coli* with consecutive inhibition zone of 3.52 mm; 8.34 mm; and 3.00 mm (Ningsih, *et al.*, 2016).

This research will be conducted to determine the minimum growth inhibitory concentration (MIC) of n-hexane extract of soursop leaves against *P. acne*, then to formulate n-hexane extract of soursop leaves as a natural antibacterial additives in handsanitizer.

EXPERIMENTAL

Materials

The materials used in this study were soursop leaves from Purbalingga, chloroform, *P. acne*, Nutrient Agar (NA), Nutrient Broth (NB), Yeast Peptone Agar, tetracycline HCl, 70% alcohol, hydrochloric acid, ether, acetic acid anhydride, sulfuric acid, FeCl₃, Mg powder, Dragendorff reagent and NaCl.

Extraction of sourshop leave

Extraction of sourshop leave was performed as described in the previous report (Ningsih, et al., 2014), the leave was extracted by maceration using n-hexane. In brief, a total of approximately 100 grams of sourshop leaves was soaked in 700 ml of n-hexane, covered and then stored in a dark room and shaken with a shaker of 120 rpm for one week. After that, the filtrate was taken and residue was macerated again with 300 ml of n-hexane for 3 days. Furthermore, the filtrate was taken. Maceration conducted obtained the n-hexane filtrate. N-hexane filtrate of sourshop leaves was concentrated by using a rotary evaporator at a temperature of $\pm 40^{\circ}\text{C}$. The concentrated extract was weighed to obtain the yield values.

Antibacterial activity test

The initial test of antibacterial activity was performed by diffusion (Ningsih, et al., 2014). A total of one loop of bacteria from the cultures stock was taken and incubated in 10 mL of liquid medium (Nutrient Broth) for 18-24 hours at 37°C and shaken using a water bath at 100 rpm. A total of 5 mL bacterial culture was taken and OD value was then measured with a value less than one at a wavelength of 620 nm. If the OD values >1 , then bacteria cultures were taken for 50 μL , if OD <1 bacteria cultures were taken for 100 μL . Pour 15 mL of media Nutrient Agar (NA) with temperature of $\pm 40^{\circ}\text{C}$ into a petri dish and let it solidify. Then the culture disseminated on the media. After that, agar was riddled with diameter of $\pm 8\text{ mm}$ using a crock bor. A total of 50 μL n-hexane extract of sourshop were put into each holes with extract concentration used for inhibitory test of 1000; 500; 250; 125; 65; 30; 15; 10; 5; and 1 ppm and incubated at 37°C for 24 hours, with the positive control of tetracycline and negative control of aquades. Clear zone is visible around the hole, indicating the antibacterial activity was subsequently formed, then clear zone was measured using a caliper. Once Growing Minimum Inhibitory Concentration (MIC) against *P. acne* bacteria would then be measured.

Identification of chemical compounds of n-hexane extract of sourshop leaves

The qualitative chemical test was used for detection of alkaloids, flavonoids, saponine, terpenoids, polyphenols and steroids (Djamil and Anelia, 2009).

Alkaloid test

Extract samples were dissolved in 2 mL of hydrochloric acid, heated for 5 minutes and filtered. The filtrate obtained was added by 2-3 drops of Dragendorff reagent. The presence of the alkaloid compounds is indicated by an orange precipitate.

Flavanoid test

A total of 2 mL sample ($\pm 0.05\%$ w/v) were dissolved in 2 mL methanol, then added by Mg powder and 5 drops of concentrated HCl. The presence of flavanoid compound is indicated by the formation of red or orange colour.

Saponin test

A total of 2 mL sample ($\pm 0.05\%$ w/v) were dissolved in aquadest in a test tube then added by 10 drops of KOH and heated in 50°C for 5 minutes in the water bath, shaken for 15 minutes. If the foam is formed steady for 1 cm and remained stable for 15 minutes, it indicates the presence of saponins.

Terpenoid test

A total of 2 mL sample ($\pm 0.05\%$ w/v) were added by 1 mL Lieberman-Burchard reagent. Terpenoid compounds are identified by the formation of a dark blue or blackish green.

Polyphenol test

A total of 2 mL sample ($\pm 0.05\%$ w/v) were dissolved in 10 mL aquadest, heated for 5 minutes and filtered. The filtrate formed was added by 4-5 drops of FeCl_3 5% (w/v). The phenol is identified by the formation of a dark blue or blackish green.

Steroid test

A total of 2 mL sample ($\pm 0.05\%$ w/v) were added by 1 mL Lieberman-Burchard reagent. The formation of a dark blue or blackish green colour indicates the presence of steroid.

Formulation of handsanitizer

Formulation of Gel Dosage

A total of 0.25 g carbopol was developed in 25 mL hot aquadest, then stirred by using a stirrer, added by 0.1 g methyl paraben. A total of 25 mL aquadest was added into the mixture, n-hexane extract of sourshop leaves was added gradually and then added by 0.5 mL Glycerin and 50 μL of TEA while stirring until it forms a gel (Sari, 2006).

Table 1. Formulation of antiseptic gel of sourshop leaves extract

| Materials | F1 | F2 | F3 | F4 |
|-----------------|------------------|------------------|------------------|------------------|
| Soursop extract | 0 ppm | 1 ppm | 5 ppm | 10 ppm |
| Carbopol | 0.2 g | 0.2 g | 0.2 g | 0.2 g |
| TEA | 50 μL | 50 μL | 50 μL | 50 μL |
| Glycerin | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| Methyl Paraben | 0.1 g | 0.1 g | 0.1 g | 0.1 g |
| Aquadest | 50 mL | 50 mL | 50 mL | 50 mL |

Antibacterial Activity Test of Dosage

The initial antibacterial activity test of dosage was performed by diffusion. A total of one loop of bacteria from the stock cultures were taken and incubated in 10 mL of liquid medium (Nutrient Broth) for 18-24 hours at 37°C while shaking by water bath sway. After that, a total of 15 mL Yeast Peptone Agar media (PYG) with temperature $\pm 40^{\circ}\text{C}$ was poured, and a number of bacterial culture was taken then OD value was measured with a value less than one at a wavelength of 620 nm. If the OD value >1 , a total of 50 mL cultures is taken. If the OD value <1 , a total of 100 mL cultures is taken, and then distributed in sterile petri dish, and then the cup was shaken in order to spread the bacteria evenly and streaked by spread plate using drugal sky. Furthermore, it was allowed to stand at room temperature in order to solidify the media. After being solid, it was riddled with diameter of $\pm 8\text{ mm}$ using crock bor. Handsanitizer gel dosage was inserted into the holes then incubated at 37°C for 24 hours. Visible clear zone around the holes indicates the antibacterial activity of handsanitizer gel dosage then visible clear zone was measured using a caliper.

Gel Stability Test

Examination of gel dosage stability was conducted at room temperature on day 0, 5, 10, 15 for organoleptic, homogeneity, pH, dispersive power, consistency test.

Organoleptic Test

Observation was seen directly in terms of shape, color and smell of the gel. Gel is usually clear with a semisolid consistency (Ansel, 1989).

Homogeneity Test

Homogeneity test was conducted is by smearing the gel sample on a piece of glass or other suitable transparent material, gel dosage should indicate the homogeneous composition and coarse grains are not observed (Kurniawan, et al., 2012).

pH test

The pH test of the dosage was conducted using the universal pH stick which was dipped in immersed gel samples. After perfectly dipped, the discolouration of universal pH stick was observed and compared to the standard universal pH. The pH value of gel dosage must conform to the skin's pH of 4.5 to 6.5 (Tranggono and Latifah, 2007).

Dispersive Power Test

A total of 0.5 g of gel sample were placed on a 15 cm diameter spherical glass, another glass was placed on it and allowed to stand for 1 minute. Dispersion of gel was measured. Then, it was added by 150 g of extra loads and allowed to stand for 1 minute and then the constant diameter was measured. Dispersive power of 5-7 cm indicates semisolid consistency which is very convenient in use (Kumesan, et al., 2013).

Consistency test

Consistency test was conducted by observing the consistency changes of a gel dosage whether there is a separation between the gelling material and its carrier, which is water. Consistency test used

the centrifugal test in which gel sample was centrifuged at 3800 rpm for 5 hours and then physical changes were observed (Djajadisastra and Mun'im, 2009).

RESULTS AND DISCUSSION

Determination of minimum growing inhibitory concentration of n-hexane extract

Determination of minimum growing inhibitory concentrations (MIC) was conducted to determine the minimum concentration of n-hexane extract of soursop leaves that can inhibit the growth of bacteria *P. acne*. Determination MIC in this study used pitting diffusion method. Pitting diffusion method is used because it will result more visible inhibition zone and samples can be absorbed by the media as a whole thus the growth of bacteria evenly spread on the agar medium. Medium used for testing is a nutrient agar medium (NA). Medium NA is a universal medium that can be used by most bacteria.

Inhibition zone of N-hexane extract of soursop leaves against *P. acne* can be determined by measuring the clear zone which is formed around the hole samples. Inhibition zone was measured using calipers vertically, horizontally and diagonally, then average in millimeters. Results of determination of MIC of n-hexane extract of soursop leaves can be seen in Figure 1.

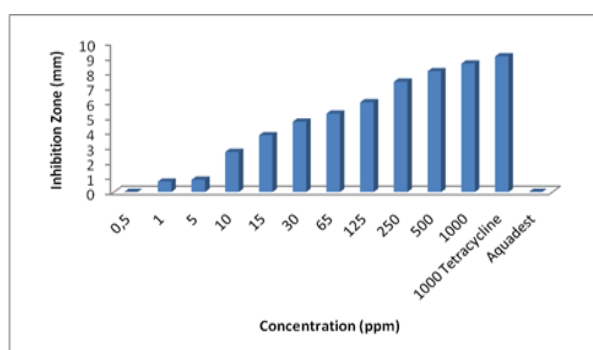


Figure 1. The growth inhibition of soursop leaves extract against *P. acne* during MIC determination.

Based on the results as seen in Figure 1, the concentration of 1 ppm is the lowest concentration that can inhibit the growth of *P. acne* with inhibition zone of 0.7 mm. Concentration of 1000 ppm is the highest antibacterial activity since it has the greatest inhibitory zone value of 8.6 mm. The negative control did not give inhibition, the positive control in the form of erythromycin gave inhibition zone of 9.08 mm.

Identification of Secondary Metabolites Compound

Colour test is used to determine the class of secondary metabolites, such as alkaloids, flavonoids, saponins, terpenoids, steroids, tannins and polyphenols. The results of the most active extracts colour test can be seen in Table 2.

Table 2 The secondary metabolites test of n-hexane extract of soursop leaves.

| Compound | Colour indicating positive result | Test Result | Description |
|------------|-----------------------------------|--------------------|-------------|
| Alkaloid | Orange precipitate | Orange precipitate | Positive |
| Flavonoid | Red or orange | Green | Negative |
| Saponin | Formation of foam | No foam | Negative |
| Terpenoid | Purple | Light green | Negative |
| Tannin | Dark bluish green | Orange | Negative |
| Polyphenol | Bluish green | Orange | Negative |

Identification of secondary metabolites in *Mangifera indica* L. leaves was conducted using phytochemicals method. Phytochemical test is used to determine the class of secondary metabolites, such as alkaloids, flavonoids, polyphenols, terpenoids / steroid, saponins and tannins of the leaves.

The active compounds obtained in n-hexane extract of soursop leaves is alkaloid. This alkaloid compounds thought to have the ability to inhibit the growth of bacteria *P. acne*. Alkaloid has the ability as an antibacterial by interfering with peptidoglycan constituent component of the bacterial cell, so the cell wall layers are not fully formed and caused the death of these cells (Ajizah, 2004).

Formulation of handsanitizer gel dosage

The formulation of handsanitizer gel dosage requires basic ingredient gelling agent which is carbomer. Carbomer is an acrylic acid polymer which is hydrophilic and stable. Carbomer generates good viscosity for gel dosage. The selection of hydrophilic polymer which is bioadhesive in handsanitizer can enhance the transferring of active substances into the skin (Pavelić, et al. 2001).

The formulation of gel dosage in this study was conducted by dispersing the carbomer in distilled water and stirred by using a magnetic stirrer. It aims to homogenize the gel regularly. The formulation of the gel does not require high speed because it aims for homogenization process. If the speed is too high, it will change the gel's color into cloudy. Beside, rapid stirring aims to prevent formation of agglomerates. Agglomerates are clumps because of mixing several compounds (Shu, 2013).

Handsanitizer gel dosage is made by mixing carbomers and aquadest until all carbomers distributed evenly. Utilization of aquadest aims to eliminate the mineral content that may affect the activity of active substances and prevent further loss of pH so that the dosage changes into acid. The acidity level of gel dosage must conform to the skin's pH which is 4.5 - 6.5. It is due to the function of skins is to protect the body from outside interferences, especially chemical disruptions. The disruptions can be mitigated by the presence of fat on skins surface from skins pallid gland which has the pH of 4.5-6.5 (Anwar, 2012).

The concentration of carbomer used is 0.2 g on 50 mL of aquadest. The higher concentration of carbomer is added, the higher viscosity will obtain that causes the gel base become rigid. Handsanitizer gel base formulation is also added by several other supporting components and additives n-hexane extract as the main ingredient of antibacterial compounds. The other components which are added in these formulations include methyl paraben, triethanolamine and glycerin.

Gel has advantages such as good dispersive power on the skin, the cooling effect caused by the slow evaporation of water on the skin, does not inhibit the physiological function of the skin, especially respiratio sensibilis which means the process of spending certain substances such as salt through sweat glands on the skin. Gel does not coat the surface of the skin-tight and did not clog the pores, easily washable with water and allow the use on the hairy body and release the medicine well. Meanwhile, as it is known, ethanol n-hexane extract of soursop leaves has loamy and abrasive consistency, so it is not efficient to be used directly in applications daily as an antibacterial.

Evaluation of hand sanitizer gel dosage

Evaluation of handsanitizer gel formulation is required to determine handsanitizer gel dosage conditions before and after stability test was conducted by using physical parameters so that physical stability and feasibility of handsanitizer gel dosage can be known. Physical stability test of gel handsanitizer in this study is conducted for 15 days. Examination of dosage stability was conducted at room temperature on day 0, 1, 5 and 10 for organoleptic test, homogeneity, pH, dispersive power, consistency to see the stability of dosage during 15 days.

Organoleptic test

The results of the organoleptic examinations conducted to gel dosage of n-hexane extract of soursop leaves performed on three bases: gel 0 ppm, gel 1 ppm, gel 5 ppm and gel 10 ppm can be seen in terms of shape changes, colour and smell. The test showed no significant change to the shape, color and smell of the gel dosage during storage for 15 days. Colour test of gel dosage has almost the same colour, at concentrations of 0 to 10 ppm, colour of gel dosage is clear. During storage from day 0 to day 15, colour of dosage at concentrations of 0, 1, 5 and 10 ppm does not change.

Concentration of n-hexane extract of soursop leaves which is added does not affect to the smell of dosage. Dosage at 0 ppm to 10 ppm are odorless. While the organoleptic test showed that the gel shape does not change from day 0 to day 15, the all dosage with concentration of 0, 1, 5 or 10 formed a gel during storage. The result of this clear gel dosage is relevant with the statement of Ansel (Ansel, 1989) which stated that the gel was usually clear with a semi-solid consistency.

Homogeneity test

Homogeneity test aims to look at the stability of the gel during storage. Homogeneity of handsanitizer gel dosage was investigated by placing it on two objective glasses and the result showed the homogeneous form of a transparent gel. During the 15 days of storage, there are no changes in stability at concentrations of 0, 1, 5 or 10 ppm. This shows that the emulsifier in gel handsanitizer is working well. Kurniawan (Kurniawan, et al., 2012) mentioned that dosage should show a homogeneous composition and absence of coarse grains.

This homogeneity test results are relevant with the previous research, that the homogeneity of dosage can be seen by using glass and showing no solid particles contained in the gel, and also the absence of gel clumping or bad distributed gel in dosage.

pH test

The pH test is a test for chemical stability. The pH test aims to analyze whether the gel has the appropriate pH value and suitable with the skin. The pH value of gel that does not conform with the skin's pH will cause irritation to the skin. Topical dosage should be in the skin's pH range of 4.5-6.5. If it is too acidic, it will cause skin irritation and if too alkaline, it can cause scaly skin. It happens due to the damaged of acidic mantle on stratum corneum layer of the skin. The pH of handsanitizer gel dosage was measured by using a pH meter. Results of dosage pH test can be seen in Figure 2.

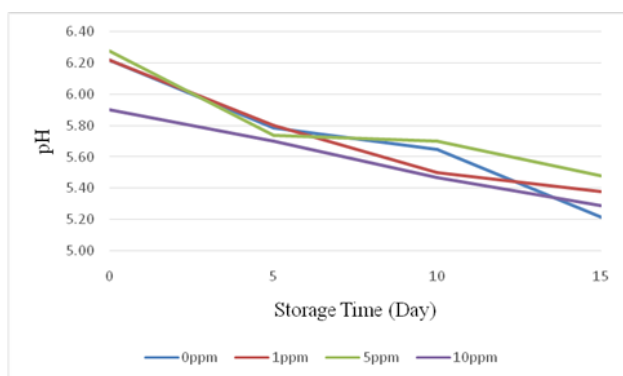


Figure 2. Graphic result of pH test on handsanitizer gel dosage.

The pH measurement of handsanitizer gel dosage was performed once in 5 days during 15 days. The pH value at day 0 is slightly more alkaline than the following days, this happens due to the neutralization of the carbomer when the addition of triethanolamine. Anwar (2012) reported that the process of neutralization and viscosity could not occur directly, neutralization and maximum viscosity could be obtained from 5 minutes to 3 hours. Decreasing pH value of handsanitizer gel dosage on following days happens due to

degradation of phosphatidylcholine that is caused by oxidation and hydrolysis. Even though pH test results on day 0 to day 15 is decreasing, but it is still appropriate with the Indonesian National Standard (SNI) which is pH value of 4.5-6.5. Gel dosage with a concentration of 0 ppm has the pH value of 5.21 - 6.22; concentration of 1 ppm has the pH value of 5.38 - 6.22; concentration of 5 ppm has the pH value of 5.48 - 6.28; and the concentration of 10 ppm has the pH value of 5.29 to 5.90.

Consistency test

Consistency is an important physical characteristics in a semisolid dosage. Value of consistency relates to the ability of a dosage to penetrate. Consistency measurement was conducted using ependoff tube and centrifuged for 15 minutes at 3000 rpm. Gel dosage at concentrations of 0, 1, 5, and 10 ppm are consistent in gel form. Storage of dosage on day 5 showed that all the dosages after the test of consistency are consistent in gel form. This is caused by enlargement of the carbomer size with the solvent in gel dosage thus increasing the consistency of a gel. During storage on day 5 to day 15, the gel dosage's performance is consistent in gel form.

Dispersive power test

The observations of dispersive power aims to see the capabilities of gel dosage to spread on the surface of the skin so that it can determine the spread of active substances that contained in the gel on the skin. This relates to the distribution of the active substances that contained in the dosage. The test results of dispersive power can be seen in Figure 3.

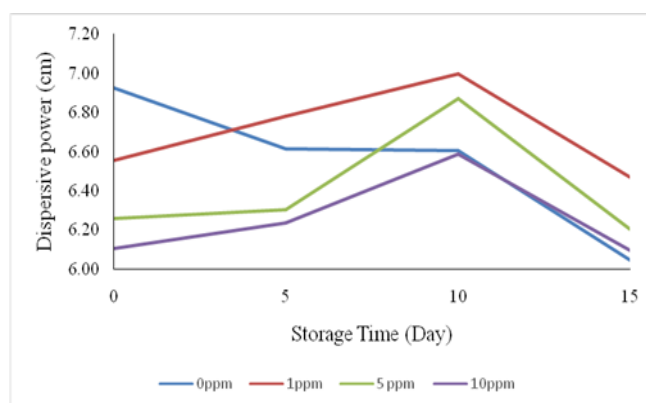


Figure 3. Graphic of dispersive power test.

The test resulted that the dispersive power of handsanitizer gel dosage corresponds with Indonesian National Standards (SNI). Kumesan (Kumesan, et al., 2013) reported that the dispersive power between 5-7 cm showed semisolid consistency which is very convenient in use. During 15 days storage, the value of the dosage dispersive power at concentration of 0 ppm is 6.05 - 6.92 cm; 1 ppm is 6.47 - 7 cm; 5 ppm is 6.20 - 6.87 cm and concentration of 10 ppm is 6.09 - 6.59 cm.

Antibacterial activity of handsanitizer gel dosage

The test results of gel dosage antibacterial activity against *P. acne* showed inhibition zone at concentration of 1 ppm, 5 ppm and 10 ppm with diameter of 3.53; 3.26 and 2.20 mm respectively. While the negative control zone does not show any inhibition zone. Whereas the positive control which is used in handsanitizer gel dosage is a commercial handsanitizer gel. Inhibition zone diameter of this positive control is 7.56 mm. The increasing concentration, the result of inhibition zone diameter gets smaller. Based on the result, it can be concluded that the handsanitizer gel dosage at concentration of 1 ppm, 5 ppm and 10 ppm have antibacterial activity that can be formulated into dosage forms antiseptic gel. The results of dosage antibacterial activity test can be seen in Figure 4.

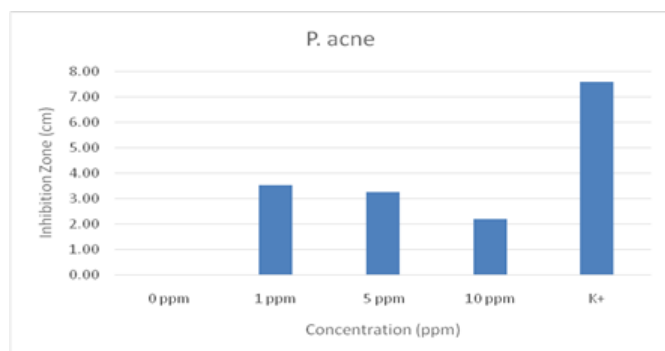


Figure 4. Graph of antibacterial activity of handsanitizer gel dosage test.

N-hexane extract of soursop leaves has antibacterial activity after being formulated into antiseptic hand gel dosage. The results generally show that the greater concentration of soursop leaves extract, then the antibacterial activity of n-hexane extract gel soursop leaf is getting smaller. This is caused by the absorption process of the active compounds that penetrate into the media in inhibiting the growth of *P. acne*.

CONCLUSIONS

The minimum growth inhibitory concentrations (MIC) of n-hexane extract of soursop leaves against *P. acne* was 1 ppm with an inhibition zone of 0.7 mm. This soursop leaves extract was formulated in handsanitizer gels with dosages of 1 ppm, 5 ppm and 10 ppm, and showed inhibition zones of 3.53, 3.26 and 2.20 mm respectively.

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