ANTIBACTERIAL AND ANTIACNE ACTIVITY OF n-HEXANE FRACTION OF ALFALFA HERB (*Medicago sativa* L.) ETHANOL EXTRACT IN VITRO AND ON RABBIT (*Oryctolagus cuniculus*)

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Abstract

Propionibacterium acnes (P. acnes) can cause inflammation of the polysebaceous glands and lead to acne. Flavonoids in alfalfa herb are thought to have antibacterial and anti-inflammatory properties. Flavonoids can be absorbed into n-hexane solvents. This study aims to determine antibacterial activity of n-hexane fraction of alfalfa herb ethanol extract (nHFAH) against P. acnes in vitro, to calculate the total flavonoid levels in the fraction, and to determine antiacne activity of the fraction on rabbit. This study is an experimental study with post-test control group design. Extraction of alfalfa herb was done by maceration method using 70% ethanol and fractionated with n-hexane. Antibacterial activity test was carried out using disk diffusion with 5 series of concentrations (15%, 20%, 25%, 30% dan 35%), positive control (clindamycin 2 µg/disk) and negative control (DMSO 100%). Diameters of the inhibition area were analyzed statistically using a Kruskal Wallis test followed by Mann Withney test with 95% of confidence level. Rabbit back skin was shaved on 5 different areas (3x3 cm2/area). Induced with 0.2 mL of P. acnes suspension (3x109 CFU/mL) intradermal. Acne formed (24 hours) on all areas, then were treated with T1 (clindamycin 2%), T2 (DMSO 100%), T3 (nHFAH 20%), T4 (nHFAH 25%), T5 (nHFAH 30%). Erythema diameter reduction on day 9 was analyzed using one-way anova, continued by post hoc tukey with 95% of confidence level. Total nHFAH flavonoid levels were tested by colorimetric method using UV-Vis spectrophotometer at λ 429.5 nm. The results showed that nHFAH has antibacterial activity against P. acnes with an average value of diameter of inhibition area for each concentration were 7.30; 7.49; 7.75; 7.92 and 8.24 mm. nHFAH exhibits antiacne activity with the percentage for each concentration were 67.01%; 77.84% and 85.84% respectively. Total nHFAH flavonoid levels were 6.09 mgQE/ gram.

***Keywords**: Alfafa herb, n-Hexane fraction, Propionibacterium acnes.*

INTRODUCTION

Propionibacterium acnes (P. acnes) causes acne by producing lipases which can convert glycerides into free fatty acids and glycerol. These free fatty acids can irritate the follicular walls and cause increased cell turnover and inflammation. P. acnes bacteria persist and proliferate in the environment formed by a mixture of sebum and keratinocytes, are antigenic and can increase the formation of antibodies that have an impact on the inflammatory response (IAI, 2011).

Some plants have natural antibacterial properties for some strains of bacteria, for example alfafa leaves (*Medicago sativa* L.). The methanol extract of alfalfa leaves showed significant antimicrobial activity and supported the use of alfalfa herbal extract as a broad-spectrum antimicrobial agent (Chavan et al., 2015). The antibacterial activity of alfalfa herb extracts has also been investigated in vitro on both Gram positive and Gram negative bacteria, resulted an antibacterial against *Staphylococcus aureus, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa,* and *Klebsiella pneumon*ia using the agar diffusion method. In addition, the flavonoid content has been confirmed in this extract (Doss et al., 2011).

The alfalfa herb ethanol extract tested in vivo on male rats induced by carrageenan has anti-inflammatory activity of 58.78% at a dose of 45 mg/KgBW and suspects that the active compound that plays a role in anti-inflammatory activity is flavonoids (Susilowati and Rosyidah, 2008).

Flavonoid compounds as secondary metabolites of a plant can inhibit arachidonic acid (cyclooxygenase) which will produce prostaglandins so that they have anti-inflammatory properties (Panche et al., 2016). The n-hexane solvent was able to extract flavonoids with amaunt level of 8.13% from 96% of ethanol extract of alfalfa herb and suspected that the flavonoids contained in the test fraction were aglycones or isoflavones (Susilowati et al., 2012).

The n-hexane fraction of alfalfa herb, which is a fabaceae family, has never been carried out in vitro research on *P. acnes*, Nevertheless, the n-hexane fraction of Chinese petai (*Laucaena leucocephala* (Lamk.) De Wit) from the family fabaceae has proven to contain flavonoid compounds and has antibacterial activity against *Staphylococcus aureus* (Abriyani and Nurfalah, 2019). Based on these descriptions, it is interesting to prove the antibacterial and antiacne activity of the n-hexane fraction of the alfalfa herb ethanol extract against Propionibacterium acnes, as well as knowing the total levels of flavonoids in that fraction.

MATERIALS AND METHODS

This study was an experimental study with post test control group design. This study has been approved by the medical/health research bioethics commission of The Medical Faculty of Sultan Agung University Semarang, with ethical clearance number 663/X/2019/Bioethics Commission.

Maceration was carried out by soaking 0.85 Kg of alfalfa herb powder in 70% ethanol solvent (technical), the amount of solvent used for maceration for 3 days was 6.37 L and remaceration for 2 days was 2.12 L of the total 70% ethanol solvent. After being filtered then the two macerates obtained were mixed and then concentrated using a rotary vacuum evaporator at a temperature of 55ºC at a speed of 70 rpm until the thick extract is obtained and the yield of the extract is calculated.

The fractionation process was carried out using a separating funnel by mixing 20 g of alfalfa herb ethanol extract in 200 mL of water and 200 mL of n-hexane (1: 1) solvent. The solution mixture was shaken out in one direction slowly then allowed to stand for a moment until two layers are formed due to differences in density and polarity. The water phase is at the bottom because it has greater specific gravity than n-hexane. The resulting filtrate is then concentrated using a rotary vacuum evaporator at a temperature of 50ºC at a speed of 60 rpm.

Antibacterial activity test of n-hexane fraction of alfalfa herb extract

Mueller Hinton gel media was weighed as much as 0.98 g and dissolved in 25 mL of aquadest in an erlenmeyer, then heated on an electric stove and stirred until the media is completely dissolved. The finished media is sterilized by autoclaving at 121ºC for 15 minutes (Qomariah, et al., 2018, and Pakekong et al., 2016)

Brain Heart Infusion (BHI) media was weighed as much as 0.74 grams and dissolved with 20 ml aquadest, heated on an electric stove until dissolved and homogeneous. The finished media is sterilized by autoclaving at a temperature of 121ºC for 15 minutes. One percent $BaCl_2$ solution of 0.05 mL and 1% sulfuric acid solution of 9.95 mL were mixed in a test tube, then closed tightly so that evaporation did not occur and were shaken until a cloudy solution was formed. This turbidity is used as the standard for the turbidity of the tested bacterial suspension which is equivalent to 0.5 Mc. Farland I (has a population of 1 × 108 CFU/mL).

The purpose of making bacterial cultures is to get new and young cultures so that they can reproduce well. *Propionibacterium acnes* were planted again in a zigzag manner on the surface of Mueller Hinton gel media in a petri dish using a sterile round loop wire and then incubated at 37ºC for 24 hours.

Bacterial cultures were taken using a sterile round loop wire, suspended into BHI media, then incubated at 37ºC for 24 hours. The amount of 200 µL of bacterial suspension was taken into 10 mL of 0.9% sodium chloride solution, the turbidity level of the bacterial suspension was compared with the standard solution of 0.5 Mc. Farland I.

The stock solution of the alfalfa n-hexane fraction was made into a series of concentrations of 15%, 20%, 25%, 30% respectively being added with 70 µL of 100% DMSO solution. A concentration of 15% is equivalent to 1500 µg/disk, 20% is equivalent to 2000 µg/disk, 25% is equivalent to 2500 µg/disk, 30% is equivalent to 3000 µg/disk, and 35% equivalent to 3500 µg/disk.

The bacterial suspension of 1.25 mL was mixed into 23.75 mL of MHA media, then poured into a petri dish and waited for the media to solidify. The test solution and negative control (DMSO 100%) were dropped as much as 10 µL on each disc paper in sterile petri then let stand for 10 minutes to allow the test solution to spread evenly on the disc paper. Disc paper of all test solution, positive control (2 µg clindamycin), negative control (DMSO 100%) were affixed to the surface of the media that had been mixed with bacterial suspension and incubated at 37ºC for 24 hours.

Determination of total flavonoid content

The maximum wavelength measurement with a quercetin comparison was read using a UV-Vis spectrophotometer. The amount of 1000 µL of 6 mg/L quercetin solution was added with 200 µL of 10% $AlCl_3$ and 200 µL of CH_3COOK 1 M. The absorbance was read on a UV-Vis spectrophotometer with a wavelength of 400-500 nm.

One thousand µL of 6 mg/L quercetin solution was added with 200 µL of 10% $AlCl_3$ and 200 µL of CH_3COOK 1 M. The absorbance was read on a spectrophotometer at a maximum wavelength to measure operating time.

One thousand µL of quercetin series concentrations (2, 4, 6, 8, 10, and 12 mg/L) as a standard solution, and 1000 µL of nHFAH was added with 200 µL of 10% $AlCl_3$ and 200 µL of CH_3COOK 1 M. The absorbance was read with a spectrophotometer at the maximum wavelength and the operating time that has been set.

Antiacne activity test of n-hexane fraction of alfalfa herb extract

Five male rabbits were acclimatized for 1 week, if there was rabbit sick or died, it would be excluded from the study and replaced with new rabbit which was then acclimatized again. Five rabbits each had their backs shaved and divided into 5 different areas with an area of 3x3 cm2 each. The five areas were then induced by 0.2 mL of *P. acnes* bacterial suspension at a concentration of 3x109 CFU/mL intradermally and left until the acne appeared (24 hours). Acne was marked by the presence of redness, swelling, and pus in the pimples that appeared. The areas then were treated with T1 (clindamycin 2%), T2 (DMSO), T3 (nHFAH 20%), T4 (nHFAH 25%), and T5 (nHFAH 30%) sequentially from top (near neck area) to down (near tail area); each of which is applied as much as 0.5 mL every day (2 times a day, morning and evening) for 9 days. Antiacne activity of nHFAH can be seen from its ability to reduce erythema diameter (Sa'diah, 2013). This antiacne test is a continuation of the antibacterial test on *P. acnes* in vitro, which used 3 concentrations in the middle range of 5 concentrations that used in the invitro antibacterial test. This is due to efficiency reason of the number of rabbit.

Data analysis

The normality data of the nHFAH inhibition area were tested using the Shapiro-Wilk test. The homogeneity of the variants data were tested using the Levene's test, and then were analyzed statistically using a Kruskal Wallis test followed by Mann Withney test with 95% of confidence level. The nHFAH would be proved to have antibacterial activity if the diameter of the inhibition area is significantly greater than the negative control.

The erythema diameter reduction on day 9 was analyzed using one-way anova, continued by post hoc tukey with 95% of confidence level. The nHFAH is said to have antiacne activity if the reduction in erythema diameter in the nHFAH treatment was significantly greater than the negative control. Total flavonoid levels were calculated in units of mgQE/gram using the formula:

$$\text{Total flavonoid levels} = (C \times V \times Fp) / (\text{Weight of extract}) \quad (1)$$

Information:

C : Flavonoid concentration (X value)
V : volume of extract used (mL)
Fp : dilution factor

RESULTS AND DISCUSSION

Antibacterial activity of n-hexane fraction of alfalfa herb extract

The viscous fraction of n-hexane obtained was 8.4 grams with a 14% yield, meaning that every 100 grams of alfalfa herb ethanol extract produced 14 grams of n-hexane fraction. Characteristics of nHFAH are blackish green in color and smells typical of alfalfa herbs. Based on the measurement results of the inhibition zone diameter as listed in **Table 1**, it shows that nHFAH has insignificant inhibition area between concentration series. This is because the increase in

concentration is not too large. The results of the diameter of the inhibition area are shown in **Figure 1**. The diameter of the inhibition zone formed is thought to be due to the presence of flavonoid compounds. Alfafa contains many chemical compounds, namely alkaloids, isoflavonoids, saponins, and coumarin (Barnes et al., 2005).

Isoflavone compounds from the flavonoid group that are widely found in *fabaceae* (Arifin and Ibrahim, 2018), have biological activity in inhibiting the growth of *P. acnes* bacteria by means of the alcohol group in isoflavone compounds that will react with bacterial cell walls consisting of lipids and amino acids so that the cell walls will be damaged and these compounds can enter the nucleus of bacterial cells. Furthermore, the bacterial cell nucleus will lysis and eventually die because the DNA in the cell nucleus will contact this compound and with the difference in polarity between the alcohol groups and the lipids making up DNA in isoflavone compounds, reactions and damage to the lipid structure of DNA can occur (Mukti, 2012). Further research is needed to ascertain the class of flavonoid compounds in alfalfa herb which act as antibacterial by TLC-bioautography.

Table 1. Diameter of the inhibition area of all treatment groups agains *P. acnes*

Group	Mean ± SD of diameter of the inhibition area (in mm)	Zone type
nHFAH 15% (1500 µg/*disk*)[a]	7,30 ± 0,77	Radical
nHFAH 20% (2000 µg/*disk*)[a]	7,49 ± 0,79	Radical
nHFAH 25% (2500 µg/*disk*)[a]	7,75 ± 0,92	Radical
nHFAH 30% (3000 µg/*disk*)[a]	7,92 ± 0,85	Radical
nHFAH 35% (3500 µg/*disk*)[a]	8,24 ± 0,72	Radical
Clindamycin 2 µg/*disk*[a]	14,52 ± 0,08	Radical
DMSO 100%	-	-

[a][There was a significant difference between nHFAH and clindamycin against negative control group ($p < 0.05$)].

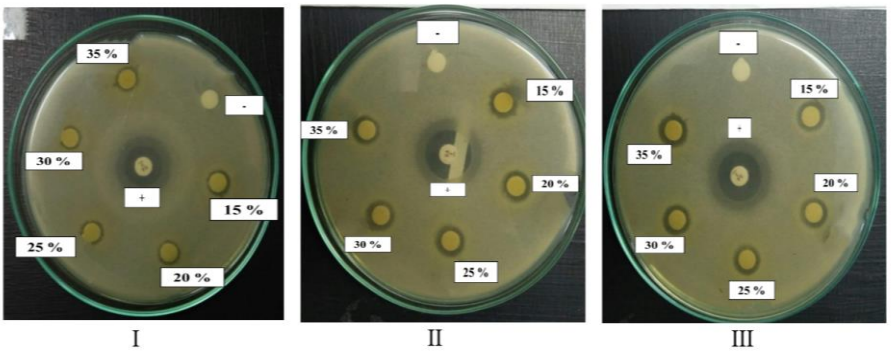

Figure 1. **Diameter of the inhibition area of all treatment groups agains *P. acnes***

Total flavonoid content

The total flavonoid content of nHFAH was obtained by reading the absorbance of the n-hexane fraction sample plotted on the quercetin standard curve. The absorbance read on a spectrophotometer should be between 0.2 - 0.8 if it is read as transmittance (Gandjar and Rohman, 2016).

The maximum wavelength of quercetin is read at a wavelength of 400-450 nm (Aminah et al., 2017). The maximum wavelength of quercetin obtained in the study was 429.5 nm and a stable absorbance was obtained at 25 minutes. The results of measuring the quercetin standard curve with absorbance readings at a wavelength of 429.5 nm and an operating time of 25 minutes can be seen in **Figure 2**. The regression equation obtained is $y = 0.0504x + 1.1537$ with an value of $r = 0.9901$.

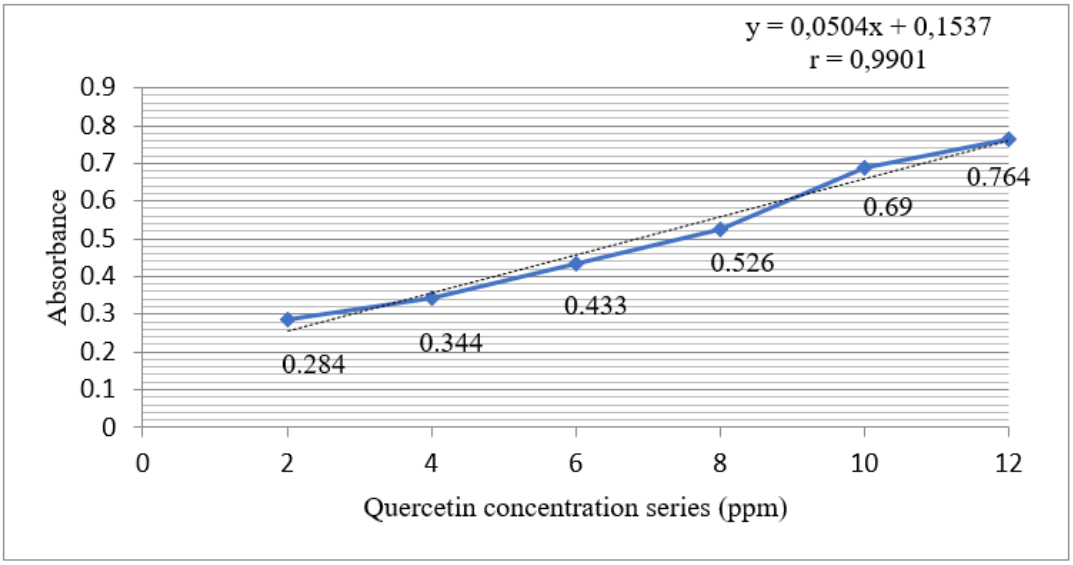

Figure 2. Quercetin standard curve

The principle of determining the levels of flavonoids is the formation of a complex between aluminum chloride with the ketone group on the C4 atom and the hydroxy group on the adjacent C3 or C5 atom from the flavone and flavonol groups. Quercetin is used as a comparison because quercetin is a flavonoid that has a ketone group on the C4 atom and a hydroxy group on the C3 or C5 atom (Azizah et al., 2014). Determination of total flavonoid levels from nHFAH obtained levels of 6.0906 ± 0.0638 mgQE/gram, can be seen in **Table 2**.

The results of this study can be compared with research by Al-Amin (2019) which determined the total flavonoid levels in *Leguminosa* plants, namely jengkol leaves, where alfalfa herbs are also in the same tribe. The total flavonoid content of jengkol leaves in the ethanol extract was 9.511 mg/g and the n-hexane extract was 4.343 mg/g, where the n-hexane solvent had total flavonoids which were not much different from the alfalfa herb.

Table 2. Total flavonoid levels of nHFAH

Replication	Absorbance	Flavonoid levels (mgQE/gram)	Mean ± SD total flavonoid content (mgQE/gram)
1	0.462	6.1171	6.0906 ± 0.0638
2	0.457	6.0178	
3	0.463	6.1369	

Antiacne activity of n-hexane fraction of alfalfa herb extract

Rabbits were induced by *P. acnes* bacteria on 5 different rabbit backs intradermally after orientation of the concentration of bacteria that could cause acne and acne was still present for 2 weeks. The bacterial density used was 3x109 CFU/mL which was adjusted to the Mc. Farland 10 turbidity standard. *P. acnes* bacteria were cultured on MHA media and grown on BHI media for 24 hours before being suspended in 0.9% physiological NaCl. The use of this physiological solution is because it adjusts to the pH of body fluids and the suspension must be made fresh or immediately before being induced (Sa'diah et al., 2013). *P. acnes* that has penetrated into or across the epithelial surface will cause inflammation around the injection site, after 24 hours. At this location, there are four main signs of inflammation, namely redness, swelling, and a feeling of warmth. In some rabbits, pus develops at the injection site. The signs of inflammation are shown in **Figure 3**.

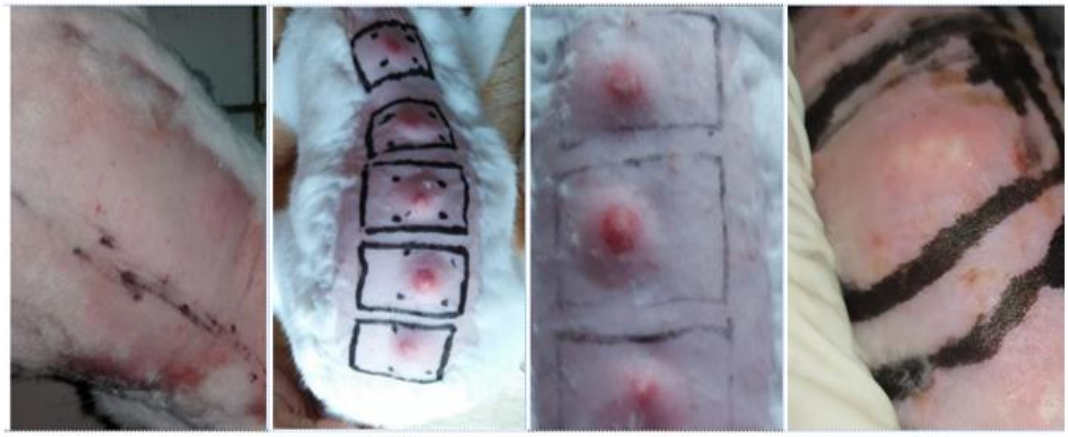

Figure 3. Acne on the skin of the rabbit's back

Information : (a) = prior to *P. acnes* induction
(b) = 24 hours after *P. acnes* induction
(c) = inflammation of the skin in the form of redness, swelling and pain (rabbits wobble when the pimple is held)
(d) = pus in the acne area

P. acnes is a normal flora of human skin, plays a role in the pathogenesis of acne by producing lipases which break down free fatty acids from lipids in the skin. These fatty acids can cause tissue inflammation and support acne. The mixture of oil and indwelling cells allows *P. acnes* to grow in the follicle, producing chemicals and enzymes that attract immune cells that cause inflammation (Portillo et al., 2013). *P. acnes* bacteria cause the secretion of lipase, protease and chemotactic factors which will produce Reactive Oxygen Species (ROS), then cause damage to keratinocytes and ultimately cause inflammation (Beylot et al., 2013).

Concentration series of 20%, 25%, and 30% n-HFAH was dissolved with DMSO, which is an organic solvent that can dissolve both polar and non-polar compounds which can be used as an extract diluent to obtain extracts of various levels. DMSO as an organic solvent which is not bactericidal. Clindamycin 2% was chosen as a positive control because clindamycin is one of the most effective antibiotics for the treatment of anaerobic bacterial infections (Brooks et al., 2005).

Application of nHFAH and control groups at the acne location was done twice a day (morning and evening). The parameters for healing acne on rabbit skin were characterized by skin that was no longer reddish, had no swelling, and was not festering. The negative control group did not experience an improvement in the condition of acne where it only had a decrease in erythema diameter of only 0.39 cm, which means that DMSO did not have anti-acne activity during the test period. The reduction in erythema diameter of nHFAH 20%, 25%, and 30% showed a significant difference compared to the negative control group ($p < 0.05$) (**Figure 4**), meaning that 20%, 25%, and 30% of nHFAH had antiacne activity with the percentage 67.01%; 77.84%; and 85.84% respectively. Concentration 30% of nHFAH had antiacne activity that was not different from positive control group.

Antiacne activity of nHFAH is strongly suspected because of the flavonoid content in it, that is 6.0906 mgQE/gram. Flavonoids cause damage to the permeability of bacterial cell walls, microsomes and microsomes as a result of the interaction between flavonoids and bacterial DNA (Cushnie and Lamb, 2005).

It is possible that the use of alfalfa herbs as a natural medicine used to treat acne may be more effective if it is formulated with suitable carriers in the form of medicinal dosage forms, whether creams, gels, ointments, or in other medicinal dosage forms. Widely developed in the field of formulations to see its potential and safety before being applied to humans. Isolation of the types of

flavonoid compounds contained in the n-hexane fraction of the alfalfa herb ethanol extract is necessary to maximize the withdrawal of compounds suspected of having activity.

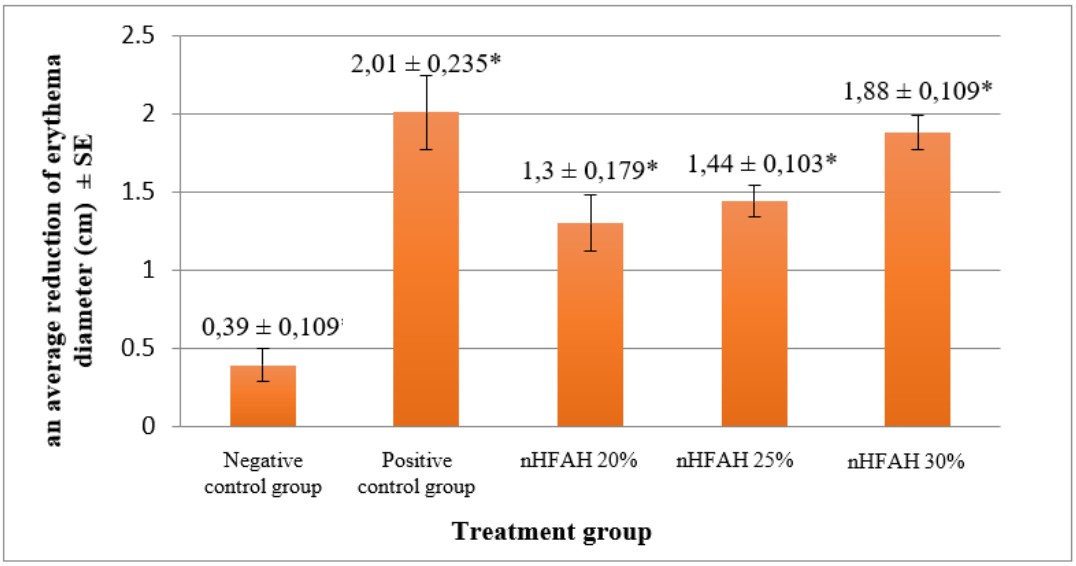

Figure 4. The average reduction in erythema diameter on rabbit back skin
Information : * = The treatment group had a decrease in erythema diameter which was different from the negative control group

CONCLUSIONS

In this work, the antiacne potential of nHFAH was studied. The results showed that nHFAH with concentration of 15%, 20%, 25%, 30% and 35% has antibacterial activity against *P. acnes* with an average value of diameter of inhibition area for each concentration were 7.30; 7.49; 7.75; 7.92 and 8.24 mm. Whereas nHFAH with concentration of 20%, 25%, and 30% exhibit antiacne activity with the percentage for each concentration were 67.01%; 77.84% and 85.84% respectively. Total nHFAH flavonoid levels were 6.09 mgQE/ gram. Based on the results, nHFAH has the potential to be further developed as a topical preparation to treat acne.

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