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RESEARCH ARTICLE

Anti-Alopecia Activity and Compound Content of n-Hexane Fraction of *Cyclea Barbata* Leaves

R. Mustarichie[1*], R. Hendriani[2], D. Gozali[3]

1. *Pharmaceutical Analysis and Medicinal Chemistry Department, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia.*
2. *Pharmacology and Clinical Pharmacy Department, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia.*
3. *Deparment of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Universiats Padjadjaran, Indonesia.*

*** Correspondence Author: R. Mustarichie**

Abstract

One of the plants empirically used by the Pasemah tribe in South Sumatra, Indonesia, as a hair grower is the green cincau (Indonesian) leaf *(Cyclea barbata* Miers.). Objective: The aim of this study was to determine the anti-alopecia activity and its compound content from the n-hexane fraction of the *C. barbata* leaves. Methods: Green grass jelly (*C. barbata* Miers.) Extracted with 96% ethanol and fractionated with water, n-hexane and ethyl acetate. The concentration of ethanol extract was diluted to 5%, 10%, 15%, 20% and 25% and all fractions were tested on rabbits by the modified Tanaka method. All data were analyzed statistically by ANOVA. Results: Test results showed that the ethanol extract of green grass jelly leaves had the activity of stimulating hair growth starting at a concentration of 15%. It was found that the n-hexane fraction had the best activity compared to other fractions and positive control (minoxidil 2%).Further tests using IR and LC-MS spectrophotometers found that the n-hexane fraction contained 1,4-dimethyl-1,2,3,6-tetrahydro-8H-pyrrolo[2',3':3,4]azepino[2,1-b]quinazolin-8-one. Conclusions: Ethanol extract and hexane fraction at concentrations of 15% and above were found to have anti-alopecia activity. The compound contained from the hexane fraction 1,4-dimethyl-1,2,3,6-tetrahydro-8H-pyrrolo[2',3':3,4]azepino[2,1-b]quinazolin-8-one] ($C_{22}H_{18}N_3O$) which was thought to cause properties hair growth.

Keywords: *Hair loss, Anti-alopecia, Green grass jelly, Cyclea barbata Miers, 1,4-dimethyl-1,2,3,6-tetrahydro-8H-pyrrolo[2',3':3,4]azepino[2,1-b]quinazolin-8-one.*

Introduction

Hair is a part of the human body that functions as a head protector from the surrounding environment and has an aesthetic function that supports a person's appearance. Hair is also a characteristic of ethnicity and serves as a symbol of social and cultural. It is only natural that severe hair loss can be annoying for those who experience it. The biggest impact that is felt due to hair loss is to reduce self-confidence that can interfere with the psychological sufferers [1].

The occurrence of hair loss is influenced by several factors both from within and outside the body. Factors in the body that cause loss is, as a result of systemic diseases, hormonal conditions, nutritional status, and genetic disorders, and external factors such as stimuli from the environment in the form of sunlight, pressure, and the use of cosmetic hair [2]. According to Noruka, about 95% of users of hair straightener in America and 53% of users in Africa, reported experiencing damage or loss and hair growth problems [3]. Some solutions to overcome hair loss have been found, one of which is by using the chemical drug Minoxidil that has been proven effective in dealing with hair loss.

However, Minoxidil has side effects of exfoliation during the first four months that cause skin discomfort [4]. To avoid these side effects, herbal ingredients can be an

alternative solution. Since ancient times, ancestors often used certain medicinal plants to treat diseases including treating hair health and growing hair that is still used today. One of the plants empirically used by the Pasemah tribe in South Sumatra as a hair grower is the green cincau (Indonesian) leaf (*C.barbata* Miers.). Cincau or green grass jelly plants are pharmacologically proven to have anti-hypertensive activity [5].

Green grass jelly leaves also have antioxidant activity with an IC50 value of 57.60 µg/m [6] Both of these activities are closely related to the mechanism of plants as hair growers. However, the activity of green cincau leaves as a hair growth stimulant until now has not been scientifically proven. This study proves the activity of green grass jelly leaves as a stimulator of hair growth, where green grass jelly leaves are extracted and fractionated, then extracts and fractions obtained will be tested for activities using the modified Tanaka (1980) method for experimental animals, rabbits [7, 8].

Materials and Methods

Materials

Green grass jelly leaves, 7 male rabbits, ethanol 96% (Merck), ammonia (Merck), chloroform (Merck), mercury (II) chloride (Merck), hydrochloric acid (Merck), potassium iodide (Merck), bismuth nitrate (Merck), nitric acid, magnesium (Merck), amyl alcohol (Merck), iron (III) chloride (Merck), 1% gelatin, ether, vanillin- sulfuric acid (Merck), anhydrous acetic acid (Merck), concentrated sulfuric acid (Merck), sodium hydroxide (Merck, toluene (Merck), sodium carboxymethyl cellulose (Sigma-Aldrich), Minoxidil 2% (Regrou). Dragendroff reagent (a mixture of solution Bi (NO3) 3.H2O in HNO3), Lieberman-Burchard reagent (a mixture of anhydrous acetic acid and concentrated sulfuric acid), Mayer reagent (a mixture of HgCl2 solution in water and KI in water), magnesium (Mg) powder (Merck), vanillin sulfate. Unless stated otherwise, all chemicals were analytical grades.

Methods

This research was carried out through several stages of work including Collection of green grass jelly leaves obtained from Sleman, Yogyakarta, Mid Java and determined at the Taxonomy Laboratory. Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran; green grass jelly leaves were processed to become simplicia, extracting green grass jelly leaves with 96% ethanol solvent by maceration, phytochemical screening of simplicia and green grass jelly extract, inspection of general standard parameters of grass jelly extract, testing of hair growth stimulant activity from ethanol extracts of green grass jelly in male rabbits using the modified Tanaka method, Liquid-liquid extraction of green grass jelly ethanol extract, testing the hair growth stimulant activity from the water fraction, n-hexane fraction and ethyl acetate green grass jelly leaves in male rabbits, and last the obtained data were analyzed statistically by the Analysis of Variance (ANOVA) method.

Results and Discussion

Processing of Plant Material: Green grass jelly leaves obtained were washed using running water, then wet sorting, then drained and dried protected from direct sunlight. Next, the plants were chopped and stored in a dry and tightly closed container [9, 10].

Extraction

The extract was made by cold maceration using ethanol 96%. The amount of sample was carefully weighed and then moistened with 96% ethanol for about 15-30 minutes, after which the solvent was added until the sample was submerged to the surface. Maceration was carried out for 3 x 24 hours and every 24 hours the solvent was replaced with a new one, during which immersion was stirred occasionally. Macerate was accommodated, put together, then concentrated using a rotary evaporator at 65 rpm at 50^0 C until a thick extract was obtained and then evaporated on a water bath to a constant weight [11, 14]. The viscous ethanol extract yield was found 16.44 % W/W.

Standard Parameters of Green Grass Jelly Extract

Examination of general standard parameters of extracts was carried out to evaluate the quality of extracts obtained based on the applicable pharmaceutical product requirements by comparing the results obtained with the applicable standards.

The general standard parameters of extracts carried out included organoleptic extract criteria, extract yield, ash content, moisture content, and TLC profile [15, 16].

The results of the standard of green grass jelly extract can be seen in Table 1.

Table 1: General standard parameters of green grass jelly ethanol extract

Parameters	Results
Moisture content	8.6 %
Total ash content	6.8%
Acid Insoluble Ash Content	0.54%

Moisture content was the amount of water that was contained and absorbed, the purpose of evaluating water content was to provide a limit or range of water content that was allowed in the extract to avoid the rapid growth of fungi in the extract. The examination of the water content of the extract was carried out by the toluene distillation method. Natasha [17] stated that the average water content in green grass jelly extract was 5.2%.

We found the average determination of water content of green grass jelly extract obtained by 8.6% (w/w). This difference possibly due to the different origins of the sample examined. Examination of total ash content and acid insoluble ash content aimed to provide an overview the mineral and inorganic content of green grass jelly leaves. Examination of total ash of content was carried out by entering 2 grams of crushed extract which has been incandescent at a temperature of 600 °C until the charcoal runs out. Next, it must be cooled and a fixed weight is obtained. Determination of ash, acid insoluble ash for plant extract parameters was also listed in Indian Pharmacopeia [18].

The total ash content found was 6.8% and the acid insoluble ash content was 0.54% which was a slight difference from other researches who mention that it contained 14.28% and 0.54% for total ash content and the acid-insoluble ash respectively [17]. Phytochemical Screening: Phytochemical screening of plants was carried out on ethanol extracts to identify groups of compounds in green grass jelly leaves. The results of phytochemical screening can be seen in Table 2.

Table 2: Phytochemical screening of green grass jelly extract

Secondary metabolites	Result
Alkaloids	+
Flavonoids	+
Saponins	+
Polyphenols	+
Tannin	+
Quinone	-
Monoterpene and Sesquiterpene	+
Steroid and Triterpenoid	+

Notes: +: detected; - : not detected

Shodiq [19] mentioned that green grass jelly extract containing steroids/terpenes, alkaloids, flavonoids, tannins, glycosides, and saponins, but as seen above we have tried several times did not detect but steroid metabolites were not detected. Other researchers mentioned that the *c. barbata* leaf extract contained flavonoids, glucosides and terpenoids [20], and other phytochemical screening of powder and 50% and 96% ethanol extract showed that tall the tested samples contained an alkaloid, flavonoids, saponins, tannins, steroids/triterpenoids [21].

These slight differences in phytochemical results might be due to the different origins of the sample examined. Hair growth stimulating the activity of Green Grass Jelly ethanol extract: Testing the hair growth stimulant ethanol extract activity of green grass jelly was carried out to find out whether the ethanol extract of green grass jelly extract has hair growth stimulating activity. In this test, it could also be seen at what concentration the extracts start to provide activity and how the activity of hair growth stimulant extracts of green grass jelly extract compared with minoxidil as a positive control. The ethanol extract of green grass jelly leaves was done by diluting the extract into several concentrations namely, 5%, 10%, 15%, 20%, and 25%.

The extract was diluted using aquadest with the help of NaCMC to increase the solubility of the extract.

NaCMC was used with a concentration of 0.5% where this material serves as a suspending agent. Besides, the addition of Na-CMC will thicken the extract preparations so that when testing the activity of hair growth stimulants, so the extract would be in longer contact with the skin and absorption of extracts to the hair follicle was more maximal [22].

Test animals: Test animals used in this study were male rabbits aged 3-5 months with a weight of 1.5 kg. The number of rabbits used is calculated based on the Federer formula [23]. Ethical approval for this study was issued by Research Ethics Committee-Universitas Padjadjaran no. 01/UN6.KEP/EC/2018. Consideration of the selection of male rabbits as test animals compared to female rabbits was because hormonal male rabbits were more stable to minimize the influence of hormonal and psychological changes in animals on hair growth [24].

The age of rabbits used ranges from 3-5 months because at this age rabbits are classified as adults so that the hair that grows was real hair or real hair. Adult rabbits have a good physiological function compared to young or old rabbits, thus minimizing their effect on research.

Before testing the test animals were quarantined for 7 days to adjust to the test environment and ensure that the test animals used were not sick. Rabbits used in this test were 3 animals where the backs of each rabbit were divided into 8 test areas, namely Extracts 5%, 10%, 15%, 20%, 25%, Negative Control: Na-CMC, Positive Control: Minoxidil 2%, Normal Control: Not treated. A recent research stated that topical administration of minoxidil could induce secretion of vascular endothelial growth factor (VEGF) which was an effector on hair growth [25].

Minoxidil grows hair by accelerating the duration of the telogen phase so that the anagen phase becomes longer and the size of the hair follicles increases. The positive control treatment was used as a comparison of test material in the activity of stimulating hair growth. Test material, positive and negative controls were given topically or applied to the rabbit's back skin twice a day.

The parameters measured in testing the growth activity of green grass jelly extract hair growth were the length of rabbit hair on the 3rd, 6th, 9th, 12th, 15th, and 18th day. The rabbit hair was measured in 6 strands using calipers. The use of calipers as a measuring instrument was done because of its accuracy of 0.05 mm so that measurements were more accurate and more reliable. The results of measurements of rabbit hair in the ethanol extract activity of green grass jelly can be seen in Fig. 1.

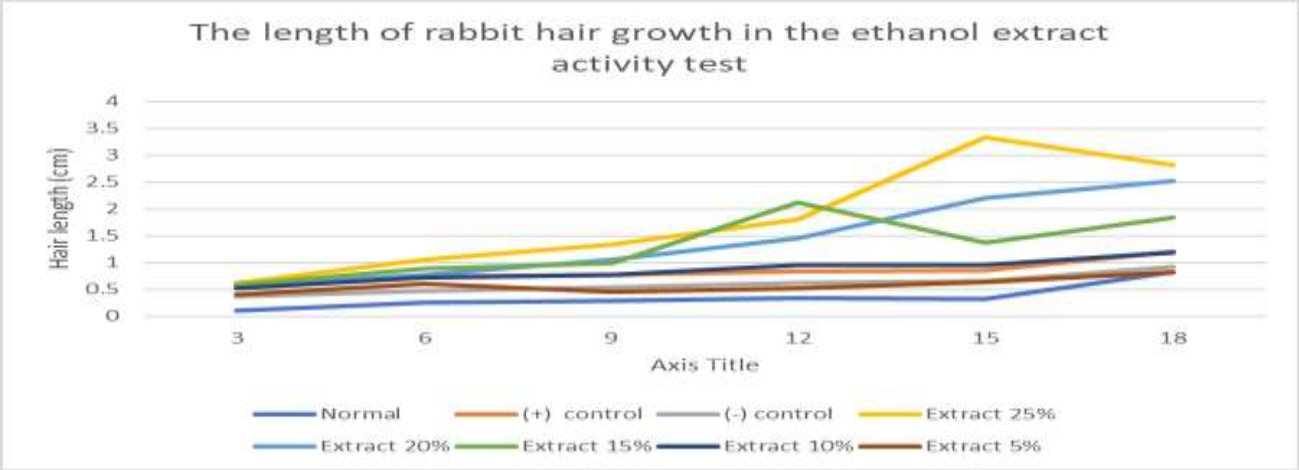


Fig. 1: Graph of test results of hair growth stimulant ethanol extract the activity of green grass jelly

The graph in Fig.1 was the average length of three rabbits on days 3, 6, 9, 12, 15, and 18. Based on the graph above it could be seen that each day the length of rabbit hair increased, rabbit hair grows in all groups.

In the hair measurement data, the longest rabbit hair growth reached 2.765 cm found in the treatment group of green grass jelly extract 25% on the 18th day of testing. The graph above showed that on the 3rd day to

the 9th day, differences in hair length between groups had not been seen. The difference in hair length between groups began to be seen clearly on the 12th day until the 18th day. Statistical analysis was performed to see differences in hair growth stimulant activity between groups so that the dose of green grass jelly leaf extract can be determined which gives hair growth stimulant activity. Statistical analysis was performed using IBM SPSS software version 22 [26].

Before further testing, the data was first tested for normality and homogeneity to determine further tests that would be used. The normality test was done by the Shapiro-Wilk method [27] and the homogeneity test uses the Levene Statistics method [28]. In the H_0 hypothesis normality test, the population of rabbit hair length data was normally distributed, while H_1, the rabbit hair length data population was not normally distributed. Shapiro-Wilk normality test results showed a significance of> 0.05 which indicates H0 was accepted so it was stated that the data were normally distributed.

Homogeneity testing of data was done by Levene statistical test where H_0 stated that the population of rabbit hair length data was homogeneously distributed and H_1 stated that the rabbit hair length data population was not homogeneously distributed. The analysis showed a significance of> 0.05 which showed that H_0 was accepted so that it was stated that the data was homogeneously distributed. Based on the ANOVA analysis, the significance value obtained <0.05 which indicated that there were differences due to the treatment given. However, ANOVA had not yet shown the extent to which differences were given between groups.

Therefore, further tests were conducted using the LSD (Least Significant Difference) method. The results of the LSD analysis showed that there were significant differences (<0.05) between the extract groups of 15%, 20%, and 25% with negative controls and normal controls. It was found that the extract of green grass jelly leaves at a concentration of started from 15% had an activity to stimulate hair growth.

Hair Growth Stimulating Activity for Water, n-hexane, and Ethyl Acetate Fractions

After the activity of green grass jelly ethanol extract was known, then it was continued by testing the activity of green grass jelly fractions to determine the most active fraction among the water, n-hexane, and water fractions. The testing of hair growth stimulant activity for the fraction was carried out using a similar testing method as ethanol extract testing using the modified Tanaka *et al*. with the following samples: water, ethyl acetate, and n-hexane fractions, positive, negative, and normal controls. Table 3 and Fig. 2 represented this test.

Table 3: Measurement results of the average length of growth of rabbit hair

Groups		The hair length on the day- (cm)					
		3	6	9	12	15	18
Positive control	Rabbit 1	0.354	0.394	0.617	0.740	1.380	1.450
	Rabbit 2	0.318	0.389	0.690	0.850	1.340	1.265
	Rabbit 3	0.315	0.446	0.645	1.150	1.234	2.038
	Rabbit 4	0.320	0.427	0.713	0.795	1.150	1.510
	Mean	0.327	0.414	0.666	0.884	1.276	1.566
SD		0.013	0.026	0.066	0.132	.0,178	0.493
Water fraction	Rabbit 1	0.199	0.361	0.720	1.065	1.350	2.130
	Rabbit 2	0.350	0.435	1.120	0.935	1.785	2.150
	Rabbit 3	0.455	0.748	1.225	1.467	1.930	2.290
	Rabbit 4	0.395	0.575	0.865	1.158	1.560	2.075
	Mean	0.350	0.530	0.983	1.156	1.656	2.161
SD		0.156	0.225	0.386	0.329	0.487	0.643
Ethyl acetate fraction	Rabbit 1	0.290	0.399	0.763	0.985	1.410	1.951
	Rabbit 2	0.300	0.390	0.985	1.250	1.485	1.875
	Rabbit 3	0.330	0.612	1.130	1.279	1.820	2.165
	Rabbit 4	0.315	0.609	0.695	0.970	1.570	2.070
	Mean	0.309	0.503	0.893	1.210	1.571	2.015
SD		0.128	0.229	0.363	0.459	0.489	0.553
n-hexane fraction	Rabbit 1	0.290	0.635	0.965	1.360	1.735	2.250
	Rabbit 2	0.403	0.655	1.150	1.168	1.515	2.400
	Rabbit 3	0.442	0.675	0.930	1.356	1.835	2.310
	Rabbit 4	0.218	0.720	0.900	1.420	1.851	2.250

	Mean	0.338	0.671	0.986	1.326	1.734	2.303
SD		0.120	0.141	0.190	0.413	0.359	0.229
Negative control	Rabbit 1	0.120	0.257	0.455	0.690	0.863	0.985
	Rabbit 2	0.080	0.150	0.480	0.635	0.600	0.780
	Rabbit 3	0.085	0.175	0.465	0.593	0.783	1.015
	Rabbit 4	0.075	0.185	0.400	0.573	0.530	0.725
	Mean	0.090	0.192	0.450	0.623	0.694	0.876
SD		0.107	0.173	0.269	0.289	0.318	0.325
Normal control	Rabbit 1	0.075	0.382	0.373	0.610	0.920	1.088
	Rabbit 2	0.099	0.159	0.341	0.650	0.445	0.820
	Rabbit 3	0.080	0.423	0.415	0.625	0.725	1.766
	Rabbit 4	0.130	0.230	0.441	0.672	0.850	1.188
	Mean	0.096	0.298	0.393	0.639	0.735	1.215
SD		0.154	0.132	0.269	0.239	0.327	0.398

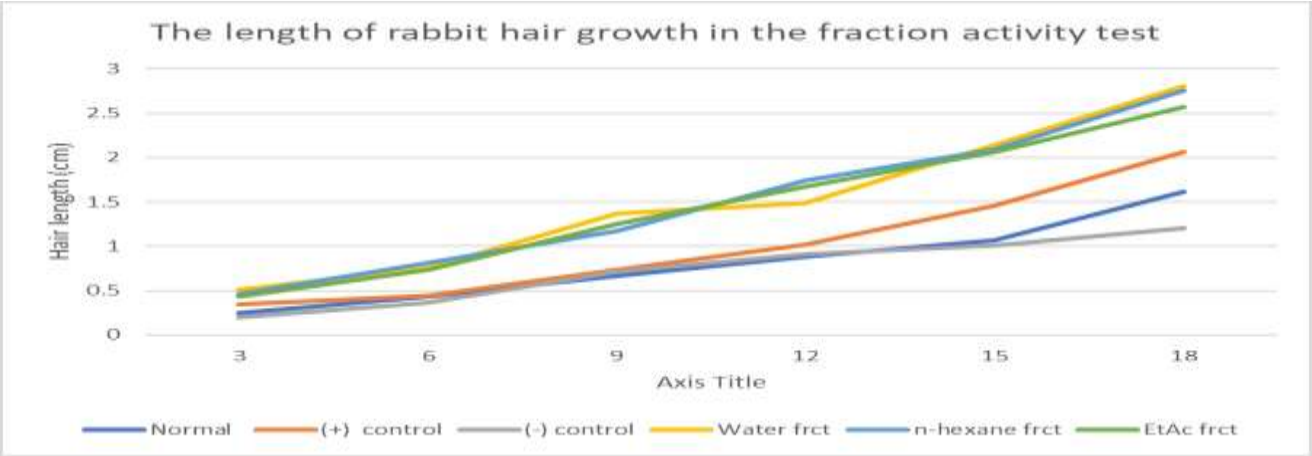


Fig.2: Rabbit hair length in the fraction activity test

In the Fig. 2 it could be seen that the fraction that showed the highest activity was the n-hexane fraction. The most active fraction after n-hexane was the water fraction then the ethyl acetate fraction. In this graph, it was shown that all green grass jelly leaf fractions had better hair growth stimulating activity than positive controls (minoxidil 2%). In the normal and negative control test groups the growth of rabbit hair was almost the same.

Results of Statistical Analysis of Hair Growth Stimulating of Ethanol Extract Fractions

Data from rabbit hair length measurements were tested for normality and homogeneity first. The normality test was performed using the Shapiro-Wilk method, while homogeneity was tested by the Levene Statistics method. The results of normality and homogeneity test data were found significance> 0.05 so that it could be stated that the data were normally distributed and homogeneous. Furthermore, the data were analysed using ANOVA, the results of the analysis showed significance <0.05 so it was stated that there were significant differences in each treatment group.

Further tests were conducted to see the extent of differences between treatment groups. Further tests were carried out by the LSD method. It was found n-hexane fraction had a significance <0.05 indicating that there was a real difference between the n-hexane fraction with negative, normal, and positive controls so that it was stated that the green grass jelly leaves n-hexane fraction showed activity hair growth stimulant and its activity was better than positive control (minoxidil 2%).

Determination of Compound Contained in the N-hexane Fraction from Ethanol Extract *C.barbata*

To find out the chemical content of the hexane fraction that might cause hair growth properties. The fraction of n-hexane from liquid-liquid extraction was then separated using Vacuum Liquid Chromatography (VLC) and Column Chromatography (CC). In VLC the stationary phase used was Silica Gel 60 and the mobile phase used was gradient n-hexane: ethyl acetate. Sub fractions obtained from the VLC were then further analyzed using TLC with the developer of n-hexane: ethyl acetate (8: 2).

Eleven sub-fractions were obtained which were then monitored their chromatogram pattern by TLC.

Sub fractions were grouped based on their separation pattern. It was found that in the combined sub-fraction group no. 23-38 using the separation process using Preparative Thin Layer Chromatography (PTLC) with Silica Gel GF254, and the mobile phase of n-hexane: ethyl acetate (8: 2), obtained compound bands separate from other bands. This band was scraped and dissolved in methanol, filtered and crystallized. In this study, the compound taken was a compound which was a separate and bright band from another band as seen in Fig. 3, so that it could be assumed that the compound or isolate obtained was the main compound of the n-hexane fraction..

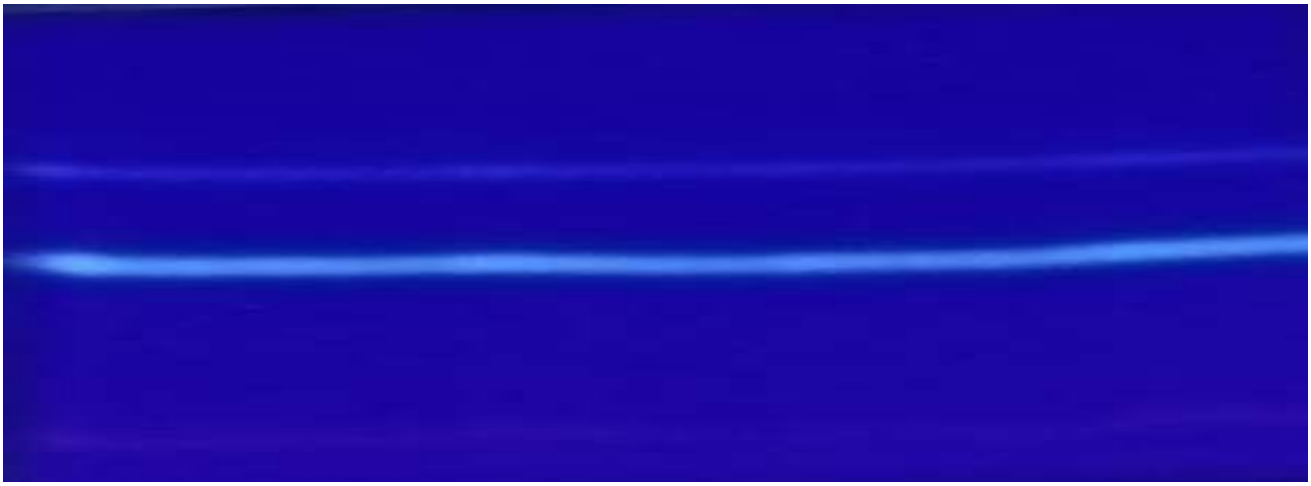


Fig.3: Results of preparative TLC of the combined sub-fraction group no. 23-38

The resulting isolates were identified by two methods, namely infrared spectroscopy, and liquid chromatography-mass spectroscopy. But before further identification, the group of isolate compounds was identified first using TLC with a variety of spotting views. Spotting viewers used were dragendorf, citroborate, KOH, anisaldehyde, $FeCl_3$, and Lieberman-Burchard. Positive results were shown by the plate which was given the appearance of the dragendorf spot, which produced an orange spot which meant the isolate was classified as an alkaloid compound.

The results of infrared spectrophotometry (see Fig. 4) showed that the isolate contains NH strain at wave number 3450.66 cm^{-1}, CH aliphatic strain at wave number 2924.09 cm^{-1}, aliphatic CH at wave number 2854.66 cm^{-1}, bending C = O at wave number 1734.01 cm^{-1}, and C = C at wave number 1631.78 cm^{-1}. After identification using an IR Spectro instrument, then proceed using the Liquid Chromatography-Mass Spectrophotometry (LC-MS) instrument.

The LC-MS analysis results in one chromatogram peak with a retention time of 3.24. (See Fig. 5) The spectrum results showed that the isolate was pure or there was only one compound. This result was directly proportional to the results of the purity test using two-dimensional TLC, where there was only one single stain. The peak at a retention time of 3.24 showed the peak of the main compound (M-H) + at 340.1442 m/z and has the presumption of the chemical formula $C_{22}H_{18}N_{3}O$. After being matched with the results of IR spectroscopy instruments and it turned out the results obtained were following the compounds published by Wei *et.al* [29].

Seen from the similarity of NH spectral infrared spectroscopic spectrum wave numbers at wave numbers 3431 and 3450.66, strain CH at 2925, 2856, 2924.09 cm^{-1}, CH, 2854.66 cm^{-1}, strain C = C 1674, 1631.78 cm^{-1} and bending C = 2925, 2856, 2924.09 cm^{-1}, CH, 2854.66 cm^{-1}, strain C = C 1674, 1631.78 cm^{-1} and bending C = O at wave number 1734.01 cm^{-1} ; it concluded that the compound obtained from this study was 1,4-dimethyl-1,2,3,6-tetrahydro- 8H- pyrrolo [2',3':3,4] azepino [2,1-b] quinazolin-8-one which had boiling point at 822.52 ^{o}K, melting point at 639.87 ^{o}K , and Gibbs energy of 615.7 kJ/mol [30].

Fig. 6 shows the structural formula of 1, 4-dimethyl-1, 2, 3, 6-tetrahydro-8H-pyrrolo [2', 3':3, 4] azepino [2, 1-b] quinazolin-8-one] ($C_{22}H_{18}N_3O$) drawn by ChemDraw 15.0.

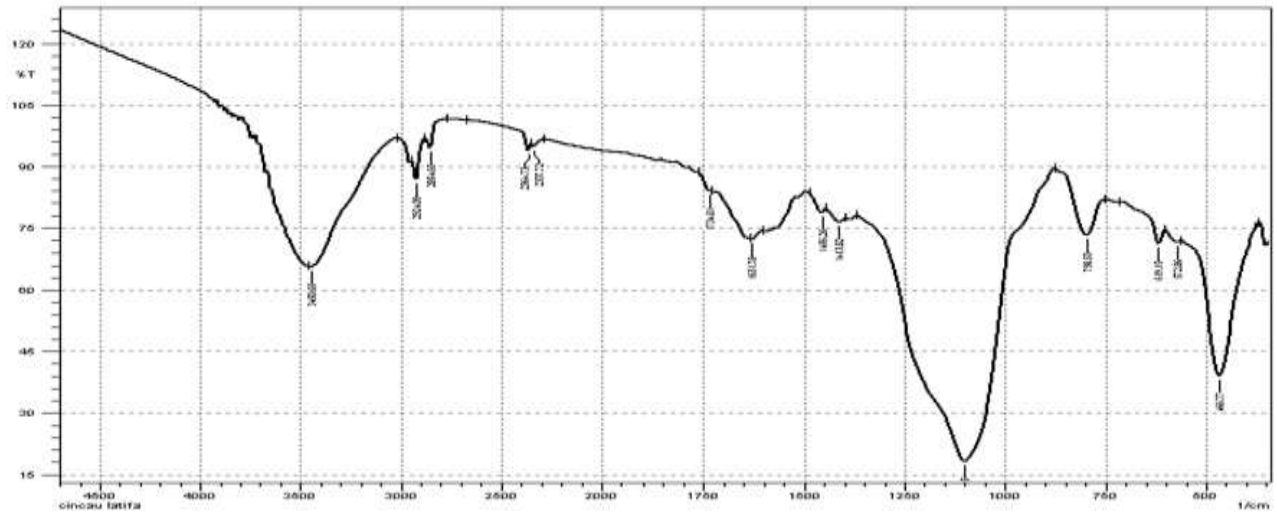
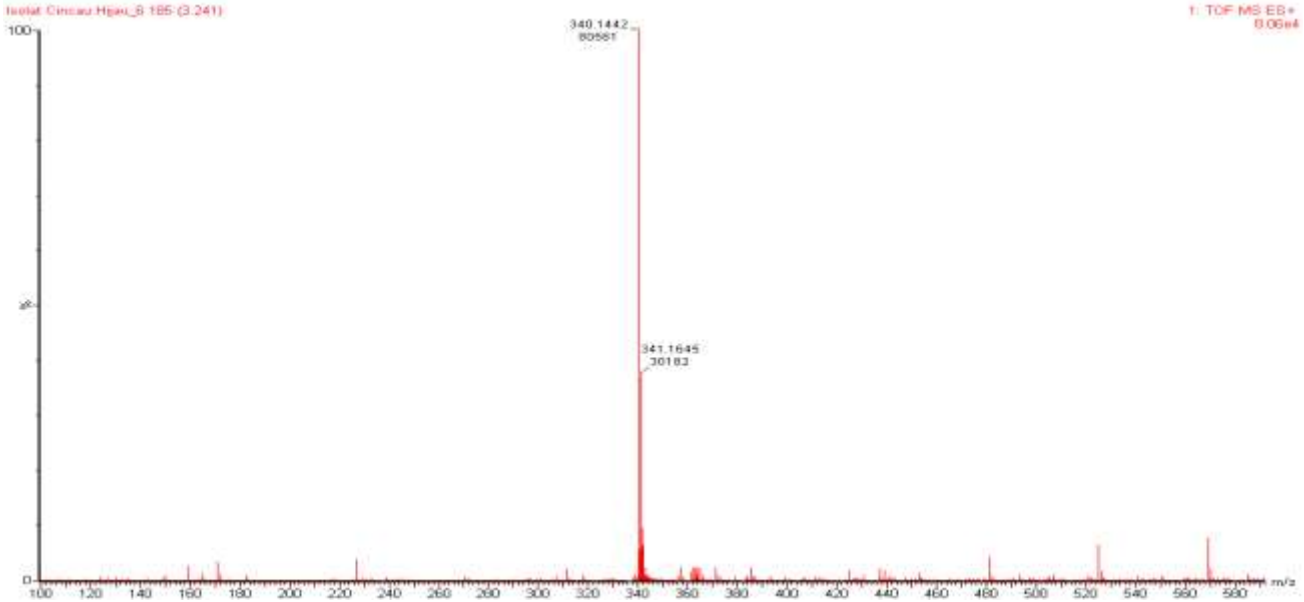


Fig. 4: IR- spectra of *C.barbata* isolate



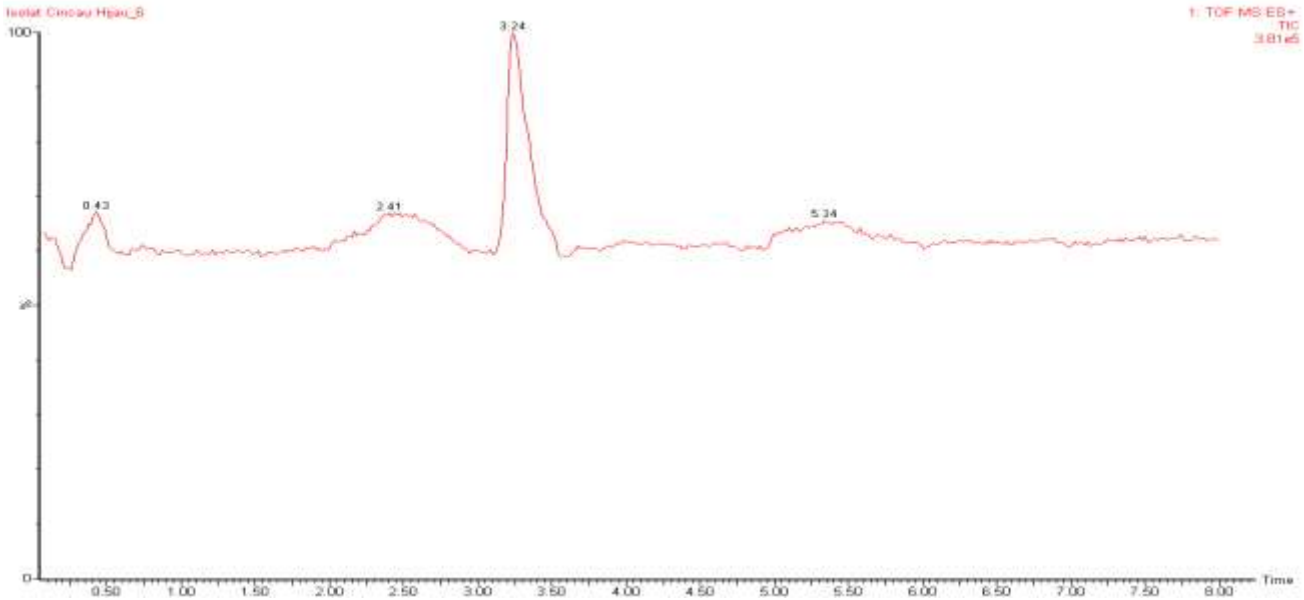
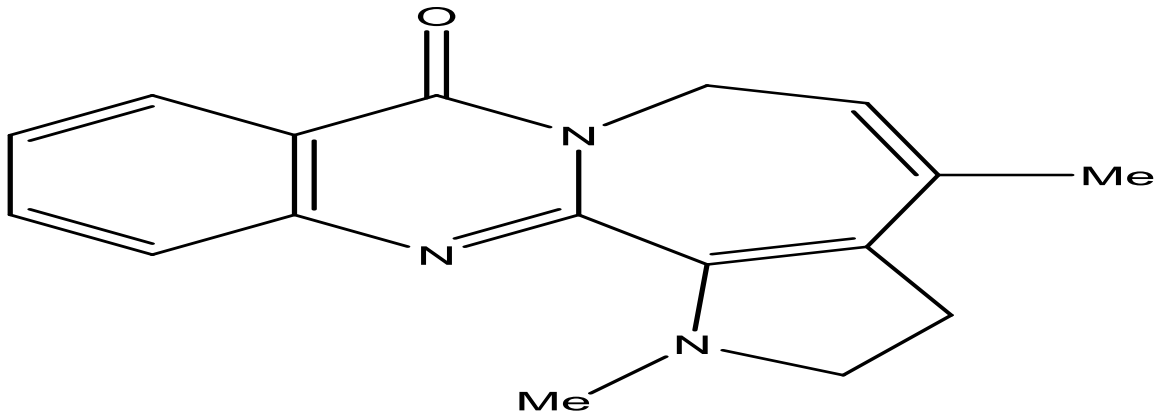


Fig. 5: LC-MS Spectra of n-hexane fraction of *C. Barbata*



1,4-dimethyl-1,2,3,6-tetrahydro-8*H*-pyrrolo[2',3':3,4]azepino[2,1-*b*]quinazolin-8-one

Fig. 6: Prediction structure of 1,4-dimethyl-1,2,3,6-tetrahydro-8*H*-pyrrolo[2',3':3,4]azepino[2,1-*b*]quinazolin-8-one (Chemdraw Profesional 15.0 drawing)

Conclusions

Based on research results testing the hair growth stimulant activity of ethanol extract and green grass jelly leaf fraction, it can be concluded that the ethanol extract of green grass jelly leaves has a hair growth stimulant activity starting at a concentration of 15%. In testing the growth stimulant activity of the green grass jelly fraction it was shown that the water, n-hexane, and ethyl acetate fractions of the green grass jelly had the activity of stimulating hair growth. The fraction that showed the best activity was the n-hexane fraction compared to other fractions and positive control (minoxidil 2%).

IR spectrophotometer and LC-MS tests found that the n-hexane fraction contained 1, 4-dimethyl-1, 2, 3, 6-tetrahydro-8H-pyrrolo [2', 3':3, 4] azepino [2, 1-b] quinazolin-8-one which possibly causing its activity.

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