Isolation of Compound Agents Anti bacteria from Bandotan (Ageratum conyzoides L.) Plants Using Bioassay Guided Fractionation Method.

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ISOLATION OF COMPOUNDS AGENTS ANTI BACTERIA FROM BANDOTAN (*Ageratum conyzoides L*) PLANTS USING BIOASSAY GUIDED FRACTIONATION METHOD

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ABSTRACT

Objectives: Bandotan (Ageratum conyzoides L) herbaceous plants have been empirically used for wound medicine and digestive disorders. The treatment is related to its pharmacological activity as an antibacterial. the purpose of this study was to isolate responsible compounds as antibacterial

Methods: The research phase is extraction using maceration method, fractionation by liquid vacuum column chromatography followed by TLC, the same profile of TLC combined and then tested for its activity against Esceresia coli and Staphylococus aureus.

Results: The results of the antibacterial activity test showed that the inhibition of ethyl acetate extract was 14 mm, the combined fraction 4 was 16 mm and subfraction gab 4 was 14.5 mm at S aureus, whereas in E coli ract the ethylacetate extract was 11 mm, the combined fraction 4 was 13 mm and sub FG4b of 10 mm. The Identification with using UV-Vis, IR and GCMS spectrophotometers showed the presence of coumarin compounds, 2 kromen compounds (precocene II and 7-methoxy-2,2-dimethyl-6-vinyl-2H-kromen), 2 palmitic acid derivative compounds (hexadecanoic acid and 9,12-octadecanoic acid), triterpenes (neopitadiene) and methyl-5,11,14,17-eikosatetraenoic compounds

Conclusions: The minimum inhibition of combined 4b subfraction against S. aureus is 25 mg / ml and 50 mg / ml for E. coli. Identification compounds of bandotan herbs was contain compounds called coumarin, kromen, fatty acids and 5,11,14,17-methyl eicosatetraenoate

Keywords: Ageratum conyzoides, isolation, Bioassay guided fractionation

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INTRODUCTION

Empirically medicinal plants have been used by one third of the world's population. One of these plants is bandotan (*Ageratum conyzoides*), including famili Asteraceae. This plant is a wild plant and as a weed. Bandotan contains compounds at alkaloid, tannins, flavonoids, steroids, terpenoids, coumarins and essential oils, with properties as medicine for wounds, hives, flu, fever, diarrhea, intestinal inflammation, and rheumatism (8). Methanol, ethanol and water extract from bandotan herbs have antibacterial activity (10, 11), until now, the search for antibacterial active compounds from natural materials is still being carried out considering that synthetic drugs such as existing antibiotics can cause problems with the emergence of resistance and not all antibiotics can be given to children (6). Therefore the search for active ingredients based on natural ingredients is unlimited, including the search for new compounds that have pharmacological properties such as

antibacterial. Some researchers have succeeded in developing the use of bandotan plants, including natural insecticides (2), biolarvasida (9), antimalarials (4), antifungals (14), and as antibacterial (12), isolation of bandotan plants produced kromen compounds, hexamethoxiflavone compounds and 7-methoxy-2,2-dimethyl-6-vinyl-2H-kromen (7), With the information on the efficacy of the bandotan plants, further isolation needs to be done to find out which compounds are responsible as anti-bacteria

METHODS

Material

Herbs bandotan, hexane, ethyl acetate (EtOAc), methanol (MeOH), and chloroform (CHCl3), TLC plate, chromatography column, Shimadzu Double Beam UV-VIS spectrophotometer, FT-IR spectrophotometer, and S. bacterial mass spectrum chromatography. *S. aureus* and *E. coli*.

Research methods

This research is an experimental laboratory research with exploratory methods of compounds based on solvents and antibacterial activity. This research is carried out through 4 stages, namely extraction, fractionation, compound identification and activity testing.

Prosedure

1. **Sample extraction**
   The extraction method used is the maceration method with different polarity solvents. A total of 500 grams of dried samples are macerated with hexane (3 x 24 hours) to remove the fat and oil contained in the sample. After filtration, the residue was macerated again with ethyl acetate (3 x 24 hours), the filtrate was concentrated using a rotary evaporator at a temperature of 40°C - 50°C so that a crude ethyl acetate extract was obtained. Then followed by phytochemical screening. The crude extract of ethyl acetate was then fractionated by Thin Layer chromatography with eluent namely chloroform: methanol (9: 1). Both eluent blends were chosen which gave the best separation. Then the eluent is used for column chromatography. Each fraction was carried out by anti-microbial test. The fraction that has the highest antimicrobial activity is then fractionated again and followed by an anti- microbial test. Sub-fraction which has antibacterial activity, then continued identification using UV-Vis, IR, and GC-MS spectrophotometers.
2. **Phytochemical Test**
   a. Alkaloids
      0.3 g of the sample was dissolved in 10 ml of chloroform and then filtered. The filtrate from the filtering was added a few drops of 2M H_2SO_4, then shaken so that it formed 2 layers. The (colorless) acid layer was pipetted into a test tube and added reagents Mayer, Dragendorff, And Wagner.
   b. Flavonoids and Phenolic Compounds
      A total of 0.5g of sample added 30% methanol until submerged then heated. The filtrate is added with 10% NaOH and H_2SO_4.
   c. Saponin
      A total of 0.5 g of sample in a goblet are added 50 ml of hot water and boiled for 5 minutes, then filtered. A total of 10 ml of the resulting filtrate was put into a closed test tube for 10 minutes, the formation of a stable foam within 5 minutes indicated the presence of saponin.
   d. Tanin
      A total of 0.5 g of sample in a goblet are added 50 ml of hot water and boiled for 5 minutes, then filtered. 10 ml of filtrate added with $FeCl_3$ 1%.
   e. Triterpenoid-Steroids
      A total of 0.5 g of sample was added 5 ml of ethanol and then heated and filtered. The filtrate is evaporated then ether is added and shaken. The ether layer was separated and added Liebermenn-burchad reagent (3 drops of anhydride acetic acid and 1 drop of H_2SO_4 solid).

3. Fractionation and Identification of Active Compounds Thin Layer Chromatography (TLC)
Samples are bottled using a capillary pipe on the TLC plate that has been marked with a starting line and finish line. The solvent is put in a closed vessel and left until the steam is saturated, then the KLT plate is put in a vessel and closed again. After the solvent rises and reaches the finish line, the TLC plate is immediately removed and dried. Dry TLC plates are then observed under ultraviolet light at 254 nm wavelength. Determination of the best eluent is done by comparing the number of spots and separation patterns resulting from several solvent systems used. Eluent which produces the highest number of spots and the best separation pattern is then used in the fractionation process. Chromatography A sample column of 2 grams is dissolved in an ethyl acetate solvent and then put into a chromatography column. The best determined eluent is poured gradually into the chromatography column. The fraction that comes out of the chromatographic column is accommodated in a test tube each of 5 ml. After the fractionation process is complete, then proceed at the stage of combining fractions using TLC. Fractions with the same spot pattern was combined into one fraction.

4. **Antibacterial Activity Test**

Preparation Media and Bacterial Suspension

forty g of TSA (tryptone soy agar) were dissolved in 1 liter of distilled water and then heated and stirred using a magnetic stirrer until homogeneous. After the homogeneous solution is put into a test tube as much as 20 ml, then covered with cotton. The media was sterilized with an autoclave at a pressure of 1.5m with a temperature of 121 0C for 15 minutes. The tubes are tilted before hardening and left for 24 hours. This media is used for the growth of *S. aureus* and *E. coli* bacteria.

Antibacterial Test

Bacterial cultures obtained were then diluted using the Mc method. Farland to obtain test bacteria with a concentration of 1 x 107. A total of 100 µl of bacteria were diluted, then poured in a petri dish containing TSA media and spread with spreader glass rods and then allowed to solidify. After solid, the media is perforated with a pipette ± 5.5mm in diameter. Samples that were diluted with ethyl acetate solvents were then dropped into a well as much as 20 µl with a concentration of 300 mg / ml. The test media were then incubated for 24 hours at 37 0C then their antibacterial activity was observed, as standard, chloramphenicol was used with a concentration of 0.4mg / mL

Determination of MIC (Minimum Inhibition Consentration).

Determination of MIC values was carried out after it was found that the ethyl acetate fraction of the leaves of the bandotan plant had antibacterial activity. The culture of the test bacteria as much as 1 ose was put into 10 ml of TSB liquid media and then incubated in a swaying incubator for 24 hours at 37 temperatures. 50 µl of bacterial culture was then mixed into 20 ml of ± 45 0C TSA media then left until solidified. The media so that the solid has been punched with the base of the drop pipette (± 5.5 mm in diameter). The concentration variations used to determine MIC are 500, 250, 100, 75, 50, 25, 10, and 5 mg / ml. A total of 50 µl of the sample was inserted into the TSB media hole that had been incubated with the test bacteria, then re-incubated for 24 hours at 37 0C. After incubation is complete, observations are made for bacterial growth. The concentration of ethyl acetate fraction of bandotan plants which caused bacteria not to grow on the subculture was the concentration chosen as the MIC value.

RESULTS

Extraction, phytochemical test and fractionation of Bandotan Leaves

Extraction was used maceration method, with n-hexane and ethyl acetate as solvent. The resulting extracts were 0.076% and 0.07%, respectively. Extraction with n-hexane was intended to separate impurities such as fat and nonpolar compounds which can interfere with fractionation by Thin Layer chromatograpy (TLC). Next, phytochemical tests were carried out with chemical reagents (table 1) and extracts were fractionated using TLC with silica gel GF254 as the stationary phase and chloroform: methanol (9: 1) as the mobile phase (table 2).

Table 1. Phytochemical test of Ethyl acetate extract from bandotan leaves

compounds	Chemical Reagens	Result	Literatur	Description
Alkaloid	wagner	No deposit	brown Deposit	-
	dragendorf	yellow	Red orange	-
	Mayer	No deposit	White deposit	-
Flavanoid	murexid	red	red	+
Tanin	FeCl3	blue	blue	+
Terpenoid/steroid	Liebermenn-burchad	red	red	+
Saponin	foam test	Constant foam in 10 minutes	Foam does not disappear in 10 minutes	+

Description: (+) ethyl acetate extract contains a class of compounds
(-) ethyl acetate extract does not contain a class of compounds

Table 2. The Results of fractionation of ethyl acetate fraction

spot	Color on visible light	Color in light UV254	Rf	Weigt (gr)
1	Blackish green	Blackish red	0,96	14.9
2	Dark green	Blackish red	0,87	22.51
3	Light green	Dark green	0,73	20.12
4	Light green	Dark green	0,58	19.32
5	Light yellow	Light yellow	0.46	5.29
6	Colorless	Light yellow	0,36	4.23
7	Colorless	Light yellow	0,21	1.87
8	Colorless	Light yellow	0.14	1.07

The separation results show the more polar fraction, the smaller the weight. The greatest weight in fraction 2 (22.51 mg), while the lowest weight in fraction 8 (1.07 mg). All fractions were tested for anti-bacterial activity

Antibacterial Activity Test

Antibacterial activity test was carried out on ethyl acetateextract, eight fractions produced, and chloramphenicol as standard antibacterial (Table 3).

Table 3. Observation of inhibitory zones on S aureus and E coli bacteria from ethyl acetate extracts and their fractions

sample	inhibition zone (mm)					
	S. aureus			*E. coli*		
	repetition			repetition		
	1	2	average	1	2	average
etil asetat solven	-	-	-	-	-	-
etil asetat extract	14,0	14,0	14,0	11,0	11,0	11,0
Fraction 1	12,0	12,0	12,0	12,0	12,0	12,0
Fraction 2	13,0	13,0	13,0	12,0	12,0	12,0
Fraction 3	15.0	15.0	15.0	13,0	13,0	13,0

Fraction 4	**18.0**	**18**	**18,0**	**15.0**	**15.0**	**15.0**
Fraction 5	12,0	11.0	11,5	11,0	11,0	11,0
Fraction 6	11,0	10,0	10,5	10,0	10,0	10
Fraction 7	10,0	10,0	10,0	10,0	9,0	9,5
Fraction 8	10,0	10,0	10,0	10,0	9,0	9.5
Kloranfenikol (0,4 mg/mL)	18,0	19,0	18,5	20,0	20,0	20,0

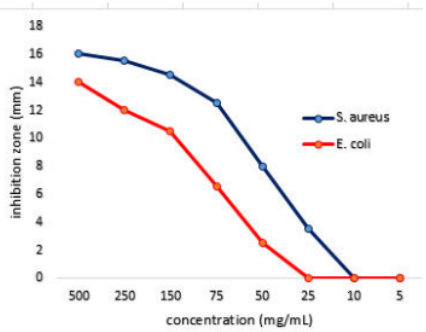
Fractionation of Active Compounds

Fraction 4 is the most active fraction then fractionated by column chromatography with the stationary phase of silica gel GF254 and the mobile phase of chloroform: methanol (9: 1). Furthermore, the fraction with the most weight was tested for its activity on the bacteria S aureus and E coli (tabl2 4, figure 1)

Table 4. The results of fractionation of active fractions

sub-fraction	weight (mg)	color
4a	7	Yellowish green
4b	36	yellow
4c	7,8	yellow
4d	5.4	yellow
4e	4,3	yellow

Description: Subfraction 4a (tube 1-10), Subfraction 4b (tube 11-28), subfraction 4c (tube 29-49), Subfraction 4d (tube 50-73) Subfraction 4e (tube 74-96).



Anti-bacterial activity test, showed a minimum dose of 4b sub-fraction of 25 mg / mL was able to inhibit S. aureus with a inhibition zone of 3.5 mm and a concentration of 50 mg / mL capable of inhibiting e coli by 2.5 mm

Identification of Active Compounds

Identification of active compounds in sub-fraction 4b using UV-Vis, IR and GC-MS spectrophotometer instruments. UV-Vis spectrophotometer results on fraction 4b produced 7 absorption peaks (table 5), Infra-red (IR) produced 6 wavelengths showing functional groups (table 6), and GCMS showed 7 peaks with different retention times so that 6 compounds were obtained (table 7)

Table 5. UV-Vis spectrophotometer results

Peak	Wavelength	Absorbansi
1	666.0	2,172
2	607,0	0,468
3	534,0	0,658
4	502,0	0,764
5	429,0	2,250

6	372,0	1,682
7	266,0	1.956

Table 6. FT-IR spectrophotometer result

Wave number	Functional groups	Wave number	Functional groups
3402,57	OH (*Stretching*)	1461,04	CH_2
3009,49	CH(*Stretching aromatic*)	1372.74	CH_3
2936.39	CH(*Stretching alifatik*)	1254,78	C-O-C (*Stretching*)
2853.06	CH(*Stretching alifatik*)	1049.37	CH(*bending*)
1734.57	C=O-OR	755,68	CH(*bending*) aromatik
1507.32	C=C (*aromatic*)	721,24	CH_2(*rocking*)

Table 7. GCMS analysis results subfraction 4 b and suspected compounds

Peak	Retention time (minutes)	**Area (%)**	Similarity (%)	Alleged compound
1	7,817	11,801	95	2H-1-benzopiran-2-one-(CAS) kumarin
2	9,465	14.188	93	2H-1-benzopiran,6,7-dimetoksi-2,2-dimetil-ageratokromon
3	9,656	9.038	81	7-metoksi-2,2-dimetil-6-vinil-2H-kromen
4	10.686	6,028	92	2,6,10-trimetil,14-1til-14-pentadecene(neopitadien)
5	11.672	31.318	94	Asam heksadekanoat (CAS)
6	12,654	8,126	95	Asam 9,12 –okta dekanoiat (CAS)
7	12.319	19.359	90	Metil-5,11,14,17- eikodekanoat

DISCUSSION

This research was conducted with the stages of extraction, phytochemical testing, fractionation, and continued activity tests. The fraction that had the highest activity was fractionated again so that the subfraction which gave the highest inhibition was obtained and continued with the identification of the active compound. The result of extraction of purified etyl acetate was 0.07%, and based on compound searches qualitatively showed the presence of flavanoid, tannin, terpenoid / steroid and Saponin compounds. other studies show the presence of plavonoids, alkaloids, terpenes, chromenes, chromones, benzofurans, koumarins, essential oils, sterols and tannins(8). fractionation of etyl acetate extract produced 8 compounds. and the more polar the fewer the numbers. the highest activity was obtained in fraction 4. then in the fraction again and the sub-fraction with the highest weight was tested for its activity test to see the MIC on Staphylococcus aureus (S. Aureus) and Esseceria coli (E.coli) bacteria. then subfractions are identified by UV-vis, IR and GCMS. the results of identification on UV-Vis have a peak at a wavelength of 666 nm indicating excitation π- π *, at this peak is characteristic of the chromophore group in the conjugated double bond system (-C = C-C = C-) or on the aromatic ring. The absorption display at a wavelength of 372 nm is an excitation of n-n * indicating conjugation (-C=C-C= O-). Furthermore, FT-IR results showed the presence of aromatic bonds C = C (ring stretching), CH aromatic (stretching), aromatic CH (bending). The appearance of the absorption band shows compounds with aromatic rings. There are 2 CH aliphatic (stretching), CH aliphatic (bending), CH2 (bending) CH3. The appearance of absorption band at wave number 3402.57 cm-1. Showed the presence of OH stretching groups and out-of-field bonding of OH at 920 cm-1 uptake which indicated the presence of carboxylic acid derivatives. Uptake at wave number 1734.77cm-1 indicates the presence of C = O-OR bonds from the ester compound. Results GC-MS identification has 7 peaks and the most dominant is the 5th peaks followed by peaks 7, 2, 1, 6, 3 and 4. The expected compounds are also supported by UV-VIS and FT-IR spectrophotometer data analysis. ased on identification on bandotan there are compounds coumarin, chromon, chromene, and fatty acids. These compounds have anti-bacterial activity, this is supported by research on coumarin activity as an anticoagulant, an antibiotic (3).The phenolic compounds such as chromene were successfully isolated from bandotan plants, including precocene II and 7-methoxy-2,2-dimethyl-6-vinyl-2H-chromene compounds (8). Another phenolic

compound with a structure similar to pre-lecturer II is escoparone, the compound has antibacterial activity (13). Whereas fatty acids such as hexadecanoic have anti-bacterial activity (5). Minor compounds such as neopitadiene also have anti-bacterial activity (1).

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