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# Isolation of bioherbicidal compounds of n-hexane extract of billygoat weed (Ageratum conyzoides L.) against the growth of spiny amaranth (Amaranthus spinosus L.)

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***Abstract:*** Spiny amaranth (*Amaranthus spinosus* L.) is an invasive species that interferes with the growth of cultivated plants and reduces crop yields. Long-term use of synthetic herbicides poses risks to the environment and human health, so environmentally friendly weed control alternatives are needed. This study aims to evaluate the potential of n-hexane extract of billygoat weeds (*Ageratum conyzoides* L.) as a bioherbicide through phytochemical tests, extraction, fractionation, and isolation. The extract was carried out using the maceration method, followed by fractionation using column chromatography, and isolation and effectiveness tests on spiny amaranth. The results of the phytochemical test showed that sub- fractions A1 and A2 contained alkaloid and terpenoid compounds. The extracts of sub-subfractions A1 and A2 showed weed control effectiveness of up to 100% 1 day after application (DAA) even at the lowest concentration of 2%. These results indicate that billy goat weeds have high potential as a source of botanical bioherbicides.

***Keywords:*** *Ageratum conyzoides* L, n-hexane, *Amaranthus spinosus* L, bioherbicide, secondary metabolites

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## 1. Introduction

Weeds are wild plants that provide losses to humans both directly and indirectly because their presence is not expected (Widaryanto *et al.*, 2021). Weeds act as competitors with cultivated plants, as a result there is a need for weed control efforts (Jabran *et al.*, 2015). The presence of weeds causes competition with corn plants, so that sweet corn production has decreased by 13-51% (Safrina *et a*l., 2024). A common control method is chemical control using synthetic herbicides. Continuous weed control using herbicides will make weeds resistant, disturbed health and problems with the environment (Rahmadhani *et al*., 2016). The results of screening of 5 types of annual plants and 5 types of weed plants through guided tests showed that one of the weeds used as a great potential bioherbicide is billygoat weeds (*Ageratum conyzoides* L.) because it has allelopathic compounds in the form of secondary metabolites in it (Erida *et al*., 2020). Based on research Muningsih and Wijaya (2024) billygoat weeds contains allelochemical compounds, one of which is phenol which is able to inhibit weed growth and phenol is dangerous if it hits the sprouts, this is due to phenol compounds being able to inhibit the metabolism of food reserves.

Research results Prasetia *et al*. (2022) billygoat weeds leaf extract subfraction A using one of the solvents, namely n-hexane, has an effect on height, stem diameter and leaf area at concentrations of 8%-10%. The same thing was reported by Ananda *et al*, (2022) application of billygoat weeds leaf extract subfraction B at a concentration of 2% was able to inhibit the growth of spiny amaranth weed as much as 100%. The billygoat weeds extraction process can use n-hexane (nonpolar) solvent. This solvent has stable, volatile, transparent, selective properties, so it is good if used as a solvent (Hadi, 2013) According to Erida *et al.* (2023) billygoat weeds extract using n-hexane solvent was able to inhibit spiny amaranth weed by 40% at concentrations of 10% and 20% and inhibit at a concentration of 15% by 90%. In contrast to solvents that have semi-polar and nonpolar levels of polarity, n- hexane extracts were able to inhibit spiny amaranth weeds equivalent to 2,4D at 21 HSA (Erida *et al*., 2021). The results of the phytochemical test of billygoat weeds extract using n-hexane solvent showed positive results of 2 secondary metabolite compounds, namely alkaloids and terpenoids, which caused disruption of spiny amaranth metabolism at 7 DAA (Zikri *et al*.,2024). Purification is the final step in the process of isolating natural material compounds. The purpose of isolation is the separation of compounds from impurities so that the resulting isolate has a high purity value (Muldianah *et al*., 2021).

Spiny amaranth (*Amaranthus spinosus* L.) is a plant that has fast and wild vegetation, so it can grow anywhere including agricultural land, and this weed is a strong competitor in competing with the main crop (Mukhtar *et al*., 2024). This is emphasized by Tarigan (2015) that spiny amaranths can germinate on the soil surface reaching 56%-78% if germinated in a germinator with a temperature of 80 $^0$C. Spiny amaranth has the ability to live in very high salt levels and in extreme drought (Yu *et al*., 2022). Based on the description above, it is necessary to conduct research to determine secondary metabolites through phytochemical tests and the effect of billygoat weeds extract isolation on the growth of spiny amaranth weed.

## 2. Methodology

### *2.1. Place and Time of Research*

This research was conducted at multiple locations within Syiah Kuala University (USK), Banda Aceh, Indonesia, including the Weed Science Laboratory (Department of Agrotechnology, Faculty of Agriculture), the Food Analysis Laboratory (Department of Agricultural Product Technology), the Sample Analysis Room (Department of Chemistry, Faculty of Mathematics and Natural Sciences), the Environmental Quality Testing Laboratory (Department of Chemical Engineering, Faculty of Engineering), and the Experimental Garden (Faculty of Agriculture). The study took place from May to December 2024.

### *2.2. Tools and Materials*

The tools used in this study included burlap sacks, knives, analytical balances, 12-mesh sieves, 500, 1000, and 2000 mL beakers, tabletop balances, large and small column chromatography apparatus (73 cm × 5 cm and 70 cm × 3 cm, respectively), chambers, UV lamps, 50 mL and 100 mL vial bottles, wooden mortar, 1 kg capacity pots, and stative stands.

The materials used consisted of billygoat weed (Ageratum conyzoides L.) leaves collected from Indrapuri District, Aceh Besar Regency; 100 g of spiny amaranth (Amaranthus spinosus L.) seeds obtained from Blang Bintang District; cotton, aluminum foil, labeling paper, distilled water, 26% ammonia, n-hexane and ethyl acetate solvents, cow manure, alluvial soil from Blang Bintang District, silica gel 60 TLC plates (0.2–0.5 mm), silica gel, and Tween 20.

### *2.3. Experimental Design*

The design used in this study is a completely randomized design (CRD) non-factorial pattern with 10 treatments and 3 replicates. So that there are 30 experimental units.

The arrangement of data treatments can be seen in Tables 1 and 2.

**Table 1: Treatment list of n-hexane extract sub-subfraction A1**

| *No.* | *Treatment Code* | *Description* |
|---|---|---|
| 1 | A1.1 | Billygoat weed n-hexane extract sub-subfraction A1 2% concentration |
| 2 | A1.2 | Billygoat weed n-hexane extract sub-subfraction A1 4% concentration |
| 3 | A1.3 | Billygoat weed n-hexane extract sub-subfraction A1 6% concentration |
| 4 | A1.4 | Billygoat weed n-hexane extract sub-subfraction A1 8% concentration |
| 5 | KA | Aquades Control |

**Table 2: Treatment list of n-hexane extract sub-subfraction A2**

| *No.* | *Treatment Code* | *Description* |
|---|---|---|
| 1 | $A_{2.1}$ | Billygoat weed n-hexane extract sub-subfraction A2 2% concentration |
| 2 | $A_{2.2}$ | Billygoat weed n-hexane extract sub-subfraction A2 4% concentration |
| 3 | $A_{2.3}$ | Billygoat weed n-hexane extract sub-subfraction A2 6% concentration |
| 4 | $A_{2.4}$ | Billygoat weed n-hexane extract sub-subfraction A2 8% concentration |
| 5 | KA | Aquades Control |
| | | |

## *2.4. Research Procedure*

### *2.4.1. Preparation of Billygoat Weed Samples*

Billygoat weed leaves were obtained from Gampong Lam Leubok, Indrapuri Subdistrict, Aceh Besar District and collected as much as ± 150 kg wet weight. The criteria for billygoat weed leaves taken are ideal age leaves, optimum growth, light green leaves, no diseases or pests and have a perfect leaf shape. Billygoat weed leaf samples were picked by pulling them up to the roots and then put into a gunny sack. The collected billygoat weed leaves were separated from the stem. Billygoat weed leaves are spread on a mat and air-dried by avoiding sunlight so as not to damage the compounds in it for 2 weeks. The fresh weight of billygoat weed leaves is 150 kg, and after drying it is 50 kg.

### *2.4.2. Extraction*

The dried billygoat weed leaves were then ground using a wooden mortar finely, then the entire billygoat weed leaf powder was moistened with 26% ammonia for ± 2 hours, after which the billygoat weed leaf sample was macerated using n-hexane solvent for 3x24 hours, then after complete soaking, the extract was filtered using a filter coated with cotton so that the filtrate was separated from the simplisia nya. The billygoat weed leaf samples were macerated repeatedly for 3x24 hours until the extracted compounds were obtained perfectly clear green in color. The filtrate that has been obtained will be evaporated using a rotary evaporator at a temperature of 40°C, so that a concentrated extract of billygoat weed is obtained which will be used to test the growth activity of spiny amaranth weed.

### *2.4.3. Fractionation*

Fractionation is a process of separating concentrated extracts from extraction results using maceration that have been evaporated and utilizing two different types of solvents but not mixing with each other, in this process using a tool called column chromatography. The bottom of the column is filled with small cotton and approximately 5 g of sand. On top of the sand, 250 g of silica gel is inserted, which has been soaked for 1 hour using n-hexane solvent to avoid air bubbles. Re-inserted 5 g of sand and then added 50 g of billygoat weed extract. The concentrated extract of billygoat weed was poured and allowed to drop slowly through the silica gel and then added n-hexane and ethyl acetate solvents in the ratio of 9:1, 8:2, 7:3, 6:4, 5:5, 3:7, 2:8 as a solvent system and kept in mind so as not to produce air bubbles on the stationary phase (silica gel). Then the column faucet is slowly opened so that the eluent (solvent liquid) will flow, each drop that comes out is 20 drops per minute. The drops will be collected using 100 ml. vial bottle. The results of the eluent that has been obtained through the collection in the vial bottle, will be put together based on the Thin Layer Chromatography (KLT) process.

The KLT plate is measured and cut according to the spot needs, the size of the thin layer chromatography plate with a height of 10 cm and width as needed, the top and bottom of the KLT plate are measured with a distance of 0.5 cm as the base line and finish line. Above the KLT plate will be bottled using a capillary pipette based on the distance between spots, which is 1 cm, bottling using the code S (without fractionation), 1, 5, 10, 15, 20, and so on until completion. The thin layer chromatography process was completed then continued with the process of rising the pattern of stains from the bottling results into the chamber, the solvents used were n-hexane and ethyl acetate in a ratio of 9 n-hexane : 1 ethyl acetate. The base line should not be covered with a solvent system, then closed tightly until airtight. Waited until the KLT plate absorbs the eluent to reach the finish line that has been marked. The stain pattern that has appeared is lifted and then the KLT plate is inserted into the ultraviolet lamp to be more accurate.

Stain patterns that look similar will be combined or put together into 1 container. Four subfractions were obtained, namely A, B, C and D.

### *2.4.4. Isolation*

The subfractions that have been obtained, namely A1, A2, A3, and A4, will be isolated, namely column re-chromatography and thin layer re-chromatography as well as in the fractionation stage. The subfraction carried out in this study is only A using a small-sized column chromatography (70 cm long, 3 cm diameter) using a stationary phase of silica gel G 60 F in an elution gradient.

### *2.4.5. Phytochemical Test*

Phytochemical tests were analyzed in the Sample Analysis Room of the Chemistry Department, Faculty of Mathematics and Natural Sciences, USK. This test is carried out to find out what major chemical compounds are contained in the sub-subfractions of billygoat weed n-hexane extract. The method used to conduct phytochemical tests is to analyze phytochemicals through the appearance of stains.

### *2.4.6. Preparation of Planting Media*

The type of soil used was Alluvial soil obtained from Blang Bintang District, Aceh Besar Regency. The soil was air dried for 3 days, then cleaned from plant debris and dirt. The soil was mixed using manure in a ratio of 2:1 and sieved using a 12 mesh sieve. The soil was put into 1 kg pots.

### *2.4.7. Planting Spiny amaranth Seeds*

Before planting, thorny amaranth seeds are soaked for 1x24 hours in order to get seeds that are nutritious, selected seeds that sink and have a shiny black color. After putting the soil into the pot, 2 thorn amaranth seeds were put per pot, when it was 2 weeks old it would be eliminated and leave only 1 best seed as an indicator.

### *2.4.8. Watering*

Watering is done once a day using clean water. The amount of water capacity is about 200 ml.

### *2.4.9. Billygoat weed Extract Application*

Billygoat weed extract was applied to spiny amaranth once when the spiny amaranth weed was 14 days after planting (HST). The application was carried out using a glass sprayer bottle to all parts of the spiny amaranth. After calibration, the need for extract per pot is 4 ml, so that in 3 replicates 12 ml is needed. The result of 4 ml is 100 sprays.

### *2.4.10. Data Analysis*

*Phytochemical Test:* Phytochemical analysis was carried out using concentrated extracts of billygoat weed sub- subfractions evaporated using a rotary evaporator which were tested using different reagents before being applied to the indicator weed of spiny amaranth, as for the purpose is to see what groups of compounds can be drawn by billygoat weed extract with n-hexane solvent.

*Percentage of Weed Control:* The percentage of weed control can be obtained based on visual observations resulting from an easy-to-understand rating system (Frans and Talbert, 1977). The percentage of weed control was observed at 1, 7, 14, and 21 HSA by means of a score system conducted by 5 people and then averaged.

**Table 3: Percentage of Weed Control**

| *Value* | *Category* | *Description* |
|---|---|---|
| 0 | No effect | No weed control<br>No effect of weed damage |
| 10-30 | Mild influence | Very poor weed control<br>Slight discoloration of the plant, stunted plant<br>Lack of control<br>Momentary crop damage occurs |
| 40-60 | Moderate influence | Lack of control<br>Moderate control, plants usually recover<br>Longer lasting crop damage<br>Moderate control<br>Long-lasting damage and no recovery |
| 70-90 | Weight effect | Heavy crop damage<br>Crops are almost wiped out and some survive<br>Excellent for weed control |
| 100 | Very heavy influence | - Weed mortality |

*Source:* Frans and Talbert (1977)

# 3. Results and Discussions

## 3.1. Phytochemical Test

The results of the phytochemical test can be seen in table 4.

**Table 4: Phytochemical Test**

| *No.* | *Secondary Metabolite Assay* | *Results* | *Billygoat weed N- Hexane Extract Sub-Subfraction A1* | *Billygoat weed N-Hexane Extract Sub-Subfraction A2* |
|---|---|---|---|---|
| 1 | Alkaloids | Meyer<br>Dragendorff Wagner | +<br>+<br>+ | +<br>+<br>+ |
| 2 | Terpenoids | Liebermann-Burchard Test | + | + |
| 3 | Steroids | Liebermann-Burchard Test | - | - |
| 4 | Saponins | Shuffling | - | - |
| 5 | Flavonoids | HCl and Metal Mg | - | - |
| 6 | Phenolic | FeCl3 | - | - |

Table 4 shows that based on the phytochemical test, the n-hexane subfractions of A1 and A2 billygoat weed contain alkaloid and terpenoid compounds, which use Meyer's reagent, Dragendorff, Wagner and Liebermann-Burchard tests. Phytochemical analysis is a method used in identifying secondary metabolite compounds in plants qualitatively (Agustina *et al*.,2017) . This shows that n-hexane solvent is able to attract alkaloid and terpenoid compounds in billygoat weed leaves. Alkaloid compounds contained in bioherbicides are able to inhibit ion transfer on cell membranes (Sari and Jainal, 2020) . Alkaloids induce plant growth inhibition by disrupting DNA processes, causing changes in enzyme activity, protein metabolism, and cytoplasmic membrane integrity, among others. This is confirmed by studies in which quinolizidine alkaloids produced by legumes, such as lupanine and spartenin, can have impaired membrane permeability and inhibition of protein synthesis. Plant alkaloids are widely distributed in four plant families, including Asteraceae, Apocynaceae, Boraginaceae, and Fabaceae (Latif *et al*., .2017)

Alkaloids are compounds that have activity as antifungals by interfering with DNA esterase and RNA polymerase (Fatma *et al*., 2021) . The content in alkaloids contains chemical components in the form of anthraquinones, glycosides and resins that can penetrate the fungal cell wall, so that there is a disturbance in the metabolic process in fungal cells which results in cell growth disorders at certain concentrations and results in mortality of the fungal cells (Utami *et al*., 2022). Terpenoids are secondary metabolite compounds in the hydrocarbon group that are widely produced in plants, especially contained in sap and cell vacuoles (Mierza *et al*., 2023). Terpenoids are compounds consisting of isoprene units that have 5 carbons (-C5) which will be synthesized from acetate through the mevalonate pathway (Hartati *et al*., 2016). Terpenoids can be extracted with nonpolar or polar solvents. Terpenoids are one of the largest and structurally diverse groups of natural compounds. Terpenoids are a class of natural products derived from mevalonic acid (MVA) which is composed of a number of isoprene (C5) structural units. Terpenoids are widely present in nature, with diverse structures. and diverse. To date, more than 50,000 terpenoids have been found in nature,1 and most of them are isolated from plants (Sun, 2017).

Some terpenoids play an important role in plant growth and development, such as gibberellins, because plant hormones regulate plant development and carotenoids play a role in photosynthesis, terpenoids also play a role in plant interactions with the environment, such as participating in the plant defense system in the form of phytoalexins and interspecies competition as interspecies sensing compounds. The results of research by Yang *et al.* (2020) showed that various terpenoids have been shown to have significant disease prevention and treatment effects, as well as having antitumor, anti-inflammatory, antibacterial, antiviral, antimalarial effects and also show potential functions in immune regulation, neuroprotection, antiallergic. The activities exhibited by terpenoids have an important role in the development of new drugs and the improvement of existing treatment options.

## 3.2. Weed Control Percentage of Spiny amaranth

The application of n-hexane extract sub-subfractions A1 and A2 successfully inhibited the growth of spiny amaranth weeds by 100% at 1 HSA (Day After Application). The application of n-hexane extracts of sub-subfractions A1 and A2 successfully inhibited the growth of spiny amaranth weeds by 100% at 1 HSA (Day After Application). The lowest concentration was able to control spiny amaranth weeds aged 14 HST (Day After Planting). The effect of applying n- hexane extracts of sub-subfractions A1 and A2 can provide vegetative damage seen at 1 HSA which has the characteristics of dwarfing, leaf fatigue, discoloration of leaves such as burning, then increasingly severe damage such

**Table 5: Mean Percentage Of Spiny amaranth Weed Control Due To The Application Of A1 And A2 Billygoat weed N-Hexane Sub-Fraction Extracts At Various Concentrations**

| *Concentration Treatment* | *Mean Percentage of Spiny amaranth Weeds (%)* | |
|---|---|---|
| | *Sub-subfraction A1* | *Sub-subfraction A2* |
| | *1 HSA* | *1 HSA* |
| Aquades | 0,00<br>(2,86) | 0,00<br>(2,86) |
| 2% | 100,00<br>(90,00) | 100,00<br>(90,00) |
| 4% | 100,00<br>(90,00) | 100,00<br>(90,00) |
| 6% | 100,00<br>(90,00) | 100,00<br>(90,00) |
| 8% | 100,00<br>(90,00) | 100,00<br>(90,00) |

as damage to weed tillers that occur, can inhibit seed germination, inhibit enzyme processes in spiny amaranth weeds so that weed mortality occurs.

The lowest concentration was able to control spiny amaranth weeds aged 14 HST (Days After Planting). Further research by Prasetia *et al*. (2022) confirmed that the results of subfraction A using n-hexane solvent gave the death of spiny amaranth weed at a concentration of 8%. The same thing was also reported by Ananda *et al*. (2022) the application of subfraction B of billygoat weed extract at a concentration of 2% was able to inhibit the growth of spiny amaranth weed by 100%. This also affects the selection of solvents. Solvents contribute to influencing the extracts used as organic herbicides because each solvent is able to attract different compounds. The number of compounds extracted in the extraction process affects the solvent used (Safitri, 2018). Cahyanti *et al*. (2015) added that terpenoids, flavonoids and phenol compounds are allelochemicals that inhibit cell division so that they can be used as bioherbicides.

## 4. Conclusion

Phytochemical tests on sub-subfractions A1 and A2 showed the presence of alkaloids and terpenoids. These compounds are known to be phytotoxic and play an important role in allelopathic activity against weeds.

Effectiveness testing showed that the extract at 2% concentration was able to control spiny amaranth by 100% on day 1. This shows that billygoat weed n-hexane extract has great potential as an environmentally friendly plant-based bioherbicide.

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