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Isolation and Characterization of Ethyl Acetate Fraction from *Abroma augusta* L as an Anti-Inflammatory Agent

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Abstract

Abroma augusta is a bush plant that lives on the edge of the river. This plant is commonly used as an anti-inflammatory drug for joints and broken bones. It contains several secondary metabolites, such as alkaloids, triterpenoids, steroids, and flavonoids, which exhibit anti-inflammatory activity. UV-visible (UV-Vis) spectrophotometry of isolate 1.3 indicated absorption at a maximum wavelength of 282 nm. The wavelength suggested that the electron transition π–π* is the absorption of UV spectra typical for triterpenoid compounds that have chromophores in the form of non-conjugated double bonds. FT-IR spectrophotometer characterization data from isolate 1.3 revealed the presence of triterpenoid compounds having carboxyl groups C-O (ester), C-$(CH_3)_2$ (gem-dimethyl), carbonyl C = O esters, and C-H (alkyl). Analyses of UV-Vis, FT-IR, GC-MS, ^{1}H-NMR, and ^{13}C-NMR spectra showed that the compound obtained was the triterpenoid *β*-amyrin. Activity test demonstrated that isolate 1.3 with a 10 mg dose showed stronger anti-inflammatory activity than the positive control of sodium diclofenac.

Keywords: *Abroma augusta* Linn, Anti-Inflammatory, Edema, Ethyl Acetate

1. Introduction

Plants are an essential source of medicines for human. Human uses plant parts such as roots, stems, leaves, flowers, seeds, and fruit as a safe medicine in treating various diseases. Moreover, plants containing bioactive compounds have attracted increasing attention as traditional medicine [1]. The research in the ethnobotanical field addresses the characteristics of traditional Indonesian knowledge to establish priorities together with the local community. Then, it ensures that local values are translated into rational uses of resources and effective conservation of biological diversity and cultural knowledge [2,3]. The "Patah Kemudi" plant *Abroma augusta* Linn is a small tree from the Malvaceae tribe; it spreads from India, Southeast Asia, South China, through the archipelago to the Pacific. In Southeast Asia, *A. augusta* is found in Thailand, Vietnam, Malaysia, Indonesia, and the Philippines. In Jambi Province, this plant spreads in the forest area around Bungo, Tebo, and Muaro Jambi Regencies. *A. augusta* generally lives along the edge of the water, in the bush, or an open area at an altitude of 5–1100 mdpl. It is also commonly found in secondary forests, abandoned lands, village edges, and roadsides [2].

Some parts of *A. augusta* are used to cure various diseases. For example, traditional communities in Bungo and Tebo Regencies, Jambi Province use the roots of this plant as a fracture remedy [3,4]. In addition, the Talang Mamak community in Bukit Tigapuluh National Park has long used the plant as an effective fracture medicine [2]. Furthermore, *A. augusta* leaves have several active compounds with antimicrobial, cytotoxic, antidiabetic, and anti-inflammatory activities [5,6].

Anti-inflammatory drugs can suppress or reduce inflammation, a natural immunological response to injuries and infections resulting from harmful stimuli. It includes physical injury, tissue injury, microbial pathogens, and immunological cross interactions in the body [6–8]. Effective anti-inflammatory drugs are currently available for the treatment of acute and chronic inflammatory

diseases. Based on their mechanism of action, anti-inflammatory drugs are typically divided into steroidal and non-steroidal types. These drugs work by inhibiting the release of prostaglandins into the injured tissue [9,10].

Compounds from *A. augusta* leaves have potential anti-inflammatory activity. The n-hexane fraction of *A. augusta* contains the steroid γ-sitosterol compound and exhibits good anti-inflammatory activity. The methanol fraction of *A. augusta* also contains squalene compounds with good anti-inflammatory activity compared with standard controls [11–14]. Crude extracts of *A. augusta* contain chemical compounds, such as glycosides, alkaloids, carbohydrates, and steroids, with total phenols and flavonoids of 0.29% and 0.50% w/w, respectively [15]. The other chemical compounds are taraxerol, flavonoids, and phenolics with antidiabetic activity [16]. All parts of *A. augusta* show higher anti-inflammatory activity than the standard sodium diclofenac drug because of the alkaloids and flavonoids in this plant [1]. In the present study, we evaluated the anti-inflammatory activity of *A. augusta* extract. We explored and characterized the bioactive compounds of *A. augusta* extract in the ethyl acetate fraction using UV-Vis, FT-IR, GC-MS, and NMR and evaluated their anti-inflammatory activity in infected mice.

2. Materials and Methods

Research Materials and Instruments

The material used in this study is *A. augusta*. Healthy leaves were collected from the Regency of Tebo Jambi Province, Indonesia. The chemicals used were methanol, n-hexane, ethyl acetate, acetone, aquades, silica gel 40 (70–230 mesh) ASTM for column chromatography (Perkin Elmer), 5% $FeCl_3$, 10% NaOH, Mg-HCl, H_2SO_4 (p), 6% HCl, chloroform (Sigma Aldrich), and silica gel thin-layer chromatography (TLC) plate 60 F_{254}. The absorbance of pure compounds isolated at different concentrations was obtained via UV-Visible (UV-Vis) spectroscopy (Shimadzu, Model 1800, Japan). The MS spectra were recorded using a Varian GC-MS/MS (Model Varian CP 3800) equipped with a VF-5 fused silica capillary column (30 m × 0.23 i.d., film thickness 0.25 μm). The injector and mass transfer line temperatures were set at 250°C and 300°C, respectively. In addition, ^{1}H and ^{13}C-NMR spectra were measured on a Brucker (600 MHz) spectrometer with TMS (Tetramethylsilane) as the internal standard [7].

Extraction and Isolation

A. augusta leaves were cut into small pieces, dried at room temperature for 15 days, and mashed using a grinder to obtain coarse powder, which could pass through sieve number 60. The powder (1500 g) was extracted by a multilevel maceration method with a step gradient polarity solvent (n-hexane, ethyl acetate, and methanol) for 24 h [17]. The macerate was concentrated under vacuum at 45°C using a rotary evaporator to produce crude extract. The crude extract was dissolved and analyzed by TLC to select the appropriate eluent and then separated by open column chromatography using a stationary silica gel 60 phase [18]. The column fractions with the same retention factor (Rf) were combined and evaporated. The main components contained in the active sample fraction were separated and purified using column chromatography, and then the pure compound was recrystallized. The phytochemical screening and purity of the compounds were run with three eluents [16].

Characterization of Chemical Compounds

The metabolites isolated from the ethyl acetate fraction of *A. augusta* were characterized using UV-Vis, FT-IR, GC-MS, and NMR spectrometry in accordance with previously described procedures and conditions [15,17,19]. The isolate (1 mg) was dissolved in 2 mL of methanol, and its spectrum was observed at a 200–400 nm wavelength on a UV-Vis spectrophotometer. Moreover, 1 mg

isolates were mixed with 50 mg of KBr pellets, homogenized, and then identified at 4000–400 cm^{-1} on a FT-IR (Perkin Elmer) spectrophotometer. For GC-MS (Perkin Elmer), 1 μL of isolate was injected into the GC-MS tool, which was set under appropriate conditions. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on an Agilent DD2 (500 MHz for 1H-NMR and 125 MHz for ^{13}C-NMR) with TMS as an internal standard (chemical shifts in δ, ppm) in $CDCl_3$ or $(CD_3)_2CO$ or DMSO. Coupling constants (J) were reported in Hz. UV-Visible spectrophotometry was performed using a single beam provided by Shimadzu-UV mini 1240 instrument. UV lamps used CAMAG 254 nm, radiation (λ = 254 and 365 nm). TLC was performed with silica gel 60G F254 using Merck (Art.5554), and spots were visualized by cerium sulfate vapors and ultraviolet light. All solvents were analytical reagent grade [20,21].

Anti-Inflammatory Activity of A. augusta in Carrageenan-Induced Paw Edema

Male CD1 mice (20–25 g, 7–8 weeks of age) were fasted for up 18 h before the experiment. Each mouse was marked with a marker on its tail. The initial volume of the mouse feet was measured before being treated and expressed as the base foot volume (Vo). Then, the mouse feet were injected with as much as 0.3 mL of 1% carrageenan suspension by sub-plantar. Before the injection, the foot soles of the mice were cleaned with 70% ethanol. After 15 min of carrageenan injection, the volume of the feet was measured using calipers. Then, the feet were injected again with the extracts and isolates prepared. Mice not administered with any treatment of isolate/extract (just solvent) served as the negative control. Mice administered with diclofenac sodium dose at 0.14 mg/kgBW served as the positive control. Then, the mouse foot was measured every 30 min for 180 min of observation and expressed as the final volume (Vt) [22,23]. The anti-inflammatory activity was calculated by measuring the volume of edema thickness in the feet before and after treatment. The results were verified by comparing them to the positive control.

Phytochemical data analysis was performed by analyzing the reaction between the reagent and the sample used. Whether or not color changes, sediment formation, and layer formation occurred was observed [5,19]. In the carrageenan-induced paw edema, intraperitoneal injection of carrageenan in mice showed a time-dependent increase in paw edema. The anti-inflammatory activities data were analyzed and described by monitoring the decrease in % edema formed in the feet of mice. The percentage inhibition of edema volume was before and after treatment by substances tested using the previous studies. Some irritants were used as edema inductors, such as formalin, choline, yeast, and dextran. The percent of edema calculation was measured as previously described [8,15,24].

3. Results and Discussion

A. Augusta plant has an anti-inflammatory activity that is traditionally used in humans, but the specific chemical compounds with bioactivity remain unknown. The extract of the ethyl acetate fraction from *A. augusta* leaves has a 1.165% yield. In this study, we used ethyl acetate fraction to obtain a specific secondary metabolite as an anti-inflammatory. Phytochemical screening was performed as previously described. Results confirmed that the ethyl acetate fraction of *A. augusta* leaf extract contains flavonoids, quinone, steroids, and phenolic compounds (Table 1), and the isolate has steroids.

Isolation of Active Compound of A. augusta Leaves. Bioactive compounds were isolated by column chromatography. The extracted compounds were first simplified using vacuum liquid column chromatography. Fraction 1 was subjected to gravity column chromatography to obtain four subfractions. Fraction 1.3 was selected because it had the best TLC stain pattern in the form of a single-tailed stain. Figure 1 shows the TLC results of fraction 1.3 in three eluents: n-hexane: ethyl = 9: 1; n-hexane: chloroform = 6: 4; and n-hexane: acetone = 8: 2.

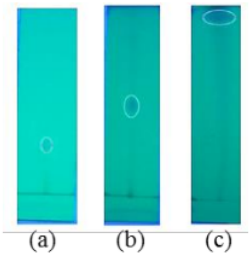


Figure 1. Thin-layer chromatography (TLC) pattern of fraction 1.3 of column chromatography (a) n-hexane: ethyl = 9: 1 (b) n-hexane: chloroform = 6: 4 and (c) n-hexane: acetone = 8: 2

Our TLC test results using three types of eluent, namely, (a) n-hexane: ethyl = 9: 1, (b) n-hexane: chloroform = 6: 4, and (c) n-hexane: acetone = 8: 2, obtained the Rf values of 0.325, 0.525, and 0.925, respectively. The staining pattern formed a single stain in the three eluents, which confirm that the compound obtained is pure enough (isolate). Then, phytochemical screening was carried out on isolate 1.3. It was used to determine the presence of secondary metabolites. The results of phytochemical tests (Table 1) showed that isolate 1.3 contains steroids.

Table 1. Phytochemical screening of ethyl acetate extracts and isolate

Screening	Testing method	Extract	Isolate
Alkaloids	• Meyer reagent	-	-
	• Dragendorff	-	-
Flavonoids	Willstatter	+	-
Tannins	$FeCl_3$	-	-
Saponins	Foam test	-	-
Quinone	KOH	+	-
Terpenoids	Liebermann Burchard	-	-
Steroids	Liebermann Burchard	+	+
Phenolic	$FeCl_3$	+	-

(+) Contains Secondary Metabolites

Characterization Anti-inflammatory Compound Using UV-Vis Spectrometry. The isolate 1.3 compound obtained from *A. augusta* leaves had UV light absorption at a maximum wavelength of 282 nm (A = 1.3562). The electron transition $\pi \rightarrow \pi^*$ indicates that this compound has a double bond belonging to the steroid ring. Moreover, the compound's absorption belongs to a triterpenoid group with chromophore in a non-conjugated double bond [15,17,19].

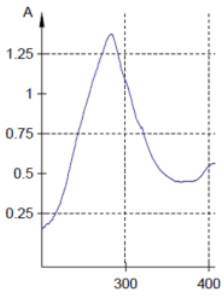


Figure 2. UV-Vis Spectrum of isolate

Characterization Anti-inflammatory Compound Using FT-IR Spectrometry. The isolated compound had absorption at 1240 and 1376 cm^{-1}, indicating the presence of the C-O carboxyl group (ester) and C-$(CH_3)_2$, respectively. The absorption suggests the presence of dimethyl gem groups as a characteristic of triterpenoid compounds. Furthermore, the absorption at 1735 cm^{-1} indicated the presence of the carbonyl group C = O ester (R-CO_2-R'). Hence, the vibration at 1465 cm^{-1} corresponds to the bending absorption of the CH_2 functional group, and that at 2924 cm^{-1} corresponds to asymmetric CH stretching bond absorption [25–27].

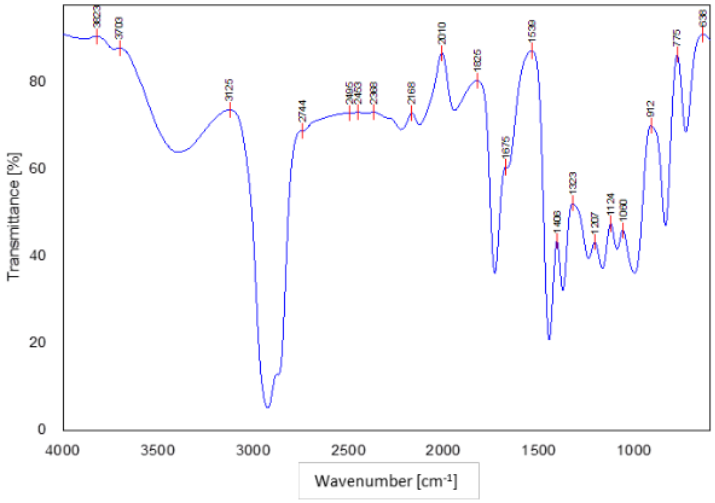
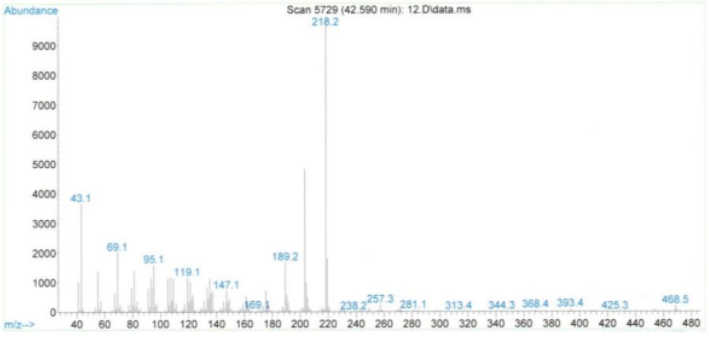
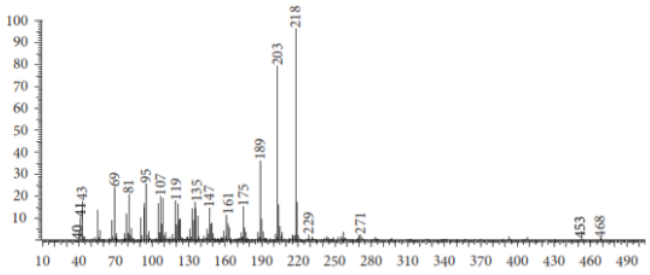


Figure 3. FT-IR spectrum of the isolate compound

Characterization Anti-inflammatory Compound Using GC-MS Spectrometry. GC-MS was used to analyze the possibility of compounds produced from the isolates (Figure 4a), then compared to β-amyrin acetate.



(a)



(b)

Figure 4. Spectrums of GC-MS of (4a) isolate 1.3 from from *Abroma augusta* (4b)β-amyrin acetate from *Manilkara subsericea* extract (Mart.) [18]

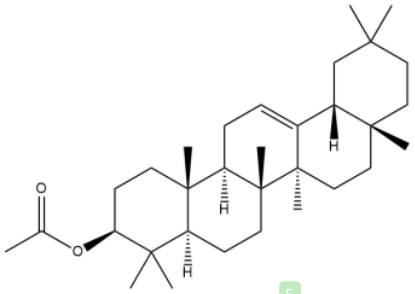


Figure 5. Chemical structures of β-amyrin acetate ([(3S,4aR,6aR,6bS,8aR,12aR,14aR,14bR)-4,4,6a,6b,8a,11,11,14b-octamethyl-1,2,3,4a,5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicen-3-yl] acetate)

The abundance of ions at *m/z* 218, 189, and 69 is typical for the fragmentation of *β*–amyrin acetate [18]. However, the MS spectrum of compound A4$_4$ indicates a molecular ion at *m/z* 426, which constitutes the frame of *β*-amyrin [18,23,28]. The characteristic of the *β*-amyrin acetate compound contained in fragment the molecular ion (M+) at m/z 218 (base peak) is characteristic to triterpenoid fragmentation [28,29], the solving molecular ion *β*-amyrin compound. Our isolate fragment showed the relative abundance of $C_{32}H_{52}O_2$: 468 (M^+,5), 218(100), 203(44), 189(17). *β*-amyrin type triterpenes presented peak at *m/z* 203 higher that peak at *m/z* 189 which is belongs to α-amyrin. By contrast, α-amyrin type triterpenes showed equal abundance [29]. These results suggest that isolate 1.3 was successfully isolated and that it is the steroid *β*-amyrin acetate.

^{1}H-NMR Spectrophotometry. The analysis with ^{1}H-NMR ($CDCl_3$; 500 MHz; δ ppm) as in Figure 6 and its expansion in Figure 7 shows eight methyl group signals each appearing at 0.80, 0.82, 0.90, 0.92, 0.96, and 1.09 ppm.

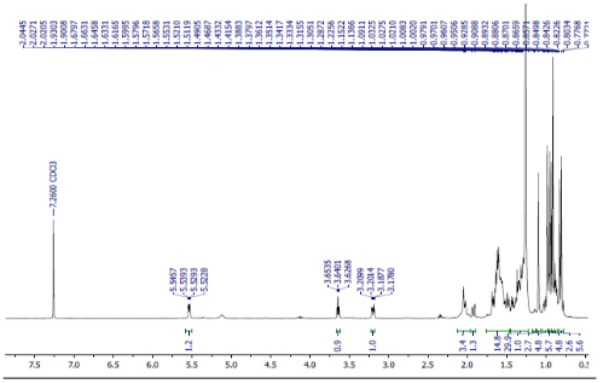


Figure 6. ^{1}H NMR spectrum of isolate 1.3 ($CDCl_3$, 500 MHz)

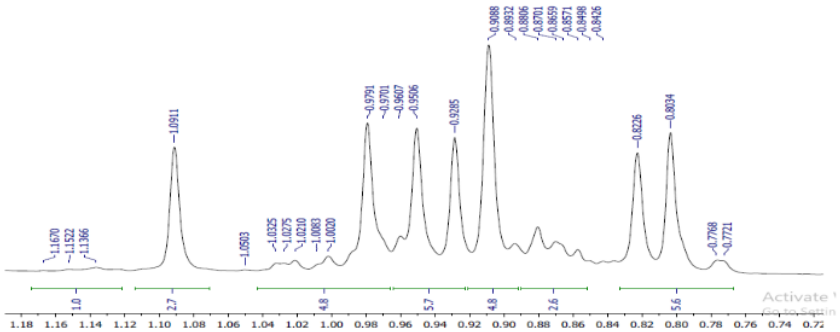


Figure 7. Expansions of the ^{1}H-NMR spectrum of isolate F.1.3 for protons at chemical shifts from 0.74 ppm to 1.18 ppm ($CDCl_3$, 500 MHz)

Table 2. Comparison of the ^{1}H-NMR spectrum data of compound F.1.3 ($CDCl_3$, 500 MHz) with compound A4 standard of *β*–amyrin acetate

Position H	Chemical Shift (δ_H) ppm (J Hz)			Position H	Chemical Shift (δ_H) ppm (J Hz)		
	F.1.3.	**A4** [18]	***β*-amyrin acetate** [18]		**F.1.3.**	**A4** [18]	***β*-amyrin acetate** [18]
1	**1.22-1.30 (m)**	1.28–1.35 (m)	1.04–1.64 (m)	**16**	**0.77 (m)**	0.79–1.91 (m)	0.79–1.98 (m)
2	**1.60**	1.67 (m)	1.60–1.88	**17**	**-**	**-**	**-**

	(m)		(m)				
3	**3.64 (t)**	4.49 (t)	4.50 (t, 8.0 Hz)	**18**	**1.91 (d)**	1.91 (d)	1.93 (d; 4.2 Hz)
4	**-**	**-**	**-**	**19**	**1.15 (m)**	1.00-1.67 (m)	1.00 -1.66 (m)
5	**0.84 (m)**	0.84 (m)	0.84 (m)	**20**	**-**	**-**	**-**
6	**1.53 (m)**	1.54 (m)	1.40–1.53 (m)	**21**	**1.32 (m)**	1.30 (m)	1.08–1.32 (m)
7	**1.38 (m)**	1.38 (m)	1.33–1.52 (m)	**22**	**-**	**-**	**-**
8	**-**	**-**	**-**	**23**	0.90 (s)	0.90 (s)	0.88 (s)
9	**1.51 (m)**	1.54 (m)	1.58 (m)	**24**	**0.95 (s)**	0.97 (s)	0.96 (s)
10	**-**	**-**	**-**	**25**	**0.92 (s)**	0.86 (s)	0.86 (s)
11	**1.60 (m)**	1.62 (m)	1.63 -1.80 (m)	**26**	**0.97 (s)**	1.00 (s)	0.97 (s)
12	**5.5 (t)**	5.11 (t)	5.18 (t; 3.5 Hz)	**27**	**1.09 (s)**	1.06 (s)	1.13 (s)
13	**-**	**-**	**-**	**28**	**0.82**	0.84 (s)	0.83 (s)
14	**-**	**-**	**-**	**29**	**0.86 (s)**	0.86 (s)	0.88 (s)
15	**0.87**	0.86 (m)	0.85–1.76 (m)	**30**	**0.80 (s)**	0.79 (s)	0.87 (s)
OCH3	**2.04**	2.03 (s)	2.05 (s)				

Table 2 shows that the compound of F.1.3 is similar to A4 compound and *β*-amyrin acetate standard. Comparison of the ^{1}H-NMR data indicates that this compound contains *β*-amyrin acetate [16,23].

^{13}C-NMR Spectrophotometry. As shown in Figures 8 and 9, magnetic resonance spectroscopy of the ^{13}C-NMR carbon core ($CDCl_3$, 125 MHz, δ ppm) revealed 29 carbon signals consisting of six methyl carbons, eleven methylene carbons, nine methylene carbons, and three quaternary carbon atoms. The signals at δ_C: 14.27 and 15.58 ppm are for methyl groups bound to the steroid base. Meanwhile, the signals at δ_C: 15.61, 17.66, 18.95, and 21.47 ppm are signals for methyl groups bound to side chains. At the chemical shift, 63.27 ppm is the signal for C that binds to the OH group. Then, the signal at the chemical shift is 117.03, and 158.23 ppm is a signal for C sp^2.

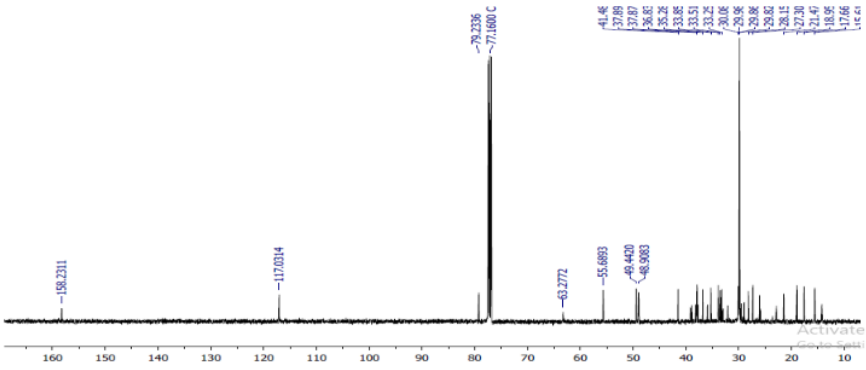


Figure 8. ^{13}C NMR spectrum of isolate 1.3 ($CDCl_3$, 125 MHz)

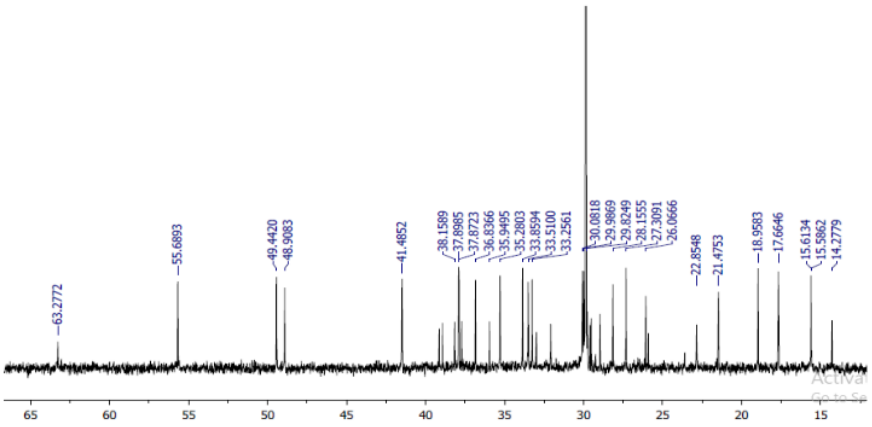


Figure 9. ^{13}C-NMR spectrum of isolate 1.3, the chemical shift for carbon in 14.27–63.27 ppm ($CDCl_3$, 125 MHz)

Anti-Inflammatory Activity. Inflammation is a common reaction of living tissues toward an injury. Steroidal anti-inflammatory agents lyse and possibly induce the redistribution of lymphocytes, which cause a rapid and transient decrease in peripheral blood lymphocyte counts to affect longer-term response. A reduction in % edema was monitored in the anti-inflammatory activity test, which was carried out on the ethyl acetate viscous extract and isolate 1.3. Carrageenan-induced inflammation is a helpful model for the estimation of anti-inflammatory effect. The development of edema in the rat's paw after the carrageenan injection is due to the release of histamine, serotonin, prostaglandin, and so on [1,6,15]. The diameters of the edema after carrageenan was injected on the feet of mice are listed in Table 3.

Table 3. Diameter of the mouse feet before and after injection with carrageenan

Treatment*	Dose (mg/kgBW)	Diameter of the mice's feet (mm)	
		Before	After
Positive Control	0.14	0.25	0.32
Negative Control	-	0.17	0.26
Extract	5.0	0.22	0.34
Extract	10	0.26	0.37
Extract	15	0.23	0.34
Isolate	5.0	0.24	0.32
Isolate	10	0.26	0.34
Isolate	15	0.23	0.34

As shown in Table 3, the edema diameter in mouse feet after being injected with carrageenan ranged from 0.26 mm to 0.37 mm. After carrageenan injection in the test animals, we analyzed the anti-inflammatory activity and then injected test samples on the mouse feet. Anti-inflammatory activity was evaluated by measuring at intervals of 30, 60, 90, 120, 150, and 180 min. The results obtained are listed in Tables 4 and 5.

Table 4. Anti-inflammatory effectiveness test results

Treatment	Dose (mg/KgW)	Feed volume after injection of the sample (mm)						
		Carrageenan (0 min)	30 (min)	60 (min)	90 (min)	120 (min)	150 (min)	180 (min)
Positive Control	10	0.32	0.31	0.30	0.27	0.27	0.25	0.25
Negative Control	10	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Extract	5	0.34	0.32	0.30	0.27	0.25	0.25	0.25
Extract	10	0.37	0.36	0.30	0.28	0.27	0.26	0.26
Extract	15	0.34	0.32	0.30	0.30	0.30	0.25	0.25
Isolate	5	0.32	0.31	0.26	0.26	0.26	0.26	0.26
Isolate	10	0.34	0.30	0.28	0.27	0.26	0.26	0.26
Isolate	15	0.34	0.32	0.30	0.28	0.27	0.27	0.27

Table 5. Calculations decrease of % edema

Treatment	Dose (mg/KgBW)	% edema					
		30	60	90	120	150	180
Positive Control	10	24%	20%	8%	8%	0%	0%
Negative Control	10	53%	53%	53%	53%	53%	53%
Extract	5	45%	36%	23%	14%	14%	14%
Extract	10	38%	15%	8%	4%	0%	0%
Extract	15	39%	30%	30%	30%	9%	9%
Isolate	5	29%	8%	8%	8%	8%	8%
Isolate	10	15%	8%	4%	0%	0%	0%
Isolate	15	39%	30%	22%	17%	17%	17%

Tables 4 and 5 shows that the isolate and ethyl acetate extracts of *A. augusta* leaves have anti-inflammatory activity. Thus, although the extract has higher anti-inflammatory activity than the isolates, the extract might contain other anti-inflammatory compounds. Furthermore, the volume of the mouse feet decreased. After 180 min, all formula doses exerted a similar effect to the initial volume of the mouse feet of mice [12,22,23].

The isolates and extracts reduced % edema to a level close to that in the positive control at the concentration (10 mg). These results indicate that the decrease in % edema improves and almost close to the positive control as the dose administered is increased. Figures 6 and 7 show the graphs of % positive edema reduction control of the isolates and extracts.

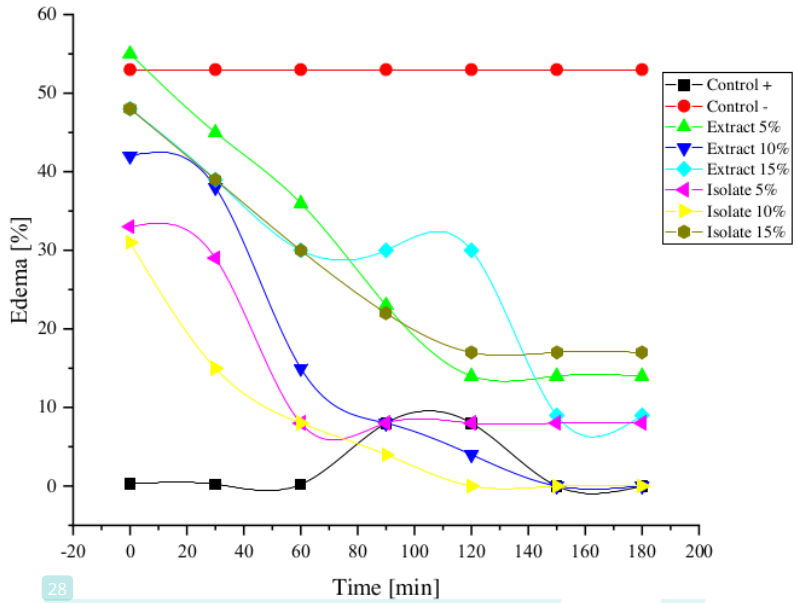


Figure 10. Inhibition of carrageenan-induced paw edema in mice. The graph is plotted between control, doses of standard drug diclofenac sodium (0.14 mg/kg), time reaction in X axis, and percent edema in Y axis. Inhibition of paw edema slightly decreased with the isolates than with the extracts.

Figure 10 shows that the decrease in % edema started from the 30th min and then optimized at the 90th min. On average, % edema decreased and began to reach the initial volume of the mouse feet

and approached positive control activity. Thus, the extracts possibly exerted their anti-inflammatory action by influencing the second phase of inflammation, namely, the cyclooxygenase pathway, rather than the lipoxygenase pathway.

4. Conclusion

The UV-Vis, FT-IR, GC-MS, and NMR characterization of isolate.1.3 of *A. augusta* showed that the compound obtained is the steroid *β*-amyrin acetate, which is the active anti-inflammatory agent. The isolate concentration of 10% produced the least % edema because of its anti-inflammatory activity against inflammation. Moreover, the anti-inflammatory activity of the isolates was better than that of the positive control.

5. Acknowledgement

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