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SECONDARY METABOLITE PROFILE OF SACHA INCHI (*PLUKENETIA VOLUBILIS* L.) SEED EXTRACT USING LC-ORBITRAP HRMS AND ITS ANTIBACTERIAL ACTIVITY

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Abstract. Research on the exploration of active compounds from Sacha Inchi (SI) seeds, *Pluketenia volubilis* L., has been conducted. The study aimed to profile the secondary metabolites of SI seeds using liquid chromatography-orbitrap high-resolution mass spectrometry (LC-Orbitrap HRMS), screen for chemical compounds, determine total phenolic and flavonoid contents, and assess antibacterial activity. The antibacterial assay included inhibition zone measurement, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The results revealed that the acetone extract of SI seeds contains steroid/triterpenoids, alkaloids, and flavonoids. According to LC-Orbitrap HRMS data, 41 steroids, 20 alkaloids, and one flavonoid were identified in the acetone extract. Neriifolin, Dehydrated antipain, and Artoindonesianin B were the predominant steroid, alkaloid, and flavonoid, respectively, representing the novelty of this research. The acetone extract of SI seeds exhibited a total phenolic content (TPC) of 28.04 ± 1.40 mg GAE/g and a total flavonoid content (TFC) of 25.98 ± ±1.40 mg QE/g. The acetone extract of SI seeds was fractionated with several solvents and tested for antibacterial activity. SI seed extract inhibited *Staphylococcus epidermidis* ATCC 25923, *Staphylococcus aureus* ATCC 12228, and *Methicillin-resistant Staphylococcus aureus* (MRSA). The chloroform fraction demonstrated the strongest inhibition against MRSA, with an inhibition zone of 16.4 ± 0.4 mm (strong), MIC 3.125 mg/mL, and MBC 25 mg/mL, while the *n*-hexane fraction showed the strongest activity against *S. aureus* (15.7 ± 0.3 mm (strong), MIC 3.125 mg/mL, MBC 6.25 mg/mL) and *S. epidermidis* (10.5 ± 0.3 mm (strong), MIC 3.125 mg/mL, MBC 25 mg/mL). This study provides a solid foundation for future research, particularly on the secondary metabolites of SI seeds as antibacterial candidates.

Keywords: Sacha Inchi seed, secondary metabolite profile, LC-Orbitrap HRMS, antibacterial, acetone extract.

1. Introduction

Sacha Inchi (SI) (*Plukenetia volubilis* L.) is a legume plant of the *Euphorbiaceae* family, genus *Plukenetia*, that bears green star-shaped fruits containing flesh and brown seeds.[1, 2] SI originated in Peru and has spread to Central and South America, as well as Thailand, China, Vietnam, and Indonesia.[3] In Indonesia, this plant is known as "Star Bean"' due to the star shape of the fruit. SI is a versatile plant that may be consumed or utilized as a raw material to make cosmetics,[4] but it is not yet well known, and therefore its applications are limited.

SI seeds have been reported to contain 25–30 % protein composed of essential amino acids, with leucine being the most abundant (64 %), followed by tyrosine, isoleucine, lysine, threonine, and valine.[5] SI seeds have a fat content of 35–60 %, with unsaturated fatty acids accounting for the majority (82 %).[6] α-Linolenic acid (ALA, omega-3) is the main fatty acid, with a content of 46.8–50.8 %, followed by linoleic acid (omega-6) of 33.4–36.2 %, and oleic acid (omega-9) of 8.7–9.6 %. In addition, SI seeds include vitamin E, flavonoids, and minerals.[7,8] SI's secondary metabolites are classified as flavonoids, steroids, or triterpenoids.[9, 10]

The presence of secondary metabolites in SI is recognized for its ability to generate a range of biological activities, such as antioxidant, antibacterial, antiproliferative, and antitumor.[11–14] However, the literature indicates that reports on secondary metabolites in SI seeds are quite limited, particularly concerning the specific

compounds within the secondary metabolite group. Recent research on the metabolite profile of SI has utilized liquid chromategraphy-mass spectrometry (LC-MS) to identify its seconddary metabolites, which were subsequently evaluated for potential anti-aging,[15] antioxidant,[16] and anti-inflammatory[17] activities. Several studies on SI husks and seed shells have shown antibacterial activity.[18, 19] It has also been reported that SI seed oil inhibits *Staphylococcus aureus* and *Enterococcus faecalis*.[20, 21] Related to its activity as an antibacterial, a comprehensive study to determine the minimum inhibitory and bactericidal concentrations of SI seeds has also not been widely carried out. Based on this background information, the purpose of this study is to profile secondary metabolites in Sacha Inchi (SI) (*P. volubilis* L.) seeds using LC-Orbitrap HRMS. This technique has advantages in terms of sensitivity and accuracy, resulting in more thorough and accurate information on the presence and characterization of chemical compounds in the sample. This study involved extraction, fractionation, phytochemical screening, and determination of total phenolic and flavonoid contents. Antibacterial activity was evaluated using inhibition zones, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC).

2. Experimental

2.1. Materials

The materials used in this research included Sacha Inchi (SI, *Pluketenia volubilis* L.), obtained from Sragen, Indonesia. The solvents used for extraction were acetone (p. a), *n*-hexane (p. a), chloroform (p. a), ethyl acetate (p. a), and methanol (p. a), purchased from Merck, Germany. Acetic acid and ammonia solutions used in various chemical processes were also obtained from Merck, Germany. The following compounds were used for further analysis: sulphuric acid, hydrochloric acid, aluminum chloride, sodium carbonate, and sodium acetate (Merck, Germany). The following chemicals were also used: magnesium powder, quercetin, gallic acid (Sigma Aldrich, USA), distilled water (deionized water), Mayer reagent, Dragendorff reagent, Wagner reagent, and Folin-Ciocalteu reagent (Merck, Germany). Bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC): *Staphylococcus epidermidis* ATCC 25923, *Staphylococcus aureus* ATCC 12228, and *Methicillin-resistant Staphylococcus aureus* (MRSA). Bacterial cultures were grown on nutrient agar (NA) and Mueller-Hinton agar (MHA), with Mueller-Hinton broth (MHB) serving as the liquid medium (HiMedia, India). DMSO (Merck, Germany) was utilized as both a solvent for the bioactive compounds and a negative control in antibacterial tests. Clindamycin and vancomycin (Oxoid, UK) were used as positive controls in the antibacterial assay.

2.2. Methods

2.2.1. Extraction and Fractionation

SI seed samples were extracted using freshly distilled acetone at room temperature and macerated for 3×24 hours. Every 24 hours, the material was filtered, and a new solvent was introduced to complete the extraction. The mixed filtrate was then evaporated to yield a crude extract. The crude extract was then subsequently fractionated using solvents of increasing polarity: *n*-hexane, chloroform, ethyl acetate, and methanol. This fractionation was intended to separate compounds based on their polarity for further analysis.

2.2.2. Phytochemical Screening

Phytochemical screening was performed using several standard tests. The alkaloid test involved mixing the sample with ammonia and chloroform, followed by the addition of HCl and specific reagents.[22] Flavonoids were detected using the Shinoda test, which employed the extract, Mg powder, and concentrated HCl. The terpenoid test involved the addition of chloroform and concentrated H_2SO_4 to the extract. The steroid / triterpenoid test used a mixture of the extract with chloroform, CH_3COOH, and concentrated H_2SO_4. The saponin test was conducted by mixing of the extract with distilled water and HCl, followed by observation of foam formation. Tannins were identified by mixing the extract with $FeCl_3$.[23]

2.2.3. Metabolite Profile Analysis

Secondary metabolite profiling of SI seed extract was carried out using LC-Orbitrap HRMS. 61 mg of sample was dissolved in 1 mL of HPLC-grade methanol, vortexed for 2 minutes, and then sonicated at 30 °C for 30 minutes. The mixture was vortexed for 2 minutes, centrifuged at 1400 × *g* for 5 minutes, and filtered through a 0.2 µm nylon filter before being analyzed using the LC-Orbitrap HRMS system.[24]

LC-HRMS parameters:

a. Liquid Chromatography: Thermo Scientific™ Vanquish™ Horizon UHPLC with Binary Pump (Germering, Germany)
   Analytical Column: Thermo Scientific™ Accu-core™ Phenyl Hexyl 100 mm length × 2.1 mm ID × 2.6 µm particle size (Lithuania)
   Mobile Phase: A: MS grade Water with 0.1 % Formic Acid
   B: MS grade Acetonitrile with 0.1 % Formic Acid

b. High Resolution Mass Spectrometry: Thermo Scientific™ Orbitrap™ Exploris 240 HRMS (Bremen, Germany)
Ionization Mode: Both positive and negative ionization modes (switching)
Ion Source: H-ESI (Heated Electrospray Ionization)

2.2.4. Total Phenolic Content Assay

To generate a 100 ppm solution, 5 mg of extract was added to a 50 ml measuring flask, and the volume was made up with 96 % ethanol. 0.2 ml of sample solution was mixed with 1 ml of Folin-Ciocalteu reagent, agitated, and left for 8 minutes. The solution was mixed with 3 mL of Na_2CO_3 and left for two hours, then the mixture was adjusted to the calibration mark with ethanol. The absorbance was measured at 770 nm using a UV-Vis spectrophotometer.[25, 26]

2.2.5. Total Flavonoid Content Assay

15 mg of extract was dissolved in 10 ml of ethanol to obtain a concentration of 1500 ppm. From this solution, 1 mL was pipetted, mixed with 1 mL of 2 % $AlCl_3$ solution and 1 ml of 120 mM sodium acetate. The sample was incubated for one hour at room temperature. UV-Vis spectrophotometry was used to determine absorbance at the maximal wavelength (430 nm).[27, 28]

2.2.6. Antibacterial Activity Determination

2.2.6.1. Inhibition Zone Measurement

The well-diffusion method was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations. The bacterial strains were spread on MHA, and 20 µL of the sample was placed into the wells. Plates were incubated at 37 °C for 24 hours, and the inhibition zones were then measured.[29, 30] Clindamycin is used as a positive control for *S. aureus* and *S. epidermidis*, vancomycin as a positive control for MRSA, and DMSO as a negative control.

2.2.6.2. Minimum Inhibitory Concentration (MIC)

The microdilution method[29] was used to determine the MIC measurement. Each well of a 96-well microplate was initially filled with 100 µL of MHB. Then, 100 µL of the sample (previously diluted with DMSO) was added to the wells (each sample was evaluated in duplicate), followed by serial dilution. Each well then received 100 µL of the pre-diluted bacterial suspension prepared in 30 mL of MHB (Fig. 1). The microplate was sealed firmly and incubated for 24 hours on a shaker at 200 rpm at room temperature (30 °C). After incubation, bacterial growth was assessed. Wells showing visible bacterial growth were considered to have concentrations above the MIC, whereas wells with no visible growth indicated the MIC.[31]

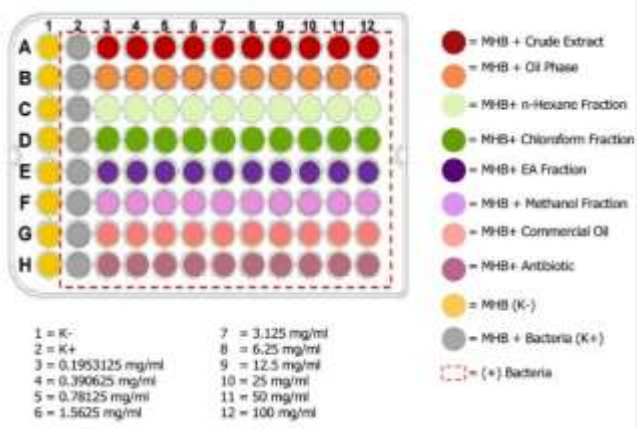


Fig. 1. Sample formation in a 96-well plate in the MIC measurement

2.2.6.3. Minimum Bactericidal Concentration (MBC)

To determine the MBC, 10 µL of the sample concentration (MIC or higher) was plated onto a NA. The plates were incubated at room temperature for 24 hours. Following incubation, the plates were examined for bacterial growth. If no bacterial colonies were observed, the concentration was recorded as the MBC value.[27, 29–31] All experiments were performed in triplicate (n=3), and data are presented as mean ± standard deviation (SD).

3. Results and Discussion

The maceration of SI seeds with acetone yielded two distinct layers: an oil phase and an organic phase. This separation occurs because non-polar lipophilic molecules (such as triglycerides and fatty acids) remain in the oil phase, whereas the organic phase contains more polar bioactive compounds, such as flavonoids and tannins.[4, 12] These compounds are distinguished for their pharmacological properties, encompassing antioxidant and anti-inflammatory activities. To isolate and investigate the bioactivity of chemicals present in the organic phase, a series of fractionation steps was carried out using solvents of increasing polarity, including *n*-hexane, chloroform, ethyl acetate, and methanol. This stepwise polarity-based separation guarantees that substances with comparable solubility characteristics are

divided into separate fractions, making it easier to identify and assess their bioactivity.[8] To successfully separate substances based on their chemical characteristics, the solvents were selected according to a systematic polarity gradient.[32]

3.1. Phytochemical Screening

Phytochemical screening was performed by observing the color changes produced after adding specific reagents or reactants to the SI seed acetone extract and its fractions.[33] The phytochemical screening targeted alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, and triterpenoids. According to the results, SI seed contained alkaloids, flavonoids, and terpenoids; however, saponins and tannins were not detected in all samples. Table 1 shows the phytochemical screening results for SI seeds.

3.2. Metabolite Profile

The phytochemical screening indicated that the crude extract and its fractions contained secondary metabolites from the same group; consequently, the secondary metabolite profile of Sacha inchi seeds was established using the acetone crude extract analyzed by LC-Orbitrap HRMS.

LC-Orbitrap HRMS combines liquid chromatography, which separates substances with high-resolution mass spectrometry to identify and characterize them. LC-Orbitrap HRMS has specific advantages in terms of sensitivity and accuracy, especially in the identification and quantification of molecules.[34] In contrast to low-resolution mass spectrometry (LRMS), which has a limited ability to differentiate between similar molecules and is susceptible to interference at low concentrations, HRMS gives more detailed and accurate information on chemical composition.[34] The Orbitrap detector in LC-Orbitrap HRMS has an extremely high mass resolution capacity, allowing for the study of molecules with similar masses.[35] This characteristic is particularly important when evaluating complex chemicals in biological or environmental matrices, as it allows for more exact identification and characterization of molecules in an unknown sample. This includes metabolites that may be undetectable by other approaches.[36]

The analytical results revealed that the secondary metabolites in SI seeds primarily comprised steroids, alkaloids, and flavonoids, aligning with prior phytochemical screening findings. A total of 41 steroids, 20 alkaloids, and 1 flavonoid compound were identified in the acetone extract of SI seeds (Table 2). This outcome is a novel finding of this investigation. Unlike previous research, which only reported the classes of secondary metabolites in SI seeds, this study successfully identified individual compounds within each group, particularly steroids, alkaloids, and flavonoids.

The predominant secondary metabolites in the acetone extract of SI seeds were identified as steroids, based on the overall LC-Orbitrap HRMS ions analysis. The predominant peak in the steroid category was detected at a retention time of 9.4 minutes, displaying a molecular ion peak at *m/z* $[M+H]^+$ 535.32782 with a peak area of 159,858,758.1 (Table 2). This signal corresponds to Neriifolin ($C_{30}H_{46}O_8$, calculated MW 534.3102) (Fig. 2, *a*).

Table 1. Phytochemical Composition of SI Seed

Sample	Alkaloids	Saponins	Flavonoids	Steroid / Terpenoid	Tannins
Acetone Crude Extract	+	–	+	+	–
Oil Phase	+	–	+	+	–
n-Hexane Fraction	+	–	+	+	–
Chloroform Fraction	+	–	+	+	–
Ethyl Acetate Fraction	+	–	+	+	–
Methanol Fraction	+	–	+	+	–

Table 2. Secondary Metabolites of Acetone SI Seed Extract Identified by LC-Orbitrap HRMS

No	Compounds	Formula	Calc. MW	*m/z*	RT (min)	Peak Area (Max)	Reference Ion
1	2	3	4	5	6	7	8
Steroids							
1	Neriifolin	$C_{30}H_{46}O_8$	534.31902	535.32782	9.40	159858758.1	$[M+H]^+$
2	Cimicifoetiside B	$C_{39}H_{60}O_{11}$	704.41307	705.42035	12.56	137178182.9	$[M+H]^+$
3	Progenin II	$C_{39}H_{62}O_{12}$	722.42345	723.43073	12.09	112031967.4	$[M+H]^+$
4	Turkesterone 22-acetonide	$C_{30}H_{48}O_8$	536.33489	537.34216	11.56	109232632.7	$[M+H]^+$
5	Zymosterol	$C_{29}H_{46}O_2$	426.34947	427.35675	14.96	93371236.02	$[M+H]^+$
6	Venustone	$C_{31}H_{50}O_9$	566.34526	567.35254	11.03	93076289.95	$[M+H]^+$

Continuation of Table 2

1	2	3	4	5	6	7	8
7	Aspacoside C	$C_{32}H_{50}O_{11}$	610.33517	611.34222	11.14	92892902.21	$[M+H]^+$
8	Cholic Acid Glucuronide	$C_{30}H_{48}O_{11}$	584.31935	583.31207	8.93	85683693.33	$[M+H]^+$
9	5α-Dihydrotestosterone	$C_{19}H_{30}O_2$	290.22453	291.23181	12.21	82596471.57	$[M+H]^+$
10	5α-Dihydrotestosterone glucuronide	$C_{25}H_{38}O_8$	466.25484	467.26212	8.69	82596471.57	$[M+H]^+$
11	Penicisteroid F	$C_{30}H_{50}O_7$	522.35539	523.36267	11.99	81971386.88	$[M+H]^+$
12	Linckoside G	$C_{32}H_{52}O_{10}$	596.35596	597.36319	12.83	78030182.55	$[M+H]^+$
13	3β,15α,16α,24α-Tetrahydroxy-25,26,27-trinor-16,24-cyclo-cycloartane-23-one-3-O-β-D-xylopyranoside	$C_{32}H_{50}O_9$	578.34502	579.35229	11.39	76709783.38	$[M+H]^+$
14	Arvenin III	$C_{36}H_{54}O_{12}$	678.36046	679.36774	10.72	76268687.56	$[M+H]^+$
15	Tetrahydrocortisone	$C_{21}H_{32}O_5$	364.22469	365.23199	7.04	56058355.23	$[M+H]^+$
16	Oleandrin	$C_{32}H_{48}O_9$	576.32762	577.3349	11.6	55086204.23	$[M+H]^+$
17	7α-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20369	305.21097	9.73	54780021.58	$[M+H]^+$
18	Kurilensoside H	$C_{32}H_{54}O_{11}$	614.36659	597.36322	10.62	54606791.13	$[M+H-H_2O]^+$
19	25-Hydroxy-atrotosterone A	$C_{28}H_{46}O_8$	510.31902	511.32629	10.88	51784718.37	$[M+H]^+$
20	Kurilensoside G	$C_{32}H_{54}O_{10}$	598.37111	581.36774	11.47	51285781.6	$[M+H-H_2O]^+$
21	Fasciculic Acid B	$C_{36}H_{60}O_9$	636.42131	637.42859	14.01	50507566.17	$[M+H]^+$
22	Penicisteroid G	$C_{30}H_{50}O_6$	506.36049	507.36777	13.81	45657162.56	$[M+H]^+$
23	Mantuoluoside G	$C_{35}H_{54}O_{11}$	650.36608	651.37335	11.99	45589629.19	$[M+H]^+$
24	27-Norcholestanehexol	$C_{26}H_{46}O_6$	454.32932	477.31857	13.29	44910206.51	$[M+H]^+$
25	6β-hydroxytestosterone	$C_{19}H_{28}O_3$	304.20363	305.21091	9.31	41895542.44	$[M+H]^+$
26	11β-Hydroxyandrosterone	$C_{19}H_{30}O_3$	306.21904	307.22632	10.68	41600278.26	$[M+H]^+$
27	Ecdysone 25-O-D-glucopyranoside	$C_{33}H_{54}O_{11}$	626.36522	627.3725	12.31	40493439.44	$[M+H]^+$
28	Colocynthenin E	$C_{31}H_{48}O_9$	564.32707	565.33435	11.33	35448821.3	$[M+H]^+$
29	2-deoxy-20-hydroxy-5alpha-ecdysone 3-acetate	$C_{29}H_{46}O_7$	506.32292	507.3302	11.89	28874563.06	$[M+H]^+$
30	Punicesterone B	$C_{31}H_{50}O_6$	518.35989	519.36717	15.02	26717342.91	$[M+H]^+$
31	Cucurbitacin C4	$C_{31}H_{50}O_8$	550.34868	551.35596	12.51	26358763.98	$[M+H]^+$
32	Brainesteroside C	$C_{33}H_{50}O_{11}$	622.33318	623.34045	10.48	24403872.25	$[M+H]^+$
33	Fasciculic Acid F	$C_{36}H_{60}O_{10}$	652.41655	653.42383	12.87	23720501.12	$[M+H]^+$
34	Fomitoside H	$C_{42}H_{66}O_{11}$	746.4594	747.46667	14.11	22208645.94	$[M+H]^+$
35	β-Estradiol	$C_{18}H_{24}O_2$	272.1776	273.18488	8.36	22150949.74	$[M+H]^+$
36	Combretic Acid C	$C_{32}H_{50}O_7$	546.35314	547.36041	13.54	21278467.45	$[M+H]^+$
37	Withanoside XI	$C_{34}H_{52}O_{11}$	636.3496	637.35687	10.39	18124868.73	$[M+H]^+$
38	Lobophysterol B	$C_{31}H_{52}O_7$	536.3701	537.37738	14.29	17511759.45	$[M+H]^+$
39	Acetylpinnasterol	$C_{29}H_{44}O_6$	488.31248	487.30521	4.54	13152702.28	$[M-H]^-$
40	Certonardosterol I	$C_{29}H_{50}O_5$	478.36556	479.37283	12.16	11811592.16	$[M+H]^+$
41	3beta,11beta-dihydroxy-24,25,26,27-tetranor-cycloart-7-en-23,16beta-olide-3-O-beta-D-xylopyranoside	$C_{31}H_{46}O_9$	562.31139	563.31866	12.97	8334161.04	$[M+H]^+$
	Alkaloids						
1	Dehydrated antipain	$C_{27}H_{42}N_{10}O_5$	586.33507	587.34222	10.575	316197798.7	$[M+H]^+$
2	Asterriquinol E	$C_{25}H_{22}N_2O_4$	414.15619	208.08546	5.03	146285094	$[M+2H]^{2+}$
3	Tryptoquivaline O	$C_{23}H_{18}N_4O_5$	430.12865	431.13593	5.039	128532183.3	$[M+H]^+$
4	Trigonelline	$C_7H_7NO_2$	137.04761	138.05489	0.777	88148022.49	$[M+H]^+$
5	Netropsin	$C_{18}H_{26}N_{10}O_3$	430.21994	413.21671	7.401	83499709.82	$[M+H^-H_2O]^+$
6	Ricinine	$C_8H_8N_2O_2$	164.05853	165.06583	2.662	74687207.92	$[M+H]^+$
7	Lucentamycin C	$C_{26}H_{46}N_6O_5$	522.35295	523.36023	14.237	72399589.03	$[M+H]^+$
8	Cereusitin A	$C_{25}H_{34}N_4O_6$	486.24635	487.25363	7.387	65425530.74	$[M+H]^+$
9	Microginin 590B	$C_{31}H_{50}N_4O_7$	590.36616	589.35889	9.169	63855265.2	$[M-H]^-$
10	Rimosamide C	$C_{28}H_{42}N_4O_7$	546.30374	547.31104	8.831	47225447.97	$[M+H]^+$
11	Rimosamide D	$C_{27}H_{40}N_4O_7$	532.28818	513.27026	8.419	42837722.58	$[M-H-H_2O]^-$
12	L-Pyroglutamic acid	$C_5H_7NO_3$	129.04256	130.04982	1.058	32510070.16	$[M+H]^+$

Continuation of Table 2

1	2	3	4	5	6	7	8
13	3H-Dibenz[f,ij]isoquinoline-2,7-dione	$C_{23}H_{22}N_2O_2$	358.16667	359.17395	4.194	32011537.44	$[M+H]^+$
14	Diatretol	$C_{16}H_{22}N_2O_4$	306.15907	307.166d 35	8.967	19798195.4	$[M+H]^+$
15	Benhamycin	$C_{23}H_{18}N_2O_4$	386.12509	194.06985	4.054	17169021.04	$[M+2H]^{2+}$
16	Cladoquinazoline	$C_{23}H_{22}N_4O_4$	418.16521	419.17249	4.581	11726882.1	$[M+H]^+$
17	Pyridoxal	$C_8H_9NO_3$	167.05841	168.06569	1.056	10574747.84	$[M+H]^+$
18	Mycemycin D	$C_{15}H_{11}ClN_2O_3$	302.04515	303.05243	3.872	9943536.236	$[M+H]^+$
19	Aniquinazoline D	$C_{24}H_{22}N_4O_4$	430.16539	429.15811	7.293	8504030.831	$[M-H]^-$
20	Brintonamide A	$C_{39}H_{60}N_6O_9$	756.44194	757.44922	12.688	7884352.437	$[M+H]^+$
	Flavonoid						
1	Artoindonesianin B	$C_{26}H_{28}O_8$	468.1761	469.18338	7.31	10541728.54	$M+H]^+$

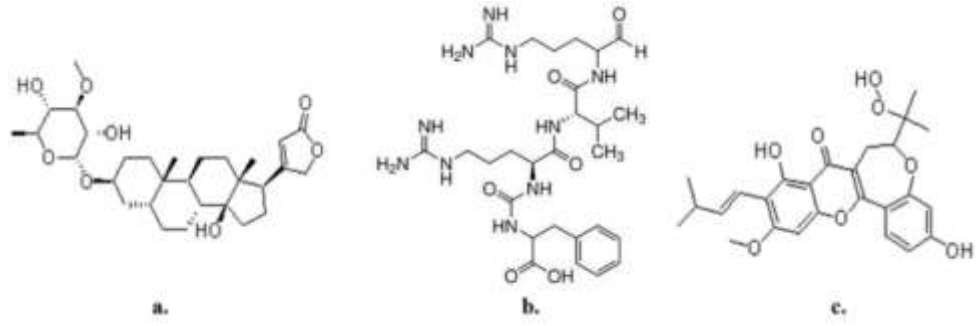


Fig. 2. Structure of: a – Neriifolin; b – Dehydrated antipain; c – Artoindonesianin B

The mass spectrum of Neriifolin exhibits a fragmentation pattern (Fig. 3) originating from the parent molecular ion $[M+H]^+$ $C_{30}H_{47}O_8$ at *m/z* 535.32782. The fragment $C_{18}H_{25}O_3$ at m/z 289.17993 corresponds to the fundamental structure of the steroid, and the fragment C_7H_9O at *m/z* 109.06473 signifies the existence of an unsaturated lactone ring, a hallmark of cardenolides. The diminutive fragment, C_5H_7O at *m/z* 83.04913, constitutes a component of the steroid ring. Neriifolin has shown promise as an anticancer compound, with low IC_{50} values across many cancer cell lines, including those linked to breast and ovarian cancer.[37, 38] It has been noted to bind to the Na^+/K^+ ATPase enzyme, altering the production and localization of its subunits within cells and thereby triggering apoptosis in cancer cells.[37, 38]

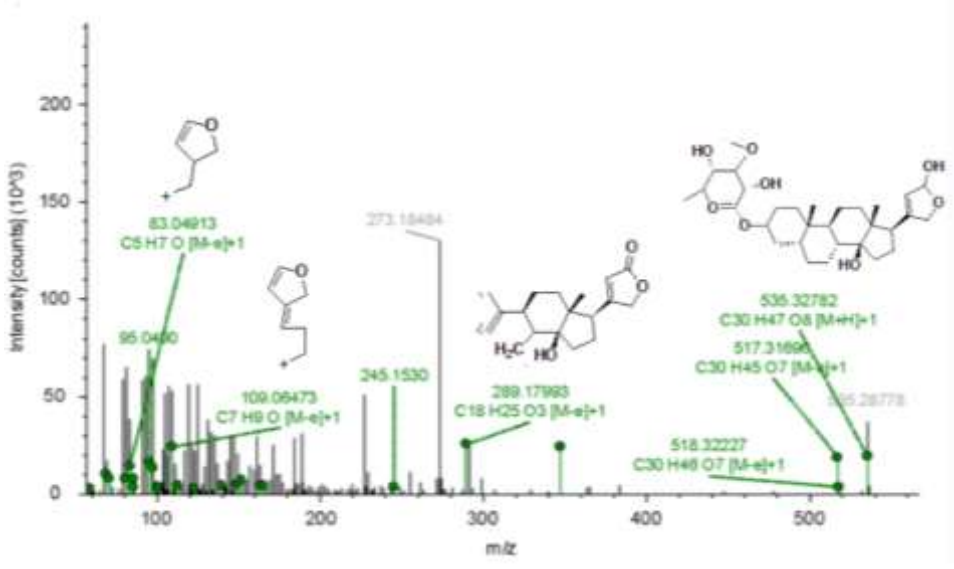


Fig. 3. Fragmentation Pattern of Neriifolin

Additionally, LC-Orbitrap HRMS analysis detected 20 alkaloids in the acetone extract of SI seeds. Dehydrated antipain is the predominant predicted alkaloid, detected at a retention time of 10.57 minutes with an area of 316,197,798 (Table 2). This peak aligns with the chemical formula of Dehydrated antipain ($C_{27}H_{42}N_{10}O_5$, MW calc. 586.33507) (Fig. 2, *b*).

The fragmentation pattern of Dehydrated antipain, as indicated by its analytical spectrum, originates from the parent molecular ion $[M+H]^+$ at *m/z* 587.3422, corresponding to the chemical formula $C_{27}H_{42}N_{10}O_5$ (Table 2). Subsequent fragmentation yielded an ion at *m/z* 569.33020, resulting from the elimination of H_2O, and confirming the presence of hydroxyl groups in its structure. The fragment at *m/z* 397.22180 corresponds to the cleavage of the peptide bond, while the fragment at *m/z* 228.13184 ($C_8H_{16}N_6O_2$) denotes a partial peptide framework. The fragment at *m/z* 105.06966 (C_8H_9) indicates the presence of an aromatic ring, whereas the fragment at *m/z* 79.05416 (C_6H_7) denotes a disrupted alkyl chain (Fig. 4). This fragmentation pattern offers definitive evidence corroborating the structure of Dehydrated antipain, which comprises peptide chains, aromatic rings, and diverse nitrogen-containing functional groups.[39]

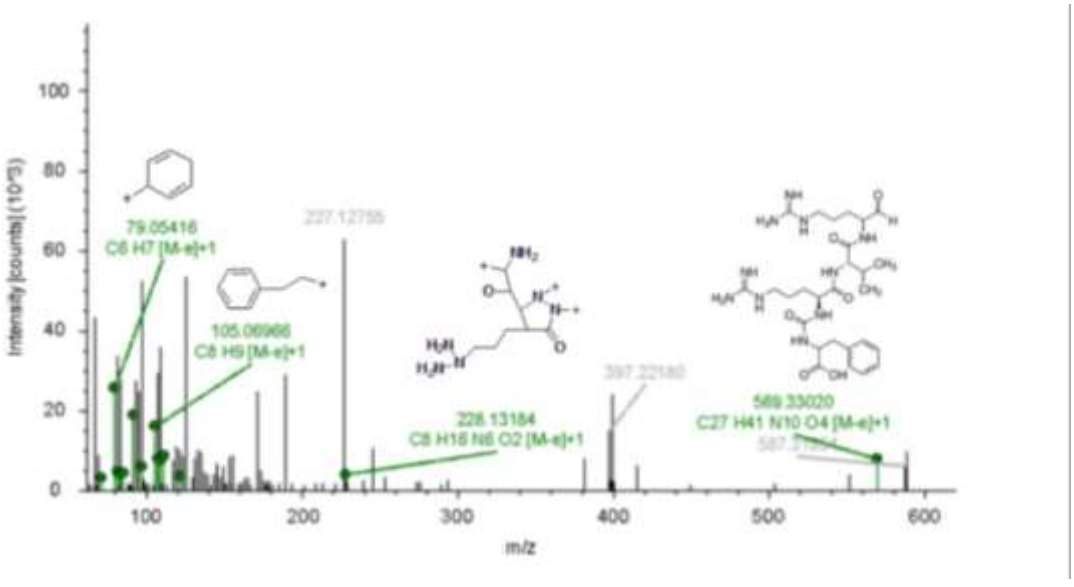


Fig. 4. Fragmentation Pattern of Dehydrated Antipain

A flavonoid was detected in the sample, in addition to alkaloids and steroids. The molecule was detected with a retention time of 7.31 minutes, exhibiting an area of 10,541,728.54 and a molecular ion at *m/z* $[M+H]^+$ 469.18338 (Table 2), corresponding to Artoindonesianin B ($C_{26}H_{28}O_8$). The initial fragmentation produced an ion at *m/z* 451.22845, which was ascribed to the loss of H_2O. Subsequent fragmentation generated distinctive ions, signifying structural components with hydroxyl groups and aromatic ring substituents (Fig. 5). Artoindonesianin B is a natural flavonoid, originally recognised as an isolated constituent in the roots of *Artocarpus champeden* (Lour.). This compound exhibits considerable cytotoxic action against murine leukaemia cells, P-388, with an IC_{50} value of 3.9 µg/mL.[40, 41] It possesses unique features as a natural hydroperoxide. Thus, the compounds identified in the acetone extract of SI seeds may serve as a foundation for further research aimed at isolating the principal bioactive constituents.

3.3. Total Phenolic Content and Total Flavonoid Content

3.3.1. Total Phenolic Content

A study was performed to examine the total phenolic content (TPC) of the samples, aiming to identify significant variations in phenolic content extracted using different solvents (Table 3). The crude extract displayed a TPC of 28.04 ± 1.40 mg GAE/g. The *n*-hexane fraction showed a TPC of 15.50 ± 0.78 mg GAE/g, indicating a considerable presence of phenolic compounds.[42] This finding is unusual since phenolic compounds are generally polar (due to hydroxyl groups) and are usually extracted with polar or semi-polar solvents (methanol, ethanol, acetone, ethyl acetate). The unexpectedly high TPC in the non-polar *n*-hexane fraction can be explained by the presence of structurally modified and lipophilic phenolic derivatives in

Sacha Inchi seeds. This phenomenon aligns with findings by Mannoubi,[42] who reported that non-polar solvents can yield high TPC due to the affinity of modified phenolic compounds such as prenylated, acylated, or methylated derivatives for non-polar environments. The primary factor governing solubility is not merely solvent polarity but the specific structural characteristics of the solute itself.[43] These structural modifications, involving the addition of non-polar groups, reduce molecular hydrophilicity and enhance solubility in non-polar solvents like *n*-hexane.[43, 44] The TPC values of the chloroform and ethyl acetate fractions were 8.29 ± 0.41 mg GAE/g and 5.31 ± 0.27 mg GAE/g, respectively, indicating that semi-polar solvents like ethyl acetate are effective in extracting certain phenolic compounds.[45] In contrast, methanol produced relatively low TPC (1.39 ± 0.07 mg GAE/g), possibly due to structural incompatibility with the predominant phenolic compounds in the sample.[46] The solubility of phenolic compounds depends not only on solvent polarity but also on their molecular structure and specific characteristics.[47] This may be related to the limited presence of hydroxyl groups or the occurrence of non-polar groups (*e. g.*, methoxy groups) in certain phenolic compounds, which makes them less polar and therefore more soluble in less-polar solvents.[41]

3.3.2 Total Flavonoid Content

The evaluation of total flavonoid content (TFC) across multiple samples demonstrated notable variance in flavonoid extraction depending on the solvent used (Table 4).

The crude extract displayed a total flavonoid content (TFC) of 25.98 ± 1.30 mg QE/g. *n*-Hexane fraction showed a TFC of 13.45 ± 0.67 mg QE/g. The effectiveness of *n*-hexane in flavonoid extraction is due to its non-polar characteristics, which aid in dissolving lipophilic substances.[46]

Table 3. Total Phenolic Content of SI Seed

Fraction	Absorbance	GAE(x), mg/ml	TPC, mg GAE/g
Acetone Crude extract	0.422	0.0334	28.04 ± 1.40
n-hexane	0.310	0.0185	15.50 ± 0.78
Chloroform	0.179	0.0019	8.29 ± 0.41
Ethyl acetate	0.219	0.0063	5.31 ± 0.27
Methanol	0.184	0.0017	1.39 ± 0.07
Oil phase	0.173	0.0002	0.16 ± 0.01

Table 4. Total Flavonoid Content of SI Seed

Fraction	Absorbance	QE(x), mg/ml	TFC, mg QE/g
Acetone Crude extract	1.464	0.0779	25.98 ± 1.30
n-hexane	0.776	0.0404	13.45 ± 0.67
Chloroform	0.551	0.0281	9.35 ± 0.47
Ethyl acetate	0.377	0.0186	6.18 ± 0.31
Methanol	0.045	0.0004	0.14 ± 0.01
Oil phase	0.164	0.0069	2.30 ± 0.12

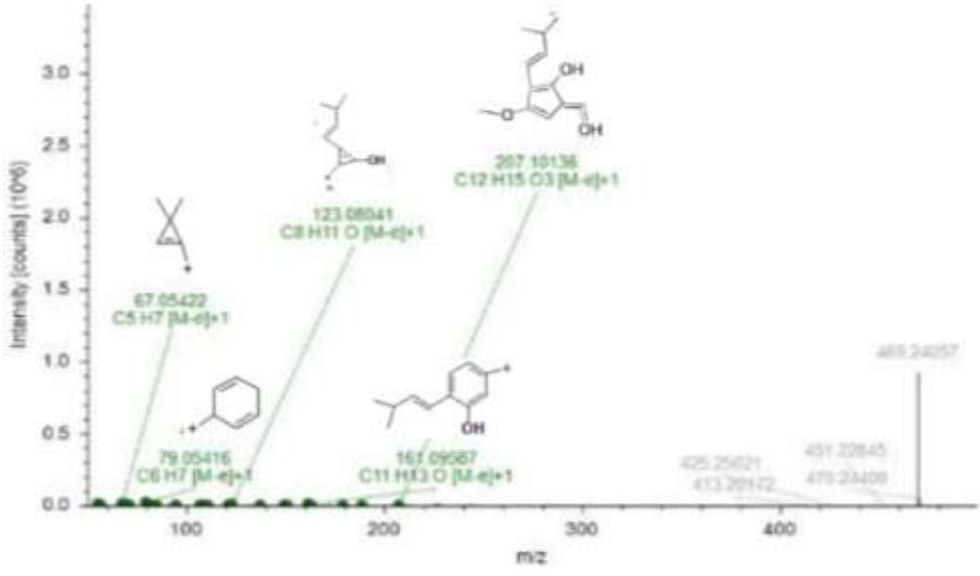


Fig. 5. Fragmentation Pattern of Artoindonesian B

The chloroform fraction showed a TFC of 9.35 ± 0.47 mg QE/g; which was lower than that of *n*-hexane, likely due to its increased polarity, which limits the extraction of lipophilic molecules.[48] Ethyl acetate, a semi-polar solvent, yielded a TFC of 6.18 ± 0.31 mg QE/g, indicating some ability to extract both polar and non-polar flavonoids, though not as efficiently as *n*-hexane. Methanol, a polar solvent, demonstrated a very low TFC (0.14 ± 0.01 mg QE/g), possibly due to its incompatibility with the predominant flavonoid compounds in the sample.[49] The oil phase demonstrated the lowest flavonoid concentration (2.30 ± 0.12 mg QE/g), indicating insufficient solubilization of flavonoids in that phase. These results highlight the importance of solvent selection in the extraction process to maximise flavonoid yield. The choice of a suitable solvent significantly influences the extraction efficiency because flavonoids differ in polarity and therefore require appropriately matched solvents for effective extraction.

3.4. Antibacterial Activity

3.4.1. Antibacterial Activity Against *S. epidermidis* ATCC 25923

According to the antibacterial assay (Fig. 6, Table 5), the *n*-hexane fraction was the most effective sample against *S. epidermidis*, with a strong inhibition zone (10.5 ± 0.3 mm), the lowest MIC (3.125 mg/mL), and a low MBC (25 mg/mL). Despite the inhibitory zone value being inferior to that of the chloroform fraction (13.3 ± 0.4 mm), the MIC value is likewise reduced, suggesting that this fraction is more efficacious in inhibiting bacteria. The *n*-hexane fraction necessitates a reduced concentration to inhibit bacteria, hence enhancing its efficacy in inhibition. This activity is likely attributable to the presence of lipophilic bioactive chemicals in the *n*-hexane fraction, such as terpenoids, alkaloids, or fatty acids, which effectively disrupt bacterial cell membranes or impair vital metabolic processes. Owing to their hydrophobic characteristics, these compounds can readily penetrate the bacterial cell membrane, resulting in the disruption of cellular functions, hence inhibiting bacterial growth. For comparison, the commercial oil, which refers to industrially processed Sacha Inchi oil sold in the market, showed no inhibitory activity (0 mm inhibition zone). This suggests that the industrial refining process may remove or inactivate antibacterial constituents present in the crude and fractionated extracts. Positive controls, clindamycin and vancomycin, exhibited clear inhibition zones confirming the assay's validity, whereas DMSO, used as a negative control and solvent, showed no inhibition. These results collectively confirm the reliability of the antibacterial test procedure.

The low minimum bactericidal concentration (MBC) of the *n*-hexane fraction against *S. epidermidis* ATCC 25923 (Fig. 7) in comparison to other samples signifies this fraction's efficacy in killing bacteria at comparatively low concentrations. The MIC and MBC indicate that the *n*-hexane fraction possesses significant bacteriostatic and bactericidal properties, making it a promising candidate for the development of natural antibacterial drugs against *S. epidermidis*.[27, 31] The findings align with the phytochemical screening results, indicating that the *n*-hexane fraction comprises alkaloids, steroids, flavonoids, triterpenoids, and terpenoids. These chemicals exhibit notable antibacterial activity, including alkaloids that disrupt bacterial protein synthesis and terpenoids that compromise the integrity of bacterial cell membranes. This bioactive composition likely contributes to the strong bacteriostatic properties (low MIC) of the *n*-hexane fraction and enhances its overall inhibitory potency.

Table 5. Antibacterial Activity of SI Seeds

Sample	*S. epidermidis*			*S. aureus*			*MRSA*		
	IH*	MIC*	MBC*	IH*	MIC*	MBC*	IH*	MIC*	MBC*
Acetone Crude Extract	14.2 ± 0.2 (*S*)	6.25	50	9.7 ± 0.0 *(M)*	6.25	25	0 *(I)*	0	–
Oil Phase	8.6 ± 0.3 (*M*)	12.5	100	0 *(I)*	0	–	7.8 ± 0.3 *(M)*	12.5	50
n-hexane	10.5 ± 0.3 (*S*)	3.125	25	15.7 ± 0.3 *(S)*	3.125	6.25	17.6 ± 0.3 *(S)*	6.25	25
Chloroform	13.3 ± 0.4 (*S*)	6.25	25	13.8 ± 0.4 *(S)*	6.25	6.25	16.4 ± 0.4 *(S)*	3.125	25
Ethyl Acetate	11.3 ± 0.2 (*S*)	6.25	50	11.6 ± 0.2 *(S)*	6.25	12.5	12.2 ± 0.2 *(S)*	6.25	25
Methanol	10.7 ± 0.3 (*S*)	12.5	25	11.8± 0.3 *(S)*	12.5	12.5	13.4 ± 0.3 *(S)*	12.5	25
Commercial Oil	0 *(I)*	0	–	0 *(I)*	0	–	0 *(I)*	0	–
Positive Control**	32.27 ± 0.4 (*S*)	–	–	27.5 ± 0.5 (S)	–	–	19.55 ± 0.3 (S)	–	–
Negative Control (DMSO)	0 (*I*)	–	–	0 (I)	–	–	0 (I)	–	–

IH = inhibition zone, MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, *S* = strong, *M* = moderate, *I* = inactive; * IH in mm, MIC and MBC in mg/mL; ** Clindamycin for *S. aureus* and *S. epidermidis*, vancomycin for MRSA

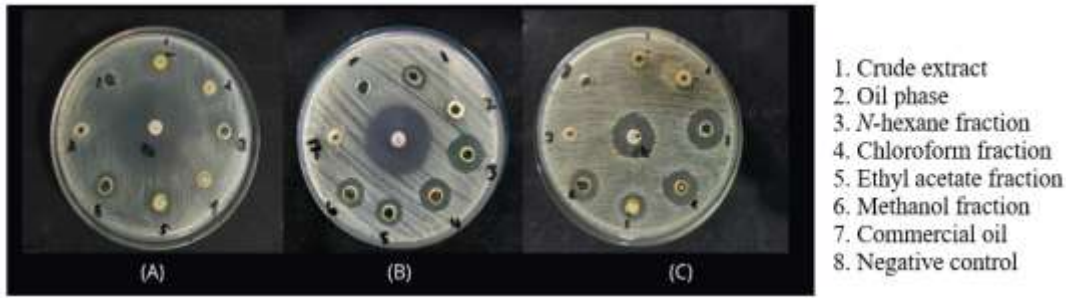


Fig. 6. Agar plates showing inhibition zones of different fractions against A. *S. epidermidis* ATCC 25923, B. *S. aureus* ATCC 12228, C. MRSA

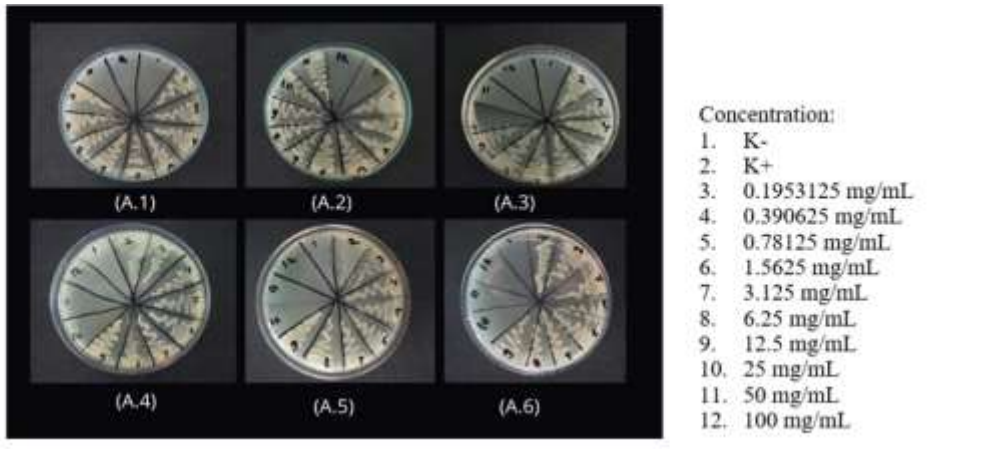


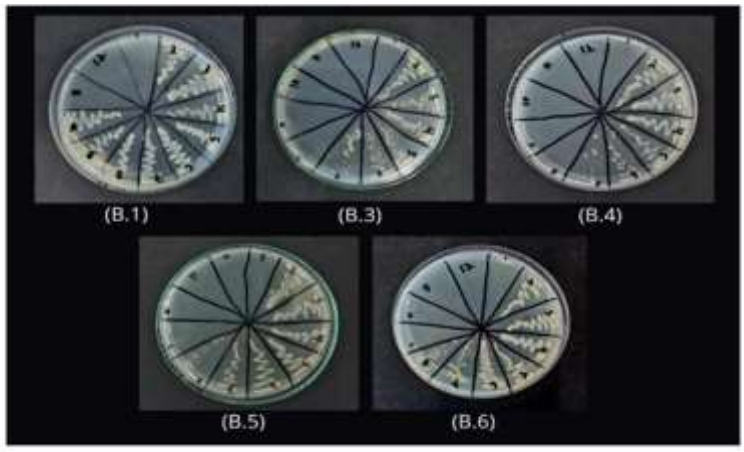
Fig. 7. Minimum Bactericidal Concentration on *S. epidermidis* ATCC 25923

3.4.2. Antibacterial Activity Against *S. aureus* ATCC 12228

The *n*-hexane fraction also showed the highest antibacterial effectiveness, both in inhibiting growth and killing *S. aureus* among all tested samples. In addition to having the most significant inhibitory power, the *n*-hexane fraction also showed the lowest MIC (3.125 mg/mL). Furthermore, its low MBC (Fig. 8), 6.25 mg/mL, indicated the ability of this fraction to kill bacteria at lower concentrations compared to other samples. The *n*-hexane fraction was superior compared to other fractions, such as the chloroform and ethyl acetate fractions, which had higher MIC and MBC values. This indicates that the *n*-hexane fraction not only has strong bacteriostatic activity but also efficient bactericidal activity.[27, 31] The combination of large inhibition zones and low MIC and MBC values indicates that this fraction contains highly potent bioactive compounds, such as lipophilic or hydrophobic compounds, which may be more effective in damaging bacterial cell membranes or inhibiting essential bacterial metabolic processes, which are the basis for strong antibacterial activity. Based on these results, the *n*-hexane fraction has advantages in terms of effectiveness and efficiency. Compared with other samples, this fraction requires a lower concentration to achieve optimal inhibitory and bactericidal effects against *S. aureus* ATCC 12228.

3.4.3. Antibacterial Activity Against MRSA

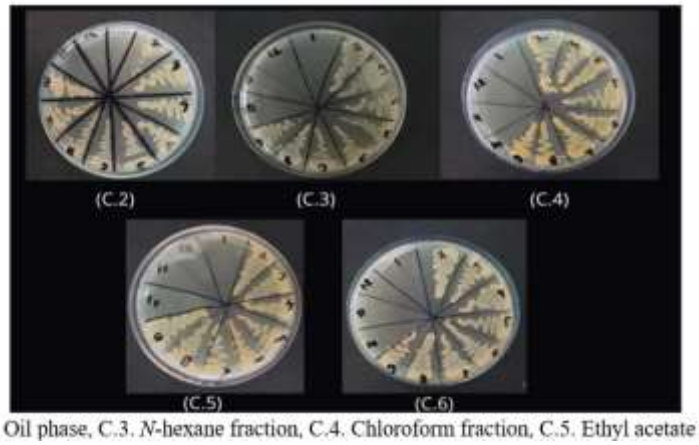
The chloroform fraction is the most effective candidate for inhibiting and killing MRSA (Table 7). This fraction shows an inhibition zone of 16.4 ± 0.4, which is categorized as strong. This confirms that the chloroform fraction has a high ability to inhibit the growth of MRSA, showing greater effectiveness than the acetone crude extract and slightly higher activity compared to the oil phase, which demonstrated moderate antibacterial activity according to Table 5. The MIC of the chloroform fraction is 3.125 mg/mL, which is the lowest MIC value among all samples tested. This low MIC indicates high antibacterial potential. This is important because the ability to inhibit bacteria at low concentrations can reduce the risk of side effects or toxicity that are usually associated with the use of compounds at high concentrations.



B.1. Crude extract, B.2. Oil phase, B.3. *N*-hexane fraction, B.4. Chloroform fraction, B.5. Ethyl acetate fraction, B.6. Methanol fraction

Fig. 8. Minimum Bactericidal Concentration on *S. aureus* ATCC 12228

The MBC of the chloroform fraction was 25 mg/mL (Fig. 9), demonstrating that this fraction can kill MRSA at a higher concentration than its MIC. This effectiveness indicates that after inhibiting bacterial growth at lower concentrations, the chloroform fraction can still exert bactericidal effects at higher concentrations.[27, 31] In comparison, the *n*-hexane fraction produced one of the largest inhibition zones against MRSA among all samples.[27, 31] However, despite its larger inhibition zone, the *n*-hexane fraction showed slightly lower effectiveness than the chloroform fraction, particularly in terms of MIC and MBC values.



C.2. Oil phase, C.3. *N*-hexane fraction, C.4. Chloroform fraction, C.5. Ethyl acetate fraction, C.6. Methanol fraction

Fig. 9. Minimum Bactericidal Concentration on MRSA

The results of antibacterial activity indicate the suitability of the presence of bioactive compounds, which were detected through phytochemical screening. These compounds are known to have strong bacterial activities. Alkaloids, for instance, can disrupt cell membrane function and inhibit bacterial protein synthesis. Flavonoids are capable of inhibiting enzymes and damaging bacterial cell membranes, while steroids and terpenoids, due to their lipophilic nature, can more easily penetrate bacterial membranes. This interaction may interfere with bacterial metabolism or cause direct damage to the cell structure.[17] These findings are consistent with the TPC and TFC measurements; the *n*-hexane fraction showed slightly lower effectiveness than the chloroform fraction, particularly in terms of MIC and MBC values. This study provides a solid foundation for further research, particularly in the exploration of antibacterial compounds in SI seeds.

4. Conclusions

The acetone extract of SI seeds was found to contain various chemical compounds, including steroids / triterpenoids, alkaloids, and flavonoids. LC-Orbitrap

HRMS analysis identified 41 steroids, 20 alkaloids, and one flavonoid in the acetone extract of SI seeds. The total phenolic content (TPC) and total flavonoid content (TFC) of the acetone extract of SI seeds were 28.04 ± 1.40 mg GAE/g and 25.98 ± 1.30 mg QE/g, respectively. The predominant steroid, alkaloid, and flavonoid were Neriifolin, Dehydrated antipain, and Artoindonesianin B, respectively. Furthermore, SI seeds showed inhibitory activity against three pathogenic bacteria: *S. aureus*, *S. epidermidis*, and *MRSA*. The chloroform fraction showed the most significant inhibitory activity against MRSA, while the *n*-hexane fraction exhibited the strongest inhibitory activity against *S. aureus* and *S. epidermidis.*

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ПРОФІЛЬ ВТОРИННИХ МЕТАБОЛІТІВ ЕКСТРАКТУ НАСІННЯ SACHA INCHI (*PLUKENETIA VOLUBILIS* L.) ЗА ДОПОМОГОЮ LC-ORBITRAP HRMS ТА ЙОГО АНТИБАКТЕРІАЛЬНА АКТИВНІСТЬ

Анотація. *Досліджено активні сполуки насіння Sacha Inchi (SI), Plukenetia volubilis L. Цілі здійсненого дослідження: скласти профіль вторинних метаболітів насіння SI за допомогою рідинної хроматографії з Orbitrap-мас-спектрометрією високої роздільної здатності (LC-Orbitrap HRMS), здійснити скринінг хімічних сполук, визначити загальний вміст фенолів і флавоноїдів, а також оцінити антибактеріальну активність. Антибактеріальний аналіз передбачав вимірювання зони пригнічення, мінімальної інгібувальної концентрації (МІК) та мінімальної бактерицидної концентрації (МБК). Результати показали, що ацетоновий екстракт насіння SI містить стероїди / тритерпеноїди, алкалоїди та флавоноїди. За даними LC-Orbitrap HRMS, в ацетоновому екстракті ідентифіковано 41 стероїд, 20 алкалоїдів та один флавоноїд. Переважали з-поміж стероїдів, алкалоїдів і флавоноїдів, відповідно, нерійфолін, дегідрований антипаїн та артоіндонезіанін B, що й становить наукову новизну цього дослідження. Ацетоновий екстракт насіння SI характеризувався загальним вмістом фенолів (ЗВФ) 28,04 ± 1,40 мг GAE/г та загальним вмістом флавоноїдів (ЗВФл) 25,98 ± 1,40 мг QE/г. Ацетоновий екстракт насіння SI було фракціоновано з використанням кількох розчинників і протестовано на антибактеріальну активність. Екстракт насіння SI пригнічував ріст Staphylococcus epidermidis ATCC 25923, Staphylococcus aureus ATCC 12228 та метицилінрезистентного Staphylococcus aureus (MRSA). Хлороформна фракція виявила найсильніше пригнічення щодо MRSA: зона інгібування 16,4 ± 0,4 мм (сильна), МІК 3,125 мг/мл, МБК 25 мг/мл; тоді як н-гексанова фракція виявила найвищу активність проти S. aureus (15,7 ± 0,3 мм (сильна), МІК 3,125 мг/мл, МБК 6,25 мг/мл) та S. epidermidis (10,5 ± 0,3 мм (сильна), МІК 3,125 мг/мл, МБК 25 мг/мл). Це дослідження створює міцне підґрунтя для подальших наукових досліджень, зокрема щодо вторинних метаболітів насіння SI як потенційних антибактеріальних агентів.*

Ключові слова: *насіння Sacha Inchi, профіль вторинних метаболітів, LC-Orbitrap HRMS, антибактеріальна активність, ацетоновий екстракт.*