Secondary metabolites from the leaves and stem of the Nigerian mangrove plant -*Acrostichum aureum* possess wound healing and antimicrobial properties

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Abstract

Acrostichum aureum is a medicinal plant used for the treatment of several disease conditions and infections. In Nigeria, its use is limited to the management of stomach pain and skin infections. This research aims to evaluate the wound healing and antimicrobial properties of the secondary metabolites from the stem and leaf of *Acrostichum aureum*. The crude extract was subjected to liquid- liquid partitioning successively with n-hexane, ethylacetate, butanol and water. Wound healing activity was determined using wound excision model while the antimicrobial activity was evaluated using the Inhibition Zone Diameter (IZD) model. Metabolomics was carried out using High performance Liquid Chromatography coupled with Diode Array Detector (HPLC-DAD). Result from the study revealed that the crude extract and butanol fractions of both stem and leaves of *Acrostichum aureum* elicited significant ($p<0.05$) reduction in wound size on progressive administration of the extract at all doses when compared to the controls. From the result, models treated with 2% AAL-BF, 6% AAS-BF and povidone had at least 94% wound contraction on day 12 respectively, while models treated with 2% AAS-BF and 6% AAL-BF had 89.5% and 85.0% wound contraction respectively on the 12th day. The crude extract and fractions of *Acrostichum aureum* showed a dose dependent response against the pathogens. The crude extract of both AAS and AAL showed the best antibacterial activities. AAL showed an IZD of 10.0 ± 0.7, 6.00 ± 0 and 12.0 ± 0 for *Staphylococcus aureus, Pseudomonas*

aereginosa, and *Escherichia coli* respectively at the highest dose (1 mg/mL), while AAS showed an IZD of 10.0 ± 0.7, 7.0 ± 0 for *Staphylococcus aureus* and *Pseudomonas aereginosa* respectively at dose of 1.0 mg/mL. Fractions of AAL also showed activity against *Staphylococcus aureus* and *Escherichia coli.* The HPLC-DAD identified compounds; quercetin-3-galactoside, rocaglamide, palitantin and isorhamnetin diglycoside proposed to be responsible for the bioactivities observed. In conclusion, the secondary metabolites from the leaf and stem part of *Acrostichum aureum* growing in Nigeria possess wound healing and antibacterial properties and can be optimized for the development of potent therapeutic agents.

Keywords: Wound healing, antimicrobial activities, mangrove fern, antibacterial, HPLC, *Acrostichum aureum*.

Introduction

Wounds generally refers to any break in the continuity of the normal anatomical structure and function of the skin (Chhabra *et al.,* 2017). According to Sen (2019), about 8 million individuals around the globe suffer from wounds. In the United States, chronic wounds impact 2% of the population (Järbrink *et al.,* 2017), a statistic that is also observed in various European nations (Phillips *et al.,* 2016). Research also indicates that the cost of wound care is particularly high in low- and middle-income countries, exacerbated by disparities in health insurance coverage (Aregbeshola & Khan, 2018; Cleopatra & Eunice, 2018). In Nigeria, many patients in teaching hospitals struggle to afford wound care, with most coming from low socio-economic backgrounds (Cleopatra & Eunice, 2018; Ogundeji *et al.,* 2020). Although wound healing is a natural phenomenon which is easily achieved with acute wounds like cuts, burns, abrasion and surgical wounds, wound infections often prevent healing, consequently leading to prolonged clinical conditions (Hurlow and Bowler, 2022; Puca *et al.,* 2021). Infected wounds negatively impact quality and slows down the healing process (Pallavali *et al.,* 2017). Among surgical patients, wound infections account for one-third of hospital-acquired infections and contribute to 70-80% of deaths (Pallavali *et al.,* 2017). Furthermore, irrespective of wound type, wound infections are linked to both morbidity and mortality, especially in developing countries like Nigeria (Anguzu *et al.,* 2007). A study conducted by Puca *et al.,* 2021, revealed the presence of microorganisms like Escherichia *coli*, *Pseudomonas aureginosa* and *Staphylococcus aureus* infestations in chronic wounds. They also highlighted the growing concern of antimicrobial resistance in the treatment of wound infection, thus necessitating the need for novel wound healing and antibacterial agents with little or no side effect. Plants have served as a reservoir for bioactive compounds over the years due to their ability to synthesize complex

compounds required for their survival in certain climate, habitat or against predators (Chaachouay and Zidane, 2024, Umeokoli *et al*., 2016). A plant material that possesses both antibacterial as well wound healing properties will have great potential in the management of wounds of all types. *Acrostichum aureum*, commonly known as the golden leather fern, is a mangrove species traditionally used in various medicinal practices across tropical and subtropical regions (Khan *et al.,* 2013). It is an evergreen plant which occurs worldwide in mangrove swamps, salt marshes, canal margins and low hammocks. The plants are exposed to unfavorable environmental and climatic conditions, which make them develop adaptive defense mechanisms and produce secondary metabolites that protect them against stressful abiotic and biotic factors. Some of the traditional uses of *Acrostichum aureum* includes the treatment of non-healing ulcers, boil, wounds, snakebite, bleeding, worm infection, asthma, sore throat, constipation and elephantiasis (Akinwunmi *et al.,* 2022). This study seeks to identify viable alternatives to conventional antimicrobial and wound healing treatments for low- and middle-income countries like Nigeria, while also contributing to addressing the increasing challenge of antibiotic resistance.

Materials and methods

Collection of plant materials

Fresh leaves and stem of *Acrostichum aureum* (family, Pteridaceae) were collected from the swampy area of the university of Lagos on March, 2021. The plant was identified and authenticated by Dr. Akinnibosun Henry Adewale, a Taxonomist in the department of Plant Biology and Biotechnology, University of Benin and was given a voucher number UBH-A650.

Preparation of Sample and Extraction

Fresh leaves and stem of *Acrostichum aureum* were rinsed in running tap water to remove debris and then air dried under a shade. The leaves and the stem were pulverized using a mechanical blender to coarse powder. About 800 g each of the pulverized leaves and stem were macerated in 3.2 L of methanol with intermittent agitation using a magnet stirrer for 72 hours. At every 24 hours interval, the miscella is decanted from the marc and replaced with fresh menstruum for maximal extraction. After 72 hours, total extract obtained were filtered with a muslin cloth and subsequently with Whatman filter paper No. 1 (125mm). The filtrates were pulled together and concentrated using a rotary evaporator at 45°C to obtain the crude extract.

Solvent-solvent fractionation of secondary metabolites from the leaves and stem

The solid extract was subjected to liquid-liquid fractionation using n-hexane, ethyl acetate and

butanol in increasing polarity sequence to simplify the matrix. The fractions were concentrated, dried and stored for further studies.

Antimicrobial assay

Preliminary antimicrobial screening of the extracts was carried out using the agar well diffusion assay method as described by Aida *et al.*, (2001). The test was conducted using four standardized broth cultures of the test bacteria (*S. aureus, B. subtilis, P. aeruginosa, and E. coli*) and two clinical isolates of the fungi (*Aspergillus niger and Candida albicans*). Each test isolate's 0.5 McFarland standard bacterial and fungal suspension was applied using a sterile swab stick to sterile Mueller- Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (LS BIOTECH, USA), respectively. Then, a sterile cork borer was used to make five wells (8 mm in diameter) on each of the MHA and SDA plates. Aliquots of 80 µL of each extract dilution, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL was applied in each of the wells in the culture plates previously inoculated with the test organisms. The positive controls utilized were ciprofloxacin (5 g/mL) and miconazole (50 g/mL), whereas DMSO was used as the negative control. The cultures in MHA and SDA plates were incubated for 24 and 48 hours, respectively, at 37 and 27 ℃. The antimicrobial potential of each extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). The Minimum Inhibitory Concentration (MIC) of the active endophytic fungal extract was determined for each of the test organisms that were sensitive in the preliminary screening. Stock solution of 10 mg/mL for each of the active samples in the preliminary assay was prepared, followed by a two-fold serial dilution in other to get graded (working) concentrations including 5, 2.5, 1.25, 0.625 mg/mL. Then, 1 mL from each of this dilution (concentration) was transferred into sterile petri dishes and 9 mL of molten agar cooled to 40℃ was added to it and the mixture stirred clockwise and anticlockwise to ensure proper mixing [The addition of the agar diluted the concentration of the extract to a final concentration of (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL). Thereafter, the base of each plate was divided according to the number of the test organisms then a loopful of each of the test organisms previously standardized to McFarland turbidity was streaked on its respective segment. Also, for negative control, the organisms were streaked on the sterile molten agar that did not contain the extract. The culture plates were then incubated for the bacterial and fungal isolates at 37°C for 24 hours and 25°C for 3 days, respectively. After incubation, the plates were examined for microbial growth by checking for visible growth, using a plus sign (+) indicating growth (resistance) while a negative sign (-) indicates no growth (inhibition / susceptible).

Wound healing assay

Formulation of herbal ointment

After preparation of extract, the next step was to formulate herbal preparation for each of the plant extracts. The herbal extracts formulation was prepared in the form of an ointment and administered topically. The ointment base (petroleum jelly) was used 3g of the extracts were incorporated into the base using the triturating method of preparing medicated ointments. The required quantity of the ointment base was measured in grams separately and melted at a temperature of about 70 ° C in a hot water bath. The designated quantity of each extract was added to the melted base at 40°C, mixed and swirled gently and continuously until a homogenous dispersion was obtained.

Wound induction and treatment using excision wound model

Wound excision was done according to the method of Mukherjee *et al.,* (2001). Thirty animals were anesthetized prior to and during creation of the wounds, with chloroform by open mask method. The rats were inflicted with excision wounds. The dorsal fur of the animals was shaved with a sterilized razor blade and the anticipated area of the wound to be created outlined on the back of the animals using a permanent marker. A full thickness of the excision wound of 283 mm was created along the latissimus dorsi markings using toothed forceps, scalpel and pointed scissors under aseptic conditions. Homeostasis was achieved by blotting the wound with cotton swab and soaked in normal saline. The animals were divided into six groups containing three rats each. The first group was treated with 2%w/w conc. of the leaf extract (6 g of leaf extract in 30 ml of petroleum jelly). The second was treated with 6%w/w conc. of the leaf extract (6 g of leaf extract in 10 ml of petroleum jelly). The same was carried on group 3 and 4 using 2% and 6% w/w concentrated of the stem extract respectively. The fifth group was treated with bland ointment containing only petroleum jelly (negative control). The final group was treated with a standard ointment known as povidone (positive control). The same process was carried for the crude extract and butanol fractions of the leaves and stem. The respective therapeutic treatment was administered once daily topically to the animals of respective groups. percentage wound contraction was calculated from the days of measurements of wound area. The wound contraction was calculated as % reduction in wound area with respect to initial wound area.

Wound Contraction (%) = [(WDO-WDT)/WDO] × 100

WDO= the wound diameter or area on day zero.

WDT= the wound diameter or area on day t.

Wound size for each animal of group was determined in several days until the final analysis of the results were concluded.

Bioactive Compound detection

Analytical HPLC was carried out using a Dionex P580 HPLC system coupled with a photodiode array detector (UVD340S). Routine detection wavelengths were set at 235, 254, 280, and 354 nm. The separation column (125 × 4 mm, length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. Vacuum Liquid Chromatography (VLC) was carried out using silica gel (230-400 mesh, Merck).

Statistical analysis

Data obtained were analyzed using GraphPad Prism version 8 and the results expressed as mean ± standard deviation. Significant differences of the results were established by one-way analysis of variance (ANOVA) and Tukey multiple range comparison. The acceptance level of significance for the results was $p < 0.05$.

Results

Wound healing assay

The result of the wound healing effect of the crude and fractions are showed in tables 1 -4 below:

Table 1: Wound healing activity of crude extracts (AAL and AAS)

	Basal	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
2% AAL	1.90 ±0.06	2.10 ±0.09 ns	1.70 ±0.12 ns	1.40 ±0.35 *	1.20 ±0.12 **	1.30± 0.07 *	1.17 ±0.03 **	0.87 ±0.03 **	0.40± 0.05 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
6% AAL	1.80 ±0.7	1.97 ±0.15 ns	1.77 ±0.15 ns	0.80 ±0.06 **	0.20 ±0.12 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
2% AAS	1.90 ±0.06	1.90 ±0.06 ns	1.60 ±0.03 **	1.70 ±0.03 ns	1.40 ±0.17 **	0.70 0 **	0.30 ±0.03 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
6% AAS	1.60 ±0.03	2.10 ±0.09 **	1.90 ±0.03 ns	1.40 ±0.06 ns	1.10 ±0.17 **	0.70 ±0.17 **	0.30 ±0.12 **	0.10 ±0.05 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
Negative control	1.90 ±0.06	2.10 ±0.06 ns	2.30 ±0.09 **	2.20 ±0.09 **	2.10 ±0.03 ns	1.90 0 ns	1.70 ±0.7 ns	1.60 ±0.03 **	1.20 ±0.03 **	0.80 ±0.06 **	0.60 0 **	0.40 ±0.03 **

Povidone	1.97 ±0.09	1.80 ±0.09 ns	1.80 ±0.12 ns	0.90 ±0.07 **	0.50 ±0.07 **	0.30 ±0.07 **	0.10 ±0.06 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **

*Values are expressed as Mean ± SD, *n=3*. Mean values with asterisk (*) and double asterisk (**) as superscript across the row are considered statistically significant at $p<0.05$ and $p<0.01$. Mean values with "ns" as superscript are not significant ($p>0.05$). Where AAS: *Acrostichum aureum* crude stem extract, and AAL: *Acrostichum aureum* crude leave extract.

Table 2. Percentage of wound contraction prior to the day of 100% wound healing

Concentration of extract	Percentage (%)	Day
2% AAL	78.9	Day16
6% AAL	89.0	Day8
2% AAS	84.2	Day12
6% AAS	93.8	Day14
Negative control	78.9	Day22
Povidone	94.9	Day12

From the table above, 6% AAL achieved the fastest wound healing rate, reaching 89.0% in just 8 days, outperforming other extracts and controls. In contrast, the negative control took the longest time to reach 78.9% healing, requiring 22 days.

Table 3. Wound healing activity of butanol fractions of AAL and AAS

	Basal	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
2% AAL-BF	1.8 ±0.06	2.10 ±0.06 **	2.00 ±0.07 ns	1.60 ±0.06 ns	1.30 ±0.06 **	0.80 ±0.07 **	0.50 ±0.06 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
6% AAL-BF	2.00 ±0.03	2.30 ±0.09 *	1.50 ±0.09 ns	1.40 ±0.09 **	0.80 ±0.09 **	0.40 ±0.09 **	0.40 ±0.03 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
2% AAS-	1.90 ±0.06	1.90 ±0.15	1.80 ±0.10	1.50 ±0.15	0.90 ±0.18	0.50 ±0.17	0.20 ±0.12	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00

BF		ns	ns	ns	**	**	**	**	**	**	**	**
6% AAS-BF	1.70 ±0.03	1.80 ±0.06 ns	1.80 ±0.03 ns	1.60 ±0.06 ns	1.20 ±0.09 **	0.80 ±0.06 **	0.20 ±0.10 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **
Negative control	1.90 ±0.06	2.10 ±0.06 ns	2.30 ±0.09 **	2.20 ±0.09 **	2.10 ±0.03 ns	1.90± 0.04 Ns	1.70 ±0.70 ns	1.60± 0.03 **	1.20± 0.03 **	0.80± 0.06 **	0.60± 0.07 **	0.40 ±0.03 **
Povidone	1.97 ±0.09	1.80 ±0.09 ns	1.80 ±0.12 ns	0.90 ±0.07 **	0.50 ±0.07 **	0.30 ±0.07 **	0.10 ±0.06 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **	0.00± 0.00 **

*Values are expressed as Mean ± SD, *n=3*. Mean values with asterisk (*) and double asterisk (**) as superscript across the row are considered statistically significant at $p<0.05$ and $p<0.01$. Mean values with "ns" as superscript are not significant ($p>0.05$). Where AAS-BF: *Acrostichum aureum stem butanol fraction*, and AAL-BF: *Acrostichum aureum* crude leave butanol fraction.

Table 4 Percentage wound contraction with last day before the 100% wound healing

Concentration of butanol fraction	Percentage (%)	Day
2% AAL-BF	94.4	Day12
6% AAL-BF	85.0	Day12
2% AAS-BF	89.5	Day12
6% AAS-BF	94.1	Day12
Negative control	78.9	Day22
Povidone	94.9	Day12

From the table above, 2% AAL-BF had the best wound healing effect on day 12 when compared to 6% AAL-BF and other extract concentration, suggesting that the extract may be more effective at low doses. Contrarily, 6% AAS-BF achieved 94.8% wound closure on the 12th day. All extract achieved higher degree of wound healing in a shorter time (at least 10 days) when compared to the negative control.

Antimicrobial assay

The results of the Preliminary antimicrobial activity of the leaf extract, stem extract and fractions as well as MIC and FIC combined effect of the leaf extract and stem extract against *some isolated microbes are* shown in Tables 5- 13 below;

Table 5 Preliminary antimicrobial activity of leaf extract of *A. aureum* (AAL)

Test organisms	Concentration (mg/mL) / Inhibition Zone Diameter (mm)					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	10±0.7	6±0.7	4±0	3±0	0±0	18
P. a	6±0	4±0	0±0	0±0	0±0	8
E. c	12±0	9±0	7±0	0±0	0±0	12
C.a	4±0	0±0	0±0	0±0	0±0	14
A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; A. n*: Aspergillus niger*; C. a: *Candida albicans*.

Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 5 above shows that the leaf extract of *A. aureum* (AAL) demonstrated antibacterial effects that increased with the dose. Among the tested clinical isolates, *Escherichia coli* was the most susceptible, showing a 7 mm inhibition zone diameter (IZD) at a concentration of 0.25 mg/mL. This is closely followed by *Staphylococcus aureus,* with an IZD of 3mm at 0.13 mg/mL. However, the extract exhibited minimal antifungal activity, only affecting *Candida albicans* and showing no effect against *Aspergillus niger*.

Table 6 Preliminary antimicrobial activity of stem extract of *A. aureum* (AAS)

Test organisms	Concentration (mg/mL) / Inhibition Zone Diameter (mm)					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	10±0.7	6±0	2±0	0±0	0±0	18
P. a	7±0	5±0	4±0	2±0	0±0	8
E. c	0±0	0±0	0±0	0±0	0±0	12
C. a	0±0	0±0	0±0	0±0	0±0	14

A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; A. n*: Aspergillus niger*; C. a: *Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

From table 6, the crude stem extract of *A. aureum* (AAL) exhibited antibacterial activity that increased with the dose. Among the clinical isolates tested, *Staphylococcus aureus* was the most susceptible, with a 2 mm inhibition zone diameter (IZD) observed at a concentration of 0.25 mg. However, the extract did not show any antifungal activity against the tested clinical fungal isolates.

Table 7 Preliminary antimicrobial activity of ethylacetate fraction of *A. aureum* leaf extract (AAL-EF)

Test organisms	**Concentration (mg/mL) / Inhibition Zone Diameter (mm)**					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	5±0	5±0	4±0	3±0	0±0	18
P. a	5±0	4±0	3±0	3±0	2±0	8
E. c	2±0	0±0	0±0	0±0	0±0	12
B. s	4±0	4±0	3±0	0±0	0±0	18
C. a	0±0	0±0	0±0	0±0	0±0	14
A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; B. s: *Bacillus subtilis;* A. n*: Aspergillus niger*; C. a: *Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 7 above shows that the ethyl acetate fraction of *A. aureum* leaf extract (AAL-EF) demonstrated antibacterial activity that increased with the dose. Among the clinical isolates tested, *Pseudomonas aeruginosa* was the most susceptible, with a 2 mm inhibition zone diameter (IZD) at a concentration of 0.06 mg/ml. Additionally, the extract showed minimal antifungal activity, affecting only *Candida albicans* and having no effect against *Aspergillus niger* when compared to the positive control.

Table 8 Preliminary antimicrobial activity of ethylacetate fraction of *A. aureum* stem extract (AAS-EF)

Test organisms	Concentration (mg/mL) / Inhibition Zone Diameter (mm)					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	5±0	4±0	0±0	0±0	0±0	18
P. a	0±0	0±0	0±0	0±0	0±0	8
E. c	5±0	0±0	0±0	0±0	0±0	12
B. s	5±0	4±0	3±0	2±0	2±0	18
C. a	0±0	0±0	0±0	0±0	0±0	14
A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; B. s: *Bacillus subtilis;* A. n*: Aspergillus niger*; C. a: *Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 8 shows that the ethyl acetate fraction of *A. aureum* stem extract (AAS-EF) showed dose-dependent antibacterial activity, with *Bacillus subtilis* being the most susceptible, exhibiting a 2 mm inhibition zone at 0.06 mg/ml. The extract also had minimal antifungal effects, only affecting *Candida albicans* and showing no activity against *Aspergillus niger* compared to the positive control.

Table 9 Preliminary antimicrobial activity of n-Hexane fraction of *A. aureum* leaves extract (AAL-HF)

Test organisms	Concentration (mg/mL) / Inhibition Zone Diameter (mm)					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	2±0	0±0	0±0	0±0	0±0	18
P. a	3±0	2±0	2±0	0±0	0±0	8
E. c	2±0	0±0	0±0	0±0	0±0	12
B. s	4±0	4±0	3±0	2±0	0±0	18
C. a	0±0	0±0	0±0	0±0	0±0	14
A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; B. s: *Bacillus subtilis;* A. n*: Aspergillus niger*; C. a: *Candida albicans*. Antimicrobial potential was

indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 9 above shows that the n-hexane fraction of *A. aureum* leaf extract (AAL-HF) exhibited dose-dependent antibacterial activity, with *Bacillus subtilis* being the most susceptible, showing a 2 mm inhibition zone at a concentration of 0.13 mg/ml. The extract also demonstrated minimal antifungal activity, affecting only *Candida albicans* and showing no activity against *Aspergillus niger* compared to the positive control.

Table 10 Preliminary antimicrobial activity of n-Hexane fraction of *A. aureum* stem extract (AAS-HF)

Test organisms	**Concentration (mg/mL) / Inhibition Zone Diameter (mm)**					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	4±0	2±0	0±0	0±0	0±0	18
P. a	0±0	0±0	0±0	0±0	0±0	8
E. c	5±0	0±0	0±0	0±0	0±0	12
B. s	5±0	4±0	3±0	2±0	2±0	18
C. a	4±0	0±0	0±0	0±0	0±0	14
A. n	0±0	0±0	0±0	0±0	0±0	13

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; B. s: *Bacillus subtilis;* A. n*: Aspergillus niger*; C. a: *Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 10 above shows that the n-hexane fraction of *A. aureum* leaf extract (AAL-HF) displayed dose-dependent antibacterial activity, with *Bacillus subtilis* being the most susceptible, showing a 2 mm inhibition zone at a concentration of 0.06 mg/ml. The extract also had minimal antifungal activity, affecting only *Candida albicans* and showing no activity against *Aspergillus niger* compared to the positive control.

Table 11 Result of Minimum Inhibitory Concentration (MIC) of crude extracts and fractions

Test organisms	Minimum Inhibitory Concentration (mg/mL)					
	AAL	AAL-EF	AAL-HF	AAS	AAS-EF	AAS-HF
S. a	0.25	>1.00	0.50	0.50	>1.00	0.25
P. a	>1.00	0.12	1.00	0.25	0.06	>1.00
E. c	0.25	>1.00	0.50	>1.00	0.25	>1.00
C. a	1.00	>1.00	0.12	>1.00	0.50	>1.00

Key: S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; C. a: *Candida albicans*.

The MIC data from table 11 showed that the organisms had varying degree of sensitivity to the different extracts. However, AAS-EF and AAL-HF showed high sensitivity below 1.0 mg/mL for three out of four organisms more than any other extract.

Table 12 MIC and FIC of leaf and stem extracts of *Acrostichum aureum* combined against *Pseudomonas aureginosa*

Combinations	Ratios	MICa	MICc	FIC Index	Remark
Leaf:Stem extract	4:1	50:200	10:10	1.7	Indifference
Leaf:Stem extract	3:2	50:200	15:40	2.6	Antagonism
Leaf:Stem extract	2:3	50:200	5:30	0.95	Synergism
Leaf:Stem extract	1:4	50:200	1.25:20	0.3	Synergism

Key: MICa: MIC of sample alone; MICc: MIC of the most effective combination: FIC Index: Fractional Inhibitory Concentration Index

From Table 12, the results indicate varying interactions between the leaf and stem extracts at different ratios. At a 4:1 ratio, the combination was indifferent with an FIC index of 1.7. At a 3:2 ratio, the combination exhibits antagonism with an FIC index of 2.6. However, at both 2:3 and 1:4 ratios, the combination shows synergism, with FIC indices of 0.95 and 0.3, respectively, suggesting enhanced antibacterial activity at these ratios.

Table 13: MIC and FIC of leaf and stem extracts of *Acrostichum aureum* combined against *Staphylococcus aureus*

Combinations	Ratios	MICa	MICc	FIC Index	Remark
Leaf:Stem extract	4:1	50:200	40:5	1.2	Indifference
Leaf:Stem extract	3:2	50:200	15:10	0.2	Synergism
Leaf:Stem extract	2:3	50:200	5:3.125	0.2	Synergism
Leaf:Stem extract	1:4	50:200	10:20	1.8	Indifference

Key: MICa: MIC of sample alone; MICc: MIC of the most effective combination: FIC Index: Fractional Inhibitory Concentration Index.

Table 13 shows that different combinations of leaf and stem extracts have varying effects on antibacterial activity. At ratios of 4:1 and 1:4, the combinations were indifferent with FIC indices of 1.2 and 1.8, respectively, indicating no significant enhancement or reduction in activity. However, at 3:2 and 2:3 ratios, the combinations show strong synergism with FIC indices of 0.2, indicating significantly enhanced antibacterial activity at these ratios.

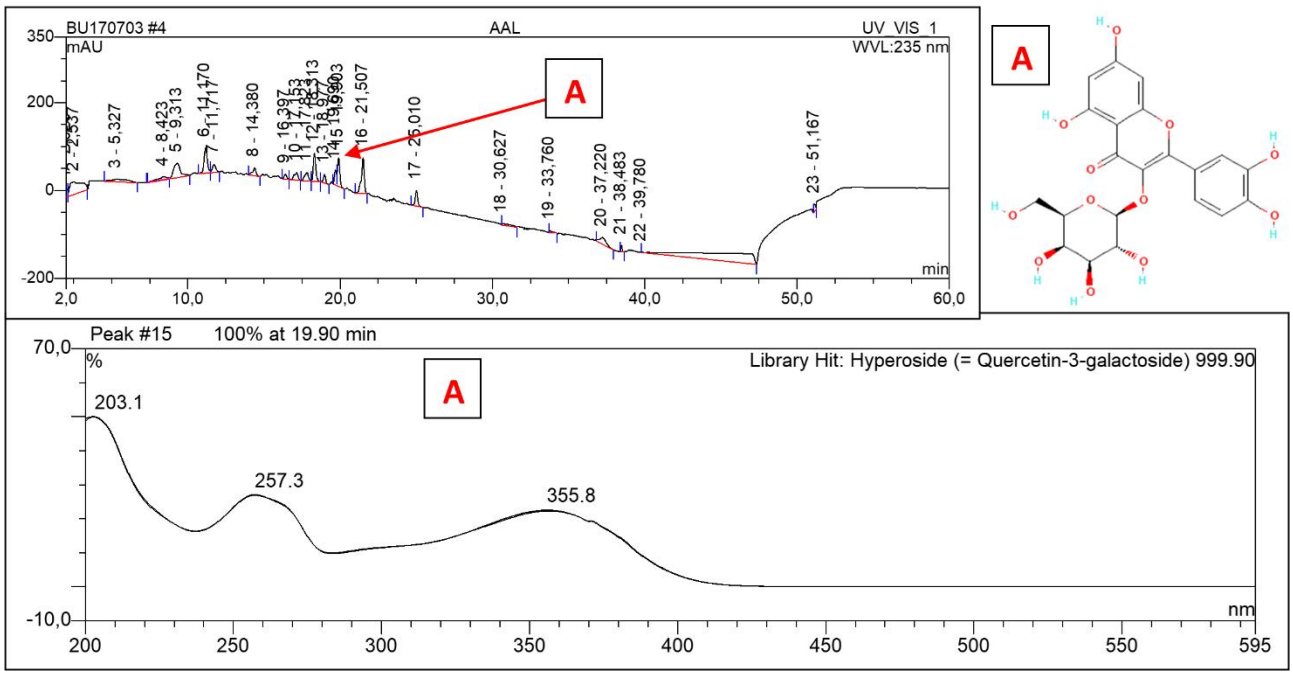


Figure 2. HPLC Chromatogram and UV – Spectra of A= Quercetin-3-galactoside (RT=19.90, 999.90, $C_{21}H_{20}O_{12}$, MW= 640.5 g/mol) from crude *Acrostichum aureum* extract.

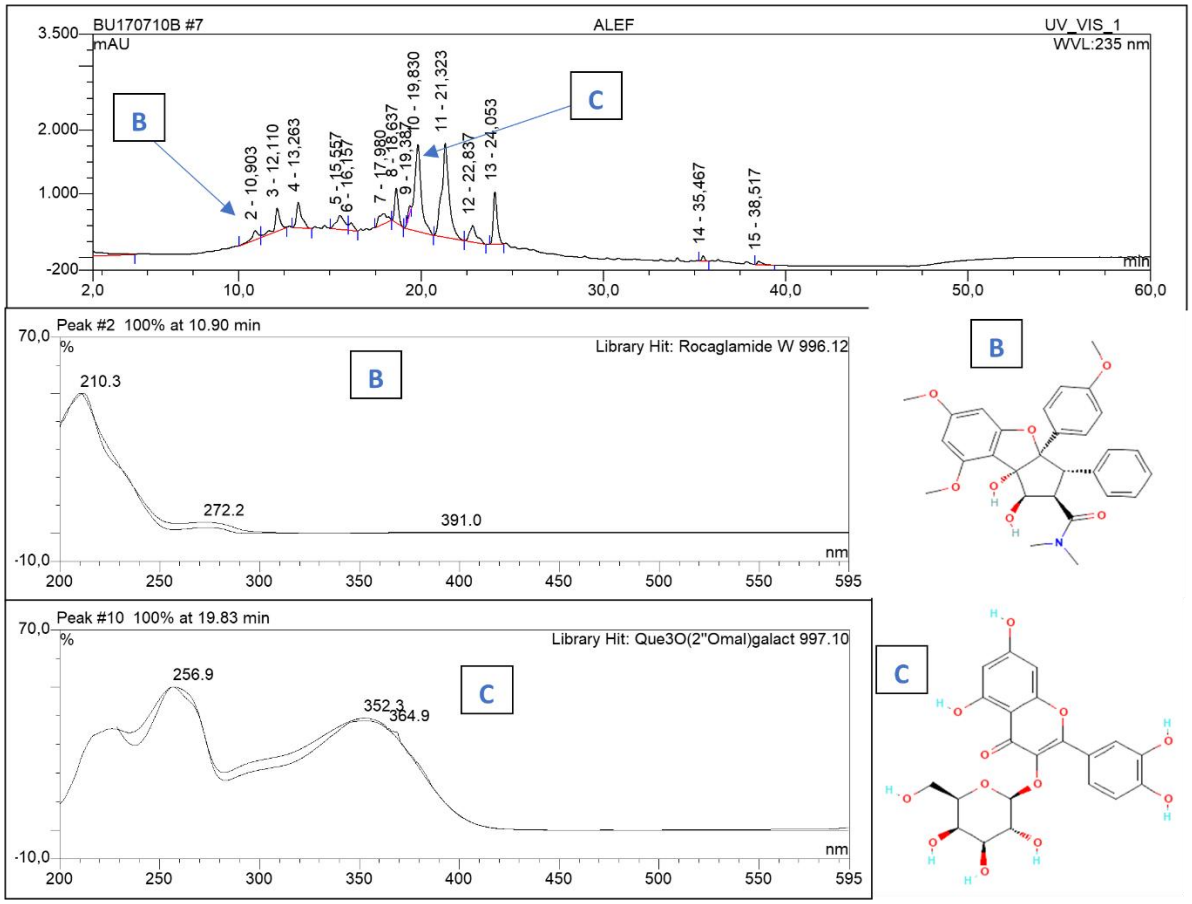


Figure 3. HPLC Chromatogram and UV – Spectra of B = Rocaglamide (RT=10.90, 996.12, $C_{29}H_{31}NO_7$, MW = 505.6 g/mol) and C = Quercetin glycoside derivative (RT = 19.83, 997.10) from ethylacetate fraction of *Acrostichum aureum*.

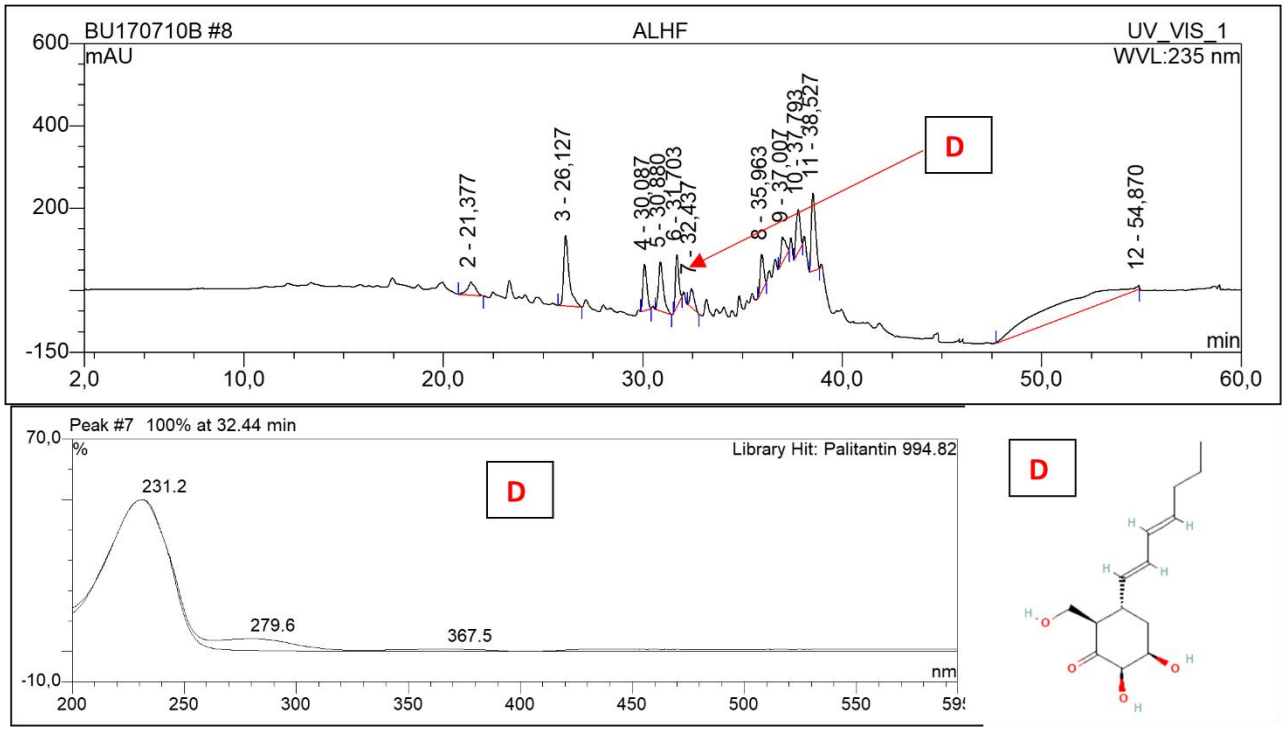


Figure 4. HPLC Chromatogram and UV – Spectra of D = Palitantin (RT=32.44, 994.82, $C_{14}H_{22}O_4$, MW = 254.32 g/mol) from n-Hexane fraction of *Acrostichum aureum*.

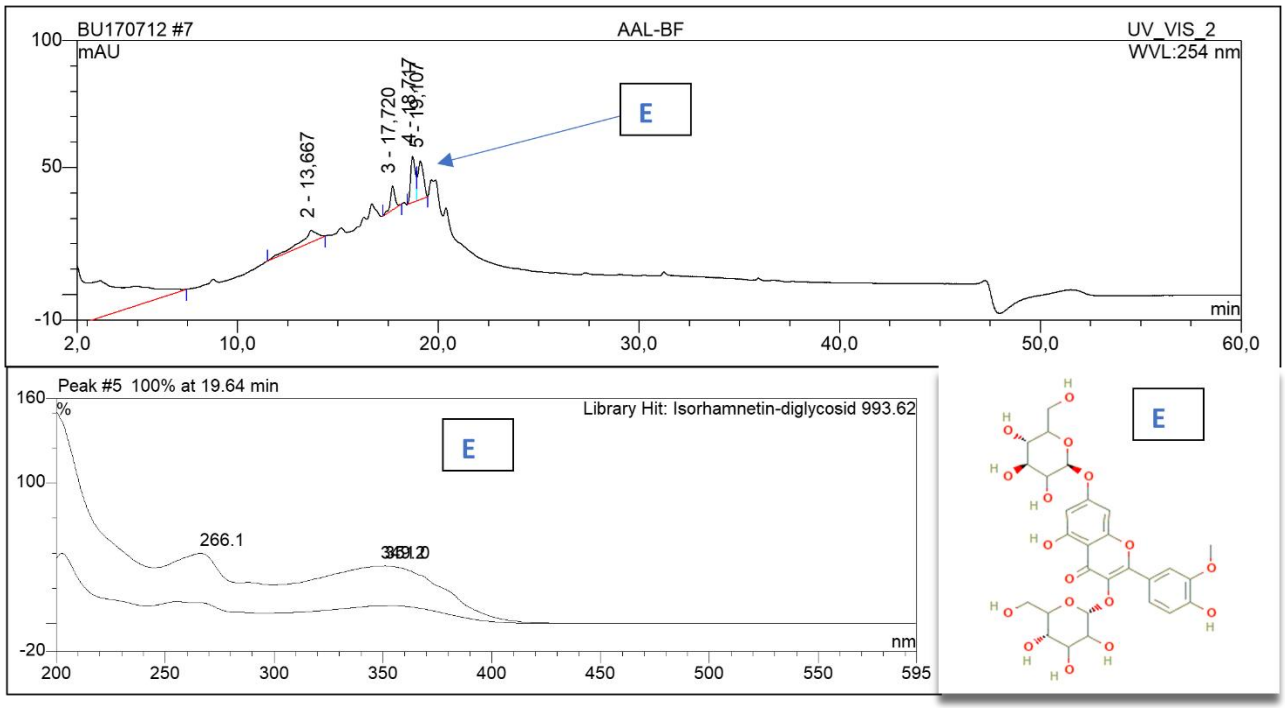


Figure 5. HPLC Chromatogram and UV – Spectra of E = Isorhamnetin-diglycoside (RT=19.64, 993.62, $C_{28}H_{32}O_{17}$, 640.5 g/mol) from butanol fraction of *Acrostichum aureum*.

Discussion

The results of the experiments showed that the crude extract and fractions of *Acrostichum aureum* leaf and stem possess wound healing properties comparable to the wound-healing property of the rhizomes of the plant which was previously reported (Herman *et al.,* 2013). Results from the study revealed that crude extract and butanol fractions of both stem and leaf portions of *Acrostichum aureum* had significant ($p<0.05$) reduction in wound size on progressive administration of the extract at all doses when compared to the controls. Specifically, the 6% AAL had the fastest wound contraction, achieving 89% healing on the eight day and 100% healing before the 10th day. This performance far exceeds the standard drug povidone which achieved 94.9% wound contraction on the 12th day. The crude stem extract also showed significant ($p<0.05$) reduction in wound size with 6% of the crude stem extract achieving 93.8% wound contraction on the 14th day. The butanol fractions of both crude and stem extract showed complete healing by the 14th day however there were variable percentages of healing prior to total healing. For instance, 2% AAL-BF, 6% AAS-BF and povidone, all had at least 94% wound contraction on day 12, whereas, 2% AAS-BF and 6% AAL-BF had 89.5% and 85.0% respectively on the 12^{th} day. The wound healing activity of the evaluated crude and fractions may be due to the anti-inflammatory activities of compounds such as isorhamnetin diglycoside in the butanol fraction of the leaves (Figure 5) and quercetin-3-galactoside in the crude fraction of the leaves (Figure 2). According to Moulin *et al.,* (2000) acceleration of wound contraction is associated with fibroblast activation. Quercetin and its derivatives have been shown to promote the recovery of cutaneous wounds through the inhibition of inflammatory response, acceleration of angiogenesis, proliferation and mobilization of fibroblasts and collagen deposition (Huang *et al.,* 2024), thus explaining the observed activities and the possible heightened activity of the crude extract. The antimicrobial activities of the plant and fractions determined using the Inhibition Zone Diameter (IZD) method are presented in tables 5-10. The crude extract and fraction of *Acrostichum aureum* showed a dose dependent response against the pathogens. The crude extract of both AAS and AAL showed the best antibacterial activities. AAL showed an IZD of 10.0 ± 0.7, 6.00 ± 0 and 12.0 ± 0 for *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli* respectively at the highest dose (1 mg/mL) (Table 5), while AAS showed an IZD of 10.0 ± 0.7, 7.0 ± 0 for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively at 1.0 mg/mL. There was no activity against *E. coli* (Table 6). The observed activities in AAL maybe due to the presence of Quercetin -3- galactoside. Quercetin is a plant flavonoid which possess several pharmacological activities including the

inhibition of several Gram-positive, Gram-negative bacteria and drug-resistant microorganisms (Nguyen and Bhattacharya, 2022, Jaisinghani, 2017). Similarly, Li *et al.,* (2012), previously reported that quercetin exhibited antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-sensitive *Staphylococcus aureus*. The antibacterial activity of quercetin is linked to the presence of hydroxyl groups which contributes to its solubility (Hooda *et al.,* 2020) and disruption of the bacterial cell membrane (Cushnie and Lamb, 2005). Furthermore, the ethylacetate fraction of the leaf (AAL-EF) and stem (AAS-EF) showed a mild activity against *Staphylococcus aureus, E. coli,* and *Bacillus subtilis* while no activity was observed with AAS-EF against *Pseudomonas aeruginosa.* This result suggests that the AAL-EF fraction which inhibited all the bacteria pathogen possess compounds with broad range activity. These activities may be attributed to the two identified compounds in the fraction; rocaglamide and quercetin derivative (Figure 3). In addition to the antibacterial activity of quercetin described above, the compound rocaglamide which was also identified may have also contributed to the antibacterial activity. Rocaglamide (cyclopenta [b] benzofuran) is a flavagline first isolated by king and colleagues (1982) from *Aglaia elliptifolia.* Fractions, AAL-HF and AAS-HF also showed mild activity against *Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis.* However, AAS-HF also failed to inhibit the activity of *Pseudomonas aeruginosa.* The activity observed with AAL-HF may be due to the presence of palitantin (Figure 4). Palitantin, a metabolite derived from cyclohexane, initially isolated from the Penicillium species, has been reported to possess antiprotozoal properties and moderate antimycobacterial activity (Fuska *et al*., 1970). According to Ola *et al.,* (2018), palitantin inhibited the growth of *Enterococcus faecalis* UW 2689 and *Streptococcus pneumoniae* with MIC value of 64 µg/mL. Generally, the crude extract and fractions showed a poor antifungal activity against *Aspergillus niger* even at 1 mg/mL (Table 5-10). However, only AAL (Figure 1) and AAS-HF (Figure 10) showed mild activity against *Candida albicans.* This result suggests that *Acrostichum aureum* is a poor inhibitor of fungal growth. Result of the synergistic study showed significant synergism between the crude leaf and stem extract combined in a ratio of 2:3 and 1:4 against *Pseudomonas aeruginosa.* Similarly, the combination of crude leaf and stem of the plant in the ratio 3:2 and 2:3 respectively showed synergistic effect against *Staphylococcus aureus.* The onset of any injury stimulates the wound healing process (a physiological process) involving different cell types (platelets, macrophages, fibroblast, and epithelial and endothelial cells), extracellular matrix components, cytokines, growth factors, and regulatory molecules.

However, the speed of healing often depends on the degree of the injury and any agent that can fight infections (antimicrobials) or lead to coagulation of blood (wound healing) (Kondo *et al.,* 2010; Cañedo-Dorantes & Cañedo-Ayala, 2019; Veith *et al.,* 2019; Fitridge & Thompson, 2011). Cutaneous wounds are particularly susceptible to bacterial infections, and provide an entry point for systemic infections (Vittorazzi *et al.,* 2015). Consequently, the presence of microorganisms on wound can slow the healing process leading to formation of unpleasant exudates and toxins and prolong the inflammatory phase (Agyare *et al*., 2013; Houghton *et al*., 2005; Misic *et al*., 2014). Therefore, antimicrobial agents can be used to prevent wound infection and hence accelerate the wound healing process as shown in our research above. This observation can be attributed to the action of the different bioactive compounds present in both extracts. Natural compounds from plant extracts and fractions have shown to possess certain bioactivities which can improve the wound healing process by reducing scar formation. Also, bioactive secondary metabolites with antimicrobial, antioxidant, and wound healing potentials can induce blood coagulation, fight infection, and speed up wound healing (Ti *et al*., 2022; Vitale *et al.,* 2022). The presence of bioactive compounds such as quercetin, rocaglamide, isorhamnetin diglycoside in the leaf portion, which have been shown to elicit either or both antimicrobial and wound healing activities, validates the utilization of *Acrostichum aureum* leave in wound and infection management. Furthermore, the result of the study it can be inferred that the speedy wound healing observed is a result of factors such the fibroblast mobilization, and anti-inflammatory properties of the flavonoids (Quercetin, Isorhamnetin diglycoside) and the antibacterial activity of palitantin and quercetin which serves to prevent and control wound infection and prolonged healing.

Conclusion

We conclude from the study that the crude extract and ethylacetate fraction of *Acrostichum aureum* growing in Nigeria's mangrove and coastal region can be utilized in treatment of wounds and management of infections from bacteria such as *Staphylococcus aureus* and *Escherichia coli* which are usually associated with wound infections. The active principles such quercetin-3-galactoside, isorhamnetin diglycoside, and palitantin, detected using HPLC-DAD are most likely responsible for both the wound healing and antibacterial properties of the plant. Thus, compounds from *Acrostichum aureum* leaf and stem can serve as a potent and selective lead compound for the development of improved therapeutic agents for the management of chronic wounds and bacterial infections.

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Conflict of interest

The authors declare no conflict of interest.

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