Herbal gel of *Alchornea cordifolia* polyphenols-rich fractions displayed promising antimicrobial and wound healing activities

Constance Nneoma Asimole[1], Chukwubuikem C. Okolo[1]*, Nkeoma Nkasi Okoye[2], Ebere Innocent Okoye[3], Festus Basden C. Okoye[1]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Pure and Industrial Chemistry, Faculty of Physical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.
[3]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

Submitted: 24th Sept., 2022; Accepted: 7th Nov., 2022; Published online: 31st Dec.,2022
DOI: https://doi.org/10.54117/jcbr.v2i6.1
**Corresponding Author: Chukwubuikem Okolo; cc.okolo@unizik.edu.ng*

ABSTRACT

Alchornea cordifolia leaves are used ethno-botanically for the treatment of bacterial and fungal infections. The present study is aimed at investigating the antimicrobial and wound-healing effects of gel formulation from the ethyl acetate liquid fractions of the ethanol leaf extract of *Alchornea cordifolia*. The ethanol extract was subjected to liquid-liquid fractionation using n-heptane and ethyl acetate. The ethyl acetate fraction was further subjected to vacuum liquid chromatography (VLC) by gradient elution with different solvents. The ethyl acetate fraction and its VLC fractions were subjected to analytical HPLC. The chemical constituents of the ethyl acetate fraction were detected by dereplication using HPLC-DAD. Herbal gels were formulated using the Ethylacetate fraction (EF) and ethyl acetate/ethanol VLC fraction. The physical parameters, antimicrobial activity, Wound healing effect and three months stability study of the gel were evaluated. Eight (8) compounds were identified in the fractions of *Alchornea cordifolia* and three (3) compounds were identified from the optimized gel by HPLC analysis. The fractions and the gels were found to possess broad-spectrum antimicrobial activity. The gels were found to be stable as there was no change in the parameters at end of the three (3) months. Even though, the antimicrobial activity of EV3 herbal gel was lost at the end of the stability studies. Herbal gel formulated from the leaf extract of *Alchornea cordifolia* possesses antimicrobial and wound healing properties and thus could be a better alternative for the treatment of wounds compared to orthodox medicine.

Keywords*:* Herbal product, gel formulation, HPLC-DAD, Antimicrobial Activity

INTRODUCTION

Natural product research provides opportunities for new drug discoveries because of their availability and chemical diversity (Cos *et al*., 2006; Anyanwu *et al*., 2018; Kamenan *et al*., 2013; Okolo *et al*., 2021). Natural products are used to treat a wide range of diseases because they possess minimal side effect compared to synthetic

products (Iwu *et al*., 1999; Duraipandiyan *et al*., 2006; Eswarappa, 2009). Interest in utilizing natural sources in the development and formulation of skin products such as gels and creams as an alternative to conventional drugs contributes to increased interest in research of medicinal plants (Mukherjee *et al*., 2011). The therapeutic activity of a plant is due to the complex chemical nature of its different parts (Cook *et al*., 2015). Leaves of *Alchornea cordifolia* act as powerful antimicrobial and wound-healing agents mainly due to the presence of flavonoids and polyphenols and tannins (Manjunatha *et al*., 2007; Shivananda *et al*., 2007; Osadebe *et al*., 2013; Okoye *et al*., 2015). Extracts from leaves of *Alchornea cordifolia* have been reported to inhibit the growth of bacteria (Ebi, 2001). Several authors (Abo *et al*., 1999; Setzer *et al*., 2000; Osadebe and Okoye, 2003; Mavar-Manga *et al*., 2004; Mavar-Manga *et al*., 2008; Okoye *et al*., 2015) reported other activities of *Alchornea cordifolia leaves extract* like Anti-inflammatory activities, antifungal activities and cytotoxic against tumor cell lines.

Although *Alchornea cordifolia* has been used in the treatment of bacterial and fungal infections, no report exists on the development of suitable formulations from an extract and/or fraction of the plant. Hence, the present study is aimed at evaluating the antimicrobial and wound-healing properties of herbal gels formulated from the ethyl acetate fraction of *Alchornea cordifolia* ethanol leaf extract.

MATERIALS AND METHODS

Plant material

Leaves of *Alchornea cordifolia* were collected from Nsukka, Enugu State of Nigeria, in July 2015. The leaves were authenticated by Mr. Alfred Ozioko, a taxonomist at Bioresources Development and Conservation Program, Nsukka. The leaves were dried at room temperature for one week, pulverized into a coarse material using a mechanical grinder. The pulverized leaves were used for the experiments.

Extraction and Liquid-Liquid fractionation

About 500g of the pulverized leaves was subjected to extraction by cold maceration in 4 L of ethanol with continuous stirring using a magnetic stirrer for 48 hours. The ethanolic extract was concentrated almost to dryness (Ethanol fraction) using a rotary evaporator. That ethanol fraction was subjected to liquid –liquid fractionation using different solvents: n-heptane, ethyl acetate, and n-butanol. All of the fractions so obtained were filtered twice using Whatman No.1 filter paper and concentrated at room temperature.

Vacuum Liquid Chromatographic separation of ethyl acetate fractions:

The ethyl acetate fraction (14.95 g) was divided into two; 6.47g of the fraction was dissolved in 10mL of ethanol. 20g of silica gel (200-400 mesh) was added to the mixture. The admixture was triturated carefully in a mortar to form a homogenous mixture. The glass column (diameter 2.5 cm × 30 cm height) was packed with silica gel to about 14 cm height. The adsorbed fraction admixture was introduced from the top of the silica gel –packed column. A small amount of silica gel (in place of sea sand) was consequently added on top of the adsorbed admixture. Cotton wool was used to cover the silica gel to prevent distortion of the silica gel bed when the solvent system is introduced. A vacuum pump was connected to the column to create a negative pressure for the elution. The column was eluted gradually with increasing polarity of solvents from non-polar to highly polar solvents (n-Heptane, n-Heptane/ethyl acetate mixture, ethyl acetate, ethyl acetate/ethanol mixture, and ethanol) at different ratios.

Each system was collected separately in a 1 litre beaker and labeled appropriately. The ethyl acetate/ethanol fraction were selected for further studies due to literature result of previous researchers (Manjunatha *et al*., 2007; Shivananda *et al*., 2007; Osadebe *et al*., 2013; Okoye *et al.,* 2015). The ethyl acetate/ethanol fraction (50:50) was filtered twice using Whatman No.1 filter paper, concentrated with a rotary evaporator at 45°C and stored for further analysis.

Analytical High-Performance Liquid Chromatography (HPLC) analysis.

The crude ethyl acetate fraction and its VLC fractions were subjected to analytical HPLC. The solvent gradient used started from 10: 90 Methanol: Water (adjusted to pH 2 with phosphoric acid) increasing to 100 % Methanol in 45 minutes. The compounds were detected by ultraviolent visible diode array detector (UV-VIS DAD). About 2 mg of each of the extract and fractions were reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 minutes and thereafter centrifuged at 3000 RPM for 5 minutes. About 100 µL of the dissolved samples were transferred into HPLC vials containing 500 µL of HPLC-grade methanol. HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector. Detection was done at 235, 254, 280, and 340 nanometres. The separation column (125 x 4 mm; length x internal diameter) was prefilled with Eurospher C-18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by the addition of formic acid) and methanol was used as eluent.

Formulation of topical herbal gel of *Alchornea cordifolia* leaves

The herbal gel was formulated using two different fractions; this was done in other to obtain the optimized formulations which will be used for further analysis. Varied ratios of the polymers (Carbapol-934 and NaCMC) were to check for the drug-excipient interaction.
The fractions used for the formulation includes;

- Ethylacetate fraction of the ***Alchornea*** cordifolia leaf extract (EF)
- Ethylacetate/Ethanol VLC fraction from the Ethylacetate fraction (EA5)

Three doses were used for the formulation which are;

- 40mg/10g
- 80mg/10g
- 120mg/10g

A total of 42 formulations were obtained (21 for each of the fractions formulated) as shown in Table 1 and 2 below. The herbal gels formulated with ethyl acetate fraction are coded as EF while the ones formulated with EA5 are coded as EV. The different formulations for EF and EV are shown below in Tables1 and 2 respectively.

Table 1: Formulations used for the herbal gel (EF)

Ingredient / Fractions	**Carbopol (mg)**	**Sodium CMC (mg)**	**Ethyl Acetate Fraction (mg)**			**Glycerol (mL)**	**Ethanol (mL)**	**Potassium Sorbate (mg)**	**Triethanolamine (mL)**	**Distilled Water (mL)**
F1	100	100	40	80	120	0.50	0.25	5	0.5	q.s
F2	133	67	40	80	120	0.50	0.25	**5**	0.5	q.s
F3	67	133	40	80	120	0.50	0.25	5	0.5	q.s
F4	200	0	40	80	120	0.50	0.25	5	0.5	q.s
F5	0	200	40	80	120	0.50	0.25	5	0.5	q.s
F6	100	100	40	80	120	0.50	0.25	0	0.5	q.s
F7	100	100	0	0	0	0.50	0.25	5	0.5	q.s

Table 2: Formulations used for Herbal gel (EV)

Ingredient / Fractions	**Carbopol (mg)**	**Sodium CMC (mg)**	**Ethyl Acetate Fraction (mg)**			**Glycerol (mL)**	**Ethanol (mL)**	**Potassium Sorbate (mg)**	**Triethanolamine (mL)**	**Distilled Water (mL)**
F1	100	100	40	80	120	0.50	0.25	5	0.5	q.s
F2	133	67	40	80	120	0.50	0.25	**5**	0.5	q.s
F3	67	133	40	80	120	0.50	0.25	5	0.5	q.s
F4	200	0	40	80	120	0.50	0.25	5	0.5	q.s

F5	0	200	40	80	120	0.50	0.25	5	0.5	q.s
F6	100	100	40	80	120	0.50	0.25	0	0.5	q.s
F7	100	100	0	0	0	0.50	0.25	5	0.5	q.s

Procedure for formulating the gel

Carbopol and Sodium Carboxymethyl Cellulose were separately dispersed in 5 mL and 10 mL of water. The polymers (Carbapol-934 and NaCMC) were gently stirred to hydrate. The two dispersions were mixed together and stirred further for 10 minutes at room temperature. The relevant amounts of extract and potassium sorbate were mixed separately in minimal volumes of distilled water and gently stirred until complete dissolution. The solution of extract was added in aliquot amounts to the polymer dispersion, each addition was allowed to mix completely before another. This was done until all the solution was exhausted. The glycerol and ethanol were mixed and added gradually. The mixture was stirred to achieve homogeneity. The pH was checked and adjusted to 6.8-7.2 with triethanolamine. The weight of the formulation was made up to the final weight (10 grams) with distilled water and stirred again for 10 minutes. The final product was stirred for 5 minutes and then homogenized for another 15 minutes. The product was allowed to stand, protected from dust and light for adequate time to enable the evolution of air bubbles that may be present. It was packed in a suitable container and labeled appropriately.

Evaluation of topical herbal gel formulation

Physical parameters such as colour, appearance, and feeling on the application were checked and recorded. The pH of the gel was measured using a pH meter. The Viscosity of the gels was measured by using a Brookfield viscometer with a spindle and the results were recorded as a mean value in milliPascal per second (mPa.s).

Determination of antimicrobial Activity (Agar-well Diffusion Assay) of *Alchornea cordifolia* leaf fractions and the formulated herbal gels.

Test Organisms

Twenty-four hours broth cultures of six human pathogenic bacteria were used for the antimicrobial assay. They include Gram-positive bacteria (*Staphylococcus aureus and Bacillus subtilis*), Gram-negative bacteria (*Pseudomonas aeruginosa and Escherichia coli*), and fungi (*Candida albicans and Aspergillus niger*). Antibacterial activity of the fractions (ethyl acetate and ethyl acetate/ethanol VLC) and formulated gels of *Alchornea cordifolia* were evaluated by the cup plate Agar–well diffusion method (Aida *et al*., 2001). The bacterial and fungal cultures were adjusted to 0.5 McFarland turbidity standard and 0.1 mL of each of the test organisms was seeded onto sterile Mueller-Hilton Agar MHA (Oxoid, Difco USA) and Sabouraud Dextrose Agar (Titan, biotech) plates, (Diameter: 90 mm). A sterile cork borer was used to make wells (8mm in diameter) labeled EF1-EF7 and EV1-EV7 respectively on each of the MHA and SDA plates. Aliquots of 0.2 mL of each of the formulations and 80 µL of each of the graded concentrations of the fractions (20, 10, 5, 2.5, 1.25 mg/mL) were applied in each of the wells in duplicates in the cultured plates previously seeded with the test organism. The cultures were incubated

at 37 ^{o}C for 24 hours and 25 ^{o}C for 48 hours for bacteria and fungi plates respectively. Ciprofloxacin served as the positive control while a blank gel served as the negative control. Antimicrobial activity was determined by measuring the Inhibition zone diameter (IZD) around each well (excluding the diameter of the well).

Determination of antimicrobial Activity (Agar dilution Assay) of EA5 fraction

The Minimum Inhibition Concentration (M.I.C) of the EA5 fraction of *Alchornea cordifolia* leaves against the test organisms was also determined using the agar dilution method

Analytical High-Performance Liquid Chromatography (HPLC) analysis on the herbal gels

The gels (EV1, EV2 & EV3) were subjected to analytical HPLC. About 2 mg of each gel was used. The solvent gradient used started from 10: 90; Methanol: Water (adjusted to pH 2 with formic acid) increasing to 100% Methanol in 45min. The compounds were detected by a UV-VIS diode array detector.

Wound healing analysis on the optimized gels

Experimental Animal:

Adult albino rats obtained from the Animal house of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Awka were used for the study. All the microorganisms were obtained from the laboratory stocks of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. All animal experiments were conducted in compliance with the NIH guide for the care and use of laboratory animals (National Institute of Health (NIH) (2011) Pub. No: 85-23).

Evaluation of the wound healing property of the optimized herbal gels

The wound healing property of the optimized herbal gels (EV2 & EV3) was studied using the excision wound model as employed by Rashed *et al*., (2003). A total of 20 adult albino rats weighing 25 -30 g grouped into four (4) groups of five rats each were used. All the animals in each group were anaesthetized with intravenous administration of 10 mg/kg ketamine HCL, and the furs of their back were shaved with scissors. The residual fur on the shaved area was removed with hair remover. Then 70% ethanol was used to clean the shaved portion before creating the wound. A full-thickness excision wound of uniform 1.5cm^2 diameter circular areas was created along a marking using toothed forceps, a surgical blade, and pointed scissors. Then the wound was left undressed in an open environment; no local or systemic anti-microbial agent was applied over 24 hours for the wound size to be stable. The herbal gels were applied once daily for a maximum of 20 days as follows: Group 1 (Negative Control (Gel without drug), Group 2 (Positive Control (Gentamicin gel)), Group 3 (EV3 (1.20 % w/w)), and Group 4 (EV2 (0.8% w/w)). The selection of the gel for the wound healing activity is from the antimicrobial activity of the herbal gel (it was guided by the activity in view). Then, the wound diameter was measured after every 2 days of treatment using the meter rule (cm) until complete epithelization. The degree of wound healing was calculated using the formula below;

$$\text{Percentage wound contraction} = \left(1 - \frac{\text{wound area on corresponding day}}{\text{Wound area on day (0)}} \times 100\right) \quad \text{Eqn. 1}$$

Stability Study on the optimized gel (EV3)

The optimized gel EV3 (1.2 % w/w) was subjected to a stability study for 3 months

following ICH guidelines. The formulated gel was filled in the container and placed in a 200 mL saturated solution of sodium chloride inside desiccators at 35 ± 2°C and 75% Relative humidity for a period of three months. Then studies for appearance, pH, viscosity, colour, homogeneity and antimicrobial activities were carried out.

Statistical Analysis

The antimicrobial results in triplicate were analyzed with Statistical Package for Social Sicences (SPSS) version 16.0 and presented as mean ± standard error of mean (SEM) inhibition zone diameters (IZD) using One-Way ANOVA and further subjection to Tukey's post hoc test. Values of $p<0.05$ (5%) and $p<0.01$ (1%) were regarded as significant.

RESULTS

Yield of extracts and fractions of *Alchornea cordifolia* leaves

The percentage yield of the Crude extract and fractions of *Alchornea cordifolia* are as follows: crude ethanolic extract (39.30 g /7.86 % w/w), ethyl acetate fraction (14.95 g/38.04 % w/w), n-heptane fraction (13.42 g /34.15 %w/w) and the butanol fraction (10.93g/ 27.81 %w/w).

Yield of ethyl acetate VLC fractions of *Alchornea cordifolia* leaf extract

The percentage yields from the 6.47 g ethyl acetate fraction are as follows; EA1 had the highest yield of 35.55% while EA6 had the lowest yield of 0.50 %.

3.3. Bioactive compounds identified from ethyl acetate (EF) chromatographic fraction

The ethyl acetate chromatographic fraction (EF) of *Alchornea cordifolia* revealed four compounds: Epicatechin O-3, 4-dimethyl gallate, Aerophobin-2, myricetin 3-O-rhamnoside and quercetin with retention times of 10.95 min, 14.14 min, 14.42 min, 15.38 min, 18.52min and 20.50 min respectively at 100%. The HPLC chromatogram of the EF fractions and UV spectra of the identified compounds are shown in figures1 below.

Bioactive compounds identified from ethyl acetate/ethanol VLC fraction

The fraction EA5 from ethyl acetate/ethanol VLC fraction of *Alchornea cordifolia* revealed five compounds: aerophobin-2, 1-O-galloyl-6-O-luteoyl-α-D-glucoside, apigenin glycoside, quercetin-3-O- β-D-glucopyranoside and ellagic acid with retention times of 10.92 min, 14.24 min, 18.41 min, 19.62 min, and 21.38 min respectively at 100%. The HPLC chromatogram of EA5 fraction and UV spectra of the identified compounds are shown in figures 2 below.

Result of antimicrobial screening of ethyl acetate fractions of *Alchornea cordifolia.*

The result of the antimicrobial assay of ethyl acetate chromatographic (EF) and EA5 fractions are shown in tables 3, 4 and 5 below.

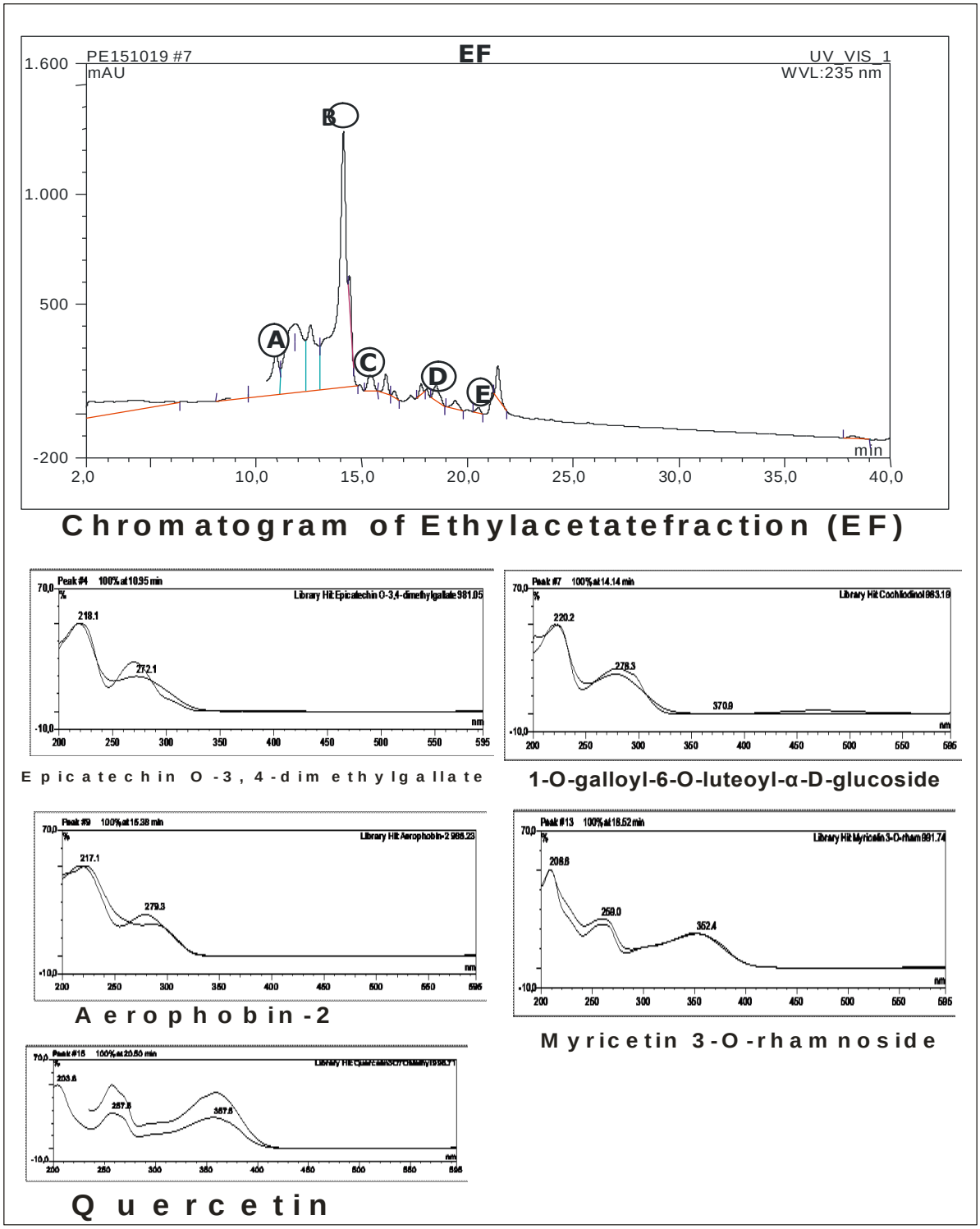


Figure 1: HPLC Chromatogram of Ethylacetatefraction (EF) and UV spectra of detected compounds

A = Epicatechin3, 4-O-dimethyl gallate B = 1-O-galloyl-6-O-luteoyl-α-D-glucoside

C = Aerophobin-2 D = Myricetin 3-O-rhamnoside E = Quercetin

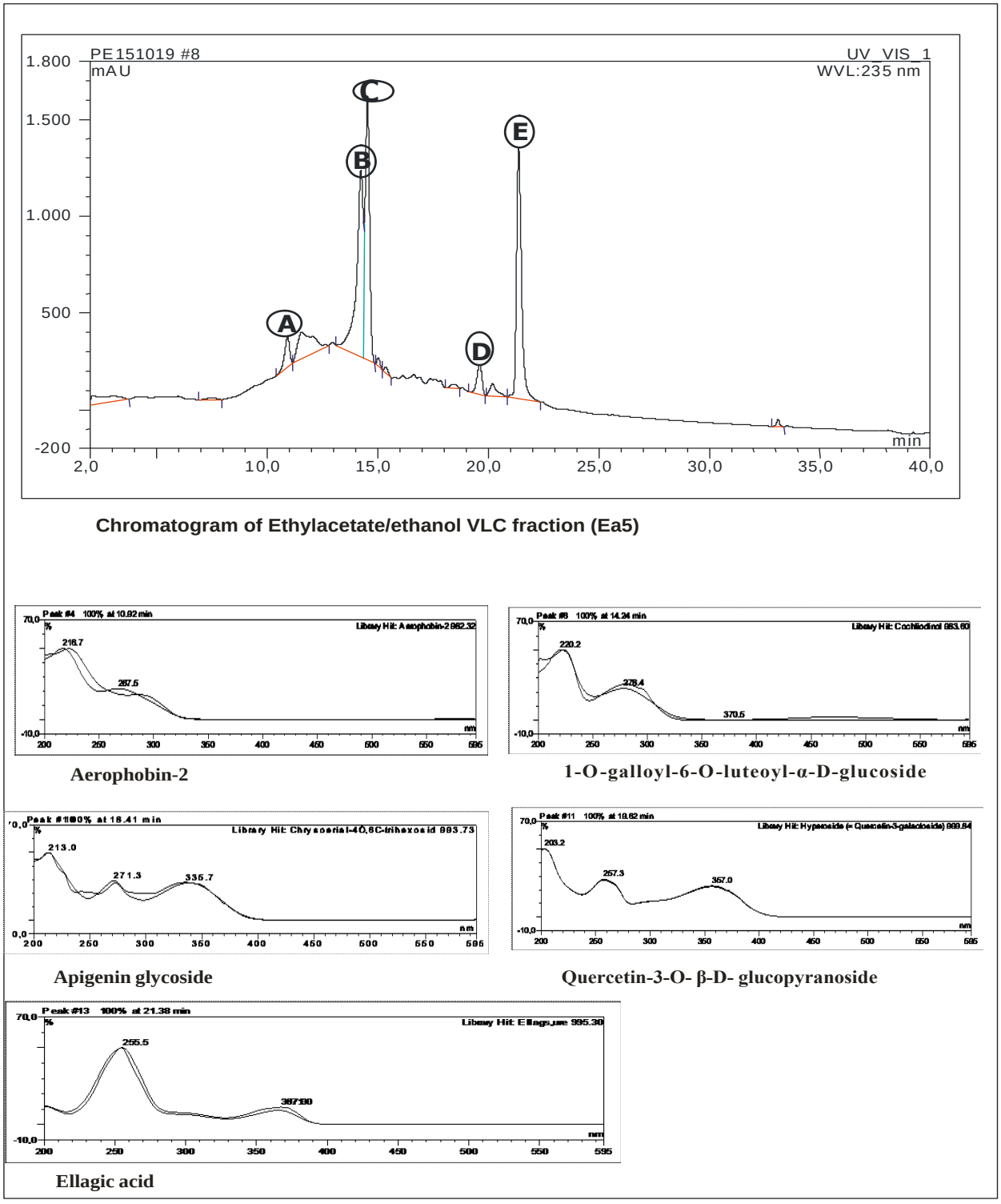


Figure 2: **HPLC Chromatogram of Ethylacetate/ethanol VLC fraction (EA5)**
A = Aerophobin-2 **B = 1-O-galloyl-6-O-luteoyl-α-D-glucoside**
C = Apigenin glycoside **D = Quercetin-3-O- β-D- glucopyranoside**
E = Ellagic acid

Table 3: Result of Inhibition Zone Diameter of Ethylacetate chromatographic fraction (EF)

Test Organisms	**Concentration (µg/mL) of EF**					**Ciprofloxacin (5µg/mL)**	**DMSO**
	20	**10**	**5**	**2.5**	**1.25**		
	IZD (mm)						
1.) Staphylococcus aureus	6±0	4±0	3±0	2±0	2±0	6±0	-
2.) Bacillus subtilis	4±0	3±0	0±0	0±0	0±0	9±0	-
3.) Escherichia coli	4±0	3±0	2±0	0±0	0±0	6±0	-
4.) Pseudomona aeruginosa	3±0	3±0	2±0	2±0	0±0	4±0	-
						Ketoconazole (50µg/mL)	
1.) Aspergillus niger	4±0	3±0	2±0	0±0	0±0	6±0	-
2.) Candida albicans	-	-	-	-	-	4±0	-

All values are Mean ± SEM (n = 3),* = p <0.05 compared to negative control (one way ANOVA; Turkey's post hoc), - = No inhibition, IZD = Inhibition zone diameter, EF = Ethylacetate fraction

Table 4: Result of Inhibition Zone Diameter of EA5 fraction

Test Organisms	**Concentration (µg/mL)**					**Ciprofloxacin (5µg/mL)**	**DMSO**
	20	**10**	**5**	**2.5**	**1.25**		
	IZD (mm)						
1.) Staphylococcus aureus	5±0	3±0	3±0	2±0	0±0	6±0	-
2.) Bacillius subtilis	14±0	11±0	10±0	9±0	9±0	9±0	-
3.) Escherichia coli	6±0	3±0	0±0	0±0	0±0	6±0	-
4.) Pseudomona aeruginosa	4±0	3±0	2±0	0±0	0±0	4±0	-
						Ketoconazole (50µg/mL)	
1.) Aspergillus niger	4±0	3±0	2±0	0±0	0±0	6±0	-
2.) Candida albicans	-	-	-	-	-	4±0	-

All values are Mean ± SEM, n = 3,* = p <0.05 compared to negative control (one way ANOVA; Turkey's post hoc), - = No inhibition, IZD = Inhibition zone diameter, EA5 = ethyl acetate/ethanol (50:50) fraction.

Table 5: Minimum Inhibition Concentration of the ethyl acetetate Chromatographic fraction (EF) against the test bacteria species

Tested Organism	Minimum Inhibitory Concentration (µg/ml) (mm) EF
	1.25 (2)
	5 (3)
Staphylococcus aureus	10 (2)

Bacillus subtilis	2.5	(2)
Escherichia coli	-	
Pseudomonas aeruginosa	2.5	(2)
Candida albicans		
Aspergillus niger		

***Values in bracket are the inhibition zone diameter (in mm)**

Optimization and selection of gel

Different ratios of the gelling agents were tried to select the best ratio for the gelling agent. Gels containing Carbopol (934) and Sodium CMC in the ratio of 2:1 showed high viscosity and were rejected. Gels containing Carbopol (934) and Sodium CMC in the ratio 1:2 showed low viscosity and were rejected. Gels containing only Carbopol (934) formed very thick and sticky gels that could not be properly spread out and were rejected too. Gels containing only Sodium CMC formed thin gels, poor in consistency and low viscosity. Gels containing Carbopol (934) and Sodium CMC in the ratio of 1:1 formed uniform and smooth gels that didn't liquefy on standing and they also showed uniform viscosity and homogeneity. Thus, gels with gelling agents in the ratio of 1:1 were selected as the optimized concentration of gelling agents. The gels are as follows: EF1, EF2, EF3, EV1, EV2, and EV3.

Physical evaluation of the herbal gels (EF1 – EV3)

Forty-two (42) formulations were prepared and evaluated based on the following physical parameters: pH, colour, Viscosity, and Homogeneity. Six (6) of the formulations turned out best and their results are reported in Table 6 below.

Table 6: Result of the physical evaluation of the herbal gels (EF1-EV3)

S No.	Parameter	EFV[a]	EF1 (40 mg)	EF2 (80 mg)	EF3 (120 mg)	EV1 (40 mg)	EV2 (80 mg)	EV3 (120 mg)
1.	pH	6.98	6.85	6.90	6.92	6.90	6.88	6.87
2.	Colour	Transparent	Light Brown	Light Brown	Light Brown	Dark Brown	Dark Brown	Dark Brown
3.	Viscosity (mPa.S)	173	174	174	173	175	174	174
4.	Homogeneity	Good	Good	Good	Good	Good	Good	Good

EFV[a] is the plain gel used as the negative control

Result of antimicrobial activity on the herbal gels

The results of the inhibition zone diameter of the herbal gels are reported in Table 7.

Table7: Result of Inhibition Zone Diameter (IZD) of the herbal gels (EF1-EV3)

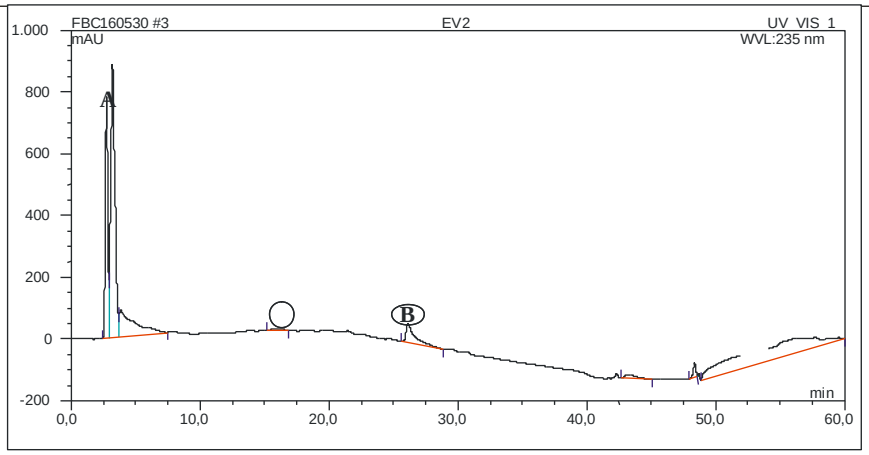
Herbal Gels	*S. aureus*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*C. albicans*	*A. niger*
	Inhibition zone diameter (mm)					
	0±0	0±0	0±0	2±0	0±0	0±0
	0±0	6±0	6±0	1±0	0±0	0±0
EF1 (40 mg)	0±0	14±0	0±0	0±0	0±0	0±0
EF2 (80 mg)	0±0	0±0	0±0	0±0	0±0	0±0
EF3 (120 mg)	0±0	0±0	20±0	19±0	0±0	15±0
EF7 (0 mg)	15±0	12±0	25±0	22±0	10±0	19±0
EV1 (40 mg)	16±0	18±0	17±0	22±0	15±0	23±0
EV2 (80 mg)	0±0	0±0	0±0	0±0	0±0	0±0
EV3 (120 mg)						
EV7 (0 mg)						

Identification of compounds present in the EV2 and EV3 gels by High-Performance Liquid Chromatography (HPLC).

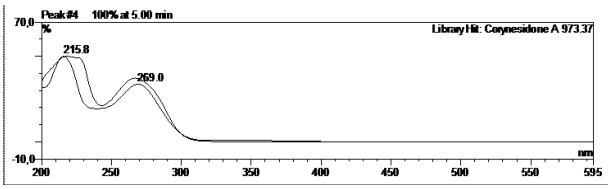
The chromatograms of Two (2) compounds identified from the optimized gels EV2 and EV3 by HPLC are shown in figure 3 below.

Result from the wound healing analysis

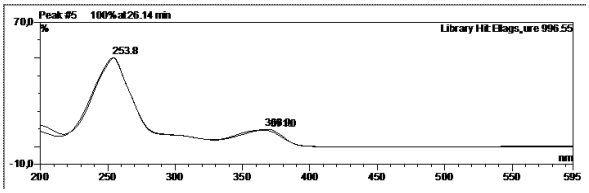
The result of the wound-healing effect of gel which showed significant healing up to the 20th day of treatment is shown in figure 4 below.



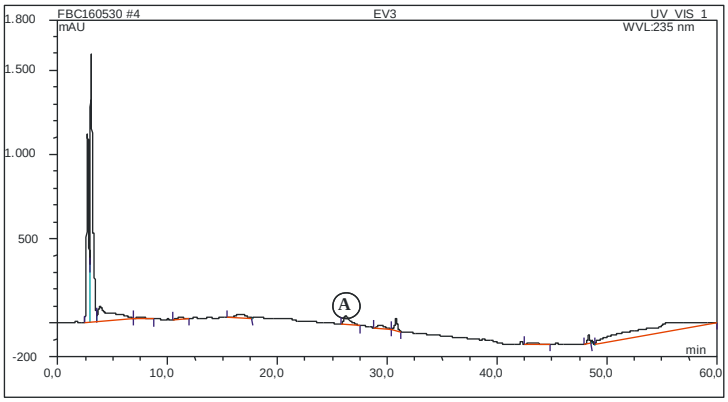
HPLC Chromatogram of gel EV2 (80mg)



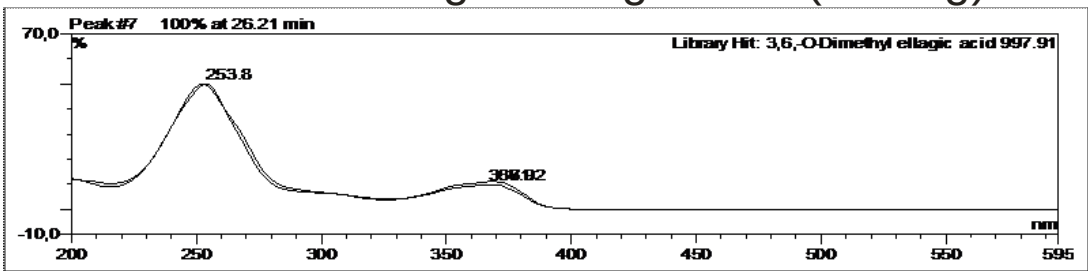
1-O-galloyl-6-O-luteoyl-α-D-glucoside



Ellagic acid



HPLC Chromatogram of gel EV3 (120mg)



3, 6-O-Dimethyl ellagic acid

Figure 3: HPLC Chromatogram of gel EV2 (80mg) and gel EV3 (120mg)

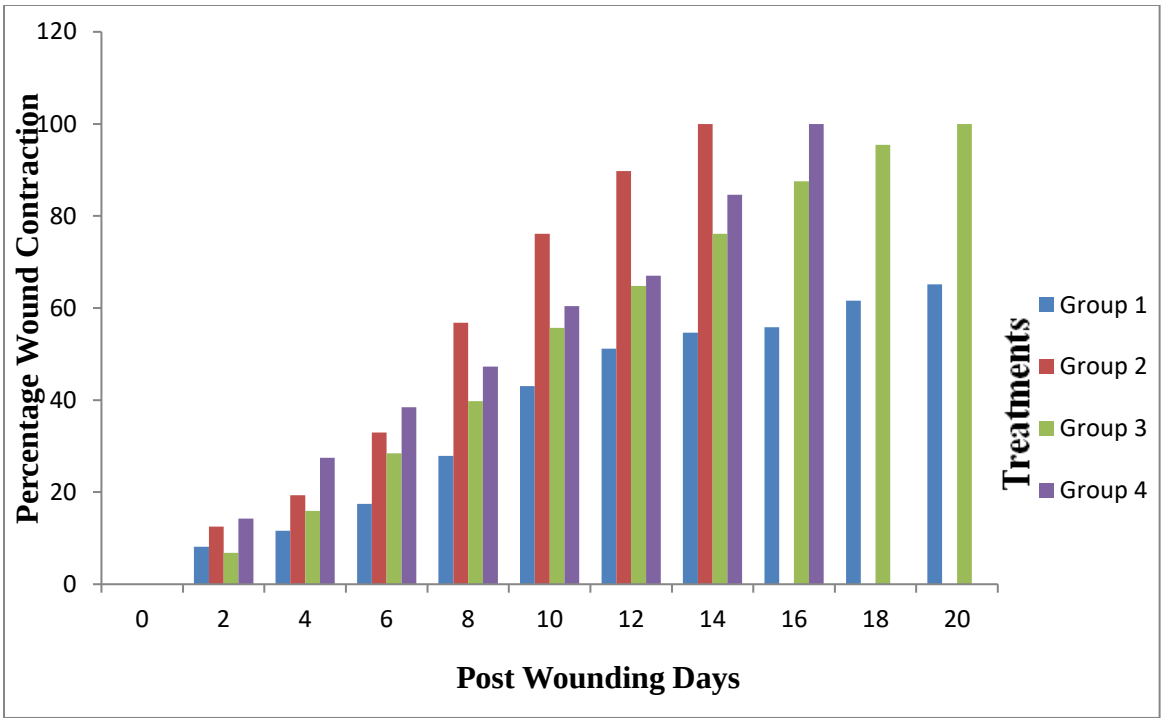


Figure 4: Wound healing analysis of the gel
Group 1 = Negative control,
Group 2 = Gentamicin gel (positive control),
Group 3 = EV2 (80mg drug),
Group 4 = EV3 (120mg drug).

Results of the Stability study

The result of three (3) months stability study of EV3 herbal gel is shown in Table 9

Table 9: Result of the physical evaluation of the herbal gel (EV3) after stability study.

S No.	Parameter	EV3	
		Values at Day 0	Values after 3 months
1.	pH	6.87	6.87
2.	Colour	Dark brown	Dark brown
3.	Viscosity (mPa.S)	174	174
4.	Homogeneity	Smooth	Smooth
5.	Appearance	Clear	Clear

Result of the Antimicrobial Screening (Post stability studies)

After three (3) months, the EV3 herbal gel was subjected to antimicrobial screening but there was no activity noted.

DISCUSSION

The ethyl acetate fraction and its VLC sub-fraction (EA5) have been shown in a previous study (Okoye *et al*., 2015) to exhibit antimicrobial activities due to the presence of phenolic compounds. In the present study, these fractions were further subjected to HPLC-DAD analysis and eight phenolic compounds namely; Epicatechin 3, 4-O-dimethyl gallate, Aerophobin-2, myricetin 3-O-rhamnoside, quercetin, galloyl-6-O-luteoyl-α-D-glucoside, apigenin glycoside, quercetin-3-O- β-D-glucopyranoside and ellagic acid were identified by dereplication. These compounds have all been previously reported to exhibit one or more of antimicrobial, antioxidant, antitumor, immunomodulatory and anti-inflammatory activities (Teeyaphant *et al*., 1993; Ong *et al*., 1997; Osadebe and Okoye 2003; Mavar-Manga *et al*., 2008; Akiyama *et al*., 2011; Okoye *et al*., 2011; Li *et al*., 2012; Gupta *et al*., 2014; Okoye *et al*., 2015). The ethyl acetate fraction and EA5 fractions of *Alchornea cordifolia* showed antimicrobial activity against both Gram-positive and Gram-negative bacteria with inhibition zone diameters ranging from 2 mm – 14 mm, which indicates a broad spectrum of antimicrobial activity. EA5 showed stronger activities against these test pathogens than EF with the Minimum Inhibition Concentration (M.I.C) values against the test organism ranging from 1.25µg/mL to 10µg/mL. The activities of EA5 against all the test pathogens are comparable to that of the positive control ciprofloxacin (Tables 3 and 4). HPLC analysis of EA5 showed the presence of quercetin and ellagic acid derivative respectively in large quantities. The strong antimicrobial activity of EA5 can be attributed to the high presence of these compounds, which have been shown to possess antimicrobial activity in previous studies (Anyanwu *et al*., 2018a (b); De *et al*., 2018; Okoye *et al*, 2015). The herbal gels prepared with EA5 (EV1-EV3) showed better antimicrobial activity than those prepared with EF (EF1-EF3) and this corroborated with the results of HPLC-DAD analysis carried out on the gels (figure 2). Formulations (EV2 and EV3), which showed better antimicrobial activity and gel properties, were selected for further tests. Three (3) compounds Ellagic acid, 3, 6-O-dimethylellagic acid and 1-O-galloyl-6-O-luteoyl-α-D-glucoside were identified from the optimized gels (EV2 and EV3) by HPLC-DAD analysis. The susceptibility of these pathogens to the herbal gels could therefore be a result of the presence of these phenolic compounds which have been previously reported to possess antimicrobial activity. The result of the wound healing studies showed that there was significant healing up to the 20th day. It was observed that treatments with the EV3 gel healed rapidly and were comparable to the Positive control until the 14th day when the group treated with Gentamicin gel showed 100% healing. Group treated with EV3 gel showed complete wound healing on the 18th day, the group treated with EV2 herbal gel showed complete healing on the 20th day while the group treated with the plain gel showed only 60 % healing up to the 20th day. The wound-healing effect of the gel may be associated with the anti-inflammatory and antimicrobial activities of the detected phenolic compounds. Ellagic acid derivatives have been reported to exhibit strong anti-inflammatory properties in previous studies (Anyanwu *et al*., 2018b). Furthermore, the observed wound-healing activity of the herbal gels explains the reason behind the utilization of *Alchornea cordifolia* extract in traditional medicine as an anti-inflammatory and wound-healing agent. The EV3 herbal gel was found stable

as there was no change in the parameters at the end of the three (3) months stability study. The loss of antimicrobial activity by EV3 herbal gel could be that the compounds from the fractions are not stable in gel form. This work agrees with the previous report of El-Mahmood *et al*., 2007 that herbal medicinal products prepared from plant and animal origins are subject to contamination and deterioration after a certain period.

CONCLUSION

Herbal gels formulated from the leaf extract of *Alchornea cordifolia* possess antimicrobial and wound-healing properties. These herbal gels, when formulated with a suitable preservative can be a good alternative to conventional gels used in wound healing.

Compliance with ethical standards

ACKNOWLEDGMENTS

We are thankful to Prof. Dr. Peter Proksch of the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Düsseldorf, Germany for making their facilities available for the HPLC analysis. The author is also grateful to the Department of Pharmaceutical and Medicinal Chemistry; Department of Pharmaceutics and Pharmaceutical Technology, Nnamdi Azikiwe University, Awka for allowing their laboratories to be used for the studies.

DISCLOSURE OF CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Abo KA, Ashidi JS (1999). Antimicrobial screening of Bridelia micrantha, *Alchornea cordifolia* and *Boerhavia diffusa*: *African Journal of Medicine Med Sci*. 28, 167-169.

Agbor C, Leopold T, Jeanne (2004). The anti-diarrhoeal activity of *Alchornea cordifolia* leaf extract. Phytother. Res. 18 (11), 873-876.

Aida P, Rosa V, Blamea F, Tomas A, Salvador C (2001). Paraguayan plants used in traditional medicine. Short communication. J. Ethnopharm. 16, 93-98.

Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K (2001). Antibacterial action of several tannins against Staphylococcus aureus, J. AntimicrobChemother. 48(4): 487-491.

Anyanwu OO, Eze PM, IE N, Ngwoke KG (2018a). Antimicrobial Properties of Jatrophacurcas L. against Dental Pathogens. *Glob. J. Med. Res*. *18*, 12-15.

Anyanwu OO, Ngwoke KG, Okoye FBC (2018b). Bioactive Constituents Responsible for the Anti-inflammatory actions of Jatropha curcas. Journal of Medicinal Botany. 2, 07-15.

Brewer D, Jerram WA, Meiler D, Taylor A (1970). The toxicity of cochliodinol, an antibiotic metabolite of Chaetomium spp. *Canadian Journal of Microbiology*. *16*(6), 433- 440.

Boniface PK, Ferreira SB, Kaiser CR (2016). Recent trends in phytochemistry, ethnobotany and pharmacological significance of Alchornea cordifolia (Schumach. &Thonn.) Muell. Arg. *Journal of Ethnopharmacology*, *191*, 216-244.

Bouamama H, Noel T, Villard J, Benharref A, Jana M (2006). Antimicrobial activities of the leaf extracts of two Moroccan Cistus

L. species. Journal of ethnopharmacology, 104(1-2), 104-107.
Choi HJ, Kim JH, Lee CH, Ahn YJ, Song JH, Baek SH, Kwon DH (2009). Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus. *Antiviral Research*, 81(1), 77-81.

Cook MT, Smith SL, Khutoryanskiy VV (2015). Novel glycopolymer hydrogels as mucosa-mimetic materials to reduce animal testing. Chem. Commun. 51 (77): 14447–14450.

Cos P, Vlietinck AJ, Berghe DV, Maes L (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept. *Journal of Ethnopharmacology*, 106:290302.

De R, Sarkar A, Ghosh P, Ganguly M, Karmakar BC, Saha DR, … Mukhopadhyay AK (2018). Antimicrobial activity of ellagic acid against Helicobacter pylori isolates from India and during infections in mice. Journal of Antimicrobial Chemotherapy. 73(6), 1595-1603.

Duraipandiyan V, Muniappan A,Savarimuthu I (2006). Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India.

Ebi GC (2001). Antimicrobial activities of *Alchornea cordifolia*. Fitoterapia. 72: 69–72.

El-Mahmood AM (2009). Antibacterial activity of crude extracts of *Euphorbia hirta* against some bacteria associated with enteric infections. J. Med. Plants Res. 3(7): 498-505.

Eswarappa SM (2009). Location of pathogenic bacteria during persistent infections: Insight from an analysis using game theory. PLoS ONE 4(4): e5383. doi:10.1371/journal.pone.0005383.

Feldman KS, Saharabudhe K, Smith RS, Scheuchenzuber WJ (1999). Immune-stimulation by Plant polyphenols: Relationship between tumor necrosis factor-production and tannin structure. Bioorganic Med. Chem. Lett. 9(7): 985-990.

Gupta S, Tyag IA, Deshmukh-Tasker P, Hinojosa M, Prasad S, Aggarwal *B. (2014).* Down regulation of tumor necrosis factor and other pro-inflammatory biomarkers by polyphenols*: Archives of Biochemistry and Biophysics. 559: 91–99.*

Iwu M, Duncan A, Janick J, Okunji O (1999). New antimicrobials of plant origin in Perspective on New Crops & New Uses: pp. 457–462, ASHS Press, AlexandriaVa, USA.

Kamenan A, Kouakou-Siransy G, Irié-Nguessan, Dally I, KablanBrou J (2013). Anxiolytic activity of an aqueous extract of *Alchorneacordifolia*(Euphorbiaceae) leaves. *African Journal of Pharm. Pharmacol. Extract.* 7(16):816-821.

Li Y, Ding Y (2012). Minireview: Therapeutic potential of myricetin in diabetes mellitus. *Food Science and Human Wellness. 1, 19–25.*

Manjunatha BK, Krishna V, Vidya SM, Mankani KL, Manohara YN (2007). Wound healing activity of Lycopodium serratum. Indian Journal of Pharmaceutical Sciences. 69(2): 283-287.

Mavar-Manga H, Brkic D, Marie DEP, QuetinLeclercq J (2004). In vivo anti-inflammatory activity of *Alchornea cordifolia* (Schumach. &Thonn.) Mull.Arg.

(Euphorbiaceae). *Journal of Ethnopharmacology*. 92: 209– 214.

Mavar-Manga H, Haddad M, Pieters L, Baccelio C, Penge A, Quetin Leclercq J (2008). Anti-inflammatory compounds from leaves and root bark of *Alchornea cordifolia* (Schumach and Thonn) Mull. Arg. *Journal of Ethnopharmacology* 115(1): 25-29.

Mukherjee PK, Maity N, Nema NK, Sarkar BK (2011). Bioactive compounds from natural resources against skin aging. Phytomedicine 19: 64-73.

Okolo CC, Mbachu NA, Anyanwu OO, Ngwoke KG, Okoye FB (2021). Screening for anti-inflammatory activity of extract and fractions of Morinda lucida stem bark and detection of bioactive secondary metabolite. *GSC Biological and Pharmaceutical Sciences*, *16*(1), 031-039.

Okoye FBC, Osadebe PO, Nworu CS, Okoye NN, Omeje EO, Esimone CO. (2011): Topical anti-inflammatory constituents of lipophilic leaf fractions of *Alchornea floribunda* and *Alchornea cordifolia*, Natural Product Research, DOI:10.1080/14786419.2010.512272

Okoye FBC (2015). A new antioxidant and antimicrobial compound isolated from Alchornea leaves. African Journal of Pharmaceutical Research and Development. Vol. 7 No. 2; pp. 87 – 94.*Ong K, Khoo H (1997).* Biological Effects of Myricetin. *General Pharmacology. 29(2): 121-126.*

Osadebe PO, Okoye FBC (2003). Anti-inflammatory effect of crude methanolic extract and fraction of *Alchornea cordifolia* leaves. Journal of Ethnopharmacology. 89 (1) 19- 24.

Osadebe PO, Okoye FBC, Nnamani NR, Uzor PE, Adiele IE, Obiano NC (2012). Phytochemical analysis, hepatoprotective and antioxidant activity of Alchornea cordifolia methanol leaf extract on carbon tetrachloride-induced hepatic damage in rats. Pacific Journal of Tropical Medicine. Pp. 289-293.

Rashed AN, Afifi FU, Disi AM (2003). Simple evaluation of the wound healing activity of crude extract of Portulaca oleracea L. (growing in Jordan) in Mus musculus JVI-1. *Journal of Ethnopharmacology*, *88* (2-3), 131-136.
Setzer WN, Shen X, Bates RB, Burns JR, McClure KJ, Zhang P, ... Lawton RO (2000). A Phytochemical investigation of Alchornea latifolia. *Fitoterapia*, *71*(2), 195-198.

Sepúlveda L, Ascacio A, Rodríguez-Herrera R, Aguilera-CarbóA,… Aguilar CN (2011). Ellagic acid: Biological properties and biotechnological development for production processes. *African Journal of Biotechnology*. 10(22), 4518-4523.

Shivananda B, Sivachandra-Raju S, Chalapathi-Rao AN (2007). Wound healing activity of Matricariarecutita L. extract. J. Wound Healing 16(7): 298-302.

Sujatha S, Kantida SR (2012). Cytotoxicity and antimicrobial studies of quercetin-3-O-galactoside (hyperoside) isolated from the leaves of Psidium guajava (Linn.). *Novus International Journal of Pharmaceutical Technology*. 1(30), e37.

Teeyapant R, Woerdenbag HJ, Kreis P, Hacker J, Wray V, Witte L, Proksch P (1993). Antibiotic and cytotoxic activity of brominated compounds from the marine sponge Verongiaaerophoba. *Zeitschrift für Naturforschung C*. *48*(11-12), 939-945.

Wu CR, Huang MY, Lin YT, Ju HY, Ching H (2007). Antioxidant properties of Cortex Fraxini and its simple coumarins. *Food Chemistry*. 104(4), 1464-1471.

Zhou J, Liang S, Fang L, Chen L, Tang M, Xu Y, ... Wei Y (2009). Quantitative proteomic analysis of HepG2 cells treated with quercetin suggests IQGAP1 involved in quercetin-induced regulation of cell proliferation and migration. *A Journal of Integrative Biology*. 13(2), 93-103.