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Pharmacognostic and *in vitro* antimicrobial evaluation of the sub-fractions of the ethyl acetate fraction of the methanol leaf extract of *Anthocleista djalonensis*

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Abstract

Anthocleista djalonensis has many ethnomedicinal claims, one of which is the treatment of infections. The study evaluated the phytochemical, physicochemical profile and antimicrobial properties of the vacuum liquid chromatographic (VLC) sub-fractions of A. Djalonensis leaves. The plant was collected from Umuoji, Idemili North, Anambra State. The plant leaves were identified, authenticated and the herbarium specimen was deposited, with herbarium number PCG/474/A/057. The leaves were dried, pulverized and extracted using cold maceration with methanol, and fractionated successively into n-Hexane, ethyl acetate and butanol. To obtain the ethyl acetate fraction, it was subjected to vacuum liquid chromatography (VLC). The phytochemical and physicochemical analyses were carried out, using standard protocols. The VLC sub-fractions were subjected to HPLC-DAD analysis. The antimicrobial assay of the VLC sub-fractions was carried out using the agar well diffusion assay. The antimicrobial activity of the VLC sub-fractions was tested against three standard clinical bacterial isolates (Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli) and one fungal isolate (Candida albican). Phytochemical screening showed the presence of alkaloids, flavonoids, tannins, proteins, glycosides, saponins, carbohydrates and steroids. The physicochemical evaluation showed that the plant contains a total ash value of 4.55 % w/w, acid insoluble ash value of 6.80 % w/w, water-soluble ash value of 2.65 %w/w, moisture content of 7.90 %w/w and water-soluble extractive value of 1.02 %w/w. The HPLC-DAD analysis of the VLC sub-fractions suggested the presence of isovitexin, septicine, vanillin, 3-methyl-2, 3, 4- pentanetriol, indo-3- carboxylic acid, Cerebroside and bromohexylamide. The VLC sub-fractions had activity against Pseudomonas aeruginosa only, and there was no zone of inhibition for the fungi. The VLC sub-fractions have very little antimicrobial activity, showing antibacterial activity against Pseudomonas aeruginosa only, with no antifungal activity against Candida albican.

Keywords: Anthocleista djalonensis, Phyto-constituents, Antimicrobial, Pharmacognostic, Pseudomonas aeruginosa.

INTRODUCTION

Over thousands of years, plants have been used because of their good source and medicinal [1,2]. In the past few years, studies have shown that several plants have ethnomedicinal properties that could be important to the world.

Many of the plant materials in rural areas are relatively cheap and used in traditional medicine [3,4]. Several metabolites are produced by plants which comprise an important source of pharmaceutical products. Plants remain the principal source of pharmaceutical agents used in traditional medicine and natural products [5].

For aromatic and other medicinal plants, there is an increased interest in the scientific research industry because of their medicinal (antimicrobial and antioxidant) properties, which are due to some phytocompounds such as carotenoids, coumarins, curcumins, flavonoids, and terpenoids, these phytoconstituents are confirmed using modern analytical techniques [6].

Most essential oils with phenolic compounds possess antimicrobial activity and are classified as generally recognized as safe (GRAS) [7]. Plants contribute to a good source of anti-infective agents and are highly effective instruments in the fight against microbial infections, therefore traditional healthcare systems are dependent on medicines derived from plants [8]. The world populations (80%) rely on the use of herbal medicines to meet their primary health care needs although; up to 90% of the developing nations rely on

the use of different medicinal plants [9]. In recent years, infections have increased to a great extent and antibiotics resistance effects have become a therapeutic problem [10]. Natural products are new sources of antimicrobial agents with possibly novel mechanisms of action; therefore, it is important to carry out a screening of these plants to authenticate their use in folk medicine, by isolation and characterization we reveal their constituents [11,12]. The discovery of novel active compounds is a result of systemic reviews [13].

Medicine is the science and practice of preventing and curing illness and disease, many useful phytomedicines come from plants and they have been used as medicine for centuries [14,15]. Medicinal plants involve secondary metabolites with their chemical structures which are useful for maintaining good health in both human and animals [16]. It has been identified by researchers that plants produce active constituents to form drugs because of their use in traditional medicine [17].

Different parts of plants such as the root, stem, bark, fruit, leaf, flower or, seed have been recognized to have medicinal properties or values and a large percentage of these plants is found in the forest [18]. It is on the basis that the topic Pharmacognostic and in vitro antimicrobial evaluation of the sub-fractions of the ethyl acetate fraction of the methanol leaf extract was formed.

Figure 1: Picture of *Anthocleista djalonensis* leaves

MATERIALS AND METHODS

Collection of Plant Sample and Identification

Fresh leaves of *Anthocleista djalonensis* were collected in May 2016 from Umuoji village, Idemili North local Government. It was authenticated by Pharmacognosy Department Nnamdi Azikiwe University Agulu Campus. The stalk and stem were washed, and removed after drying, and the dried leaves were pulverized.

Qualitative and Quantitative Phytochemical Screening

The crude extract of *Anthocleista djalonensis* were screened for the presence of alkaloids, cardiac glycosides, flavonoids, steroids, saponins, tannins, terpenoids, proteins and carbohydrates using standard phytochemical methods [19,20,21].

Physicochemical Parameters

Anthocleista djalonensis leaves were tested for moisture content, ash values, extractive values and crude fiber [22,23,24].

Preparation of Vacuum Liquid Chromatographic Sub-Fractions of Ethyl Acetate Fraction of Methanol Extract

A 500g powdered leaves were weighed and extracted in 1.5l of methanol in a conical flask. The mixture was placed on an electrical orbital shaker 200rpm for 48h at room temperature. The mixture was filtered, and the extract evaporation to dryness. The concentrated extract was dissolved in 50ml of water and poured into an Erlenmeyer flask clamped onto a retort stand. 500ml of n-Hexane was poured into the flask and shaken gently. The flask was clamped onto the retort stand and allowed to stand undisturbed until a clear separation was obtained between the two layers. The lower layer was run off and the upper layer was collected. The process was repeated to ensure exhaustive partitioning of the n-Hexane. This same procedure was repeated for ethyl acetate and labeled as ethyl acetate fraction. Furthermore, different sub-fractions were obtained using gradient vacuum liquid chromatographic separation. To achieve a uniform layer, the silica gel was compressed under a vacuum for better separation. nHexane and ethyl acetate, Dichloromethane and methanol were used as mobile phases in different ratios. The fractions were collected in a separate screw-capped test tube and concentrated on a rotary evaporator. and tested for their antibacterial and antifungal activities.

Microbiological Evaluation

Anti-microbial screening (*In vitro*)

The antimicrobial screening was determined by agar plate diffusion assay [25].

HPLC Analysis

High-Performance Liquid chromatography was performed with an Agilent 1260 infinity liquid chromatographic system (Agilent Technologies, Santa Clara, USA) fitted with a variable (200- 800 nm) ultraviolet-visible detector and a quaternary pump. The column was Hypersil ODS (C18) 3.5µm, 4.6 X 100 mm reversed phase stainless steel type (Agilent Technologies, Santa Clara, USA) [26].

Microscopy Analysis

0.5g of the powdered plant was poured into a beaker, few drops of chloral hydrate were added to cover the plant. This was left for 24hrs to enable total clearing and then placed on a glass slide. Two drops of glycerol were added to the slide and viewed under a microscope [27].

Statistical Analysis

The statistical analysis was estimated using one-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparisons Test. Means of triplicate measurements and standard deviation were determined for each sample using standard operating procedures for antimicrobial activity.

RESULTS

The phytochemical analysis of the methanol leaf extract of *Anthocleista djalonensis* showed alkaloids and reducing sugars to be highly present. Flavonoids, Proteins, cardiac glycosides, and steroids to be moderately present, tannins and saponins, to be mildly present.

Table 1: Phytochemical Analysis of *A. djalonensis*

Phytochemicals	Presence/ absence of constituents
Alkaloids	+++
Flavonoids	++
Tannins	+
Proteins	++
Cardiac glycosides	++
Saponins	+
Reducing sugars	+++
Steroids	++

(+) Trace/mildly present (++) moderately present

(+++) highly present (++++) abundantly present

The quantitative phytochemical analysis of the methanol leaf extract of *Anthocleista djalonensis* showed alkaloids (4.47%), flavonoids (10.49%), tannins (7.05%) and saponins (0.08%). Therefore, Alkaloid is confirmed to be highly present in the plant.

Table 2: Quantitative Phytochemical Analysis of *A. djalonensis*

Phytochemicals	% Composition
Alkaloids	4.47
Flavonoids	10.49
Tannins	7.05
Saponins	0.08

The result of the physicochemical properties showed the percentage of total ash value of the powdered plant as 4.55%, which is low. The percentage water-soluble extractive value of the plant sample was found to be 1.02% while the percentage of acid-insoluble and water-soluble ash were 6.80% and 2.65% respectively. The percentage loss on drying was also found to be 7.90%. Therefore, this herbal preparation is satisfactory according to National Agency for Food and Drug Administration and Control

Table 3: Physicochemical parameters of the powdered plant sample of *A. djalonensis*

Physicochemical parameters	%(w/w) composition
Total ash	4.55
Acid insoluble ash	6.80
Water soluble ash	2.65
Moisture content	7.90
Water soluble extractive	1.02

From the results obtained from the *in-vitro* evaluation of the antimicrobial activity of the different VLC sub-fractions of the ethyl acetate fraction, it was seen that at a concentration of 100 mg/ml, the various fractions exhibited inhibition of *Pseudomonas aeruginosa* only and there was no zone of inhibiting seen in *Staphylococcus aureus* and *Escherichia coli*. The highest zone of inhibition seen on *Pseudomonas aeruginosa* was observed in the n-hexane: ethyl acetate (20:80) fraction, while the lowest zone of inhibition seen on *Pseudomonas aeruginosa* was observed in DCM: methanol (20:80) fraction at 100mg/ml. Ciprofloxacin, the antibacterial agent has a higher inhibitory zone diameter on the test bacteria than the plant extract.

Table 4: *In vitro* antimicrobial activity of n-Hexane: Ethyl acetate (80:20) of *A. djalonensis*

Test organisms	Concentration (mg/mL) / IZD (mm)					
	100	50	25	12.5	6.25	Cipro 5 µg/mL
S. aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
E. coli	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
P. aeruginosa	4 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0	18
C. albicans	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Miconazole 50 µg/mL 27

Table 5: *In vitro* antimicrobial activity of n-Hexane: Ethyl acetate (20:80) of *A. djalonensis*

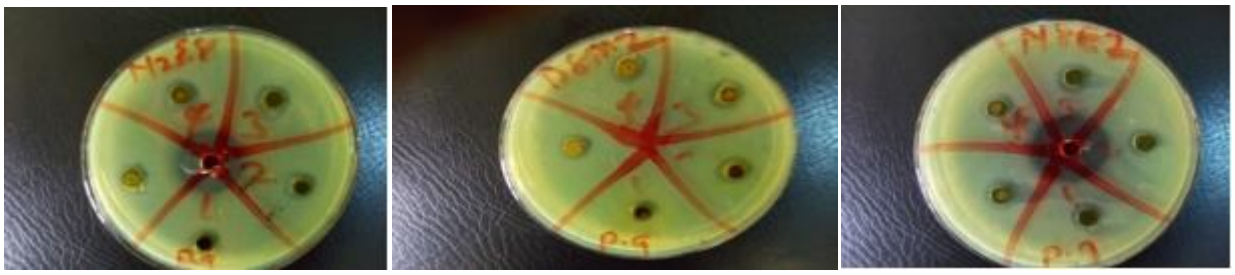
Test organisms	Concentration (mg/mL) / IZD (mm)					
	100	50	25	12.5	6.25	Cipro 5 µg/mL
S. aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
E. coli	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
P. areuginosa	7 ± 0	6 ± 0	6 ± 0	6 ± 0	5 ± 0	18
C. albicans	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Miconazole 50 µg/mL 27

Table 6: *In vitro* antimicrobial activity of DCM: Methanol (80:20) of *A. djalonensis*

Test organisms	Concentration (mg/mL) / IZD (mm)					
	100	50	25	12.5	6.25	Cipro 5 µg/mL
S. aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
E. coli	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
P. aeruginosa	6 ± 0	5 ± 0	4 ± 0	2 ± 0	2 ± 0	18
C. albicans	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Miconazole 50 µg/mL 27

Table 7: *In vitro* antimicrobial activity of DCM: Methanol (20:80) of *A. djalonensis*

Test organisms	Concentration (mg/mL) / IZD (mm)					
	100	50	25	12.5	6.25	Cipro 5 µg/Ml
S. aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
E. coli	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
P. aeruginosa	2 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18
C. albicans	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Miconazole 50 µg/mL 27



A. B. C.

Figure 2: A= n-Hexane: Ethyl acetate (80:20) against *P. aeruginosa*; **B**=n-Hexane: Ethyl acetate (20:80) against *P. aeruginosa*; **C**=DCM: Methanol (80:20) against *P. Aeruginosa*

HPLC Chromatogram

The analytical HPLC suggested the presence of some secondary metabolites. Septicine, vanillin, 3-methyl-2, 3, 4-pentanetriol, indol-3-carboxylic acid and cerebroside in n-Hexane: Ethyl acetate (80:20) fraction; septicine, isovitexin, bromhexylamide and cerebroside in DCM: Methanol (80:20) fraction; and isovitexin in DCM: Methanol (20:80) fraction.

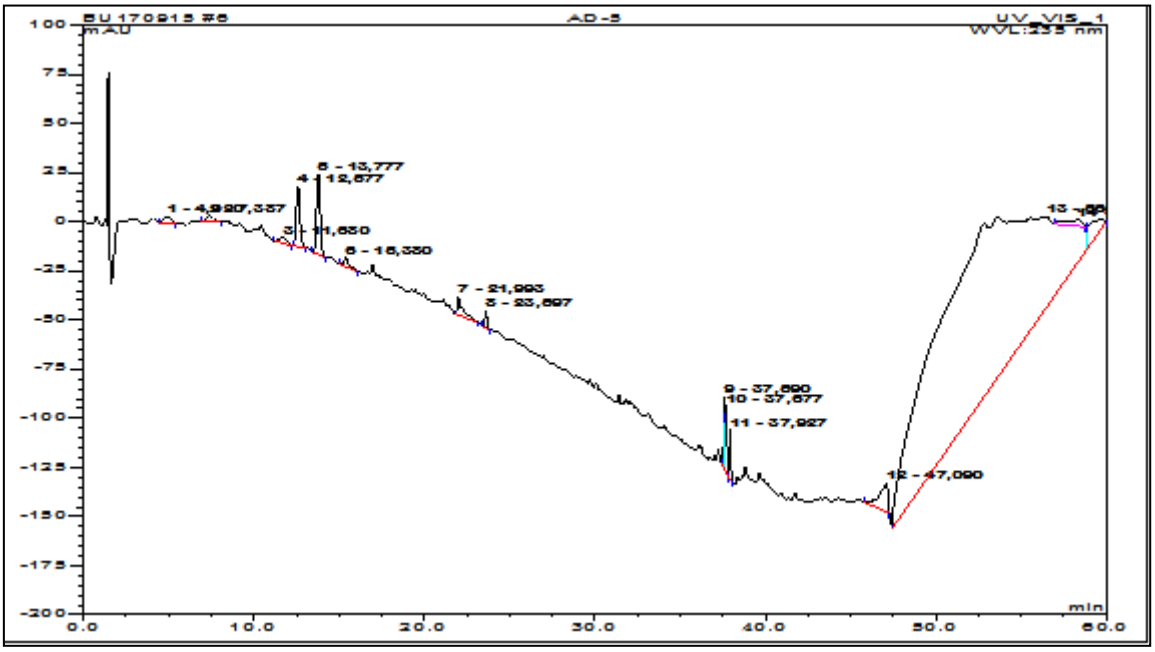


Figure 3: HPLC Chromatogram of n-Hexane: Ethyl acetate (80:20) VLC Sub-fraction

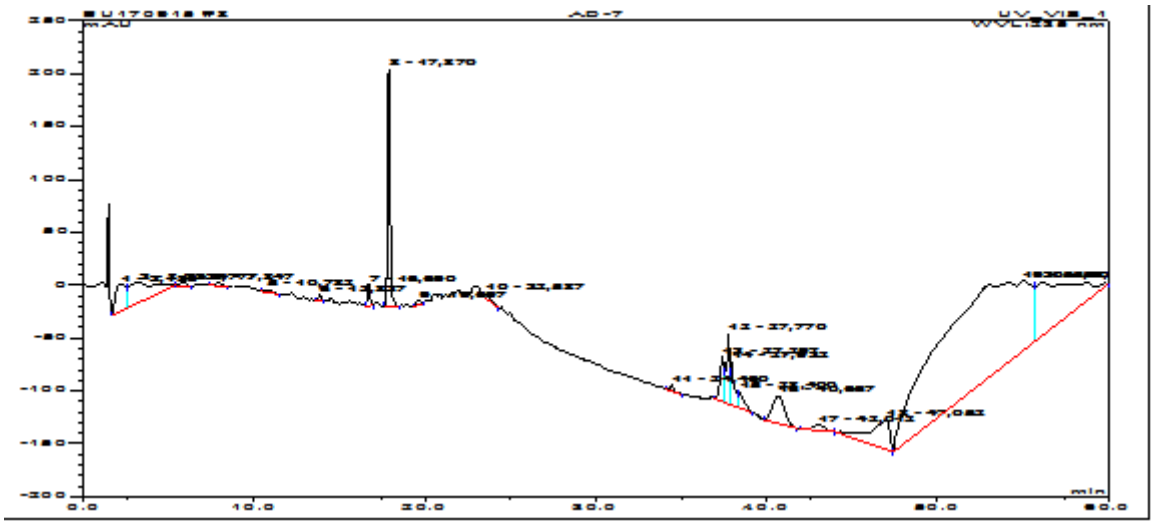


Figure 4: HPLC Chromatogram of Dichloromethane: Methanol (80:20) VLC Sub-fraction

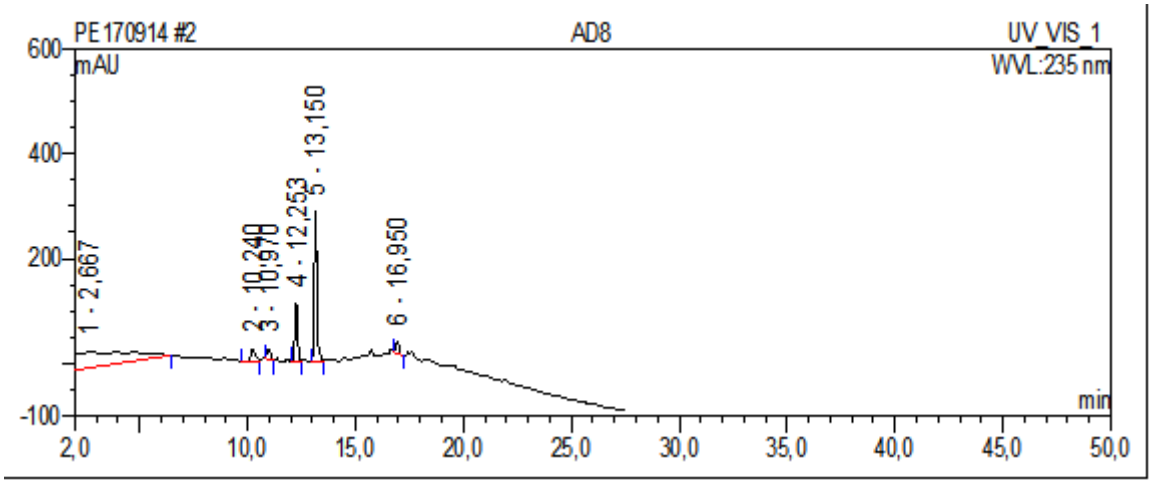


Figure 5: HPLC Chromatogram of Dichloromethane: Methanol (20:80) VLC Sub-fraction

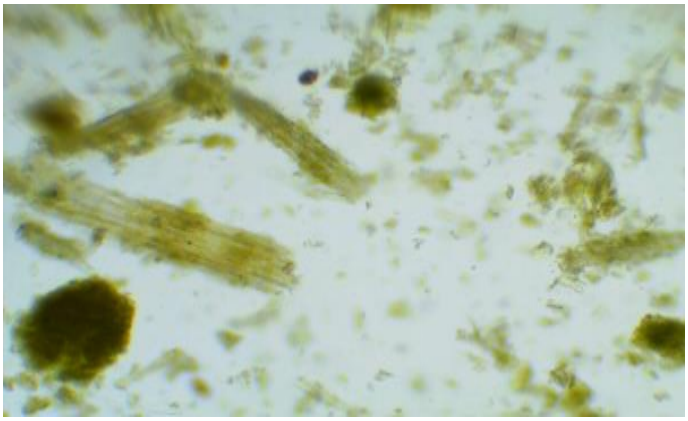


Figure 5: Microscopic analysis of powdered leaves of *Anthocleista djalonensis*

DISCUSSION

According to the Clinical and Laboratory Standard Institute (2015), standard drugs with an inhibition zone diameter (IZD) of ≥ 20 are sensitive; 15-19 are intermediate, and ≤ 14 are resistant [28]. From the results obtained from the *in-vitro* evaluation of the antimicrobial activity of the different VLC sub-fractions of the ethyl acetate fraction, it was seen that at a concentration of 100 mg/ml, the various fractions exhibited inhibition of *Pseudomonas aeruginosa* only and there was no zone of inhibiting seen in *Staphylococcus aureus* and *Escherichia coli*. The highest zone of inhibition seen on *Pseudomonas aeruginosa* was observed in the n-hexane: ethyl acetate (20:80) fraction, while the lowest zone of inhibition seen on *Pseudomonas aeruginosa* was observed in DCM: methanol (20:80) fraction at 100mg/ml. Ciprofloxacin, the antibacterial agent has a higher inhibitory zone diameter on the test bacteria than the plant extract. Miconazole, the antifungal agent showed a higher inhibitory zone diameter against all the fungi showing that the fungi organism used is viable, but the VLC fractions does not have any activity against it. The VLC sub-fractions showed no activity against the tested fungi (*Candida albicans*). The VLC sub-fractions of the ethyl acetate fraction of the methanol extract of *Anthocleista djalonensis* leaves have very little antimicrobial activity, compared to what has been said about its root extracts, which even has activity against some mycobacterium [29,30].

The result of the phytochemical analysis of the methanol leaf extract of *Anthocleista djalonensis* showed the presence of alkaloids, flavonoids, tannins, cardiac glycosides, proteins, saponins, reducing sugars and steroids. The presence of these constituents can be linked to the beneficial effects of the plants. The phytochemical study of *Anthocleista djalonensis* has also shown that the antimicrobial property of this plant could depend on certain active constituents especially alkaloids, saponins, tannins, and flavonoids. Flavonoids, saponins, and alkaloids were present and are an indication that they can be used to treat diarrhoea and dysentery. Flavonoids improve the exchange of nutrients and oxygen between the blood and the tissues in human and animals by reinforcing capillary walls [31, 32]. Tannins which are metal chelator and weak organic acidic groups are known to have anti-inflammatory and antibacterial properties [33, 34]. Alkaloids are used to treat gastritis, pancreatitis and chronic urethritis and used for their action in circulation in cases of the collapse of pneumonia [35].

The justification of phytochemical screening is determined by the colour, precipitate, interfacial film, and frothing formation. Tannins contain phenolic hydroxyl groups which are reduced by reducing agents, such as ferric chloride solution in a blue-black, brown or red precipitate formation. Reducing sugars produce a deep blue or green coloration on the addition of Fehling's reagent to copper (11) oxide, for example rhamnose, while non-reducing sugars do not convert copper sulphate to copper (11) oxide. Foam formation in saponin is based on the emulsion test. Glycosides in the presence of mineral acids undergo hydrolysis into sugars and glycones. Steroids contain keto functional groups which are reduced by sulphuric acid in the Salkowski reaction [34].

The result of the physicochemical properties showed the percentage of total ash value of the powdered plant as 4.55%, which is low. This shows that the plant has a high percentage of volatile constituents and phytochemicals that evaporated on heating. This means that the plant sample contains a low percentage by mass of mineral components. The water-soluble extractive value plays a very dominant role in the evaluation of crude/ herbal drugs. Extractive values indicate the addition of exhausted material, and adulteration which can result in incorrect processing during drying, storage, and use. The percentage water-soluble extractive value of the plant sample was found to be 1.02% while the percentage of acid-insoluble and water-soluble ash were 6.80% and 2.65% respectively. The percentage loss on drying was also found to be 7.90%. The moisture content of 8%/g of herbal preparations is satisfactory according to National Agency for Food and Drug Administration and Control [36], suggesting that high moisture contents enhance the growth of pathogenic bacteria as well as non-pathogenic ones in herbal preparations while the low moisture contents can attribute to low bacterial growth. These data can provide valuable information on the standardization and evaluation of the plant *Anthocleista djalonensis.*

The analytical HPLC suggested the presence of some secondary metabolites. Septicine, vanillin, 3-methyl-2, 3, 4-pentanetriol, indol-3-carboxylic acid and cerebroside in n-Hexane: Ethyl acetate (80:20) fraction; septicine, isovitexin, bromhexylamide and cerebroside in DCM: Methanol (80:20) fraction; and isovitexin in DCM: Methanol (20:80) fraction. Septicine is an alkaloid (indolizidine) and a cytotoxic agent [37,38,39]. Vanillin is a phenolic aldehyde and a flavouring agent. Acetoside is a glycoside and has immunosuppressive, antineoplastic, chelating, antiinfective, antioxidant and antimicrobial activities [28, 33, 34] . Indol-3-carboxylic acid has been found in patients with Liver diseases and it is also a urinary indolic tryptophan metabolite [34]. Cerebroside is the common name for a group of glycosphingolipids called monoglycosylceramides which are important components in animal muscle and nerve cell membranes [34]. Isovitexin is a flavone and has antioxidant property [34].

Plant extracts with high antioxidant activity may show a significant antimicrobial activity. These properties such as plant composition, survival within the biological system, transport properties, compound structures, affinity for the target site, and state of the target organism determines its biological activity [40]. Only aceteoside and Septicine had reported antimicrobial activity out of all the compounds present, which may have contributed to the little antimicrobial activity of the sub-fractions.

CONCLUSION

This study showed that the VLC sub-fractions of ethyl acetate fraction of the methanol extract of *Anthocleista djalonensis* leaves have very little or no antimicrobial activity, showing antibacterial activity against *Pseudomonas aeruginosa* only, no antifungal activity against *Candida albicans*. The results of this study suggest that the sub-fractions be further purified and tested for possible antibacterial potential.

APPENDIX

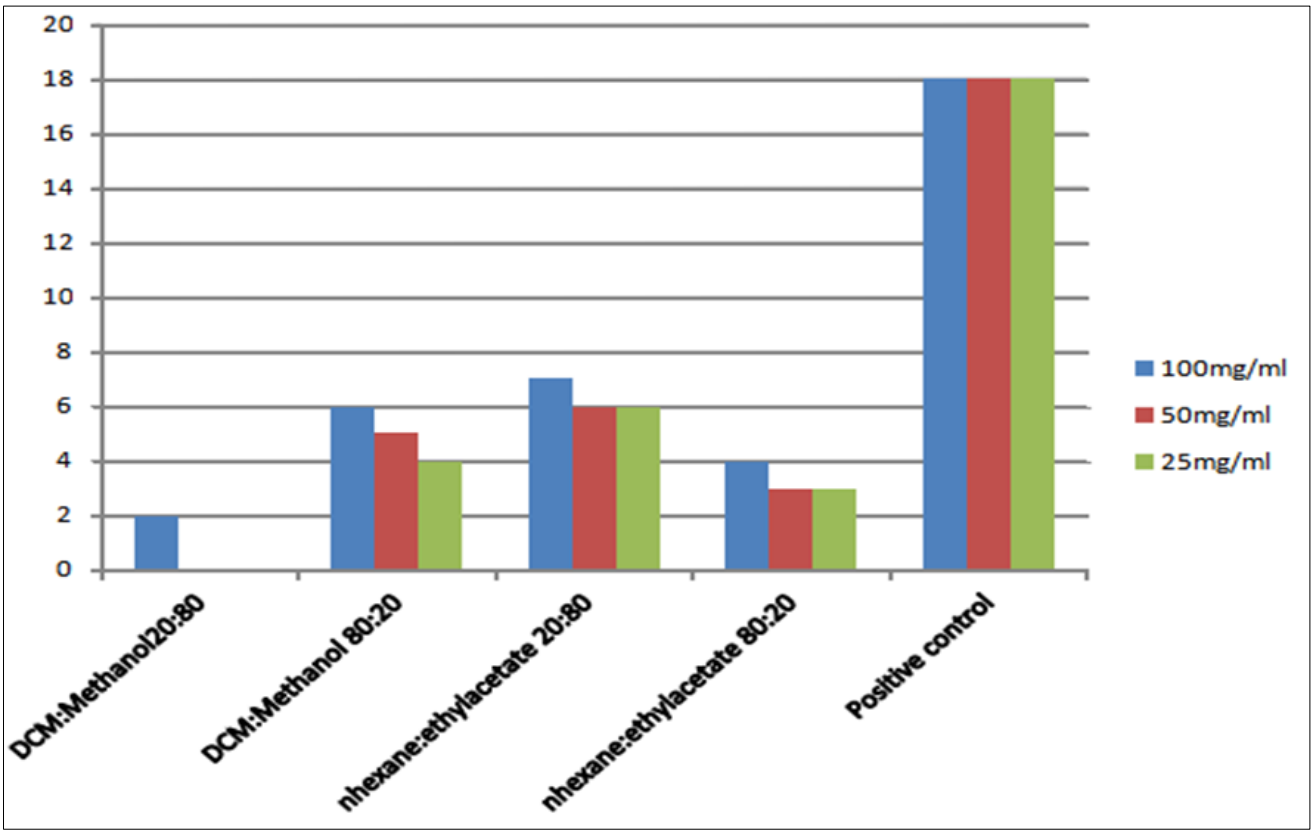


Figure 6: A chart of in hibition zone diameter against concetration on Pseudomonas aeruginosa

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