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Isolation and Partial Characterization of the Most Bioactive Metabolite from the Hexane Extract of the Aerial Part of *Hydrocotyle Verticillata* (WHORLED MARSHPENNYWORTH)

Nathalie L. Daminar [α] & Lydia M. Bajo [σ]

Abstract - Several of thousands of bioactive compounds have been mined from the natural product resources both from the marine and terrestrial environment all over the world. Natural product has proved potentiality of many plants for diverse clinical conditions. Many plants were found to contain compounds, the bioactive components which are used in natural medicine. In this study, the plant of interest is *Hydrocotyle verticillata*, mistakenly identified to be "Gotu ko la" (*Centella asiatica*) by local folks of Iligan City and Butuan City, Philippines. The natural components of the aerial part of *H. verticillata* were extracted using solvent extraction. The crude ethanolic extract of the aerial part of *Hydrocotyle verticillata* was further subjected to solvent partitioning according to increasing polarity: n-hexane, ethyl acetate (EtOAc), dichloromethane (DCM) and water. Bioassays like Brine Shrimp Lethality Test was then conducted to test which of the extracts consist of bioactive components. Among the four partitioned solvent extracts, result showed that n – hexane extract showed to be the most bioactive since it has the lowest LC_{50} .

Fractionation and purification of the hexane extract was done through series of Gravity Column Chromatography (GCC). Four isolates (HF5B1, HF5B2, HF5B3 and HF5B4) were obtained. Analysis using High Performance Liquid Chromatography (normal phase HPLC) showed three of the isolates (HF5B2, HF5B3 and HF5B4) to have similar profile, two peaks of closely similar retention time. HF5B2 was further purified in HPLC and yielded a subfraction HF5B2a with a single sharp peak on the HPLC chromatogram and with a retention time of 8.135 min.

This subfraction was partially characterized using Perkin Elmer FTIR Spectrometer. The spectrum showed peaks for the following functional groups: NH, C=O, C-C(O)-C, and CN stretch (aliphatic amine). Considering the presence of these functional groups particularly the amine, the isolated metabolite HF5B2A from hexane extract could be an alkaloid derivative. Alkaloids containing basic nitrogenic compounds were known as DNA binders.

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I. Introduction

Medicinal plants are regularly used to treat ailments because of minimal side effect and cost effectiveness. The potential for developing antimicrobial, high toxicity property from higher plants appears rewarding as it may lead to the development of phytomedicine. Hence, there is a worldwide interest to isolate biologically active compounds from higher plant species that are possible sources of medicine. The bioactive components of these plants are great sources for new therapeutic agents. These therapeutic agents are of great importance nowadays because these pose new hope for disease prevention.

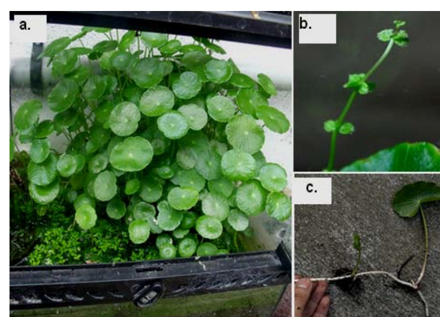

Figure 1 : *Hydrocotyle verticillata* plant shows its (b) flower and (c) roots

The plant of interest in this study is the *Hydrocotyle verticillata* (Figure 1) which has been mistakenly identified to be "Gotu kola" (*Centella asiatica*) by local folks of Iligan City and Butuan City. *Hydrocotyle verticillata* is one of the 100 species of genus hydrocotyle.[1] This is a flowering plant found in South and North America, West Indies and Bangladesh.[2] People claim that *H. verticillata* can effectively treat inflammation, anemia, asthma, blood disorders, bronchitis, fever, urinary discharge and splenomegaly associated to *C. asiatica*.[3]

Toxicity test using Brine Shrimp Lethality Test was performed to obtain the bioactive components of

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this plant, *H. verticillata* which is great source for new therapeutic agents. To isolate the most bioactive metabolite different chromatographic techniques were used in this study such as gravity column chromatography (GCC), thin-layer chromatography (TLC), and high performance liquid chromatography (HPLC). The most pure isolate then was partially characterized using the Perkin Elmer Fourier Transform Infrared Spectrometer.

a) *Objective of the Study*

The primary aim of this study is to isolate the bioactive metabolites from the plant sample *Hydrocotyle verticillata* and conduct phytochemical screening on the most bioactive extract.

This attempts to achieve the following specific objectives:

1. to extract the bioactive components possibly present in the aerial part of the plant sample *H. verticillata* using hexane, ethyl acetate, dichloromethane and distilled water;
2. to determine cytotoxicity of the sample using brine shrimp lethality test on the extracts;
3. to further fractionate the most bioactive crude extract of the plant sam H. verticillata by Thin Layer Chromatography (TLC), Gravity Column Chromatography (GCC), High Performance Chromatography (HPLC);
4. to partially characterize the purified extract by Fourier Transform Infrared (FTIR) Spectroscopy

II. Materials and Methods

a) *Chemicals and Reagents*

The chemicals used in this study were: 95% ethanol (EtOH), hexane (Hex), ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH), chloroform ($CHCl_3$). All chemicals were purchased locally and signed out from Chemistry stockroom of Mindanao State University – Iligan Institute of Technology (MSU-IIT), Philippines.

b) *Test Organisms*

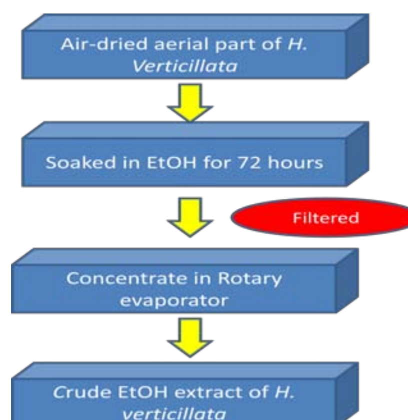
The brine shrimp, *Artemia salina* Leach were used as test organism in Brine Shrimp Lethality Test (BSLT). These test organisms were purchased as eggs in the local pet shop.

c) *Plant Material*

Fresh samples of aerial part of *Hydrocotyle verticillata* were collected by hand from a local area of Iligan City, Philippines. The samples were washed with water several times to remove dirt and other contaminants. These aerial parts were then air-dried for about three weeks.

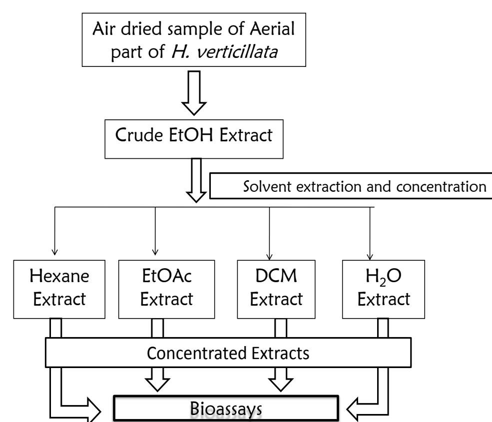
d) *Extraction*

The air – dried aerial part of the plant sample *H. verticillata* were ground using mechanical grinder. Six hundred sixty-eight grams of the ground sample were extracted with 95% ethanol for 72 hours. The resulting mixture was filtered and the solvent removed by rotary evaporator at 40°C to yield the crude ethanolic extract as shown in Scheme 1.



Scheme 1 : Preparation of Crude Extract

The crude ethanolic extract was separated by solvent partitioning according to increasing polarity: n-hexane, EtOAc, DCM and water to obtain four extracts (Scheme 2). Hex, EtOAc and DCM extracts were dried under a *vacuo* while the aqueous extract was freeze-dried. The concentrated extracts were subjected to Brine Shrimp Lethality Test (BSLT). The extracts with the highest toxicity or lowest LC_{50} were considered as bioactive extracts.



Scheme 2 : Schematic Diagram for Solvent Extraction

e) *Fractionation and Purification*

The crude hexane extracts (H) having the highest bioactivity was subjected to gravity column chromatography (GCC) using silica gel (silica gel 60, mesh 70 – 230) as adsorbent. The column used in this study was packed using the slurry method which has an

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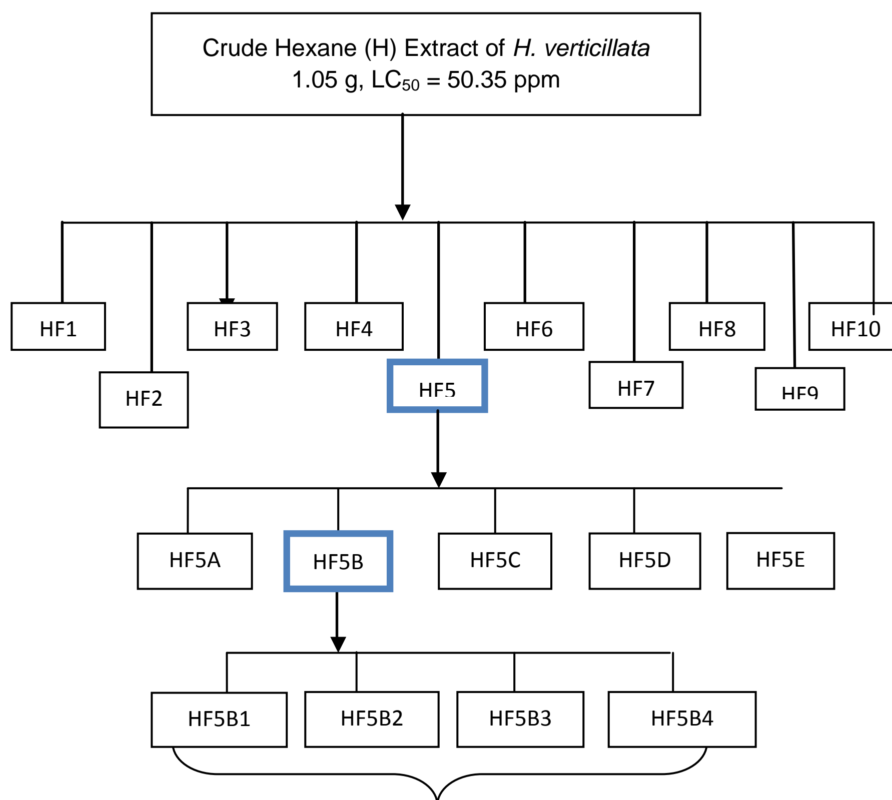
internal diameter of 25 mm and a length of 350 mm. A gradient step elution was required to elute the compounds from the column and was previously equilibrated with pure hexane. Hexane and ethyl acetate were first used as eluant . Ethyl acetate in methanol was next to be used as an eluant starting with 5-100% EtOAc in MeOH (increment of 5% in each step). The fractions obtained were subjected to Thin Layer Chromatography using Fluka TLC plate. Detection of the spots was done using UV lamp (short and long wave) and iodine vapour. Fractions with the same TLC profile were pooled together as one fraction. There were 10 fractions obtained from the first column chromatographic separation. All of these fractions were subjected to BSLT at LC_{50} of 50 ppm and antimicrobial assay to obtain the highest bioactivity. Fraction 5 (HF5) was selected for further purification because of its highest toxicity among the fractions (Scheme 3).

Second GCC was performed for further purification of HF5 using a column with smaller internal diameter of 15mm. EtOAc in Hex was used as eluting solvent starting with 35 mL of 10%, 20%, 25%, 30% of the mobile phase followed by 70% - 30% of EtOAc in Hex (increment of 2% in each step). There were five pooled fractions obtained. Pooling was done based on the result of TLC.

From among the five fractions obtained in the second GCC, the second was further purified (HF5B) for third gravity column chromatography since it has the greatest weight and highest toxicity. The column used in the third GCC had the smallest diameter of 7 mm. Four pooled fractions using 20% - 40% of EtOAc in Hex (increment of 1% in each step) as mobile phase.

The profile of each of the four fractions (HF5B1, HF5B2, HF5B3, HF5B4) was examined from the resulting peaks produced by high performance liquid chromatography (normal phase HPLC) at the Chemistry Department, Xavier University, Ateneo de Cagayan. Twenty microliters of each fraction was injected to the HPLC (Perkin Elmer) at 254 nm using a wakopak wakosil column (normal phase) with a flow rate of 1 mL per minute as stationary phase, ethyl acetate as mobile phase, and UV detector (254 nm).

Among four fractions were subjected to HPLC HF5B2, HF5B3, HF5B4 showed similar retention time. The three fractions had similar HPLC profile, thus, they had similar composition and bioactivity. HF5B2 was chosen to be further purified to represent the entire fraction by means of HPLC instrument to yield a subfraction isolate with a single sharp peak in its chromatogram (Scheme 4). Collection of the subfraction from the HPLC was guided by the appearance of the chromatogram on the computer screen. This was collected in vial and labeled as HF5B2a and partially characterized using FTIR.



Scheme 3 : Purification of Crude Hexane Extract

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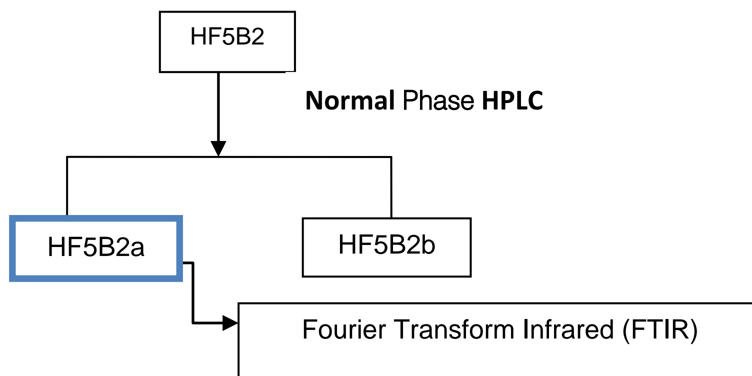
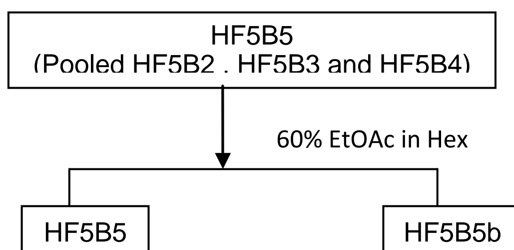
Moreover, melting point determination was then conducted to purified isolate HF5B5a corresponds to HPLC isolate (HF5B2a which was partially characterized using FTIR) to determine its property. Melting point determination was done using Fisher John Melting Point Apparatus



Scheme 4 : HPLC Purification of fraction HF5B2



Scheme 5 : TLC Purification of HF5B5 (Pooled HF5B2, HF5B3 and HF5B4 Fractions)

f) Partial Caharacterization

The HPLC subfraction labeled HF5B2a was analyzed using a Perkin Elmer FTIR spectrometer. The IR spectrum produced was examined to identify the functional groups present in the compound. This was done by making a portion of the liquid sample using KBR window, spacer of 0.015 mm and loading it into the FTIR instrument thru its injection port. Analysis of the sample was done at the Analytical Laboratory Services of Mindanao University of Science and Technology, Philippines.

III. Results and Discussion

a) Solvent Extraction

From the 668 g of air dried sample, 99.765 g of ethanolic crude extract was obtained. The crude ethanolic extract was separated by solvent partitioning according to increasing polarity and four solvent extracts were achieved. These were hexane (Hex), ethyl acetate (EtOAc), dichloromethane (DCM) and aqueous (Aq) extracts. Table 1 shows the consistency and appearance of each extract. Hex and EtOAc extracts were observed to be dark green and sticky. DCM was light green and has oily consistency while the aqueous was light orange and solid.

Table 1 : The Appearance and Consistency of the Solvent Extracts from the Aerial Part of Plant Sample *H. verticillata*

Organic Extracts	Appearance	Consistency	Weight (grams)
Hex	Dark green	sticky	28.4594
EtOAc	Dark green	sticky	1.6624
DCM	Light green	oily	12.5324
Aq	Light orange	solid	19.7625

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The colored appearance observed for each extract was due to the presence of pigment found in plants, example chlorophyll. The great abundance of chlorophyll in leaves and its occasional presence in other plant tissues, such as stems, caused these plant parts to appear green. In addition to chlorophyll, leaf cells also may contain other pigments such as yellow *xanthophylls,* yellowish-orange carotenes, and red and purple *anthocyanins.* Leaves also may contain which give them a golden-yellow color.[4]

b) Brine Shrimp Lethality Test

The toxicity of all crude extracts was evaluated by brine shrimp lethality test.[5] Toxicities of test extracts were tested at 10, 100, 1000 ppm and LC_{50} value was determined from the 24-hour death counting using probit analysis. The results of the assay are presented in Table 2.

Table 2 : Brine Shrimp Lethality Test Results of the Crude Extracts from Aerial Part of *H. verticillata*

Extraction Solvent	LC_{50} values (ppm) from the different crude extracts
EtOH	94.34
Hexane	50.35
EtOAc	76.98
DCM	223.87
Aq	377.45

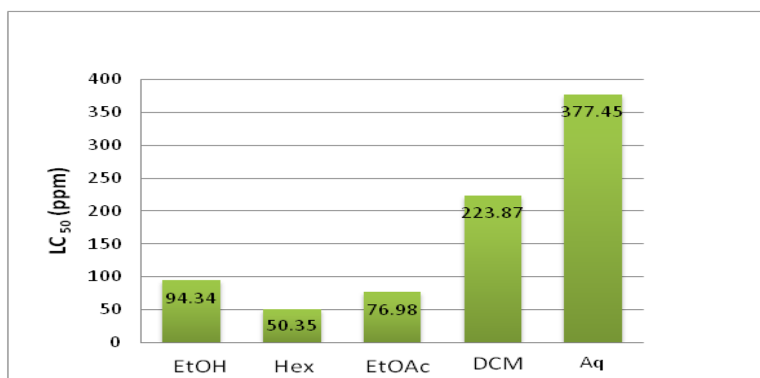

Figure 2 : Graphical Comparison of LC_{50} Values of Crude Extracts

Crude extracts of EtOH, Hex and EtOAc gave lower LC_{50} compared to DCM and the Aq extracts as shown clearly in Figure 2. But the hexane extract gave the lowest LC_{50} value of 50.35 ppm thus, it is the most active. Further- more, the bioassay indicates that the aqueous extract showed very little toxicity at LC_{50} value of 377.45 ppm.

c) Bioassay-Guided Fractionation of Hexane Crude Extracts

The most bioactive extract, the crude hexane extract which gave the lowest LC_{50} value was subjected to further purification and partial characterization.

Table 3 : The Appearance and Weight of Pooled Fractions Obtained from First GCC

Crude hex extract pooled fractions	Color	Weight (mg)
HF1 HF2 HF3	Colorless Orange Yellow	31.1 202.13 30.7
HF4	Dark green	119
HF5	Dark green	79.9
HF6	Dark reddish brown	49.6
HF7	Dark green	41.4

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HF8	Dark green	44.3
HF9	Yellow	79.8
HF10	Pale yellow	72.4

- *bold most bioactive fraction and further purified*

There were 10 pooled fractions (Table 3) obtained from the first column chromatographic separation of the crude hexane extract. All of these were subjected to BSLT at LC_{50} of 50 ppm. Results are tabulated in Table 4.

Table 4 : Percent mortality of 10 Pooled Fractions (Crude Hex Extract)

Fraction	Mean % mortality±SD	*Corrected mean % mortality at 50 ppm
HF1	3.33 ± 0.58	0.00
HF2	6.67 ± 1.15	3.45
HF3	6.67 ± 0.58	3.45
HF4	3.33 ± 0.58	0.00
HF5	80.00 ± 1.00	79.31
HF6	23.33 ± 2.08	20.69
HF7	16.67 ± 2.08	13.80
HF8	6.67 ± 0.58	3.45
HF9	13.33 ± 1.15	10.35
HF10	13.33 ± 1.15	10.35
Blank	3.33 ± 0.58	

** calculated using Schneider-Orelli's Formula*

Out of the 10 fractions only fraction 5 (HF5) showed the highest toxicity at 79.31 % mortality rate at 50 ppm. HF5 was further purified and subjected to second GCC. There were five pooled fractions obtained from the second gravity column chrom- atography. Pooling was done based on the result of TLC. Solvent EtOAc in Hex was used as eluant starting with 35mL of 10%, 20%, 25%, 30% of the mobile phase followed by 70% - 30% of EtOAc in Hex (increment of 2% in each step). To test for toxicity, BSLT assay was again done to all of these fractions using LC_{50} of 50ppm.

The results of the five fractions obtained from second GCC and their weight in milligrams (mg) are shown in Table 5. Percent mortality of these pooled fractions is also tabulated on Table 6.

Table 5 : The Appearance and Weight of Pooled Fractions Obtained from Second GCC

Crudehexextract pooled fractions	Color	Weight (mg)
HF5A	Colorless	10.5
HF5B	Dark green	30.6
HF5C	Dark green	10.2
HF5D	Dark green	11.8
HF5E	Yellow	9.98

Table 6 : Percent Mortality of 5 Pooled Fractions

Fraction #	Mean % mortality at 50 ppm ± SD
HF5A	23.33 ± 0.58
HF5B	83.33 ± 1.15
HF5C	50.00 ± 1.00
HF5D	33.33 ± 1.53
HF5E	10.00 ± 1.00
Blank	0

Results reveal that HF5B has the highest mortality rate of 83.33%; coupled to its greater weight among the five fractions, it was further purified and subjected to third GCC using the smaller diameter of a pipette serving as the column. This time 20% - 40% of EtOAc in Hex (increment of 1% in each step) was used as the eluting solvent. The results revealed four pooled fractions which are presented in Table 7. Due to the very small quantity of the samples collected in the third GCC, weights were not measured.

Again, all of these fractions were subjected to TLC detection of the shade of spots. It was done under the UV lamp (254 nm and 365nm). The results revealed one spot for each fraction.

The profile of each four fractions (HF5B1, HF5B2, HF5B3, HF5B4) was examined also through the resulting peaks obtained using high performance liquid chromatography (normal phase HPLC at 254 nm) employing wakosil wakopak column with a flow rate of 1mL/minute.

Table 7 : HPLC Results of the Pooled Fractions in the Third GCC

Isolate	#of prominent peaks	Retention time for the prominent peaks (respectively)
HF5B1	2	8.130 , 8.417
HF5B2	2	8.400 , 9.122
HF5B3	2	8.450 , 9.165
HF5B4	2	8.257 , 9.003

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HPLC results showed that HF5B1 had two shouldered peaks. For fractions HF5B2, HF5B3, HF5B4 two peaks (Appendix E. Fig.,10,11,12,) were also observed which interestingly showed closely similar retention times (Table 7). Hence, the molecules were eluted nearly all at the same time. Moreover, the appearance of first eluted peak was long and followed by a shorter peak. These results suggested that all the fractions were of similar composition and bioactivity.

Among four fractions, HF5B2, HF5B3 and HF5B4 were decided to be chosen to be further purified. This does not suggest that HF5B1 did not possess biological activity. However, due to time constraints and large number of variables that could be explored when using solvent system, thus, investigation would be focused to the three fractions mentioned above. As had been discussed similar HPLC profiles of HF5B2, HF5B3 and HF5B4 suggested that all the fractions were of similar composition and bioactivity. The fraction H5B2 was chosen to be subjected to normal phase HPLC for further purification since it represents already the entire fraction. Moreover, the two peaks of HF5B2 were both sharp which indicates that the components of this fraction were much more concentrated. Purification was done using normal phase HPLC instrument to yield a subfraction, HF5B2a with a sin- gle sharp peak on its chromatogram and with a retention time of 8.135 min. Collection of the subfraction from the HPLC was guided by the appearance of the chromatogram on the computer screen. The HPLC isolate was collected in a vial and was partially characterized using FTIR to analyze the functional groups present in this sample.

Melting point determination was then conducted to purified TLC isolate HF5B5a which corresponds to HPLC isolate HF5B2a (the first eluted peak done by HPLC and this was partially characterized using FTIR). The melting point of the isolate obtained 102-103^{0}C. It was determined by using Fisher-Johns Melting Apparatus. The short range of melting point implies its possibility of being pure.

d) Partial Characterization

IR spectrum of subfraction HF5B2a is shown in Figure 3. Absorption peaks and the possible functional groups present are tabulated below in Table 8.

Table 8 : IR Absorption Peaks for HF5B2a and Inferences[6]

Wavenumber, cm^{-1}	Inferences
3464.15 2994.38	NH secondary stretch C-H stretch
1762.44 , 1722.93	C=O stretch
1466.10	CH_2 bend
1447.66, 1382.69	CH_3 bend
1276.76	C-C(O)-C stretch
1213.41	CN stretch, aliphatic
1098.55,1062.07, 1034.99	C-C stretch
938.54	Out of plane CH bend
786.92	NH secondary wag

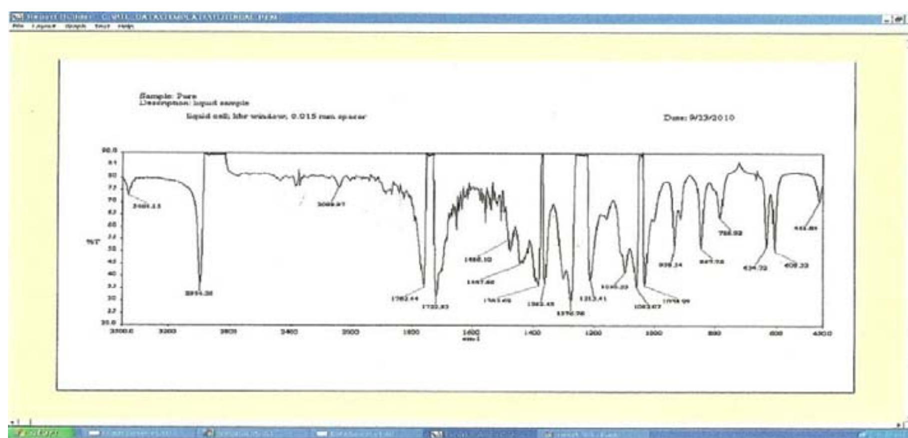

Figure 3 : FTIR Spectrum of HF5B2a

IV. Conclusion

Partial characterization of HF5B2a the most purified bioactive component of the Hexane Extract of *H. verticillata* was done using the Perkin Elmer FTIR. The following are the possible functional groups present that was deduced from the IR spectrum: NH secondary stretch, C=O stretch, CH_2 and CH_3 bend, C-C(O)-C stretch, CN stretch (secondary aliphatic amine), out of plane CH bend and NH wag. Considering the presence of these functional groups particularly the amine, the isolated metabolite HF5B2a from hexane extract could be an alkaloid derivative since alkaloid is composed of amines or nitrogen-containing compound. Alkaloid which was a DNA binder, play an important role in cancer therapy.[7]

V. Recommendations

1. HF5B2a will be subjected to reverse-phase HPLC
2. Frractions HF5B1, HF3B2 and HF5B3 must be further purified and the results will be tested for DNA-binding property

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3. HF5B2a must be further recovered to characterize and eventually elucidate their structures that may also have a pharmacological significance.
4. Subfraction HF5B2a be further characterized by EIMS, 1D and 2D-NMR, H-NMR, ^{13}C-NMR and various correlations by ^{1}H-^{1}H HMQC and HMBC.

Acknowledgement

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