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Isolation, Bioactivity and Charaterisation of 3-Ethynyl-5-(2,3-dehydropyrrole) Pyridine from the Stem Bark of *Adenanthera pavonina*

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Authors' contributions

This work was carried out in collaboration between both authors. Author KA designed and supervised the study. While, author MA carried out the laboratory works and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Natural products from medicinal plants either as pure compounds or standardized extracts, provide unlimited opportunities for new drug leads, because of the unmatched availability of chemical diversity. Due to an increasing demand for therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. The phytochemical screening was carried out via standard procedures while the isolation and characterization was done using different solvents via thin layer and column chromatography. The bioactivity studies of the purely active compound isolated was achieved using different clinical bacterial isolates, gram negative (*E. coli* and *Salmonella typhi*) and positive (*Staphylococcus aureus*); the radical scavenging power of the purely active compound was assayed using 2,2-diphenyl-1-picrylhydrazyl(DPPH) and characterization using GCMS, ^{1}HNMR, ^{13}CNMR and FTIR was carried out to facilitate structure elucidation. The focus of this paper is on the analytical and biological methodologies, which includes the extraction, isolation, bioactivity studies, and characterization of the purely active ingredients in the stem bark of *Adenanthera pavonina*.

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1. INTRODUCTION

Natural products from medicinal plants, either as pure compounds or standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity [1]. Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purposes, including treatment of infectious diseases [2]. The phytochemical research based on ethnopharmacology is considered an effective approach in the discovery of novel chemicals entities with potential as drug leads. Plant extracts/decoctions, used by folklore traditions for treating several diseases, represent a source of chemical entities but no information are available on their nature [3].

Adenanthera pavonina is a plant naturally grown in the southern part of Borno, Nigeria and is well known to Babur/Bura people by a local name 'Ghrini'. It is a medium-sized to large deciduous tree about 8-14 m tall and 40-45 cm thick, depending on location. Generally, the plant is erect with a dark brown to greyish bark and a soft-pale brown inner bark. It bears leaves, flowers and seeds. The leaves are bi-pinnate in nature with 8-21 leaflets on short stalks, which turns yellow with age.

Considerable work has been done to explore the biological activity and medicinal applications of the plant, still there are available countless possibilities of pharmacological applications which needs to be explored [4].

The aim of the research was to carry out bioactive guided chromatographic isolation and purification and to further characterized the pure active compound(s) from the stem bark of *Adenanthera pavonina* using different spectroscopic technics.

2. MATERIALS AND METHODS

2.1 Sample Collection and Authentication

The fresh stem bark sample of *Adenanthera pavonina* was collected at Kwaya Bura Village, Hawul Local Government Area, Borno State, Nigeria. It was identified and authenticated at the Department of Plant Biology, Bayero University, Kano, Nigeria, herbarium accession No. 0493.

2.2 Sample Preparation and Extraction

The stem-bark sample of *Adenanthera pavonina* was cut into smaller pieces and air dried under shade at ambient temperature for three weeks. It was pulverized mechanically (using mortar and pestle) to form a coarse powder. After pulverization the powdered sample was stored in an air tight container, in cool and dry place away from light and was later subjected to ethanol extraction [5].

About 500 g of the ground powdered sample was percolated with 1.5 L of absolute ethanol with shaking at regular intervals for one week, after which the extract was separated from the debris by filtration. The filtrate was then concentrated using a rota vapour (R110 at 40ºC) and was coded AP_1 (ethanol crude extract), weighed and kept in a cool dry place away from any form of contaminant [6].

The crude ethanol extract (AP_1) was macerated with n-Hexane, Chloroform and ethyl acetate to afford n-Hexane fraction (AP_2), Chloroform fraction (AP_3) and ethyl acetate fraction (AP_4) respectively.

2.3 Phytochemical Screening

The phytochemical screening of alkaloid, flavonoid, glycoside, phenol, protein, saponin, steroid, terpenoid, and tannin for all the four fractions were carried out according to standard protocols [7,8].

2.4 Biological Assay

2.4.1 Test organisms

Clinical bacterial isolates including *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* as well as three fungal isolates like: *Aspagillus flavus*, *Candida albican* and *Mucor specie* obtained from Department of Microbiology, Bayero University, Kano, were used for the study.

2.4.2 Preparation of stock solution and other concentrations

The stock solutions of the extracts (AP_1, AP_2, AP_3 and AP_4) were prepared by dissolving 60 mg of each extract in 1 mL of dimethyl sulphuroxide (DMSO) to produce 60 mg/mL. Lower concentrations of 30 and 15 mg/mL was obtained

from the stock solution by serial double dilution. The concentrations of isolated pure compound (AP-44) was also prepared in the same manner.

2.5 Antimicrobial Screening for the Extracts

Antibacterial and antifungal activity was screened by agar well diffusion method [9]. Nutrient agar (NA) and Potatoes dextrose agar (PDA) plates were swabbed with eight-hours-old broth culture of bacteria and fungi using sterile cotton swab respectively. On each sensitivity disc, three wells (6 mm) were made using a sterile cork borer. Three different concentrations (60, 30 and 15 mg/mL) of each extract were impregnated (100 µL) in to each well using a sterile micropipette to investigate the dose dependent activity of the extracts on both organisms used. At same time gentamicin, 125 mg/mL and ketoconazole, 100 mg/mL were used as standard. After this, the sensitivity plates were incubated at 37ºC for about 24 hours. The diameter of zones of inhibition around each well was noted and the values were measured for the eventual antimicrobial activity [10].

For all the four extracts used in this assay, ethyl acetate extract (AP_4) was the most active fraction and was selected for column chromatographic separation.

2.6 Column and Thin Layer Chromatography

About 5.0 g of the ethyl acetate extract (AP_4) was mixed with silica gel (10 g) dissolved in 10 mL of solvent (ethyl acetate) in a 50 mL glass beaker. The content was stirred thoroughly, mixed and dried. It was loaded on top of the preloaded column. Furthermore, 10 g of the silica gel was added on top of the slurry to serve as a protection. The content of the chromatographic column was eluted with different solvent in different ratio (n-Hexane-ethylacetate and ethylacetate-methanol) of increasing polarity. Fractions (F1-F61) were collected in labeled bottles based on colour and quantity at different time intervals and were monitored using TLC with appropriate solvent systems. Fractions that appeared as a single spot on the TLC plate were selected and their R_f values calculated using the equation one (1) below.

$$R_f = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent front}} \rightarrow (1)$$

2.7 Antibacterial Screening for Pure Isolated Compound

The bioactivity screening of the isolated pure compound was screened using agar well diffusion method, which was carried out in the same manner as those of extracts. In the case of pure compound isolated, standard clinical bacteria (*S. aureus, E. coli and S. typhi*) were the only organisms used. They were also obtained from Department of Microbiology, Bayero University, Kano, Nigeria and used as received.

2.8 Minimum Inhibition Concentration of the Isolated Pure Compound (AP-44)

The minimum inhibition concentration of the pure compound was determined by weighing 1 mg each of the pure compounds and dissolved in 1 mL DMSO to give 1 mg/mL concentration. Series of solutions of lower concentrations (0.5, 0.25, and 0.125 mg/mL) were obtained from the stock using two-fold serial dilution with Muller Hinton nutrient broth, 100 µl culture *E. coli, S. aureus and S. typhi* was added in each glass tube along with the positive and negative controls and the glass tubes were incubated at 37ºC for 24 hours. The positive control is a test tube containing only the nutrient broth and the test organism, while a negative control is a test tube containing only the nutrient broth [11].

2.9 Antiradical Power of Pure Isolated Compound

The antiradical power of pure isolated compound (AP-44) was carried out using DPPH free radical according to the method described by [12,13]. A solution of 80 µg/mL DPPH was used for the assay. It is prepared by dissolving 2 mg of DPPH in 25 mL of methanol in amber bottle covered with aluminium foil paper and kept below 25ºC. The Stock solutions of the compound was prepared by dissolving 0.02 g (20 mg) in 2 mL of methanol to give 20,000 µg in 2 mL. From the stock solution, concentrations of 1000 µg/mL was prepared by measuring 0.2 mL of the stock solution and then diluted with 1.8 mL of methanol. The concentrations of 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL was also prepared by serial double dilution. A standard blank solution (100 µL) of the compound was then impregnated into a 96-well micro-plate using a sterile micro pipette in triplicate according to concentration gradient. After impregnation, absorbance of blank sample solution was taken at 517 nm using a micro-plate reader. Standard

solution of DPPH (40 µL) was added to the blank solution and allowed to react in complete darkness for 30 minutes at room temperature. The absorbance of the mixture (sample + DPPH) was also measured at same wavelength using same machine and was converted to percentage inhibition using equation two below.

$$\%\text{Inhibition} = 100 - \frac{(\text{ABS}_{\text{sample}} - \text{ABS}_{\text{blank}}}{\text{ABS}_{\text{control}}} \times \frac{100}{1} \quad (2)$$

Ascorbic acid was used as standard and its concentrations were prepared in the same way and magnitude as that of the sample solutions.

3. RESULTS AND DISCUSSION

3.1 Result of Extraction and Fractionation

3.1.1 Results of phytochemical screening

Preliminary phytochemical investigations on methanol extract of stem-bark of *Adenanthera pavonina* showed the presence of alkaloids, glycosides, carbohydrates, phenols, tannins, flavonoids, terpenoids, saponins, sterols, proteins and resins.

Present study on the ethanol crude extract (AP_1) and other fractions (AP_2, AP_3 and AP_4) of stem bark of *A.Pavonina* revealed the presence of secondary metabolites which can be seen in Table 2.

3.1.2 Results of bioactivity tests

The ethanol crude extract (AP_1) and ethyl acetate fraction (AP_4) displayed good activity by inhibiting the growth of *E. Coli* (E.C), *S. typhi* (S.T), *S. aureus* (S.A), *Aspagillus flavus* (A.F), *Candida albican* (C.A) *and Mucor specie* (M.S) by observing their zones of inhibition, with ethyl acetate having a remarkable activity. The measured zones of inhibition at two different concentrations (30 and 60 mg/well) for ethyl acetate and ethanol extracts were, *S. aureus* 12 and 14; 10 and 10, *E. coli* 11 and 12; 08 and 10, *S. typhi* 13 and 15; 09 and 10, *A. flavus* 16 and 25; 08 and 09, *C. albican* 10 and 14; 09 and 10, and *Mucor specie* 10 and 14; 09 and 10, respectively.

In 2015, Albin and co-workers reported that, Chloroform, ethyl acetate, and ethanol extracts of *A. pavonina* showed antibacterial activity at same concentrations (2 mg) against *s. aureus* and *E. coli* with zones of inhibition 12, 18, 19 and 14, 10, 19 respectively, but did not show any activity against *C. albican* and *Asp. nigrican* [10].

The Fig. 5 represents the antibacterial zones of inhibition of the ethanol crude extract (AP_1) and three soluble fractions (AP_2, AP_3 and AP_4) at different concentration.

3.1.3 Results for column chromatography

From the column chromatographic elution of ethyl acetate extract (AP_4), a total of 61 eluate were collected based on colour and quantity. Out of these numbers, column fraction 44 was selected for antibacterial test, antioxidant test and characterization (FTIR, GC-MS, ^{1}HNMR and ^{13}CNMR), because it appeared as a single spot on a thin layer chromatographic plate with R_f of 0.68.

3.1.4 Antibacterial activity test results of the pure isolated compound

The highest zones of inhibition noted for the pure isolated compounds (AP44) were 28 mm against *Staphylococcus aureus* and *Escherichia coli*, and 19 mm against *Salmonella typhimurium* at 60 mg/mL, while the least zone of inhibition was found to be 11 mm against *Salmonella typhimurium* at 15 mg/mL. The antibacterial activity of the isolated pure compound appeared more promising due to the absence of antagonist which may block the action of agonist chemical by binding to a receptor but does not produce a physiological effect. As can be seen from the chart.

3.1.5 Result of MIC and MBC of isolated pure compound (AP-44)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were for the isolated pure compounds (AP-44) against *Escherichia Coli, Salmonella typhimurium* and *Staphylococcus aureus* is shown in the Table 3.

Table 1. Physical properties of the extract fractions

Fraction	Colour	Texture	Weight (g)	%Yield
AP_1	Reddish brown	Solid crystal	83.48	16.70
AP_2	Orange	Oily	0.07	0.09
AP_3	Whitish-blue	Gummy	0.08	0.099
AP_4	Reddish brown	Solid crystal	19.3	23.98

Table 2. Phytochemical ingredients of stem bark of *Adenanthera pavonina L.*

Phytochemicals	AP_1	AP_2	AP_3	AP_4
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Flavoniods	+	+	-	+
Phenols	+	-	-	+
Protein	+	+	+	-
Saponins	+	-	-	+
Steroid	+	+	+	+
Tannins	+	+	-	+
Triterpenoids	+	+	+	+

F1= Ethanol fraction, F2= n-Hexane fraction, F3= Chloroform fraction, F4=Ethyl acetate fraction

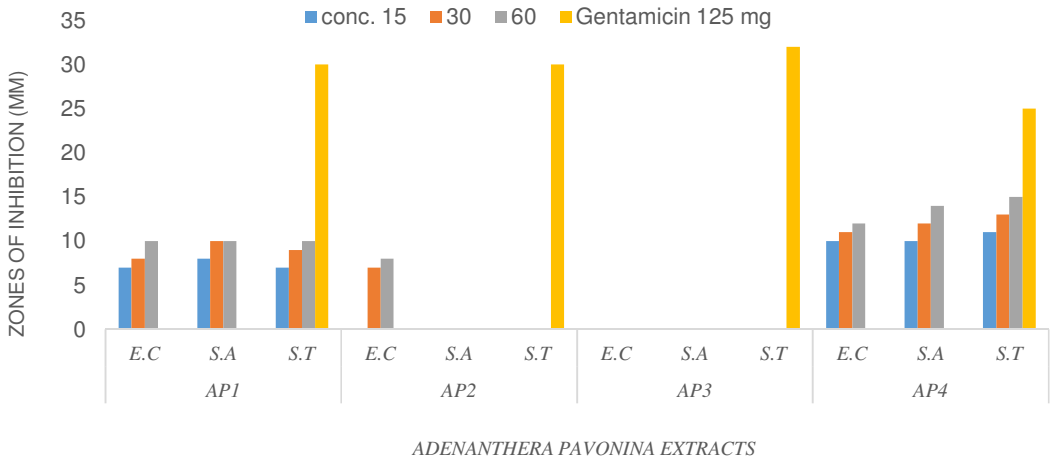


Fig. 1. Activity of extract and fractions on bacteria using gentamicin as control

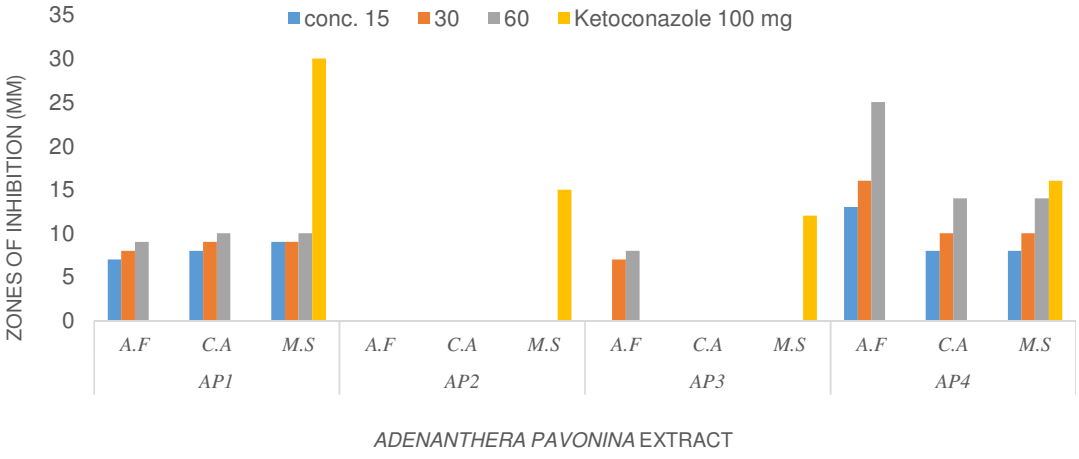


Fig. 2. Activity of extract and fractions on Fungi using Ketoconazole as control

This results is due to visible growth of bacteria (turbidity of the nutrient broth) in the glass vial. The MIC values against all the three bacteria showed a very promising activity when compared with the MIC value (12.5 mg/mL) reported by Tanveer, in 2017 for Isolated pure compound (β-sitosterol glucoside) from the stem bark of *Adenanthera pavonina* [11].

3.1.6 Result of antiradical power of the compound (AP-44)

The antiradical power of the pure isolated compound and ascorbic acid on free radical (DPPH) was concentration dependent, with high antiradical power (86.38 and 90.7%) and least antiradical power (66.5 and 50.55 %) at 1000 and 7.8125 µg/mL respectively.

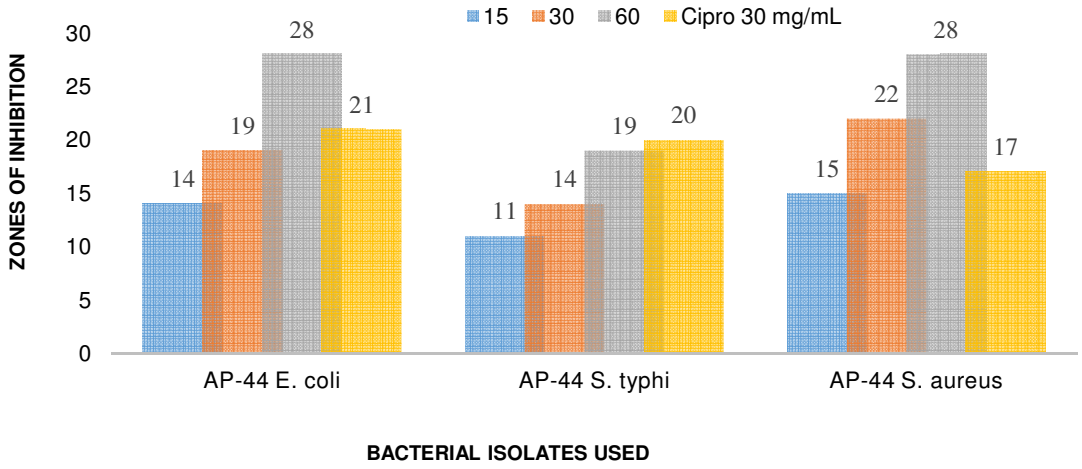


Fig. 3. Antibacterial activity of the isolated pure compounds (ap-44) and ciprofloxacin

Table 3. MIC and MBC of isolated pure compound (AP-44)

Fraction	MIC/MBC(µg/ml)	Bacterial Strain		
		EC	ST	SA
AP-44	MIC	0.25	0.5	0.25
	MBC	0.5	1000	500
	MIC	0.5	0.25	0.25
CIPRO	MBC	1000	500	500

EC: Escherichia coli, ST: Salmonella typhy, SA: Staphylococcus aureus

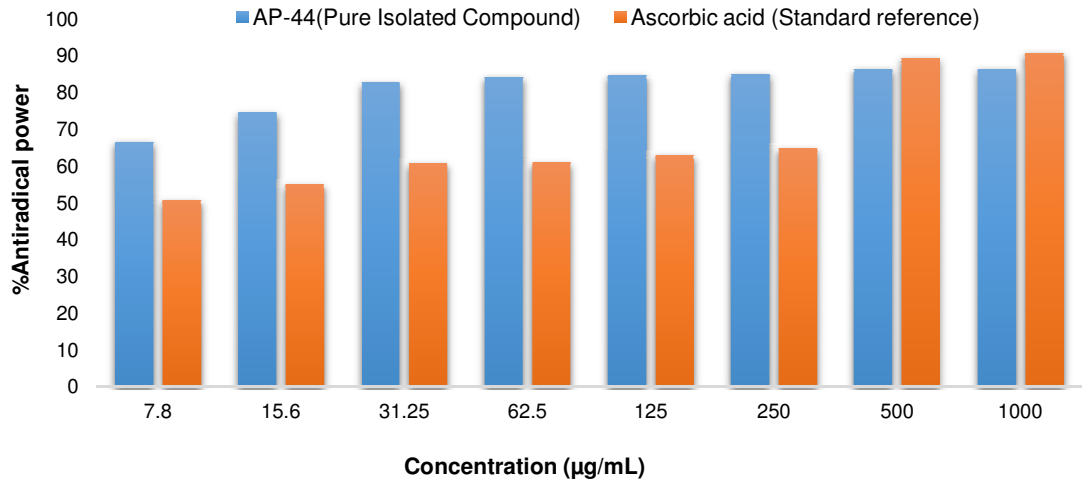


Fig. 4. Antioxidant activity of pure isolated compounds and ascorbic acid

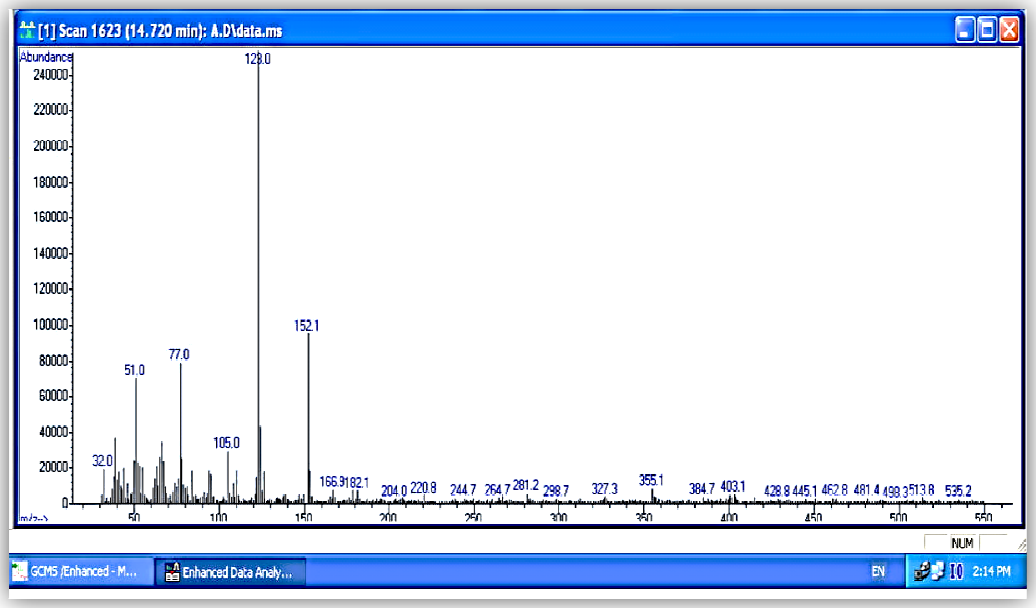


Fig. 5. GC-MS Spectrum of AP-44

Table 4. ^{1}HNMR spectral result for the isolated compound (AP-44)

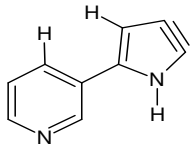
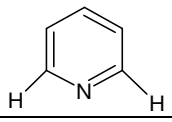
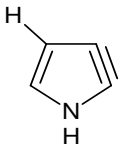
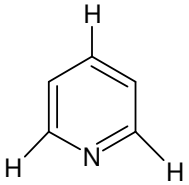
S/N	δ ^{1}H-NMR (ppm)	Type of proton	No. of proton
1	6.3	N–H	1
2	1.99211	C ≡ C–H	1
3	6.67445		2
4	8.92593		2

Table 5. ^{13}CNMR spectral result for the isolated compound (AP-44)

S/N	δ ^{13}C-NMR (ppm)	Type of Carbon	Number of Carbon
1	63.19999 and 69.88933	C ≡ C–H	2
2	108.65953, and 108.73917 114.89333 and 114.96235		4
3	145.11234, 145.03905, 144.92203, 144.69345 and 144.46333		5

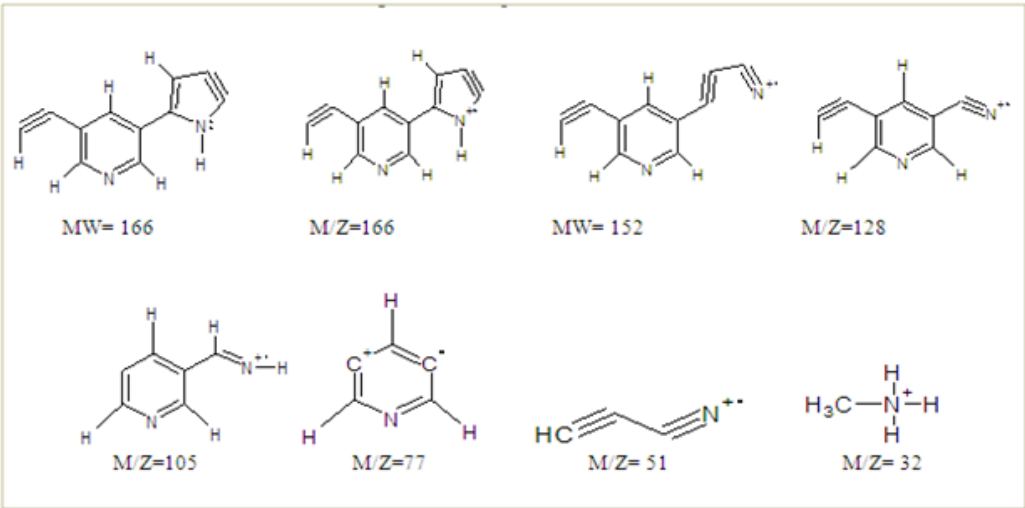


Fig. 6. Molecular Ion and fragments of AP-44 obtained from GC-MS spectrum

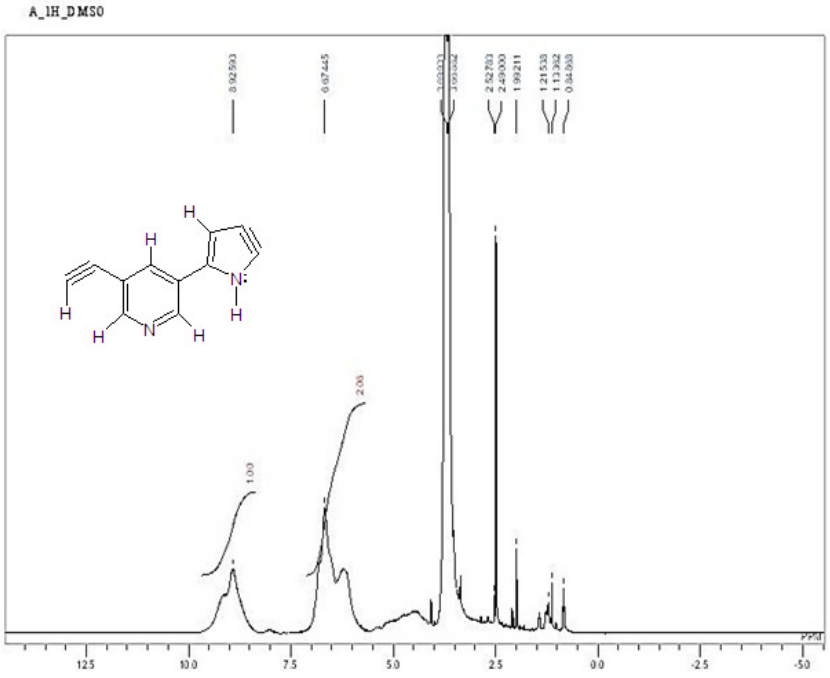


Fig. 7. ^{1}HNMR for Isolated Compound (AP-44)

Table 6. Result for FT-IR absorption band of the compound (AP-44)

S/N	Absorption Values (Cm^{-1})	Functional Group	Type of vibration causing IR absorption
1	3273	N–H and ≡C–H	Stretching
2	2348	C≡N	Stretching
3	2121	C≡C	Stretching
4	1998, 1897	C–H	Aromatic Bending
5	1607	N–H	Bending (2^{o} amine)
6	1447	C–C=C	Asymmetric stretch

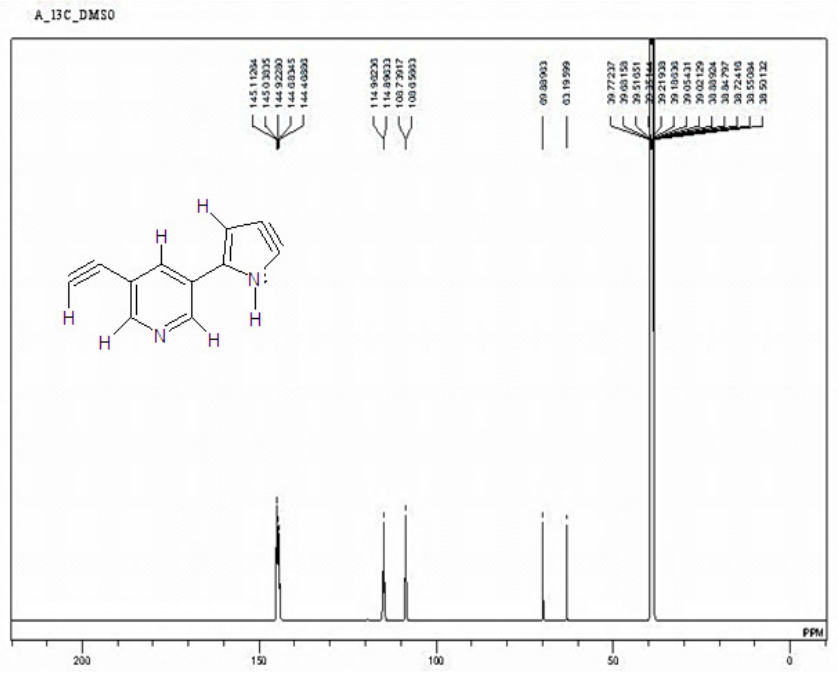
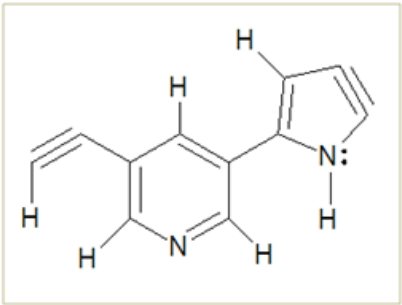


Fig. 8. ^{13}CNMR for Isolated Compound (APLF44)



{3-ethynyl, 5(2,3-dehydropyrrole) pyridine}
5-(5-ethynylpyridin-3-yl)-2,3-didehydro-1*H*-1λ^5-pyrrol-1-ylidene

3.1.7 Spectral interpretation and proposed structure of AP-44

The following are GC-MS, ^{1}HNMR, ^{13}CNMR and FT-IR spectra and tables of spectral values for the isolated pure compound (AP-44).

Characterization of bioactive isolated pure compound (AP-44) using GC-MS, ^{1}HNMR, ^{13}CNMR and FTIR unveiled the following "proposed structure."

4. CONCLUSION

Evidently the research results of the present work vividly ascertained the phytochemicals and bioactive components of the stem-bark of *Adenanthera pavonina*. It clearly present promising antibacterial, antifungal and antiradical properties of both the extracts and the isolated pure compound. However, the activity of the isolated pure compound appeared more promising than those of the extracts, this may be due to the absence of antagonist which may block the action of agonist chemical by binding to a receptor but does not produce any physiological effect. The antibacterial activity of the compound is due to the mechanisms through which the chemical compounds inhibit protein synthesis by blocking DNA replication. While the antiradical power of the pure compound on DPPH free radical is due to the mechanism through which electron(s) or hydrogen atom(s)

is/are transferred to the radical. This justifies some of the ethnomedicinal claims on stem bark extract of *Adenanthera Pavonina* as antimicrobial and antioxidant agents.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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