International Journal of Advanced Scientific Research
ISSN: 2456-0421; Impact Factor: RJIF 5.32
Received: 05-09-2020; Accepted: 20-09-2020; Published: 06-10-2020
www.allscientificjournal.com
Volume 5; Issue 5; 2020; Page No. 35-39

Isolation and Identification of 3-(3,4-Dihydroxyphenyl)-2-propenoic acid from Methyl acetate root bark extract of *Cassia sieberiana*

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Abstract
The Caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid) was isolated from *Cassia sieberiana* root bark using standard analytical methods and the main compound with [M + H] mass of 360 m/z with retention time of 0.495 in positive mode and 0.392 in negative mode, by exhibiting the base peak ion at 359 m/z was identified. Dimer of Caffeic acid was isolated and fully characterized with concerted use of FTIR, LCMS, HNMR, ^{13}CNMR spectroscopy and CHN analysis. The CHN calculations from the empirical formular C_2H_2O (42 g) and the actual mass of the compound was calculated to be 378 g/mol by comparing molecular weight obtained from MS (360 m/z). $C_{18}H18O_9$ (378 g/mol) with loss of water molecule (18 g/mol) gave a compound of mass 360 g/mol ($C_{18}H_{16}O_8$) which is the dimer of caffeic acid 180 g ($C_9H_8O_4$) **(39)** and subsequence loss of water gave the actual mass of dicaffeic acid ($C_{18}H_{14}O_7$) **(38)** are in agreements with experimental results confirmed the identity of the isolated natural product.

Keywords: *Cassia sieberiana*, Caffeic acid, Isolation, Identification and Structure elucidation

Introduction
In many of the developing countries the use of plant drugs is increasing because modern life saving drugs are beyond the reach of three quarters of the third world's population although many such countries spend 40-50% of their total wealth on drugs and health care (Geetha and Geetha, 2014) [6]. As a part of the strategy to reduce the financial burden on developing countries, it is obvious that an increased use of plant drugs will be followed in the future (Joy *et al*., 2001) [7]. That is therefore the need for continuous search for new drugs and medicinal plants are one of the useful areas of search in this regards (Odamtten, 2008) [8]. It's against this background that *Cassia sieberiana* that is widely used as herbal preparation in some parts of Nigeria was investigated. The bioactive compound from the plant was isolated and its structure elucidated.

Material and Methods
Liquid—Liquid Extraction
The separation of the crude extracts of *Cassia sieberiana (A)* was based on the solubility of plant extracts in miscible solvents with different densities like chloroform (1.480 g/ml), methanol (0.791 g/ml) and hexane (0.659 g/ml). These miscible solvents were mixed with crude extract of the plant and shake in the separating funnel and when the three or two different phases have separated into two distinct layers, and equilibrium situation or partition coefficient $K = C_2/C_1$is achieved such that the ratio of the concentration of the solute in each layer at equilibrium is in g/l of the plant extract in two solvents. When the two layers were not clearly separated NaCl was added and ammonium sulphate was also used to absorb moisture during the collection of the different fractions in the conical flask. The extraction solvents were evaporated on a water bath to dryness, weigh their mass and recorded.

Thin Layer Chromatography
Each L-L fraction was checked on TLC plate. Test samples (10mg/ml) were prepared, 2.5 µl of samples were spotted on TLC plate and allowed to dry. A TLC plate is made up of a thin layer of Silica gel 0.25mm with fluorescent indicator F_{254} with Solvent system Chloroform: methanol (9.5:0.5) was used for TLC analysis. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. The plate was removed after an optimal development time and dried and the spots/zones are detected using UV chamber and Rf value was calculated. This was followed by HPLC qualitative check.

Column Chromatography
Column chromatography was carried out on each L-L fraction, using silica gel and 5% methanol: 45% chloroform in varying concentrations to elute the column and Na_2SO_4 was used in the column to ease the elution. For each column fraction was concentrated on water bath to dryness, TLC and HPLC were carried out for qualitative check. The most pure compound was selected for elucidation of the structure.

High Performance Liquid Chromatography
Test samples (10mg/mL) were prepared from stock with HPLC grade Methanol and used for HPLC analysis. **H**PLC condition: Instrument: Shimadzhu LC- Prominence 20AT, Column: C18 column 250 mm x 4.6 mm, 5u particle, Mobile Phase: Linear A: Methanol (50%) and B: Water (50%), Flow Rate: 1ml/min, Injection volume: 10µL and Absorbance: 254nm.

Liqiud Chromatography Mass Spectroscopy
LCMS 2O1OA, SHIMADZU, JAPAN: HPLC conditions:

Column:-Phenomenox, LunaC18, 4.6*100mm, 5µm, Mobile phase: Acetonitrile: 0.1% formic acid in H_20, 60:40v/v, Flow rate: 0.5 mL/min, Column temperature: 35°C and Detector: UV 254 nm. Mass spectrometer: TSQ Quantum Access MAX Triple Quadrupole LC-MS. Mass spectrometer parameters: curtain gas 10, gas1 20 and gas2 0, needle voltage 5000 V, and declustering potential 100 V. Mode: Positive ion and negative ion mode with precursor ion mass scan from 50 – 1050 Daltons.

CHN Analysis

CHN analysis was carried out using EA 1112 Thermo Finnigan, France. This works on combustion and reduction basis which is quantitative analysis. Solid samples was weighed in tin containers (elemental analyzer) and loaded into an automatic sampler. The tin cups are then dropped in a tube where in the presence of external oxygen flash combustion occurs at a temperature of 900 °C. After combustion, the reaction gas products (CO_2, H_2O, NOx, and SO_2) are carried by helium flow to a copper reactor where excess O_2 is consumed (CuO) and NOx products are converted to N_2. They flow along a thermal conductivity detector (TCD) which produces an electrical signal proportional to the concentration of nitrogen, carbon, hydrogen and sulfur.

NMR Analysis

NMR analysis was carried out using 400MHz SUPERCON multi nuclei probe, BRUKER, West Germany. Supercon from Bruker400 MHz with multi nuclear probe commonly studied nuclei are H1,13C,P31,B11 and 2D experiments like Dept which gives the CH,CH2 and CH3 carbons. Samples were dissolved in D_2O and recorded.

FTIR Analysis (*Fourier Transform Infrared Spectroscopy)*

FTIR analysis was carried out using NICOLET 380 FT IR, Thermo Fisher Scientific - France. NICOLET 380 FT IR gives transmittance spectra in the IR range 4000 to 400 nm. 10mg of sample was mixed the KBR (which does not have any absorbance in IR range) as a supporting compound and grinded well. This powder will put in the die and apply a pressure of about 10 tons. A circular pellet was put in the IR holder and run with a scan range from 400 to 4000 per cm with a resolution of 4 per cm.

Results and Discussion

Cassia sieberiana (A), column fraction AF27 with TCL characteristics of 1 band, 0.61 retention factor, light blue (at 366 nm), blue (at 254 nm) on UV chamber and light brown on visible light and HPLC of fraction with purity of 84.90 % (Fig. 1), retention time of 2.71 mins, peak number 2 on the chromatogram was selected for further analysis (Table 1 and 2).

Table 1: TLC characteristics of Methanol Column fraction of *Cassia sieberiana (A)* **AF27**

Sample code	TLC Band	Retention Factor	TLC Profile characteristics		
			366 nm	254nm	Visible light
AF27	1	0.61	Light Blue	Blue	light brown

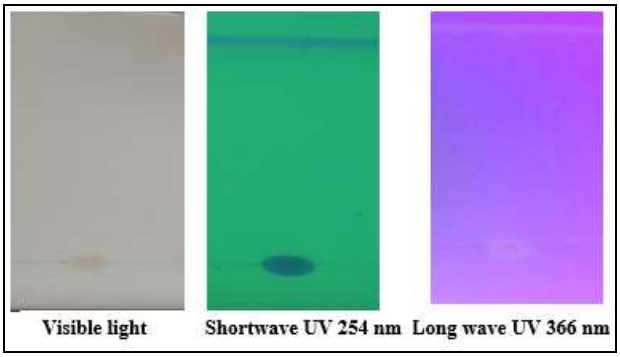


Table 2: HPLC summary of compound AF27

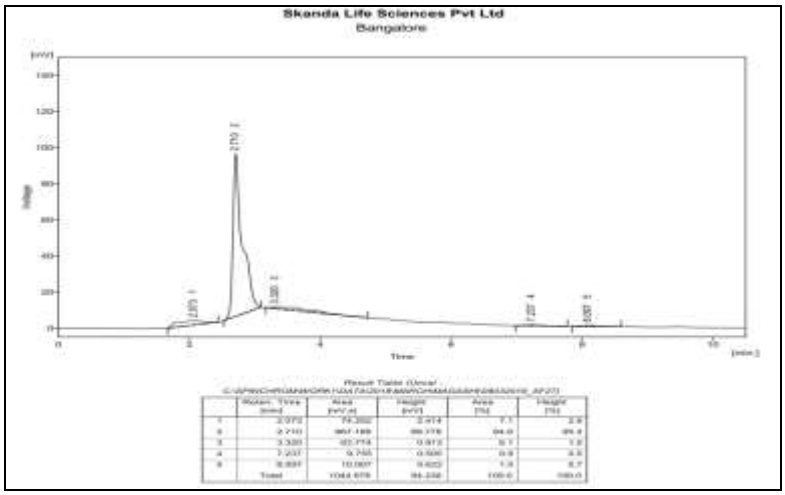


Fig 1: HPLC chromatogram of compound AF27

Table 3 and Fig. 2 shows the *Cassia sieberiana (A)* FTIR results of functional groups that complement the ^{1}H nmr (36) and ^{13}C nmr (37). In ^{1}H nmr, a bunch of signal at 6.10ppm indicate the presence of phenolic –H and the signal between 6.51 – 6.64 ppm shows the presence of few aromatic proton. Signal at 6.64 was due to H- attached to C-atom bonded to oxygen atom. 7.28 ppm was due to H-bonded to C-atom attached to benzene ring (Table 4 and Fig. 3).

Table 3: FTIR Peak values and functional groups of compound A

Absoption band (cm^{-1})	Intensity	Bond type	Functional group
1560.41	95.688	C=C	C=C stretching of alkenes
1637.56	57.748	C=C	C=C stretching of aromatic ring
1654.92	59.098	C=O	C=O Stretching of carboxylic acid
3426.43	8.141	O-H	O-H Stretching of aromatic alcohol

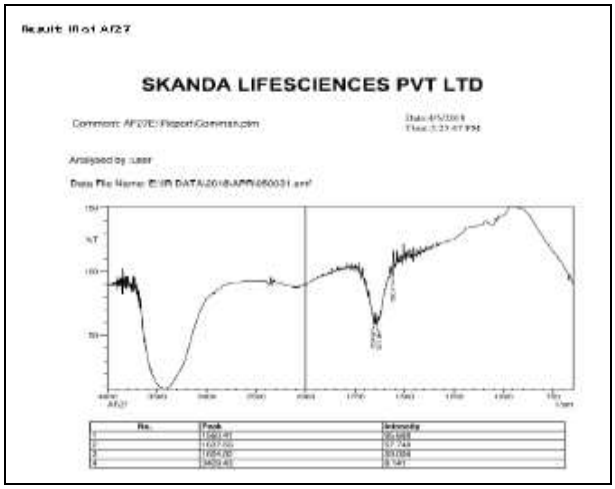
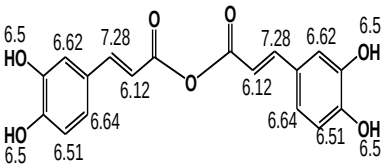
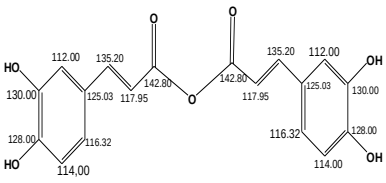


Fig 2: FTIR chromatogram of compound A (AF27)



(36)



(37)

Table 4: ^{1}H Chemical shift for type of H-atoms of compound A (^{1}H IN $CDCl_3$)

Hydrogen Identity	Chemical shift (ppm)	Type of H	Integral
Phenolic – H	6.10	ArO – H	4
Aromatic –H	6.51- 6.65	Ar – H	3
Carbonyl Cpds – H	6.64	H-C-C=O	1
Benzelic –H	7.28	Ar – C – H	3

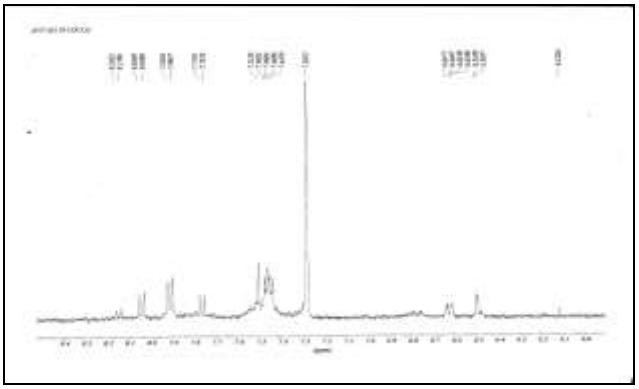


Fig 3: ^{1}H Chemical shift chromatogram of compound A (AF27 IN $CDCl_3$)

^{13}C nmr in (Table 5 and Fig. 4) shows the presence of aromatic ring system at 125.03 ppm and at 135.20 ppm revealed the presence of carbon attached to aromatic ring. Signal at 117.95 ppm indicate the presence of double bond of alkenes, whereas 142.80 ppm signal proved the presence of carboxylic acid.

Table 5: ^{13}C Chemical shift for type of C-atoms of compound A (^{13}C IN $CDCl_3$)

Carbon peaks	Chemical shift (ppm)	Type of C	ppm
A	100 – 135	Ar	125.03
B	100 – 135	Ar – C	135.20
C	100 – 150	C=C	117.95
D	140 – 180	COOH	142.80

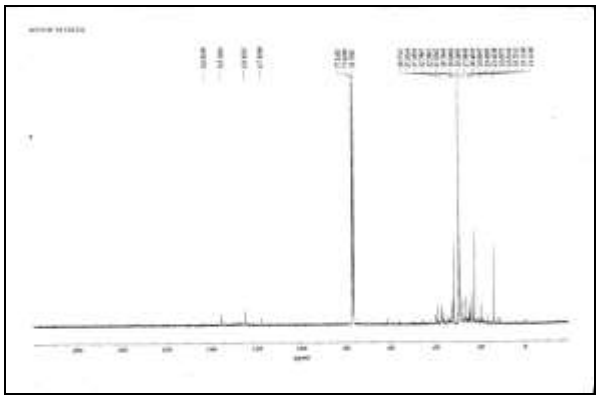


Fig 4: ^{13}C Chemical shift chromatogram of compound A (AF27 IN $CDCl_3$)

HPLC positive mode ESI spectra suggest a molecular weight 359 g/mol. with retention time of 0.495 in positive mode and 0.392 in negative mode, by exhibiting the base peak ion at 359 m/z (Table 6 and Fig. 5a & b). Subsequence fragmentations of 359 m/z ions gave ions with mass of 79, 95, 118, and 137 m/z in positive mode and the adduct ions of 375 m/z and 391 m/z, 392 m/z in the negative mode (Table 6).

Table 6: LCMS data for compound A

Mode	Name of Proposed Identity	RT	Mass Peak	Base Peak	Fragments and their relative abundance
Positive	Caffeic acid	0.495	360	359	55,64,79,95,118,137, 152,156,178,184,206, 214,224,244,253,273, 291,308,335,337,359, 370,385,395,415,429, 436,460,473,490.
Negative		0.392			59,78,94,110,140,154, 173,195,197,216,226, 251,259,274,287,301, 315,323,338,363,375, 391,392,409,434,435, 453,474,483.

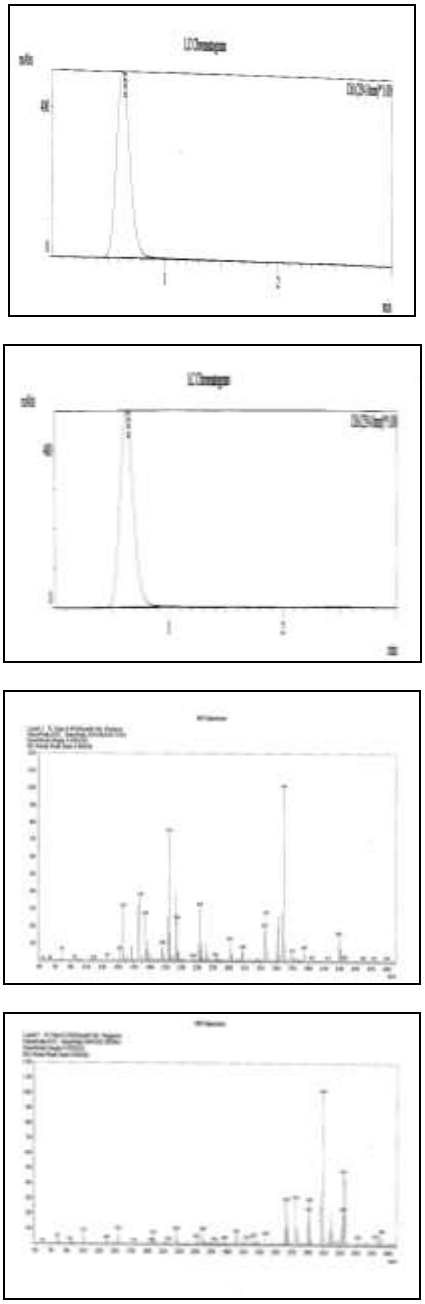


Fig 5: LCMS chromatogram of AF27 fraction. Figure 5b: LCMS chromatogram of AF27 (Positive mode) fraction. (Negative mode)

CHN analysis calculation for compound isolated from *Cassia sieberiana (A)*, gave the empirical formular of C_2H_2O (42 g) in (Table 7 and Fig. 6). The actual mass of the compound was calculated to be 378 g/mol by comparing molecular weight obtained from MS. $C_{18}H18O_9$ (378 g/mol) with loss of water molecule (18 g/mol) gave a compound of mass 360 g/mol ($C_{18}H_{16}O_8$) which is the dimer of caffeic acid 180 g ($C_9H_8O_4$) **(39)** and subsequence loss of water gave the actual mass of dicaffeic acid ($C_{18}H_{14}O_7$) **(38)** (Scheme 1).

Table 7: CHN Analysis Data for Compound A

Element name	Compound a	
	%	RT
Carbon	50.60	1.06
Hydrogen	5.01	5.67
Nitrogen	0.08	0.76
Oxygen	44.31	

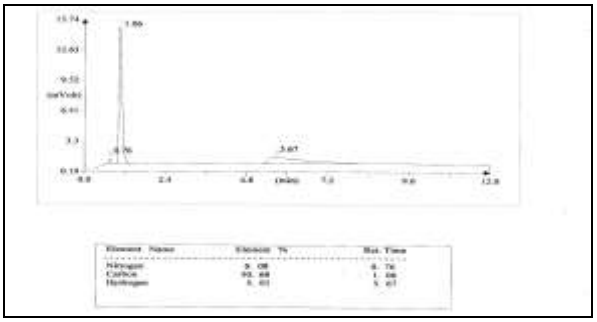


Fig 6: CHN chromatogram of AF27 fraction

Conclusion

The bioactive compound isolated from the Methyl acetate root bark extract of *Cassia sieberiana* was identified to be 3-(3,4-Dihydroxyphenyl)-2-propenoic acid (Caffeic acid) and the bioactivity, such as antibacterial, antifungal (Magashi, L.A. *et al*, 2020b and Barata *et al.*, 2007).) and free radical scavenging activities (Magashi, L.A. *et al*, 2020a) presence in *Cassia sieberiana* could be ascribed to the presence of 3-(3,4-Dihydroxyphenyl)-2-propenoic acid (Caffeic acid) in it, thereby justifying the usage of the plants in folk medicine in the treatment of ulcer, diarrhea, sinusitis, rheumatoid arthritis, asthma, pneumonia, acute sore throat, rheumatic fever, wound infections and so othersb (Kafui *et al*, 2010). Moreover, *Cassia sieberiana* was found useful in the treatment of Constipation, Common Cold, Fevers, Intestinal disorders, Aguesia and Skin Disorders (OVG, 2013).

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