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Identification of alkaloids from the methanol Leaf extract of *Acalypha wilkesiana* using HPLC/GC-MS

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ABSTRACT

Background: Different parts of *Acalypha wilkesiana* has been reportedly used in the treatment of diverse ailments traditionally. These are due to the presence of phytochemicals such as fatty acids, steroids, flavonoids, glycosides and terpenes. The aim of the study was to identify alkaloids in the leaves of *A. wilkesiana* which have been linked to some of its traditional usage by High Performance Liquid Chromatography and Gas Chromatography-Mass Spectrometry.
Methods: Dried, pulverized leaves of *Acalypha wilkesiana* were macerated with methanol and the extract obtained was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) and High Performance Liquid Chromatography (HPLC) analyses.
Results: Seven alkaloids were identified by GC-MC technique: 1-Phenyl-3,5,7-trimethyl-4,5,6,7-tetrahydropyrazolo(3,4-B)(1,4)diazepine; 1H-1,2,4-Triazole; Indolizine; Methyl-2-oxo-1H,5H,6H,7H-cyclopenta[B]pyridine-3-carboxylate; 3-Methyl-2-phenylindole; Furazano [3,4-B]pyrazin-5(4H)-one; 1H-Isoindole-1,3(2H)-dione. Also, the HPLC analysis was able to identify five classes of alkaloidal compounds; quinolinamine, benzenesulfonamide, allylamine, benzamide and indolizine.
Conclusion: The leaves of *A. wilkesiana* contain useful alkaloids that may be implicated in some of its traditional uses and as source of important bioactive molecules.

1. Introduction

Acalypha wilkesiana Mueller Argoviensis family Euphorbiaceae is an ornamental evergreen shrub, commonly known as copperleaf and Jacob's coat[1]. It is native to Fiji and other Pacific Island, however following its introduction in many tropical countries; it has become widely distributed in parts of Asia and West Africa[2]. It thrives in a humid environment and average to moderate fertile, well-drained soil. *A. wilkesiana* can produce flowers and fruits all year round in environment where the dry season is shorten. The plant height can range from 3.0 - 5.0 m with erect stem and many branches. Fine hairs are seen on the branches with closely ordered crown. The shape of the leaves vary from elliptic to wide ovate, with the margin that is coarsely tooth or serrated[1].

Different parts of *A. wilkesiana* has been reportedly used in the treatment of dysentry, skin infection, breast tumor, diabetis mellitus, diarrhoea, laryngitis, headache, swelling, cold, hypertension, malaria and as worm expellant[3-6,]. Pharmacologically, extracts from the leaf have shown activity against Gram positive bacteria. It has also been reported to possess antifungal, antimalarial, antioxidant and analgesic activities[7-9], while the seed extract exhibited immunomodulatory activity against selected tumor cells[10]. Compounds that have been isolated from the leaf include condense and hydrolysable tannins, flavonoid and

liquiritigenin[11]. Amlabu and co-workers reported the identification of 51 compounds (fatty acids, polyphenols, esters, aldehyde and terpenes) from 70% aqueous methanol extract of *A. wilkesiana* leaf with different biological activities[12]. A similar study that involved the use of ethanol in the extraction of the leaf of *A. wilkesiana* Java white, identified 13 compounds that were broadly classified into fatty acid and fatty acid ester, phytols, xanthones and sterols[13]. Studies by Anokwuru and co-workers showed that corilagin, quadrangularic acid M, geraniin and corilagin were previously isolated[14]. Other investigators also isolated 3 flavonoids (artemetin, luteolin and vitexicarpin), 5 carotenoids and 12 simple terpenes[15]. Literature search showed that alkaloidal compounds from the leaves of *A. wilkesiana* are scanty. Therefore, the aim of this study was to identify the alkaloidal phytoconstituents in the methanol leaf extract of *A. wilkesiana* by using High Performance Liquid Chromatography and Gas Chromatography - Mass Spectrometry.

Methods

Collection, identification, preparation and extraction

The leaves were collected around the Faculty of Pharmacy building (Old) in Ugbowo Campus, University of Benin, Benin-City. It was identified by Prof. H. A. Akinnibosun, Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin, Benin-City. The leaf sample was kept in the herbarium with number A311. The leaves were air dried for two weeks under shade before being placed in a hot air oven at a temperature of 45°C for 4 hours and then pulverized using electric milling machine in the Department of Pharmacognosy, University of Benin, Benin-City. The leaf powdered obtained were then kept in an airtight container until needed.

To 40.00 g of the powdered plant material in a maceration jar, 500 ml of 99 % methanol was added and shaken intermittently at 30 minutes interval for 2 hours. The jar was tightly closed and kept in a dark compartment at room temperature for 4 days. After 4 days, the content of the jar was decanted and subsequently filtered using filter paper of size 1. The filtrate obtained was then concentrated *in vacuum* at 40°C using rotary evaporator. The crude extract obtained was stored in a sample container with lid well tight and kept in a refrigerator at 4°C.

Sample Preparation for GC-MS Analysis

To 50 mg of the methanolic extract of *Acalypha wilkesiana* leaf was homogenized with a solvent mix (1:1-Hexane: Dichloromethane). This mixture was then clean-up with silica gel (mesh size 100-200 mm) which has been conditioned with hexane and 3g of anhydrous sodium sulphate on the well-packed column. The eluent obtained was then concentrated *in vacuum* using rotary evaporator. The concentrate was kept in the refrigerator at 4°C until used.

GC-MS Analysis

The GC-MS used in this study is an Agilent 6890N Gas Chromatography equipped with an autosampler connected to an Agilent Mass Spectrophotometer. One (1) µl of the cleaned-up extract was injected in the pulsed spitless mode onto a 30 m x 0.25 mm ID DB 5MS coated fused silica column with a film thickness of 0.15 µm. The carrier gas used was Helium (He) at 30 cm/s linear velocity, while the column head pressure was maintained at 20 psi to give a constant flow rate of 1 ml/min. The temperature of injection port was maintained at 280°C with the column temperature initially held at 55°C for 4 min, this was increased to 200°C at rate of 25°C/min, then to 280°C at a rate of 8°C/min and to a temperature of 300°C at a rate of 25°C/min, which was held for 2 mins. Ion source temperature in the MS is 250°C and the MS was operated at electron impact mode at 70eV ionization energy and scanned from 45 to 700 Daltons. Data were captured and processed by ChemStation software. Percentage area for each compound was determined by comparing the ratio of each area over the total area multiply by 100. Compounds were identified by comparing their base peak and two other major peaks (selected ion monitoring mode) from the fragmentation pattern for each compound and comparing with National Institute of Standard and Technology data (NIST)[16].

HPLC Analysis of the methanol extract of *A. wilkesiana*

The phytochemical contents of the plant methanol leaf extract were further identified and quantified by using reversed phase high performance liquid chromatography with direct injection. Detection and identification was done through the use of Agilent Lichrospher equipped with 100-5RP8 (250 x 4.6 mm) (18). Gradient elution was utilized using two solvent mix; solvent A 0.1 % phosphoric acid, solvent B methanol. Flow rate of the solvent was 1 ml/min, injection volume was 5 µL while the total run was 6 min. The amount and identity of the phytochemical in the extract were further determined by the external calibration curve. This was achieved by comparing the retention time with that of 19 standard compounds.

Preparation of Reference Standards

Nineteen reference standards were separately dissolved in methanol to obtain the stock solutions at concentration of 1000 µg/ml. Each of the standard aliquots was filtered through Whatman filter paper with diameter of 125 mm and the filtrate obtained was diluted with 2% ammonia and solution obtained was adjusted to neutral pH with HCl. Suitable aliquots of stock solution were pipetted out and volumes were made up to the mark with methanol. The standard (Donated by Prof Joseph K. Ebigwai) used in this study includes Quinolinamine, Benzenesulfonamide, Allylamine, Benzamide, Indolizine, Pyrazoline, Imidazole, Propargylamine, Ethylenimine, Difluoramine, Isoxazolidine, Simulansamide, Colchicine, Norethindrone, Androstane, Methanamine, Isoxazolidine, Isobutylamine and Amphetamine.

HPLC Sample Preparation

A homogenized portion of the methanol leaf extract (200 mg) was mixed with deionized water (200 ml). This mixture was then refluxed for 1 hour before it was cooled to ambient temperature. The resultant extract was then filtered with Whatman filter paper with diameter of 125 mm and the filtrate obtained was diluted to1:3 (v/v) with 2 % ammonia solution. The solution was then adjusted to neutral pH (7) using 0.01 M HCl before HPLC analysis was conducted on the final sample.

Separation and Clean-up

Dilute stock solution (80 µg/ml)(5µL) was injected into the HPLC (1260 Ultra High Pressure Liquid Chromatography) with an Agilent Lichrospher 100-5 RP8 (250x4.6mm (C18). flow rate was maintained at 1.00 ml/min, injection volume of 5µL and column temperature of 35°C was set. Mobile phases include 0.1% phosphoric acid (A) and methanol (B), run time was 50min for each sample. Separation of the peaks was optimized while the stock standards were monitored at wavelength of 242 nm. Gradient elution: 0-2.5min, 25% solvent B; 2.5-6 min, 25 % solvent B; 15-22 min, 9-11 % solvent B; 22-38 min, 11-18% solvent B; 38-43 min, 18-23 % solvent B; 43-44 min 23-90 % solvent B; 44-45 min, 90-80 %, solvent B; 45-55 min[17].

Results

Eighteen known compounds were identified, seven of which were alkaloids: 1-Phenyl-3,5,7-trimethyl-4,5,6,7-tetrahydropyrazolo(3,4-B)(1,4)diazepine (1.45%); 1H-1,2,4-Triazole (5.47%); Indolizine (9.36%); Methyl-2-oxo-1H,5H,6H,7H-cyclopenta[B]pyridine-3-carboxylate (6.13%); 3-Methyl-2-phenylindole (5.14%); Furazano[3,4-B]pyrazin-5(4H)-one (4.38%); 1H-Isoindole-1,3(2H)-dione (11.96%). The other compounds are fatty acids (steroids) and esters (Table 1).

Table 1: Relative percentage of the compounds detected in methanol leaf extract of *A. wilkesiana* by GC-MS

S/N	RT (Mins)	% Area	MW	MF	Compound
1.	3.722	2.39	54.0904	C4H6	1,3-Butadiene
2.	5.777	5.80	58.08	C3H6O	2-Propanone
3.	13.381	1.45	256.35	C15H20N4	1-Phenyl-3,5,7-trimethyl-4,5,6,7-tetrahydropyrazolo(3,4-B)(1,4)diazepine
4.	24.001	5.47	69.0653	C2H3N3	1H-1,2,4-Triazole
5.	25.569	2.06	53.0636	C3H3N	2-Propynenitrile
6.	25.804	3.77	73.09	C3H7NO	N-Ethylformamide
7.	26.056	5.41	281.4766	C18H35NO	9-Octadecenamide
8.	26.284	2.21	207.15	C7H5N5O3	Pterin-6-carboxylic acid
9.	26.536	5.12	186.34	C12H26O	4-Dodecanol
10.	26.817	1.98	207.31	C13H21NO	N-Desmethyltapentadol

11.	27.183	9.36	117.151	C8H7N	Indolizine
12.	27.366	7.39	106.1279	C7H6O	Benzaldehyde
13.	27.498	6.13	191.19	C10H8NO3	Methyl-2-oxo-1H,5H,6H,7H-cyclopenta[B]pyridine-3-carboxylate
14.	27.709	5.14	207.2704	C15H13N	3-Methyl-2-phenylindole
15.	27.944	4.38	138.06	C4H2N4O2	Furazano[3,4-B]pyrazin-5(4H)-one
16.	28.167	13.98	207.189	C8H9N5O2	Benzaldehyde, 2 - nitro,diaminomethylidenhydrazone
17.	28.396	11.96	146.12	C_8H4NO2	1H-Isoindole-1,3(2H)-dione
18.	28.722	5.99	281.31	$C_{13}H_{19}N_3O_4$	(5-Ethoxycarbonylamino-2,6-dimethylpyridin-3-yl) carbamic acid

Key: RT=Retention time, MW=Molecular weight, MF=Molecular formula

The HPLC chromatogram revealed five groups of alkaloids: allylamine, benzamide, benzenesulfonamide, indolizine and quinolinamine (Table 2). HPLC quantitative analysis of the alkaloidal content of *Acalypha wilkesiana* showed benzenesulfonamide (58.41ng/ml) as the highest alkaloidal content, allylamine (9.74ng/ml), quinolinamine (9.40ng/ml), benzamide (4.94ng/ml) and indolizine (3.40ng/ml).

Table 2: HPLC quantitative analysis of alkaloids contents of *Acalypha wilkesiana*

S/N	Retention Time (min)	Amount (ng/µl)	Compound
1	1.346	9.40588	Quinolinamine
2	1.956	58.41790	Benzenesulfonamide
3	3.139	9.74094	Allylamine
4	3.795	4.94700	Benzamide
5	4.126	3.40763	Indolizine

Discussion

Alkaloids are phytochemicals that contain at least one nitrogen in their structure and have potential to be used as analgesic and antibacterial agents. Their antibacterial activities are caused by the release of liposome from the cell, making the lipid-bilayer membrane rapture[18]. In the study conducted by Igwe and coworker on the ethanol extract of *A. wilkesiana*, twelve compounds were identified and classified as terpenes, fatty acids, fatty acyl alcohols, derivative of ester[19]. Aqueous extract of two different cultivars of *A. wilkesiana* revealed nine to ten compounds, mainly fatty acids and 5-acetyl-2-amino-4-methylthiazole[20]. In 2018, Oyebode and coworker identified twenty-five compounds and grouped them as phytol, phytol acetate, vitamin E and sterol[13]. We identified eighteen compounds, which fatty acids, alkaloids, amides, sulphonamide and nitrile.

^{1}H-isoindoline-1,3-(2H)-dione is a heterocyclic compound with a bicyclic structure in its molecule consisting of a benzene ring fused to a five member nitrogen containing ring. It derivatives are likely to inhibit inflammation due to the enhanced expression of anti-inflammatory factors such as arginase-1 and interleukin-10 (IL-10), and inhibited p65 subunit phosphorylation induced by LPS kappa B (NF-B) in macrophages[21].

Indolizine is an isomer of indole and known as pyrrole[1,2-a]pyridine, it consists of a 10π conjugated planar electronic structure. It derivatives have found application in anticancer drug development through topoisomerase I inhibition[22]. Methyl-2-oxo-1H,5H,6H,7H-cyclopenta[b]pyridine-3-carboxylate are structural derivative of alkaloids and cause inhibition of human fibroblast growth factor receptor (FGFR) family, with possible management of hyperglycemic effect and obesity[23,24]. Benzaldehyde, 2-nitrodiaminomethylidenhydrazone have been reported in *Byrsonima garneriana* extract. This extract has shown antifungal, antioxidant and cytotoxicity potentials[25]. These activities can be ascribed to its presence in the molecule of hydrazone moiety[26]. Cyclopenta[x]pyridine derivatives are highly selective and potent aldosterone synthase inhibitor, exhibit FXIIa inhibitory activity and strong melatonin receptor agonist. Also, it natural occurring derivatives possess cyto-protective activity. They act as agonist by binding to G-protein coupled receptor 40 (GPR40). Thus they can be used in treatment, suppression, and prevention of illnesses mediated by GPR40, including type 2 diabetes mellitus[27]. ^{1}H, 1,2,4 triazole is a five membered ring heterocyclic molecule with two carbon and three nitrogen atoms. This moiety has been observed in antifungal drugs like itraconazole and fluconazole; antimigraine like rizatriptan; antiviral like rivavirin[28]. Analogs of 3-methyl-2-phenylindole are cyclo-oxygenase inhibitor, this enzymes are responsible for the catalytic conversion of arachidonic acid to pro-inflammatory postaglandin and thromboxane. The inhibitory effect will result in the treatment of inflammatory conditions such as rheumatoid arthritis, ankylosing spondylitis and osteoporosis[29]. Research have suggested that pyrazolo(3,4-B)(1,4)diazepine derivatives could be used in future as promising scaffold of multiple Aurora A/B and KDR inhibitors[30]. Pterine-6-carboxylic acid is also known as 2-Amino-4-hydroxypteridine-6-carboxylic acid and belong to the class of pteridines, It is a heterocyclic compound that is made up of a fused pyrimidine ring and a pyrazine ring, and has been reported in *Foeniculum vulgare*[31]. It serves as a precursor for the formation of folate, which plays a critical role in cell growth, development, and repair. It has found application as an anticancer, antioxidant and antimicrobial agent. N-desmethyltapentadol derivatives produces its pharmacological effect by mu-opioid agonism and norepinephrine reuptake inhibition and is highly selective for the mu-opioid receptor[32].

HPLC is a versatile, robust, and widely used technique for the isolation of natural products, it can be used to separate, identify and quantify the individual compounds in phytochemical studies. Presently, this technique is currently use for fingerprinting study for the quality control of herbal plants. It resolving power is ideally suited for rapid processing multi component samples on both diagnostic and preparative scale. Researchers have designated the use of HPLC for characterization and quantification of secondary metabolites in plant extracts, mainly alkaloids[33].

Reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. Generally, RP-HPLC has a nonpolar stationary phase (C_{18}) and a moderately polar aqueous mobile phase (methanol and phosphoric acid 0.1 %). The phosphoric acid was added to the mobile phase to suppress compound ionization or to control the degree of ionization of free unreacted silanol groups to reduce peak tailing and improve chromatography[34].

Eighteen compounds were initially identified from the GC-MS analysis, most of which contain nitrogen in their structure. The HPLC analysis, probe for the presence of

these 19 standard compounds in the leaf extract of *A. wilkesiana,* showed only five; Allylamine, benzamide, benzenesulfonamide, indolizine and quinolinamine. Allylamines is a primary unsaturated alkylamine, it is the active ingredient in naftifine and terbinafine used in the treatment of dermatophyte. Terbinafine has shown effectiveness against *Aspergillus*, *Microsporum, Trichophyton* and *Epidermophyton* species. The allylamines inhibit the enzyme squalene epoxidase, which catalyze the synthesis of ergosterol. This will lead to the accumulation in toxic level of squalene, thus contribute in no small measure to their potency against fungi[35]. Benzamide is the amide derivative of benzoic acid and an inhibitor of poly(ADP-ribose) polymerase-1. Benzamide mimics the structure of the nicotinamide portion of the coenzyme NAD^+, blocking the function of ADP-ribose transfer from NAD^+ to target histone proteins performed by PARP and impeding formation of the poly(ADP-ribose) polymer. PARP activation in response to DNA strand breaks can lead to deleterious depletion of cellular NAD^+, and Benzamide was shown to block NMDA- and NO-mediated neurotoxicity in rat brain through PARP inhibition. Benzamide is further demonstrated to partially prevent decreases in striatal levels of NAD^+ and ATP produced by neurotoxic amounts of kainic acid[36-39].

Conclusion

This study showed that the major phytochemicals identified from the leaf of *Acalypha wilkesiana* by GC-MS analysis and HPLC were alkaloids (quinolinamine and indolizine) and therefore suggesting that these phytochemicals may be responsible for its different ethno-medicinal uses.

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