Phytochemical screening, proximate analysis and Chromatographic analysis of methanol leaf extract of *Chromolaena odorata* (L.) M.King & H.Rob. (Asteraceae)

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ABSTRACT

Background: *Chromolaena odorata* is a scrambling perennial shrub with strong odour that have been reported to have polar phytochemicals.

Objectives: This study was carried out to evaluate the proximate parameter, phytochemical constituents and to identify the phytochemical constituents in *C. odorata.*

Methods: Proximate analysis and phytochemical screening were evaluated using established standard methods, while High Performance Liquid chromatography (HPLC) and gas chromatography-mass spectrometry were used for the chemical profiling of the plant.

Results: Phytochemical screening revealed alkaloids, flavonoids, glycosides, tannins, saponins, terpenoids and steroids. Proximate analysis showed moisture content 7.42±0.05 %, ash value 7.90±0.04 %, crude fibre content 19.98±0.02 %, crude fat content 23.40±0.02 %, crude protein content 2.30±0.02 % and carbohydrate content 47.20±0.03 %. Thirty eight compounds were identified, with 14 of which showed percentage area of 65.18 %, these compounds are Trimethylsilyl-3-methyl-4-[(trimethysilyl)oxy]benzoate (10.56 %), 1-Bromo-8-heptadecyne (9.58 %), 2-(2-tert-butylphenoxy)-N2-(2-nitrobenzylidene)-Acethydrazide (8.55 %), N,N-Dimethyl-2-propyn-1-amine (6.36 %), 1,2-Dimethyl-3,5,5-tri(2-cyanoethyl)piperid-4-one (5.25 %), 3-ethenyl-2-(3-pentenylidene)-N-phenyl-[1.alpha.,2Z(E), 3.alpha.]-cyclopentanecarboxamide (4.84 %), Methylene-propanedinitrile (4.14 %), Imidazo(1,5-a)pyrimidine (4.10 %), 3-Methyl-3-penten-1-yne (3.37 %), 2-1-phenylethylidene-hydrazono-3-methyl-2,3-dihydrobenzothiazole (3.19 %), 1,4-Pentadien-3-one (3.18 %), Benzotriazol-1-carboxylic acid, 3-oxide, ethyl ester (3.17 %), 1-Benzylindole (3.10 %) and Pent-2-ynal (3.07 %).

Conclusion: This study shows that the leaf of *Chromolaena odorata* is a rich source of non-polar phytochemicals that are responsible for the observed activities.

Keywords: *Chromolaena odorata*, phytochemicals, proximate analysis, Gas Chromatography-Mass Spectrometry, High Pressure Liquid Chromatography.

Criblage phytochimique, analyse proximale et analyse chromatographique de l'extrait foliaire au méthanol de *Chromolaena odorata* (L.) M.King & H.Rob . (Astéracées)

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RÉSUMÉ

Introduction: *Chromolaena odorata* est un arbuste vivace brouillant à forte odeur qui aurait des composés phytochimiques polaires.

Objectifs: Cette étude vise à évaluer le paramètre proximal, les constituants phytochimiques et à identifier les constituants phytochimiques dans *C. odorata.*

Méthodes: L'analyse proximale et le criblage phytochimique ont été évalués à l'aide de méthodes standard établies, tandis que la chromatographie liquide haute performance (HPLC) et la chromatographie en phase gazeuse-spectrométrie de masse ont été utilisées pour le profilage chimique de la plante.

Résultats: Le criblage phytochimique a révélé la présence d'alcaloïdes, de flavonoïdes, de glycosides, de tanins, de saponines, de terpénoïdes et de stéroïdes. L'analyse proximale a révélé une teneur en humidité de 7,42±0,05%, un taux de cendres de 7,90±0,04%, un taux de fibres brutes de 19,98±0,02 %, un taux de matières grasses brutes de 23,40±0,02%, un taux de protéines brutes de 2,30±0,02% et un taux de glucides de 47,20±0,03%. Trente-huit composés ont été identifiés, dont 14 ont montré un pourcentage de surface de 65,18% ; ces composés sont le triméthylsilyl-3-méthyl-4-[(triméthysilyl)oxy]benzoate (10,56 %), le 1-bromo-8-heptadécyne (9,58 %), 2-(2-tert-butylphénoxy)-N2-(2-nitrobenzylidène)-Acéthydrazide (8,55%), N,N-Diméthyl-2-propyn-1-amine (6,36%), 1,2-Diméthyl-3,5,5-tri(2-cyanoéthyl)pipérid-4-one (5,25%), 3-éthényl-2-(3-penténylidène)-N-phényl-[1.alpha.,2Z(E), 3 .alpha.]- cyclopentanecarboxamide (4,84%), méthylène-propanedinitrile (4,14%), imidazo (1,5-a)pyrimidine (4,10%), 3-méthyl-3-pentène-1-yne (3,37 %), 2 -1-phényléthylidène-hydrazono-3-méthyl-2,3-dihydrobenzothiazole (3,19 %), 1,4-pentadiène-3-one (3,18%), acide benzotriazole-1-carboxylique, 3-oxyde, ester éthylique (3,17%), 1-Benzylindole (3,10%) et Pent-2-ynal (3,07%).

Conclusion: Cette étude montre que la feuille de *Chromolaena odorata* est une riche source de substances phytochimiques apolaires responsables des activités observées.

Mots-clés: *Chromolaena odorata,* produits phytochimiques, analyse proximale, chromatographie en phase gazeuse-spectrométrie de masse, chromatographie liquide haute pression.

INTRODUCTION

Chromolaena odorata (L.) M.King & H.Rob (CO), belongs to the family Asteraceae, which is one of the largest family of the flowering plant, with about 900 genera and 13000 species.[1] It is widely distributed in Australia, Europe, North and South America, Asia and tropical Africa.[2] In Africa, it has been reported in Nigeria, Ghana and Ivory Coast. It is commonly called Siam weed, Christmas bush (English), Awolowo weed (Nigeria), with synonymy; *Eupatorium odoratum* Linn. CO is a scrambling perennial shrub with a lifespan not less than ten years. It grows to a height of 2.0 to 7.0 m with brittle stem, the leaves have the shape of an arrowhead measuring 6.0 to 12.0 cm long and width of 3.0 to 7.0 cm. They are oppositely arranged in pair on the stem or branches and exhibit a strong odour, which become pronounced after been crushed due to high level of poisonous nitrate.[3]

Ethno-medicinally, the leaves are used to stop bleeding, promote healing of soft tissue and burns, treat malaria when mixed with lemon grass and also used as cough remedy. They are applied to treat skin infection and rashes.[4,5] The anticancer, anti-diabetics, anti-parasitic, anticonvulsant, anti-hepatoxic, antioxidant, antimicrobial, anti-diarrhoeal, antispasmodic, diuretic, anti-inflammatory and anti-hypertensive potential have been reported for CO plant.[6-14] The leaf has been reported to have hemostatic activity and improve fertility in male.[15]

These activities ascribed to CO could be linked to the presence of phytochemicals in the leaf, stem or root. Phytochemicals are secondary metabolites that the plant produces as a result of harsh environmental conditions, to protect itself from predators. They range from flavonoids, alkaloids, saponins, terpenoids, flavonoids and glycosides. These groups of plant compounds though may not be necessary for the daily growth or maintenance of the plant but have shown different pharmacological potentials on experimental animals and human. Their presence could be determined by screening the plant using established standard methods described by Treas and Evans.[16] However, it is possible to use gas chromatography-mass spectrometry to identify the individual compounds in the plant extract by determining the retention time from the chromatogram and fragmentation pattern in the mass spectrometry, these are then compared with existing library standards.

Plants are untapped reservoir of medicinal compounds, which could be an immediate source of cheap and readily available medicine or serve as the small molecule on which subsequent discovery will be dependent upon. The use of these plants extract for medicine is believed to be free from untoward effects. Their stability are ensured by standardizing the powdered drugs following pulverization of the plant parts by using methods described in literature. A literature search showed that the GC-MS analysis of the ethanolic leaf extract of CO have been previously carried out, with the identification of mainly flavonoids and phenols but there is paucity of information on other phytochemicaly like alkaloids present in the leaf extract of CO. Thus, this study aimed to determine the phytochemical content of CO using standard methods and chromatographic analysis and the proximate parameter of the powdered leaf.

MATERIALS AND METHODS

Collection of plant, identification and extraction

The plant was collected in the month of January within the vicnity of Ugbowo Campus of the University of Benin and identified by Prof H. A. Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin. The sample was deposited in the Departmental herbarium and UBH-C496 was assigned as the herbarium sample number.

The leaves were careful plucked from the stem and dried under shade for three weeks before the leaves were pulverized (electric milling machine in the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin) to fine powder. 141 g of the powdered leaf was macerated in 1L of methanol (95 %) for seven days in well tight jar covered with foil paper to avoid direct exposure to sunlight. There after the extractive solvent was decanted and filtered with filter paper. The solvent obtained were evaporated using rotary evaporator at 4^{o}C in vacuum. The extract was then kept in the refrigerator at a temperature of 4^{o}C until when it is to be used.

Phytochemical screening

The powdered leaf was screened for phytochemicals by using standard methods described by Sofowora[17] and Trease and Evans.[16] The methods involved the screening for alkaloids, tannins, glycosides, terpenoids, flavonoids, steroids and saponins.

Proximate analysis

Proximate profile of the leaf of *Chromolaena odorata* was evaluated by using methods described by the Association of Official Analyst Chemist (AOAC). The parameters evaluated were moisture content, crude fat content, crude fibre content, carbohydrate content, ash content and crude protein content.[18]

***Chromolaena odorata* leaf extract preparation for GC-MS analysis**

Fifty milligram of the methanol extract was dissolved in 5.0 mL of solvent mix (1:1 n-hexane: dichloromethane). This mixture was adsorbed to silica gel (mesh size 100-200 mm) by trituration using piste and mortar. The dried adsorbed extract-silica was carefully placed on an already packed column containing silica and 3 g of anhydrous sodium sulfate was placed on the adsorbed-silica in the well-packed column. N-hexane (3X10 ml) was then passed through this set-up isocratically and the eluent obtained were bulk together and evaporated using rotary evaporator *in-vacuum* at 40^{o}C to 2 mL. This was then used for GC-MS analysis.[19]

GC-MS Analysis

An Agilent 6890N gas chromatography equipped with an autosampler connected to an Agilent Mass Spectrometric Detector was used. Then 1 µL of the concentrated eluent was injected through the port into the GC-MS, 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. Helium gas was used as a carrier gas, and the column head pressure was maintained at 20 psi to give a constant flow rate of 1 mL/min. Other operating conditions were preset. The column temperature was initially held at 55^{o}C for 0.4 min, increased to 200^{o}C at a rate of 25^{o}C/mins, then to 280^{o}C at a rate of 8^{o}C/mins and to a final temperature of 300^{o}C at a rate of 25^{o}C/mins, held for 2 mins. The data obtained were compared with the National Institute of Science and Technology (NIST) library data.

Preparation of stock and working standards for HPLC analysis

The stock solution of all standards ((Quinolinamine, Benzenesulfonamide, Allylamine, Benzamide, Indolizine, Pyrazoline, Imidazole, Propargylamine, Ethylenimine, Difluoramine, Isoxazolidine, Simulansamide, Colchicine, Norethindrone, Androstane, Methanamine, Isoxazolidine, Isobutylamine, and Amphetamine), 1,000 µg/mL each, were prepared in methanol and stored at 4^{o}C when not in use.

Sample preparation for HPLC analysis

A 200-mg portion of the samples were homogenized, then mixed with 200 mL deionized water. The mixture was refluxed for 1 hour with continuous stirring, then cooled to room temperature. The extracts were filtered using a Whatman filter paper (125 mm diameter) and diluted to 1:3 (v/v) with 2% ammonia solution. The pH was adjusted to approximately 7 with 0.01 M hydrochloric acid, and then analysis was conducted using HPLC.

HPLC Analysis of the methanol extract of *Chromolaena odorata:* The methanol extract of CO was analyzed using a chromatographic system (Agilent Technologies Deutschland GmbH, Boblingen, Germany) consist of autosampler (1100TC) with 100 µl fixed loop and UV-Visible detector. The separation was performed on an Agilent Lichrospher 100-5RP8 (250 x 4.6 mm, 5µm) column at 45±1^{o}C. The mobile phase consists of methanol to phosphoric acid (0.1 %) and the separations were performed by using isocratic mode, elution performed at a flow rate of 1 ml/min. The samples were run for 9 min. and detection was done at 254 nm by UV detector. Data acquisition and integration were performed by the Analyst 1.5 software.

Separation and clean-up

A 5 µL of 80 µg/mL diluted mixed stock solution was injected into the HPLC and the separation of the peaks optimized. All stock standards were monitored at 242 nm. The HPLC conditions are as summarized in the Table 1 below.

Table 1: HPLC Conditions

Parameter	Condition			
Column	Agilent Lichrospher 100-5RP8 (250 x 4.6 mm) (C18)			
Flow rate	1.00 ml/min			
Injection volume	5µL			
Column temperature	35oC			
Mobile phase A	0.1 % phosphoric acid			
Mobile phase B	Methanol			
Run time	9 min			
Gradient	Time	0	2.5	6
	% B	25	25	50

Result and discussion

Phytochemical qualitative screening showed the presence of alkaloids, flavonoids, glycosides, saponins, steroids, tannins and terpenoids. The presence of these phytochemicals has been reported in previous studies.[20-21] Flavonoids have been reported to facilitate wound healing by either antimicrobial or antioxidant potentials through the inhibition of lipid per-oxidation which prevent cell damage and improve the viability of collagen fibril.[22] Alkaloids, terpenoids and steroids have been reported with antimalarial property, these phytochemicals form diverse group of compounds with different mechanism of action.[23] Tannins are known to prevent activation of platelet and formation of thrombus while saponins inhibit oxidative damage of cell due to its antioxidant potential.[24]

Table 1: Phytochemical screening of the leaf of *Chromolaena odorata*

Phytochemicals	Inference
Alkaloids	+
saponins	+
flavoniods	+
Terpinoid	+
Glycosides	+
Tannins	+
Steriods	+

Present = +, Absent = -

Analysis of the powdered leaf of CO for moisture and ash contents, crude fibre, fats, proteins and carbohydrate contents gave values of 7.42±0.05 %, 7.90±0.04 %, 19.98±0.02 %, 23.40±0.02 %, 2.30±0.02 % and 47.20±0.03 %, these values were similar to values obtained from previous study[25] while study carried out later showed significant difference in these values[26]. These difference could be due to varying geographical locations that the plant was collected. Moisture content gives an indication of the water content in the leaf and its susceptibility to microbial degradation, thus is an indication of its stability and how long its shelf life will be. The value obtained for the moisture content for the leaf of CO indicates that the water content is within the acceptable range of 10.00 %.[27] Ash content is essential parameter for determining the nutritional value, quality and stability of the powdered leaf of CO. It refers to the minerals and inorganic components of the powdered leaf of CO left after heating to very high temperature (600^{o}C). The process of heating removes the volatile, organic and moisture components in the powdered leaf leaving inorganic components, such as calcium, magnesium, sodium and potassium. The ash content (7.90±0.04 %) obtained was considered to be moderate and implying that the leaf of CO contains inorganic components that may be consider important though the particular minerals were not determined. Some of the biological activities ascribed to CO could be due to the presence of these minerals in the powdered leaf. Different minerals have shown activity such as immune booster (Selenium and Zinc), Haemoglobin producer (Iron) and promoter of metabolic process (Manganese). The fibre content in leaf sample promotes the palatability and enhance digestion in animal feed production. The value obtained in this study

Table 2 Proximate analysis of the leaf of *Chromolaena odorata*

Proximate analysis	Value (%)
Moisture content	7.42±0.05
Ash content	7.90±0.04
Crude fibre content	19.98±0.02
Crude fat content	23.40±0.02
Crude protein content	2.30±0.02
Carbohydrate content	47.20±0.03

following the profiling of the methanolic extract of CO, 38 compounds were identified (Table 3). Fourteen of the identified compounds showed percentage area of 65.18 % these compounds are Trimethylsilyl-3-methyl-4-[(trimethysilyl)oxy]benzoate (10.56 %), 1-Bromo-8-heptadecyne (9.58 %), 2-(2-tert-butylphenoxy)-N2-(2-nitrobenzylidene)-Acethydrazide (8.55 %), N,N-Dimethyl-2-proyn-1-amine (6.36 %), 1,2-Dimethyl-3,5,5-tri(2-cyanoethyl)piperid-4-one (5.25 %), 3-ethenyl-2-(3-pentenylidene)-N-phenyl-[1.alpha.,2Z(E), 3.alpha.]-cyclopentanecarboxamide (4.84 %), Methylene-propanedinitrile (4.14 %), Imidazo(1,5-a)pyrimidine (4.10 %), 3-Methyl-3-penten-1-yne (3.37 %), 2-1-phenylethylidene-hydrazono-3-methyl-2,3-dihydrobenzothiazole (3.19 %), 1,4-Pentadien-3-one (3.18 %), Benzotriazol-1-carboxylic acid, 3-oxide, ethyl ester (3.17 %), 1-Benzylindole (3.10 %), Pent-2-ynal (3.07 %), they showed percentage area above 3.00 % in the extract of CA.

Table 3: GC-MS analysis of the leaf extract of *Chromolaena odorata*

S/N	Retention Time	% Are a	Compound
1	3.213	2.11	Aminoacetonitrile
2	3.453	0.34	Propargylamine
3	8.088	6.36	N,N-Dimethyl-2-propyn-1-amine
4	15.092	0.15	Isoxazolidine
5	16.694	0.73	Trifluoroethene
6	16.923	1.42	Methanesulfinylfluoride
7	17.152	1.40	Ethyleniminoacetonitryl
8	17.438	3.18	1,4-Pentadien-3-one
9	17.615	3.07	Pent-2-ynal
10	17.730	0.37	Difluoramine
11	18.674	0.51	N-Ethylformamide
12	19.035	0.14	Methylguanidine
13	19.435	0.07	O-Methyl-N-methylcarbamate
14	19.595	0.08	Propanenitrile
15	21.661	0.29	(aminooxy)-Acetic acid
16	22.994	0.24	1-Methyl-3,5,-dinitro-1H[1,2,4]triazole
17	23.767	0.34	2-fluoro-Acetamide
18	23.990	4.14	Methylene-propanedinitrile
19	24.127	1.07	2-propenenitrile
20	24.997	0.51	3-chloropropanenitrile
21	25.214	1.69	1-Methyl-1H-pyrrole
22	25.472	3.37	3-Methyl-3-penten-1-yne
23	25.655	2.29	1-Buten-3-yne
24	25.815	2.53	4-Methyl-Benzaldehyde-oxime
25	26.055	4.10	Imidazo(1,5-a)pyrimidine
26	26.136	3.19	2-1-phenylethylidene-hydrazono-3-methyl-2,3-dihydrobenzothiazole
27	26.347	3.17	Benzotriazol-1-carboxylic acid, 3-oxide, ethyl ester
28	26.530	3.10	1-Benzylindole
29	26.696	10.56	Trimethylsilyl-3-methyl-4-[(trimethysilyl)oxy]benzoate
30	27.137	0.59	2-ethyl-2H-Benzotriazole
31	27.509	9.58	1-Bromo-8-heptadecyne
32	27.578	4.84	3-ethenyl-2-(3-pentenylidene)-N-phenyl-[1.alpha.,2Z(E),3.alpha.]-cyclopentanecarboxamide
33	27.766	1.91	(2-Methoxyphenyl)acetonitrile
34	28.007	1.23	2-(2H-tetrazol-2yl)Acetonitrile
35	28.390	2.75	MDMA methylene homolog
36	28.688	1.53	4-[(4-fluorophenyl)imino]-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-one
37	28.768	5.25	1,2-Dimethyl-3,5,5-tri(2-cyanoethyl)piperid-4-one
38	29.065	8.55	2-(2-tert-butylphenoxy)-N2-(2-nitrobenzylidene)-Acethydrazide

The compounds identify have been reported to possess different biological activities. Amino-acetonitrile derivatives (AADs) were discovered to express anthelmintic activity. These derivatives interfere with a unique ACR-23 nicotinic acetylcholine receptor subunit[28]. Propargylamine derivatives such as pargyline, rasagiline and selegiline are used against neurodegenerative disorders like Parkinson's and Alzheimer's diseases (2-5). (–)-Deprenyl (selegiline) is found to have an antiapoptotic function,[29] which makes it useful for symptomatic and neuroprotective treatment. N,N-Dimethyl-2-propyn-1-amine is used in the treatment of Trypanosoma cruzi infection.[30] Replacement of the furanose ring of nucleoside with isoxazolidine and isooxazoline produces a modified nucleoside with anticancer and antiviral application.[31] Moieties bearing the aforementioned nucleus were reported to possess important biological activities like; anticancer, antiviral, anti-inflammatory, antibacterial or antifungal activity.[32] Trifluoroethane: Its derivative, halothane is a potent inhalational anaesthetic.[33] Methanesulfinylfluoride is an irreversible Cholinesterase inhibitor and has been proposed for Alzheimer's therapy.[34] Ethyleneiminoacetonitryl is an amino-acetonitrile derivative and it is possessed antihelminthic properties. 1,4-Pentadien-3-one is a curcumin derivative, which has broad-spectrum biological activities such as antibacterial,[35-36] anti-inflammatory,[37] antiviral,[38] anticancer,[39] antioxidant,[40] and insecticidal.[41]

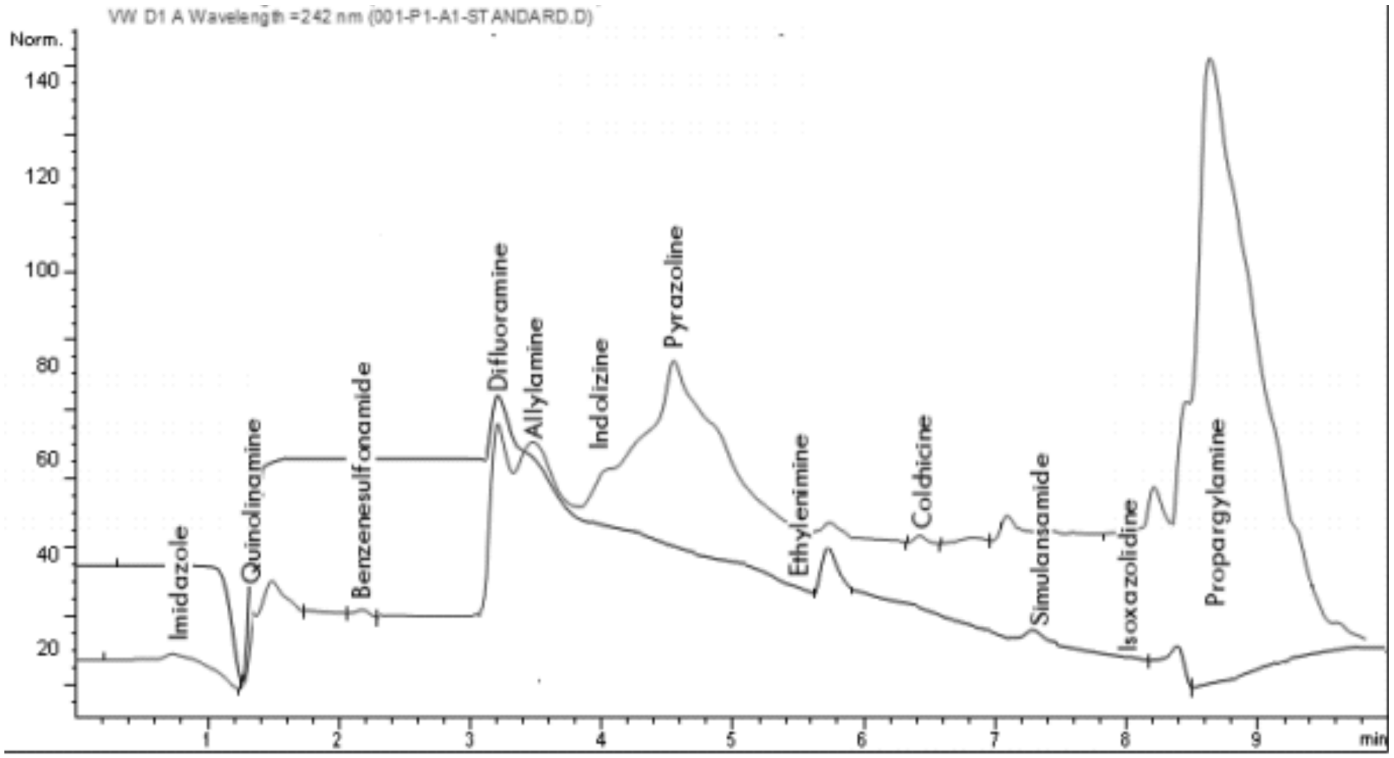

Figure 2: HPLC chromatogram of the methanol leaf extract of *chromolaena odorata.*

From figure 2 shown above, the red plot indicate the standard compounds used in the study, while the blue plot indicate the compounds in methanol extract of CO. The HPLC analysis revealed four compounds of the nineteen standards used in the probe. Simulansamide (6.032 ng/µl), difluoramine (14.975 ng/µl), isoxazolidine (8.966 ng/µl) and ethylenimine (12.483 ng/µl) respectively. Most of these compounds have nitrogen in their molecules and thus linked to the presence of alkaloids in CO leaf extract. Alkaloids are linked to some of ready mentions activities and these an been linked to some of the ethnomedicinal usage of CO. Simulansamide has been reportedly isolated from Zanthoxylum simulans, this compound shows strong inhibition of platelet aggregation.[32] Isoxazolidine derivatives have been reported with activity against species of Aspergillus, Fusarium and Botrydiplodia.[33] Prevention of platelet aggregation and antifungal activities are some of the ethnomedicinal uses of chromolaena odorata leaf. These compounds can be said to be responsible for the ascribed activity of the CO.

Table 4: HPLC analysis of the leaf of *Chromolaena odorata*

S/N	Retention Time (min.)	Amount (ng/µl)	Name
1	3.197	14.975	Difluoramine
2	5.626	12.483	Ethylenimine
3	2.170	6.032	Simulansamide
4	3.252	8.966	Isoxazolidine

CONCLUSION

The methanol leaf extract of *Chromolaena odorata* is rich in phytochemicals with different pharmacological properties. Four of such compounds were identified (Simulansamide, difluoramine, isoxazolidine and ethylenimine) with the aid of the HPLC and the ethnomedicinal usage of *Chromolaena odorata* was verified.

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