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Chemical constituent, antibacterial and antioxidant activity of crude extract and oil fraction of *L. abyssinica*

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Abstract

Theantibacterial and antioxidant activitiesof CH3Cl/MeOH crude extract and a nonpolar fraction from the $CHCl_3$ phase of *Ludwigia abyssinica*were investigated using broth dilution method and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay respectively.

Chemical composition was determined by gas chromatography coupled with mass spectrometry using Agilent Technologies 7890A GC system-Agilent Technologies 5975C inert XL EI/CI MSD. Bioassays show that *L. abyssinica*is active on the different test organisms with the MIC ranging from 0.5 to 1.5 mg/ml for the crude extract and 0.2 to 0.5 mg/ml for the non polar oil fraction. The oil extract of *L. abyssinica* exhibited a good antioxidant activity with radical scavenging activity (IC_{50}) = 89.18 µg/ml which is about 1.5 times the antioxidant activity of ascorbic acid used as control,while that of crude extract was 413.74 µg/ml. From the gas chromatography coupled with mass spectrometry analyses, 12 hydrocarbons were identifiedwith octadecane (5.67%), pentadecane(6.87%) andsqualene (57.18%) the major components.These results offer a platform of using *L. abyssinica*for alternative and complementary medicine.

Keywords: *Ludwigia abyssinica*, GC-MS, Column chromatography, antioxidant, antibacterial.

Introduction

In recent years, infectious diseases have increased to a great extent and still represent an important cause of morbidity and mortality among humans, especially in developing countries.[1] Even though pharmacological industries produced a number of new antibiotics in the last three decades,resistance to these drugs by microorganisms has increased and support the continuous search for alternative drugs. Natural productseither from plants or animalshave been a source of medicinal agents against infectious diseases.Infectious diseases, especially of bacterial origin, excite human immune cells whose action leads to an overproduction of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions, that reactwith the living tissues and cause harmful effects (such as lipid peroxidation, DNA fragmentation)which will result in a situation know as oxidative stress [2] diseases has attracted much attention of scientists and the general public [3].These antioxidants could also attenuate oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radical species.Synthetic antioxidants, such as butylatedhydroxyanisole (BHA), butylated hydroxyl toluene (BHT) and tert-butylhydroquinone (TBHQ) are commercially available and are currently in use. However, their use is now restricted due to their toxic effects [4,5].The plant kingdom beinga prolific source of a wide range of natural antioxidants,and since oxidative stress is mostly of a bacterial related disorder, it is important to develop alternative antioxidants with antibacterial activity from this source.

Ludwigiaabyssinica(Onagraceae) is a flowering plant belonging to the order Myrtales that comprises 21 genera well distributed in the tropical regions of the world [6]. In the West region of Cameroon, *L. abyssinica*is used in traditional medicine to cure many diseases among which microfilaria, malaria, urinogenital and gastrointestinal tract infections.

Previous works show that the leaves of *Ludwigia abyssinica*are used as vegetables in Nigeria, while those of *L. adscendens* are fed to livestock in Mali [7]. The seeds of *Ludwigia* species are very rich in oil [8] (Burkill, 1997). Its roots are used medicinally in infusions for treating syphilis and poulticing pimples, while a

decoction is used against diarrhoea, dysentery, flatulence, leucorrhoea and as vermifuge[9]. Although the *in vitro*antbacterial activity and oil content of *L. abyssinica* was established respectively by Oyodeji et al.[7] andBurkill [8], there is, however to the best of our knowledge, no scientific information regarding either this *in vitro* antibacterial activity in term of MIC and MBC or its antioxidant activity or the chemical composition of this oil.The present study was therefore undertaken to evaluate the *in vitro*antibacterial and antioxidant activity of the crude extract and nonpolar fraction from *L. abyssinica*aerial parts.This study also seeks to determine the chemical composition of this oily fraction by GC-MS analyses.

Methodology

Plant material

L. abyssinica (aerial part) was collected at the vegetative stage in the month of August 2011 in Fungou village, Noun Division, West Region of Cameroon. The taxonomic identification of the plant was authenticated by the Cameroon National Herbarium (CNH) where the voucher specimen (No. 27093/SRFC) is deposited.

Bacteria species and growth conditions

In this study five bacterial strains and five isolates including two Grampositive bacteria- Staphylococcus aureusATCC25922 and*Enterococusfeacalis*; and eight Gram negative bacteria-Escherichia coli ATCC11775, Klebsiella pneumonia ATCC13883, Pseudomonas aeruginosaATCC27853, Salmonella typhi ATCC6539,S. paratyphi A and S. paratyphi B, *S. typhimurium* andProteus mirabilis were used. The referencestrains (ATCC) were obtained from American TypeCulture Collection (Rockville, USA). The clinicalbacterial isolates were collected from "Centre Pasteur"(a national public health reference laboratory-Yaoundé, Cameroon). The bacteria were grown at 35 C andmaintained on nutrient agar (NA, Conda, Madrid, Spain).

Preparation of the plant extract

*L. abyssinica*was harvested and dried in a ventilated room, at room temperature and ground into powder. 1000 grams of the powder was infused in CH3CL/MeOH(1:1, v/v). The mixture was stirredevery day for 3 consecutive daysat room temperature (21±2 C) using a magnetic stirrer type IKA-MAG RCT. The homogenate obtained was subsequently filtered through Whatman paper filter No. 1, and the extract was obtained by complete evaporation of solvent with rotary evaporator at 45 C. 240 vacuum.

Column chromatography

The CH_3Cl/MeOHextract was partitioned in $CHCl_3$:H_2O (3:1) and the $CHCl_3$phase (29g) wascolumn chromatographed using glass column (40 cm 11 mm) and silica gel (0.032-0.063 mesh) (Sigma) as the stationary phases.99:1 hexane/ethyl acetate, 97.5:2.5 hexane/ethyl acetate and 95:5 hexane/ethyl acetatewere used as elution solvents.

Thin layer chromatography (TLC)was used to determine the profile of different fractions.Each fractionwas dissolved in absolute hexane and spotted on analytical TLC (silica gel G_{600}, 0.25 mm thickness)as the stationary phase. The following solvent systems and ratios wereused as mobile phase hexane/ethyl acetate 99:1;97.5**:** 2.5and 95:5.After air-drying, plates weresprayed with 2% sulfuric acid in methanol and fractions with similar bandprofiles were combined.

GC–MS analysis

The GC–MS analysis was carried out using Agilent Technologies 7890A GC system coupled to a mass detector Agilent Technologies 5975C inert XL EI/CI MSD with Triple-axis detector. The following conditions were respected for (GC-MS) analyses: column Elite-1 fused silica capillary column (30 0.25 mm ID 1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV. Helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 200 C; ion-source temperature 280 C. The oven temperature was programmed from 80 C (isothermal for 2 min), with an increase of 10 C/min, to 200 C/min, then 5 C/min to 280 C/min, and ending with a 9 min isothermal at 280 C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s.

Antibacterial assay

The broth micro-dilution method was used for susceptibility testing of bacteria species. The tests were carried out in 96-micro well sterile plates as described by Mativandlela et al.[10] The crude extract was dissolved in 5% dimethylsulfoxide (DMSO) solution and diluted with Mueller-Hinton broth(Conda) to obtain 24 mg/ml initial concentration, while the fraction was dissolved in 10% DMSO and its initial solution was prepared at 10 mg/ml. Serial two-fold dilutions of each extract were made with Mueller-Hinton broth to yield a volume of 100 µl/well. One hundred microliters of each bacterial suspension (containing about 3.0 10^6 CFU/ml) was added to respective wells containing the test substances and mixed thoroughly to give final concentrations ranging from 6 to 0.023 mg/ml and from 2.5 to 0.009 mg/ml for the crude extract and fraction respectively. Ciprofloxacin at a concentration range of 12.50 to 0.024 µg/mL was used as standard antibiotic. The cultured microplates were covered and incubated at 37 C for 24 h. The tests were carried out in triplicate. Inhibitory concentrations of the extracts were detected after addition of 40 µl of 0.2 mg/ml Thiazolyl Blue Tetrazolium Bromide (TBTB) (Sigma–Aldrich, South Africa) in two of the three well of each concentration and incubation at 37 C for 30 min. Viable bacteria change the colorless dye of TBTB to a blue color. All concentrations at which no visible color change was observed were considered as inhibitory concentrations and the lowest of these concentrations was considered as the minimal inhibitory concentration (MIC). The bactericidal concentrations

were determined by adding 50 µl aliquots from the third well (without TBTB), which did not show any visible color change after incubation during the MIC assay, into 150 µl of extract-free Mueller-Hinton broth. These preparations were further incubated at 37 C for 48 h and bacterial growth was revealed by the addition of TBTB as above. All extract concentrations at which no color change was observed were considered as bactericidal concentrations. The minimal bactericidal concentration (MBC) was recorded as the lowest of these concentrations.

Antioxidant activity

The free radical scavenging activity of the crude extract and fraction from *L. abyssinica*was measured by using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay.This assay was performed according to the procedure described by Obeid et al.[11]. DPPH solution was prepared by dissolving 3.92 mg in 100 ml of 90:10 methanol:dichloromethane.The different tests concentrationswere 2000, 1000, 750, 500, 250, 125, 62.5 and 31.25 µg/ml (for crude extract andfraction). Each test sample was added separately to DPPH solution in a test tube andwell shaken and incubated in dark at room temperature for 10 min. The absorbance was measured at 517 nm by using spectrophotometer (Shimazu). L-ascorbic acid was used as positive control. Each test was performed in triplicates and the percentage of radical scavengingactivity (% Rsa) was measured according to formula:

$$\%\ ofradicalscavenging = \frac{(ODreactionmixture - OD\ test\ sample}{ODDPPHsolution} X100$$

and Rsa_{50}values were calculated by graphical method.

Identification of phytocompounds

The components of the fraction were identified based on the comparison of their retention indices and mass spectra with the standards, using Adams library data-base.Only component which quality was greater than or equal to 80% were considered. The name, molecular weight and structureof the components of the test materials were ascertained.

Result

Chemical composition of the oils fraction of *L. abyssinica*

GC-MS analyses yielded twelve prominent peaks with retention time (Figure 1) 19.99, 21.15, 22.14, 23.15, 23.37, 24.11, 25.05, 25.41, 25.95, 26.80, 27, 63, 31.15. These peaks were identified and corresponded respectively to Hexadecane (2.40%), Tetracosane (4.01%), Octadecane (5.67%), Nonadecane (4.66%), Pentacosane (1.33%), Pentadecane (6.87%) Heneicosane (4.42%), 1-Methylcycloheptanol (1.98%), Docosane (4.97%), Tricosane (2.26%), Tetracosane (2.64%) and Squalene (57.13%) as given in Table 1.

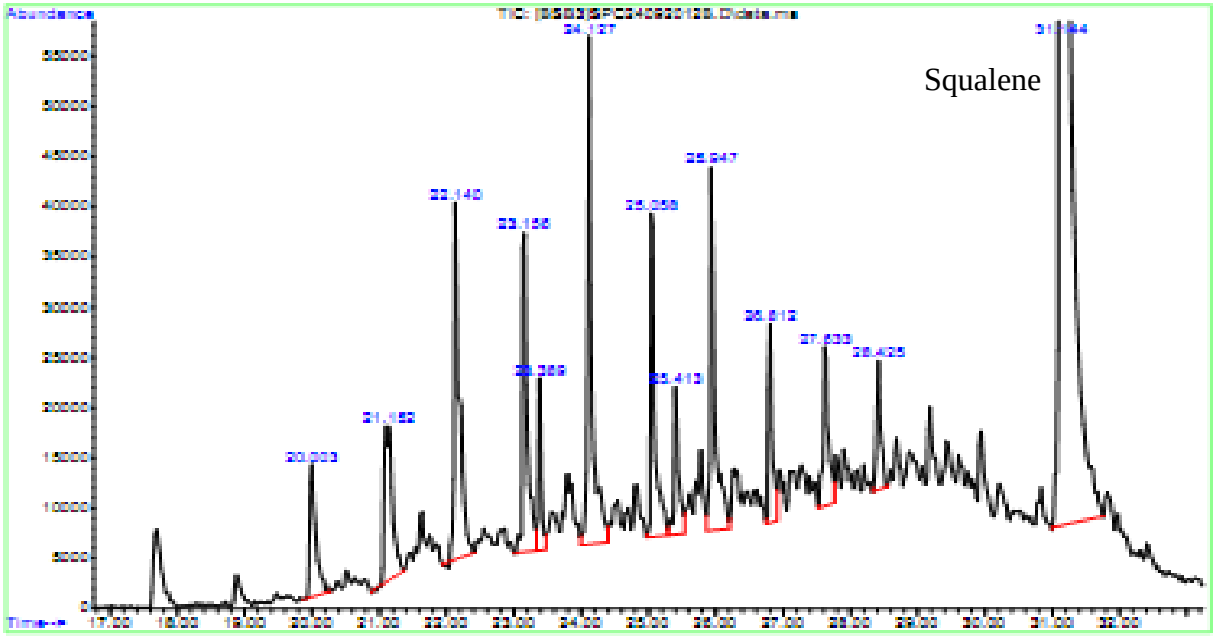

Figure 1: GC-MS chromatogram of the non polar fraction of *L. abyssinica*

Table 1: Chemical composition of non polar fraction of *L. abyssinica* by GC/MS

Peak	RT(Min)	Component	(%)	Structure
1	19.99	Hexadecane	2.40	$C_{16}H_{34}$
2	21.15	Tetracosane	4.01	$C_{24}H_{50}$
3	22.14	Octadecane	5.67	$C_{18}H_{38}$
4	23.15	Nonadecane	4.66	$C_{19}H_{40}$
5	23.37	Pentacosane	1.33	$C_{25}H_{52}$
6	24.11	Pentadecane	6.87	$C_{15}H_{34}$
7	25.05	Heneicosane	4.42	$C_{21}H_{44}$
8	25.41	1-Methylcycloheptanol	1.98	$C_{18}H_{16}O$
9	25.95	Docosane	4.97	$C_{22}H_{46}$
10	26.80	Tricosane	2.26	$C_{23}H_{48}$
11	27.63	Tetracosane	2.64	$C_{24}H_{50}$
12	31.15	Squalene	57.13	$C_{30}H_{50}$
Total			98.34	

Antibacterial activity

The *in vitro* antibacterial results (Table 2) revealed that the growth of all tested microorganisms was affected following exposure to the plant extract. This activityvariedacross thedifferentbacterial strains with MICs values ranging from 0.4 to 1.5 mg/ml for the crude extract and from 0.1 to 0.3 mg/ml for the non polar fraction.From this Table, *Escherichia coli*, *Enterococusfecalis*, *Pseudomonas aeruginosa* and*Klebsiella pneumonia* canbeconsidered asthemostsusceptiblestrains to theextractsof *L. abyssinica*since they had the lowestMIC (0.1mg/ml).

Table: 2 MIC and MBC (mg/ml) of test samples on different test bacteria

	Study parameters	Bacteria								
		E.c	**E.f**	**K.p**	**P.a**	**P.m**	**S.a**	**S.p$_A$**	**S.p$_B$**	**S.t**
Crude Extract	MIC	1.5	0.4	0.4	0.4	1.5	1.5	1.5	1.5	1.5
	MBC	6.0	3.0	3.0	6	>12	>12	12	6.0	12.0
	MBC/MIC	4	7.5	7.5	15	/	/	8.0	4.0	8.0
No polar Fraction	MIC	0.1	0.1	0.3	0.1	0.1	0.2	0.2	0.2	0.3
	MBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	MBC/MIC	5	5	2	5	5	3	3	3	2
Cypro-floxacin	CMI ($x10^{-3}$)	2	2	4	6	4	4	6	6	4
	CMB($x10^{-3}$)	4	4	6	12	8	12	24	24	12
	MBC/MIC	2	2	1.5	2	2	3	4	4	3

E.c: *Escherichia coli*; ***E.f:*** *Enterococusfecalis*; ***P.a***: *Pseudomonas aeruginosa*; ***K.p***: *Klepsiellapneumoniae*; ***S.a:****Staphyloccocusaureus,* ***P.m****: Protus mirabilis* ***S.p*A**: *Salmonella paratiphy*A, ***S.p*B**: *Salmonella paratiphy*B, ***St*'**: *Salmonella tiphy,* MIC : Minimal Inhibitory Concentration, MBC: Minimal Bactericidal Concentration.

Antioxidant activity

The results of the DPPH radical scavenging activity of the crude extract and fraction of *L. abyssinica*(Table 3) were obtained from the probit of radical scavenging percentageversus logarithm of concentration.The crude extract (IC_{50} = 141.78 µg/ml) was less active compared to its fraction (IC_{50} = 89.18 µg/ml).

Table 3: Antioxidant activity of Ludwigia abyssinica crud extrat and oily fraction and ascorbic acid.

Test Material	*L. abyssinica*		*Fraction*		Acideascorbique		
log conc	% inhibition	probit	% inhibition	probit	% inhibition	probit	log conc
3,3	94	6.56			97	6.88	2.3
3	86	6.08	99	7.33	85	6.04	2.17
2,87	75	5.67	91	6.34	77	5.74	2
2,69	63	5.33	75	5.67	55	5.38	1.87
2,39	57	5.18	54	5.1	33	4.56	1.69
2,09	49	4.97	42	4.8	7	3.59	1.39
1,79	40	4.75	20	4.16	3	3.12	1.09
IC_{50} (µg/ml)	141.78		89.18		59.31		

Discussion

In this study, we evaluated the antibacterial and antioxidant activity CH_3Cl/MeOH extract and oil fraction of *L. abyssinica*, on five bacteria strains and five bacterial isolates commonly found in urogenital and gastrointestinal tract infections. GC-MS analyses was performed to determine the different compounds present in the oil fraction from the leaves of these plants. Analyses of the different results shows that, compared to negative controls (5% and 10% DMSO), the extracts of *L. abyssinica* provoked a concentration-dependent decrease in the number of colonies of these bacteria in experimental tubes. The difference in activity observed may be either due to the constitutional or structural variability of the tested germs, or the difference in the biochemical composition of genetic transferable resistance elements between strains such as plasmids [12]. These results corroborate the work of Takeo et al. [13]who suggested that there are differences in the mechanism of action of active ingredients on bacterial isolates. Some may act at the cell wall at the level of membrane components, or may enter the cytoplasm where they would take the organelles or interfere with biochemical processes important for the microorganism life [14]. Antimicrobial substances are considered as bactericidal agents when MBC/MIC 4 and bacteriostatic when MBC/MIC > 4 [15]. The MBC/MIC ratios of the crude extract were 4 indicating the bacteriostatic nature of the tested samples on the different test bacteria, while that of fraction is bacteriostatic and bactericidal, depending on the germ. Moreover, these extracts showed activity against both Gram-negative and Gram-positive, indicating that the active principle(s) of the crude extract and oil fraction may be broad spectrum antibacterial(s).

The hexane fractions are known to contain essential compounds like essential oils[12]. Twelve compounds were identified in the essential oil amount which squalene (57.13%) was identified as the major component. Several studies have demonstrated that essential oils hold therapeutic value in the treatment of diseases and are well tolerated [16, 17]. The nonpolarfraction in our study was 15 times more active than the crude extract on the testedmicroorganisms. This shows that fractionation might have resulted to the concentration of the active principles in this fraction.

The activity of this fraction may be due to the presence of squalene or its synergetic effect with other compounds identified by GC/MS. Indeed antimicrobial activity of squalene has been shown by Senthilkumar et al. [18]. Moreover, hexadecane and octadecanepresent in the fractions had been shown to contribute to inhibitory effects on bacterial growth[19, 20], while some monoterpenes have been shown to possessantimycobacterial effect at lower concentrations [21].The lipophilic character of their hydrocarbon skeleton are of main importance in the antimicrobial action of essential oil components [22]. These compounds might act by disturbing the lipid fraction of the plasma membrane of the test bacteria, resulting in alterations of membrane permeability and leakage of intracellular materials [23]. In other to determine whether the antimicrobial effect is due to crude extract/fraction or DMSO, we tested 5% and 10% DMSO on both bacteria. The antibacterial effect was found to be due to the fraction and crude extract as DMSO did not exhibit any bacterial growth.

Many compounds structurally similar to squalene exist in nature and perform critical biological functions. Carotene is well known to be a potent $^1O_2$ quencher and unique free radical scavenger [24]. Coenzym Q10 is an important lipophilic antioxidant used in many skin care products to protect the skin from free radical damage [25] (Bando et al., 2004). Squalene and some of its related substances, including vitamin A, were shown to protected skin from sun light oxidation [26]. The high antioxidant activity of the fraction from *L. abyssinica* leaves might be principally due to squalene since it constituted more than 50% of its total contain even though the other components could have synergistic activity.

Conclusion

The results obtained from this study reveal that the aerial parts of *L. abyssinica* may be useful in the development of an antimicrobial phytomedicine which can be standardised using the oily fraction. However, further studies are needed to explore the efficiency of suitable concentration and to confirm the safety of this plant product.

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