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Chemical Composition and *in vitro* Antibacterial Activity of the Essential Oils of the Leaves, Resin and Stem-Barks of *Dacryodes edulis* (G. Don) H. J Lam growing in Cameroon on Diarrhea Associated Strains

Sara Honorine RIWOM[1], Florentine M-C NDOYE FOE[2*], Maximilienne Ascension NYEGUE [1,2,3], Reskingjoe AMBE WANKI[2], Steve VOUNDI Olugu[1], François-Xavier ETOA[1]

[1]University of Yaoundé I, Department of Microbiology, Laboratory of Microbiology, PO Box 812 Yaoundé –Cameroon. [2]University of Yaoundé I, Department of Biochemistry, Laboratory of Phytobiochemistry and Medicinal Plant Study, PO Box 812 Yaoundé- Cameroon. [3]Equipe de Recherche "Glyco et nanovecteurs pour le criblage" Institut des Biomolécules Max Mousseron (IBMM), Faculté de Pharmacie, 15 avenue Charles Flahault, Bâtiment E 2eme étage, BP 14491 34093 Montpellier-France.

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ABSTRACT

The aim of this work was to determine the chemical profile and assess *in vitro* the antibacterial activity of the leaves, resin and stem-barks of *Dacryodes edulis*. The essential oils were analyzed simultaneously by Gas Chromatography and Gas Chromatography coupled to Mass Spectrometry. Agar diffusion well and microdilution methods were used to assay the antibacterial activity. The resin essential oil contained *p*-cymene (30.32%), *α*-thujene (28.58%), *α*-phellandrene (27.14%) and *β*-phellandrene (10.16%) as the main components; the stem-barks essential oil had as abundant components *p*-cymene (35.14%), *trans*-carveol (22.60%), *α*-thujene (14.86%), *β*-phellandrene (8.65%) and *β*-elemene (5.22%). The leaves essential oil was distinct with elemol (29.22%), caryophyllene oxide (15.26%), *trans*-carveol (11.80%) and spathulenol (6.28%) as major components. The leaves essential oil was the most active with MIC and MBC value of 18.75 mg/mL on *B. cereus*; the most susceptible strain. The stem-barks essential oil had a MIC of 50 mg/mL and MBC of 100 mg/mL on all the strains meanwhile the resin essential oil had a bacteriostatic effect at 200 mg/mL. Based on these results, it emanates that the essential oils of *D. edulis* represent a potential source of antibacterial substances.

INTRODUCTION

Diarrhea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual. It remains the second leading cause of death among children under five worldwide with a surpassing six million deaths in 2012 (IVAC, 2013). The probability of a sub-Saharan African to develop diarrhea is 39.1 % as oppose to 7.2 % in developed countries (WHO, 2006). Some of the dreadful complications of diarrhea include severe dehydration, loss of weight, impaired nutrition absorption, retarded growth and recurrent infections. In the absence of proper health care, it may lead to serious complications such as the life threatening hemolytic uremic syndrome characterized by thrombocytopenia, hemolytic uremia and kidney failure (Kouitcheu *et al*., 2013). Antibiotics have been the cornerstone to diarrhea. However, the emergence of multi-drug resistant strains partly due to the indiscriminate use of antimicrobial drugs exerts a huge pressure on antibiotherapy (Ahmad *et al.*, 1998).

The present strategy used to circumvent the problem of microbial resistance to antibiotics therefore involves the search for new antimicrobial substances from plant origin (Clark, 1996).

Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. According to a report of the World Health Organization (WHO), 80% of the world populations rely mainly on traditional therapies which involve the use of plant extracts or their active substances (WHO, 1993). Essential oils (EO) have been the target bioactive substances of plant origin in recent times.

* *Corresponding Author*
Email: mail id:chantfoe@yahoo.com

Several research works confirm that they are endowed with antimicrobial, antioxidant and anti-inflammatory properties among others (Nyegue, 2006; BioactiplantBase, 2015). These properties are most often due to the fraction of EO contained in the plant (Hulin *et al*., 1998; Ghost *et al*., 2008). The works of Ndoye, 2001; Nyegue, 2006 prove that the Cameroonian flora is gifted in medicinal and aromatic plants rich in EO *Dacryodes edulis* is one of the EO bearing plants belonging to the family of Burseraceae that has a long history in folk medicine. It is a small to medium-sized tree reaching 20-25 m high (Ajibesin *et al.*, 2011).

Different parts of the plant are used in different parts of Africa in the treatment of various ailments. The decoction of the leaves is employed to relieve certain disorders of the digestive tract, toothache and earache. The leaves and stem-barks are used to cure dysentery and anaemia (Ayuk *et al.*, 1999). The resin from the bark heals scars and other skin diseases in Nigeria (Burkill, 1985). The leaves are also reported to be employed in the remedy of skin problems such as ringworm, scabies and rashes (Ajibesin *et al.*, 2011).

Most of the works carried out so far on the different parts of this plant are focused on the extracts with very few centered on the EO. The chemical profiles of the leaves, stem-barks, root-barks and fruits EO from Nigeria have been reported (Onocha *et al*., 1999; Siluo *et al*., 2013) while (Obame *et al*., 2008) reported the antibacterial activity of the resin EO from Gabon.

To the best of our knowledge, there has been no documented report on the chemical composition and antibacterial activity of the leaves, stem-barks and resin EO native to Cameroon against diarrhea strains. It is in this regard that we saw the necessity to determine the chemical constituents of the EO of *D. edulis* and investigate their antibacterial activity.

MATERIAL AND METHODS

Plant material

The plant material was harvested in February 2013 from the Centre Regional Delegation of Agriculture at Etoug-Ebe - Yaoundé. The plant was identified as *Dacryodes edulis* (G. DON) H. J Lam at the National Herbarium of Cameroon by comparison with voucher number 55498 NHC.

Bacterial strains

The antibacterial effect was tested on a panel of bacterial isolates including two Gram positive: *Staphylococcus aureus* and *Bacillus cereus* and three Gram negative *Escherichia coli, Salmonella typhi* and *Shigella spp* bacteria. All isolates were obtained from the Laboratory of Microbiology of the University of Yaoundé I.

Essential oil extraction

The fresh leaves were washed with tap water to remove the dust. The moisture-free fresh leaves were chopped into small pieces, ground in a wooden mortar and pestle. In a similar manner, the fresh stem-barks were cleaned by scabbing off the dead cells on the barks, ground in a wooden mortar and pestle. Resin was obtained by making incisions on the trunk of the plant. 220 g (resin), 500 g (stem-barks) and 500 g (leaves) were subjected to hydrodistillation for 3 hours (resin) and 4 hours (stem-barks and leaves) using a Clevenger-type apparatus as adapted by Nyegue 2006; Agnaniet *et al*., 2011. The limpid oil obtained was separated from the hydrosol by decantation, dried over anhydrous sodium sulphate, filtered and stored in amber glass vials and stored in the dark at 4 ^{o}C (AFSSAPS, 2008 ; AFNOR, 2007) prior to chemical analyses and bioassay tests

DETERMINATION OF THE CHEMICAL COMPOSITION OF THE ESSENTIAL OILS

Gas Chromatography

The GC were carried out on a Variant CP 3380 gas chromatograph equipped with a flame ionization detector (FID) adjusted at 250^{o}C coupled to two types of apolar columns (silica capillary): polar HP-5 J and W (Agilent (5%-phenyl-95% methyl polysiloxane) of capillary column 30m x 0.25mm thickness and film thickness of 0.25µm) and Supelcowax 10 (polyethylene glycol, Supelco Inc, Bellfonte, PA) fused capillary (internal diameter 30m x 0.25mm, 0.25µm film thickness). Nitrogen was the carrier gas used at a constant flow rate of 0.8 mL/min with injector regulated in split mode at 220 ^{o}C. The exit ratio was 1:100 (0.1µL of pure EO).

The injector temperature was 220 ^{o}C while that of the detector was 250 ^{o}C. The temperature was then programmed at 50 ^{o}C to 200 ^{o}C at a ramp of 5 ^{o}C/ min and then maintained at 200 ^{o}C for 10 minutes: The entire set-up was coordinated by a computer system with the COPPASS software that ensured its functioning and follow-up of the chromatographic analyses from which quantitative data were obtained from FID area percent data.

Gas Chromatography coupled to Mass Spectrometry

The GC-MS was performed on a gaseous phase chromatograph using a Hewlett-Packard (GC 5890 series II) equipped with a HP-5(5% phenyl-95%methyl polysiloxane) fused capillary silica column (internal diameter of 30m x 0.25mm, film thickness of 0.25µm) interfaced with another fused silica capillary DB-Wax (internal diameter of 30m x 0.25mm, 0.25µm film thickness). The mass detector was of the quadrupole Model 5972 and the following conditions were used: ionization energy was 70eV, column temperature programmed from 50 ^{o}C to 200 ^{o}C, ramp of 5 ^{o}C/ min and first maintained at 50 ^{o}C for 2 minutes. The injection and MS transfer line temperatures were fixed at 220 ^{o}C and 180 ^{o}C respectively.

Helium was used as the carrier gas at a flow rate maintained at 0.6 mL/ min; inlet: split, 1:10 (1 µL of a 10:100 CH_2Cl_2 solution), ionization voltage of 70eV; electron multiplier 1460eV, mass scan range 35-300 a.m.u, scan rate 2.96 scan/s. Injection of 0.1µL of pure EO. The percentage composition of the

EO was computed by the normalization method from the GC-FID peak areas, assuming an identical mass response factor for all compounds. A series of n-alkanes were used as reference points. The identification of the EO components was based on comparison of their relative retention index with published data in the literature and by matching their mass spectra with these published data (Adams, 2012).

ANTIBACTERIAL ACTIVITY

Susceptibility Test

The susceptibility of the bacterial strains to the EO was investigated as described by Valgas *et al*. (2007). Overnight cultures of the bacterial strains were spread on sterile Mueller-Hinton agar. With the aid of a sterile 6 mm cork borer, four equally spaced wells were bored in the agar plate with a fifth well at the center of the plate. Stock solutions of the EO were prepared by dissolving each EO in 10% tween-40 to a final concentration of 100 mg/mL. 50 µL of each EO was then introduced into 3 of the 5 wells.

The fourth well was filled with 50 µL of 10 % tween-40 while the central well was filled with 20 µL of the standard antibiotic (gentamicin 0.5 mg/mL) to serve as negative and positive controls respectively. The test was carried out in triplicate, incubated for 24 hours at 37 ^{o}C and examined for zones of inhibition. The diameter of the zone of inhibition was measured using a sliding caliper (mm) and the mean considered as the inhibition diameter.

Determination of the inhibitory parameters

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antibacterial agents were determined using the microdilution method as described by CLSI (2012). 100µL of sterile nutrient broth was dispensed into the 96 well microtiter plates. A serial double dilution of geometric ratio of 1/2 of the EO was realized into the broth in the wells over a concentration range of 150 mg/mL to 2.34 mg/mL for the leaves and 400 mg/mL to 6.25 mg/mL for the resin and stem-barks and 250 mg/mL to 3.91 mg/mL for gentamicin. Overnight cultures of each strain were prepared in nutrient broth and the final concentration of each well was adjusted spectrophotometrically to 1.5 x10^{6} CFU/mL.

Positive and negative control wells: nutrient broth + inoculum and EO + nutrient broth respectively were included in each test. All tests were done in triplicate and incubated at 37 ^{o}C for 24 hours. The MIC was defined as the smallest concentration of the antibacterial agent that did not allow any visible (unaided eye) color change of the medium prior to the addition of 40µL of 2,3,5-triphenyl tetrazolium chloride (TTC) at a concentration of 0.2 mg/L further incubated for 30 minutes at 37 ^{o}C.

The MBC was assessed by subculture. 50 µL of the content of wells (unrevealed) corresponding to concentrations ≥ MIC were transferred unto 150 µL of fresh nutrient broth. The plates were incubated at 37 ^{o}C for 48 hours. 40 µL of TTC was used to reveal bacterial growth in each well. The MBC was regarded as the lowest concentration of each antibacterial substance that did not allow any noticeable color change from golden yellow to red.

RESULTS AND DISCUSSION

Extraction of essential oil

Resin gave the highest yield of 11.47 % compared to those of the stem-barks and leaves which were 0.07 % and 0.01 % (w/w) respectively. In a previous study by Obame *et al*., 2008, the resin oil from Gabon gave limpid oil with a yield of 6.8 % (w/w). The fact that the resin used in this work was fresh as oppose to that used by the aforementioned authors (who bought the resin from the herbarium, probably an old one) could be the reason behind this difference in yields. In another survey carried out by Silou *et al*. (2013), a maximum yield of 0.5 % was obtained from the hydrodistillation of eight samples of *D. edulis* leaves. The difference in yield may be related to the harvest period, varied agro-climatic conditions of the regions, plant species.

Chemical compositions

Results of the chemical analysis by GC and GC-MS showed different compositions (Table 1) dominated by *p*-cymene (30.32%), *α*-thujene (28.58%), *α*-phellandrene (27.14%) and *β*-phellandrene (10.16%) in the resin EO. The stem-barks EO had as abundant components *p*-cymene (35.14%), *trans*-carveol (22.60%), *α*-thujene (14.86%), *β*-phellandrene (8.65%) and *β*-elemene (5.22%) whereas the leaf EO was distinct in elemol (29.22%), caryophyllene oxide (15.26%), *trans*-carveol (11.80%) and spathulenol (6.28%) as the major compounds. The resin and stem-barks EO exhibited some qualitative resemblance (*α*-thujene, *β*-phellandrene, and *p*-cymene) although there was a difference in levels of these compounds. The main difference was the presence of *α*-phellandrene in the resin EO as a major component but a minor compound in the stem-barks EO.

These findings do not concord with that of Obame *et al*. (2008) who reported 24 components from the resin essential oil from Gabon dominated by sabinene (21.8%), terpinene-4-ol (19.8%), *α*-pinene (17.5%) and *p*-cymene (11.3%). Moreover, Burkill (1994) found out that under steam distillation, resin yields a peppery EO rich in sabinene, *β*-phellandrene and limonene. This mismatch of data could be ascribed to the genetic variability of the plant species.

Onocha *et al*. (1999) argues that terpinen-4-ol (25.6%) and a mixture of *α*-pinene and *α*-thujene (25.2%) are the predominant components of the stem-barks EO. According to them, *β*-caryophyllene (26.4%) is the dominant constituent of the leaves EO from Nigeria. A similar result has also been published from Nigeria by Silou *et al.* (2013) who notice that the leaves EO of *D. edulis* contains mostly sesquiterpenes among which is *β*-caryophyllene (3-10%). The marked differences in the chemical constituents of the same plant across countries might be due to the

varied adaptive metabolism of the plants, season (for example before or after flowering) and time of day when harvesting is done (Perry *et al*., 1999).

Table 1: Quantitative and qualitative chemical composition of the essential oils of *D. edulis*

Kovalt's Index	Compounds (In order of elution)	Essential oil composition (%)		
		Resin	Stem-barks	Leaves
	Hydrocarbon monoterpenes	**97.64**	**68.47**	**5.37**
944	*α*-thujene	28.58	14.86	1.34
955	*α*-pinene	0.52	0.07	/
957	Camphene	/	0.77	/
984	*α*-phellandrene	27.14	1.62	/
989	*β*-phellandrene	10.16	8.65	0.64
995	*β*-pinene	0.09	1.04	/
1024	3-carene	0.49	0.93	2.46
1043	*p*-cymene	30.32	35.14	/
1064	*p*-mentha-6,8-diene (R)-(+)	0.24	2.71	0.93
1096	*Cis*-ocimene	0.10	1.24	/
1122	*Y*-terpinene	/	0.26	/
1131	Mentha-2,4-(8)-diene	/	1.18	/
	Oxygenated monoterpenes	**2.26**	**24.05**	**14.59**
1150	Linalool	/	0.90	/
1152	Pinocarveol	/	0.04	/
1187	*Trans*-carveol	1.81	22.60	11.80
1198	*p*-menth-1-en-9-ol	0.45	0.51	2.79
	Sesquiterpenes	**0.10**	**7.33**	**4.34**
1202	*β*-elemene	0.10	5.22	1.0
1209	(E)-caryophyllene	/	0.45	/
1218	*α*-guaiene	/	0.48	/
1265	*α*-humulene	/	0.46	/
1387	Germacrene D	/	0.37	/
1395	*β*-selinene	/	0.17	/
1436	*α*-selinene	/	0.18	3.34
	Oxygenated sesquiterpenes	**0.00**	**0.15**	**64.99**
1444	Elemol	/	0.15	29.22
1484	2-nerolidol	/	/	2.09
1514	Spathulenol	/	/	6.28
1541	*Trans*-isolongifolanone	/	/	1.09
1545	*Y*-eudesmol	/	/	1.16
1564	*α*-eudesmol	/	/	1.74
1570	Caryophyllene oxide	/	/	5.10
1590	Ischwarone	/	/	15.26
1610	14-hydro-4,5-dihydrocaryophyllene	/	/	3.05
	Diterpenes	**0.00**	**0.00**	**10.47**
1711	Cubitene	/	/	4.34
1714	Laurenene	/	/	0.99
1724	Rimuene	/	/	3.67
1771	Epi-laurenene	/	/	1.47

Antibacterial activity

The susceptibility pattern and inhibition parameters of the tested organisms to the EO are indicated below (Table 2 and 3). It was noticed that the most active EO was that of the leaves which exhibited an appreciable activity. Although the active only on 80% of the strains with an inhibition diameter of 13.33±0.58 mm and MIC of 18.78 mg/mL on *Bacillus cereus*. The least active EO was that of resin which demonstrated a considerable activity with an inhibition diameter of 10.33±0.58 mm and MIC of 200 mg/mL on *E. coli* which was sensitive to only 66.7% of the EO. The ratio of MBC/MIC revealed that the leaves EO was bactericidal on *B. cereus, Shigella spp* (MBC/MIC=1) and *S. typhi* (MBC/MIC=2), the stem-barks oil was also bactericidal on *S. typhi; E. coli; S. aureus and Shigella spp* (MBC/MIC=2) but bacteriostatic on *B. cereus* (MBC/MIC=4) while gentamicin was bactericidal (MBC/MIC=1) on all the five strains.

Table 2: Inhibition zone diameters of the essential oils against the tested bacteria using the well variant assay.

STRAINS	*B. cereus*	*S. typhi*	*S. aureus*	*Shigella spp.*	*E. coli*
E.O/GEN.	IZφ ± sd (mm)	IZφ ± sd (mm)	IZφ ± sd (mm)	IZφ ± sd (mm)	IZφ ± sd (mm)
Leaves	13.33±0.58	10.00±1.00	12.33±0.58	8.00±1.00	ND
Stem-bark	10.67±0.58	9.00±1.00	11.33±0.58	9.33±0.58	10.67±0.58
Resin	10.00±1.00	9.00±0.00	7.67±0.58	9.33±0.58	10.33±0.58
Gentamicin	30.00±1.73	23.00±1.00	24.33±0.58	26.00±1.00	22.00±1.00

B. cereus= Bacillus cereus;E. coli= Escherichia coli ; Gen.= gentamicin Izφ= inhibition zone diameter; *S. typhi= Salmonella typhi* ; sd= standard deviation; *S. aureus= Staphylococcus aureus*.

Table 3: Inhibition parameters of the essential oils and gentamicin on the tested bacteria.

Strains	Inhibitory Parameters (Mg/Ml)	Essential Oil			Genta.
		Resin	Stem-Barks	Leaves	
B. cereus	MIC	200	50	18.75	0.03
	MBC	ND	200	18.75	0.03
	MBC/MIC	ND	4	1	1
S. typhi	MIC	200	50	18.75	0.03
	MBC	ND	100	37.5	0.03
	MBC/MIC	ND	2	2	1
S. aureus	MIC	200	50	18.75	0.03
	MBC	ND	100	ND	0.03
	MBC/MIC	ND	2	ND	1
Shigella spp.	MIC	200	50	18.75	0.03
	MBC	ND	100	18.75	0.03
	MBC/MIC	ND	2	1	1
E. coli	MIC	200	50	/	0.03
	MBC	ND	100	/	0.03
	MBC/MIC	ND	2	/	1

B. cereus= Bacillus cereus;E. coli= Escherichia coli; Genta= gentamicin; MBC= Minimum Bactericidal Concentration; MIC= Minimum Inhibitory Concentration; ND= Not Determined; *S. typhi= Salmonella typhi* ; *S. aureus= Staphylococcus aureus*

The observed activity is probably due to the synergistic interaction between the compounds within the EO endowed with antibacterial activity just to cite hydrocarbon monoterpenes, oxygenated monoterpenes, (oxygenated) sesquiterpenes (Dorman and Deans, 2000).

These compounds act by inducing membrane protein and lipid denaturation, inhibition of DNA replication and perturbation of membrane proton motive force, loss of energy substrate (glucose, ATP), leading directly to the lysis of bacteria (cytolysis) and therefore to its death. Another mechanism of action could be the inhibition of amylase and protease production which halts the toxin production by the bacteria, electron flow and result in coagulation of the bacterial cell content (Bakkali *et al.*, 2008; Nazzaro *et al*., 2013). The bactericidal activity of the leaves EO could be attributed to the synergistic effects of the major compounds and those represented in trace amounts such as 3-carene, *α*-thujene which have been reported to have some antimicrobial actions (Dorman and Deans, 2000). Besides, the

antibacterial property of caryophyllene oxide has been reported previously (Magiatis *et al*., 2002).

Caryophyllene oxide is well recognize as a stabilizer in foodstuffs, drugs and cosmetics and also shows growth inhibiting activity against dermatophytes (Yang *et al*., 1999). The resin EO was found to be fairly similar to the stem-barks EO; an observation not unexpected given that both EO were obtained from the same source. Their moderate activity could be explained by their high content in hydrocarbon monoterpenes. It has been demonstrated that most terpenes do not possess high inherent antimicrobial activity. For instance, *p-cymene*, one of the most important components of thyme EO, does not show antimicrobial activity against many Gram-negative pathogens (Bagamboula *et al*., 2004). Other terpenes, such as limonene, α-pinene, β-pinene, γ-terpinene, δ-3-carene, sabinene and α-terpinene show a very low or no antimicrobial activity against some bacteria (Dormans and Deans, 2000). The results of this study do not concord with that in the literature documented by Obame *et al*., 2008 who holds that resin EO is characterize by terpinene-4-ol (19.8%), α-pinene (17.4%) that confer the oil a stronger and broader antimicrobial activity.

The disparity in reports could be attributed to the variation in the harvesting period, differences in soil composition and probably due to differences in the genetic variability between the plants used.

CONCLUSION

This work had as main objective to valorize the EO of *D. edulis* by establishing their chemical composition and assess their *in vitro* antibacterial activity. Hydrodistillation of the botanical material showed that the resin was the richest in EO whereas the leaves were the least. The chemical analyses revealed the resin, leaves and stem-barks EO contained 12, 21, 24 components respectively. The resin and stem-barks EO were dominated by hydrocarbon monoterpenes while the leaf EO was rich in a mixture of oxygenated monoterpenes, oxygenated sesquiterpenes and diterpenes. The leaf EO was the most active oil meanwhile the resin EO was the least active according to the susceptibility test and microdilution assay. *B. cereus* was the most sensitive strain to the EO while *E. coli* was resistant to the leaf EO. The leaf and stem-barks EO were bactericidal on most of the strains while the resin EO was bacteriostatic on all strains at a rather higher concentration. Summarily, the Gram positive bacteria were more susceptible to the toxic effects of the EO than their Gram negative counterparts. This shows that *D. edulis* EO have antibacterial activity which if well harnessed can be used in drug formulations against the usual gastrointestinal pathogens.

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