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Chemical Composition and Antibacterial Activity of Essential Oil Extraction Residue Extracts from *Hyptis suaveolens* Leaves against Resistant Bacterial Strains

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ABSTRACT

Keywords

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The aim of this study was to evaluate antibacterial properties of essential oil residues extracted from *Hyptis suaveoelens* leaves against resistant strains. Two aqueous extracts: Eaq 1 (Without essential oil extraction) and Eaq 2 (after essential oil extraction) and their fractions were tested in solid and liquid media against five resistant bacterial strains. Some secondary metabolite families were investigated using phytochemical screening. All the secondary metabolite families we were looking for were present in the crude extracts, with exception of polyterpenes and sterols. Bacterial growth inhibition diameters ranged from 0 to 21.33 mm for aqueous extract Eaq 1 and from 8 to 24 mm for essential oil residues extracted (Eaq 2). *Staphylococcus aureus* Meti. R. was the most sensitive strain. *E. coli* BLSE was resistant to both aqueous extracts and their fractions. Eaq 1 was bactericidal only against the *S. aureus* strain, with an MIC of 6.25 mg/mL. Eaq 2 was bactericidal against *S. aureus*, *P. aeruginosa* and *S. sp*, with MICs of 1.56, 3.12 and 6.25 mg/mL respectively. Aqueous extract of *Hyptis suaveolens* leaf residue, after extraction of the essential oil, has strong antibacterial activity.

Introduction

Antibiotics discovery was a major event in medicine history. Their use increased average life expectancy by some fifteen years (Fauchère and Avril, 2002). The

remarkable efficacy of antibiotics has been accompanied by heavy use in both human and animal medicine (Zilberberg *et al.*, 2017; Tello *et al.*, 2012). One of the consequences of this massive and inappropriate use of these products is bacterial antibiotic resistance

(Ouedraogo et al., 2017). The fight against antibiotic resistance has now become a major global public health issue. Antibiotic resistance is responsible for 700,000 deaths every year. And that in the absence of coordinated and effective action, this phenomenon will lead to the death of 10 million people a year by 2050 (de Kraker et al., 2016). To combat this scourge, action plans are being put in place to reduce the over-prescription of antibiotics and limit their misuse. Despite these efforts, controlling infections caused by resistant bacteria remains a challenge worldwide. Moreover, flora has already contributed enormously to the discovery of numerous active principles that have been used in the preparation of many drugs.

Modern pharmaceutical industry continues to rely on the diversity of secondary plant metabolites to find new molecules with biologically active properties (Guédé-Guina et al., 1993, 1997; Kra, 1997). A number of scientific studies have focused on volatile (essential oil) and non-volatile plant extracts, using a variety of extraction methods. However, with aromatic plants, once essential oil has been extracted, extraction residue is discarded.

And yet, some studies have shown that even after extraction of essential oil, the extraction residue still contains secondary metabolites with biological aptitudes, justifying the use of decoction in the treatment of certain ailments (Goly *et al.*, 2015; Soumahoro *et al.*, 2020).

With the aim of contributing to the fight against bacterial antibiotic resistance through the search for new molecules from plant species, this study aims to enhance value of *Hyptis suaveolens* by evaluating antibacterial properties of extracts from aerial parts of this aromatic plant; without extraction and after extraction of essential oil, against multi-resistant bacterial strains.

Materials and Methods

Plant Material

Plant material consisted of aerial parts of *Hyptis suaveolens* (Figure 1) collected in July and September 2013, in Yamoussoukro region (Côte d'Ivoire). Fresh *Hyptis suaveolens* leaves, harvested early in the morning before sunrise, were immediately transferred to the laboratory (Laboratoire des Procédés Industriels de Synthèse, de l'environnement et des énergies nouvelles (LAPISEN) at Institut national polytechnique Félix

Houphouet-Boigny (INP-HB) in Yamoussoukro (Côte d'Ivoire). They are spread out at room temperature (27 ± 2°C), out of direct sunlight, for ten days for drying. Botanical identification is carried out in the laboratory of Department of Botany, Agriculture and Agricultural and Animal Resources (ARA) at Institut National Polytechnique Félix Houphouet-Boigny in Yamoussoukro (INP-HB).

Bacterial strains

Five resistant bacterial strains isolated from patients were tested. These were: Ceft/ImpR-resistant *Pseundomonas. aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and three Broad Spectrum Beta-Lactamase (ESBL)-producing strains (*Escherichia coli, Shigella* "sp." and *Klebsiella pneumoniae*. These bacterial strains were provided by Bacteriology Department of Institut Pasteur of Côte d'Ivoire (IPCI).

Preparation of total aqueous extracts

After drying, plant material is ground using an electric grinder (RETSCH type: SK100/C Gusseinsen) (grind 1). After extraction of essential oil by steam distillation, extraction residue is dried under the same conditions as above and then ground (Grinding 2) (Figure 2). A 100 g mass of powder (grind 1 or 2) is macerated in 1 L of distilled water at room temperature (25°C) by stirring for 24 hours (Zirihi *et al.*, 2003). The macerate is filtered on cotton and then on Watmann paper (Watmann n°2). The filtrate is concentrated to 2/3 under reduced pressure at 40°C in a rotary evaporator (Buchi 461 Water Bath), then freeze-dried. Residual powders, aqueous extract (Eaq.1 and 2), are stored at 4°C in a hermetically sealed bottle.

Fractionation of Total Aqueous Extracts

Different fractions are obtained by increasing polarity extraction (Bouamama et al., 2006). Thirty-six (36) g of total aqueous extract (Eaq 1 or Eaq 2) are dissolved in 500 mL of distilled water in a separating funnel. The mixture undergoes liquid-liquid partitioning with a 500 mL volume of solvent. The operation is repeated three times. After double filtration on cotton and Whatman n°2 paper, followed by removal of the solvent, dry extracts are stored in dark containers in a refrigerator at 4°C, under nitrogen. The final aqueous phase is freeze-dried to yield the aqueous fraction. Solvents used in order of increasing polarity are hexane, dichloromethane, ethyl acetate and water.

Determination of yields

Extractions yield is determined using the relationship below:

$$R(\%) = 100 \text{ X } \frac{m_2}{m_1}$$

In this relationship, m_2 and m_1 represent respectively the masses of aqueous extract obtained (g) and of plant material used (g), on the one hand, and on the other hand the mass of fractionated extract obtained (g) and of the crude aqueous extract used (g).

Phytochemical Screening

Screening method used is that described by Harbone, based on precipitation and/or staining reactions (Harbone, 1998).

Antibacterial activity determination

Antibacterial activity in agar medium

Antibacterial activity of extracts was assessed using agar diffusion method. 6 mm diameter wells were made in Mueller-Hinton (MH) agar (Bio-Rad, France), previously prepared and poured into 90 mm-diameter Petri dishes. From young colonies (12 to 24 hours), a bacterial suspension is prepared in Mueller Hinton Broth (MHB) (Bio-Rad, France) by introducing one colony into 10 mL of MHB. A volume of 0.1 mL of the suspension obtained is then introduced into a tube containing 10 mL of MHB, corresponding to a bacterial inoculum of approximately 5.10⁶ CFU/mL. Bacterial inoculum obtained is inoculated by flooding agar surface. A volume of 80 uL of aqueous extract or test fraction is introduced into the well. Cultures are incubated at 37°C for 18 to 24 hours. Antibacterial activity determined is the average of three diameters of inhibition zone around the wells.

Determination of Minimum Inhibitory Concentrations (MIC) and Bactericidal Minimum Concentrations (BMC)

Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (BMC) were determined following the method described by Goly *et al.*, (2015). Bacterial inoculum was prepared as above. For each extract, a concentration range from 500 to 31.25 mg/mL

was prepared in distilled water. A volume of 50 μ L of test extract is added to 0.95 mL of bacterial inoculum. After homogenization and incubation at 37°C for 24 hours, the MIC corresponds to the lowest extract concentration for which no turbidity is observed in the tube. Medium turbidity is examined in each tube by looking (with human eye) through daylight. Tubes transparency indicates antimicrobial effect of extract tested.

It is used to determine the Minimum Bactericidal Concentration (MBC). MBC is determined by subculturing on new agar tubes in which no visible growth has been observed. For this purpose, control tubes were prepared by agar culture from dilutions of 10⁰, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ of the starting inoculums. These tubes correspond respectively to 100%, 10%, 1%, 0.1% and 0.01% of surviving bacteria in the culture. After incubation at 37°C for 24 hours, BMC is determined by comparing control tubes with experimental tubes. The first experimental tube in which germ count determined is less than or equal to that of 10⁻⁴ dilution, corresponds to the BMC.

Results and Discussion

Yield

Aqueous extracts without essential oil extraction (Eaq 1) and aqueous extracts after essential oil extraction (Eaq 2) were obtained in more or less equal proportions (19.68 and 19.01%) (table 1). These yields are lower than those determined by Soumahoro et al., (2020) using the same plant material. Using an ethanol-water mixture (70/30: v/v) as extraction solvent, they obtained the same extracts in proportions of 21.27 and 27.60%, respectively for extract without extraction and after extraction of essential oil. Extraction solvent used could account for this difference. According to Stanier et al., (1986), ethanol provides better extraction of water-soluble compounds. Partitioning of crude extracts gave fractions with different yields. Aqueous fraction from Eaq 2 was obtained with a yield of 26.21%, higher than that of fraction from Eaq 1 (20.97%). According to Dossa et al., (2012) some monoterpenes and sesquiterpenes are bound to sugars or other compounds such as certain phenolic compounds in form of heterosides, forming complexes that are less soluble in natural state. During extraction of terpene compounds, main constituents of essential oils, certain sugars or polar compounds initially bound to terpene compounds are released (Van Bergen et al.,

1997). The presence of these compounds in the macerate obtained after extraction of essential oil could increase yields. Yields of hexane (21.25%) and ethyl acetate (42.21%) fractions from extract Eaq 1 are higher than those of the same fractions from extract Eaq 2. However, yield of dichloromethane fraction from extract Eaq 2 is higher than that of the same fraction from extract Eaq 1 (Table 1). These differences could be explained by the solubility of compounds released during essential oil extraction in the different solvents used to partition crude extracts. These compounds are more soluble in water and dichloromethane than in ethyl acetate and hexane.

Antibacterial Activity

Inhibition Diameters

With the exception of *E. coli* strain, the other strains tested were at least sensitive to both aqueous extracts (Eaq 1 and Eaq 2) of *Hyptis suaveolens* leaves. *S. aureus* strain was extremely sensitive (inhibition diameter > 20 mm). Diameters of inhibition of bacterial growth determined were 21.33 and 24 mm, respectively for Eaq 1 and Eaq 2. *K. pneumoniae* and *Shigella sp* are highly sensitive to both aqueous extracts.

The mean growth inhibition diameter values induced by Eaq 2 against the four other strains are higher than those determined for Eaq 1, except against *Shigella sp* strain (figure 3). Our results confirm the strong antibacterial activity of hydro-ethanolic extract of *Hyptis suaveolens* leaves after extraction of essential oil, work carried out by Goly *et al.*, (2015) on solid media. Bacterial growth inhibition diameters determined were 25 and 20 mm respectively against *S. aureus* Meti. R and *P. aeruginosa* Ceft/Imp.R respectively.

Fractionation purifies extracts, enhancing their antibacterial activity. Here, we note that inhibition diameter values determined are more or less in the same order as those of crude extracts (figure 4). However, when we compare antibacterial activity of all fractionated extracts, we find that dichloromethane and ethyl acetate fractions give better activity. E. coli, which was resistant to crude extracts, was sensitive to dichloromethane and ethyl acetate fractions from Eag 1 with inhibition diameters of 10.66 and 12.66 mm, respectively. K. pneumoniae strain was more sensitive dichloromethane fractions of both extracts (Eaq 1 and 2). Inhibition diameters increased from 15 to 19.66 mm and from 18.33 to 20.66 mm respectively from crude extracts

to dichloromethane fractions. The lowest inhibition diameter values were obtained with aqueous fractions of both extracts.

Antibacterial Parameters

Antibacterial parameters determined confirm the high antibacterial activity of the aqueous extract after extraction of essential oil (Eaq 2). The lowest MIC values obtained are for this extract (Eaq 2) against *S. aureus* Meti. R (1.56 mg/mL) and *P. aeruginosa* Ceft/Imp.R (3.12 mg/mL). In addition to these two resistant strains, Eaq 2 has a bactericidal effect against *Shigella sp*, which produces extended-spectrum beta-lactamase. Eaq 1 has a bactericidal effect only against *S. aureus* Meti. R MIC determined is 6.25 mg/mL (table 2).

These results corroborate those of Goly *et al.*, (2015), who determined antibacterial activity of hydroethanol extract of *Hyptis suaveolens* leaf residues. MIC values determined for hydro-ethanolic extract after extraction of essential oil, are 0.78 and 3.12 mg/mL, respectively against *S. aureus* Meti. R and *P. aeruginosa* Ceft/Imp.R. We found no work in the literature on antibacterial activity of aqueous extracts derived from *Hyptis suaveolens* leaf residues.

The high antibacterial activity of aqueous extract obtained from *Hyptis suaveolens* leaf residues (after extraction of essential oil) could be justified by its composition. Indeed, the presence of certain volatile molecules in Eaq 1 could have an antagonistic effect on the activity of molecules with antibacterial activity (Goly *et al.*, 2015). Extraction of these volatile molecules would enhance antibacterial activity of Eaq 2. The high antibacterial activity of this extract could also be justified by possible chemical rearrangements (Bruneton, 1999) and the thermoresistance of bioactive molecules. Under effect of heat, molecular conversions could occur, accentuating antibacterial activity, justifying the choice of decoction in traditional use (Magassouba *et al.*, 2007).

Chemical Composition

All secondary metabolite families looked for are present in both crude aqueous extracts (Eaq 1 and Eaq 2), except for Polyterpenes and Sterols, which are absent from Eaq 2 and its fractions (table 3). The absence of these compounds in these extracts could be explained by their extraction during the extraction of essential oil (Van Bergen *et al.*, 1997).

Table.1 Extraction yield of aqueous extracts from *Hyptis suaveolens* leaves and their fractions.

		Aqueous e	xtract 1 an	d fractions	5	Aqueous extract 2 and fractions						
	Eaq 1	Fhex 1	Fdcm 1	Face 1	Faq 1	Eaq 2	Fhex 2	Fdcm 2	Face 2	Faq 2		
yield (%)	19,68	21,25	11,20	42,21	20,97	19,01	12,65	16,5	39,5	26,21		

Eaq 1: aqueous extract from *Hyptis suaveolens* without essential oil extraction; Eaq 2: aqueous extract from *Hyptis suaveolens* after essential oil extraction; Fhex 1: hexane fraction from Eaq 1; Fhex 2: hexane fraction from Eaq 2; Fdcm 1: dichloromethane fraction from Eaq 1; Fdcm 2: dichloromethane fraction from Eaq 2; Face 1: ethyl acetate fraction from Eaq 1; Face 2: ethyl acetate fraction from Eaq 2; Faq 1: aqueous partition from Eaq 2: aqueous partition from Eaq 2.

Table.2 Antibacterial parameters of aqueous extracts from *Hyptis suaveolens* leaves and residue

	Antibacterial parameters (mg/mL)										
Bacterial		A	queous extra	et 1	Aqueous extract 2						
strains tested	CMI	CMB	CMB/CMI	EFFECT	CMI	CMB	CMB/CMI	EFFECT			
K. pneumoniae	>25	>25	nd	nd	>25	>25	nd	nd			
S. sp	>25	>25	nd	nd	6,25	6,25	1	Bactericidal			
E. coli	>25	>25	nd	nd	>25	>25	nd	nd			
S. aureus Meti. R	6,25	12,5	2	Bactericidal	1,56	1,56	1	Bactericidal			
P. a. Ceft/Imp. R	6,25	25	4	Bacteriostatic	3,12	3,12	nd	Bactericidal			

K. p.: Klebsiella Pneumoniae BLSE; S. 'sp": Shigella 'sp" BLSE; **E. coli:** Escherichia coli BLSE; **S. aureus Meti. R**: Meticillinresistant Staphylococcus aureus; **P. a.** Ceft/Imp. R: Pseudomonas aeruginosa resistant to ceftazidine and imipenem; **nd**: not determined.

Table.3 Chemical composition of aqueous extracts from *Hyptis suaveolens* leaves and their fractions.

Secondary metabolite families	A	queous ex	tract 1 and	Aqueous extract 2 and fractions						
	Eaq 1	Fhex 1	Fdcm 1	Fac 1	Faq 1	Eaq 2	Fhe 2	Fdcm 2	Fac 2	Faq 2
Alkaloids	+	-	+	-	+	+	-	-	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+
Leuco-anthocyanins	+	-	+	+	+	+	-	+	+	+
Polyphenols	+	+	+	+	+	+	-	-	+	+
Polyterpenes and sterols	+	+	+	+	+	-	-	-	-	-
Saponosides	+	+	+	+	+	+	-	+	+	+
Catechic tannins	+	-	-	+	+	+	-	+	+	+
Gallic tannins	+	-	-	-	+	+	-	+	+	+

^{+:} Presence; -: Absence; Eaq 1: aqueous extract from *Hyptis suaveolens* without essential oil extraction; Eaq 2: aqueous extract from *Hyptis suaveolens* after essential oil extraction; Fhex 1: hexane fraction from Eaq 1; Fhex 2: hexane fraction from Eaq 2; Fdcm 1: dichloromethane fraction from Eaq 1; Fdcm 2: dichloromethane fraction from Eaq 2; Face 1: ethyl acetate fraction from Eaq 1; Face 2: ethyl acetate fraction from Eaq 2; Faq 1: aqueous partition from Eaq 2.

Figure.1 Photograph of aerial parts of *Hyptis suaveolens* (Photo Goly, 2013)



Figure.2 Diagram of extraction of essential oil (EO) and hydro-ethanolic extracts

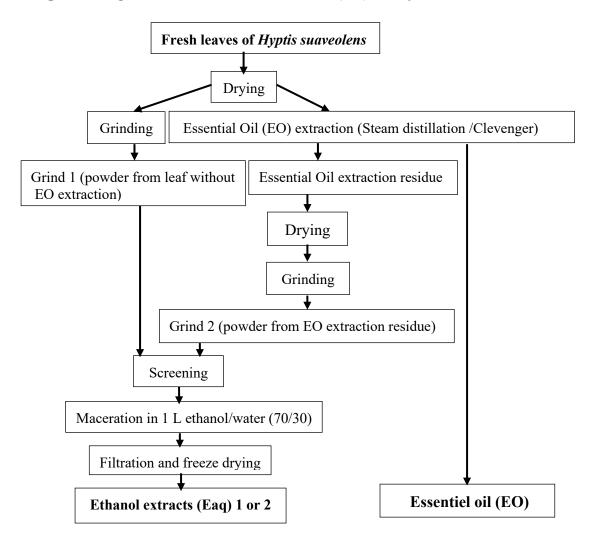
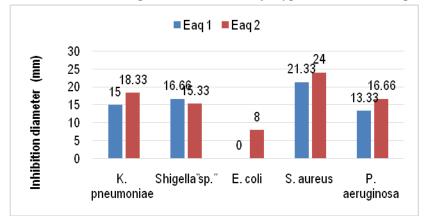
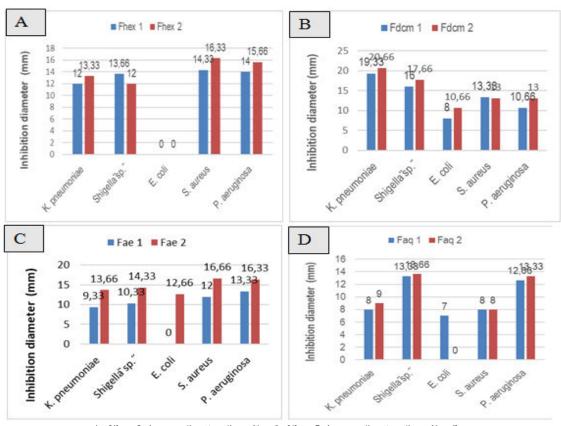


Figure.3 Diameters of bacterial growth inhibition by *Hyptis suaveolens* aqueous extracts



Eaq 1: aqueous extract from *Hyptis suaveolens* leaves without essential oil extraction; **Eaq 2**: aqueous extract from *Hyptis suaveolens* leaves after essential oil extraction.

Figure.4 Diameters of inhibition of bacterial growth by fractions of aqueous extracts from *Hyptis suaveolens* leaves.



A: Fhex 1: hexane fraction from Eqq 1; Fhex 2: hexane fraction from Eqq 2;

B: Fdcm 1: dichloromethane fraction from Eaq 1; Fdcm 2: dichloromethane fraction from Eaq 2;

C: Face 1: ethyl acetate fraction from Eaq 1; Face 2: ethyl acetate fraction from Eaq 2;

D: Faq 1: aqueous partition from Eaq 1; Faq 2: aqueous partition from Eaq 2.

Aqueous fraction is the richest in families of secondary metabolites. This could justify the interest in using decoctions in many traditional treatments. These results are in line with those of Soumahoro *et al.*, (2020).

Hexane fraction of Eaq 1 contains flavonoids, saponosides, polyphenols, polyterpenes and sterols, while that of Eaq 2 contains only flavonoids.

Dichloromethane fraction of Eaq 1 contains all families of secondary metabolites, except tannins. After extraction of essential oil, the same fraction contains tannins, but alkaloids, leuco-anthocyanins, polyphenols and polyterpenes and sterols are absent.

Alkaloids and gall tannins, absent in ethyl acetate fraction of Eaq 1, are present in ethyl acetate fraction of Eaq 2.

Phytochemical screening by Soumahoro *et al.*, (2020), using hydro-ethanol extracts obtained directly without extraction and after extraction of *Hyptis Suaveolens* leaf essential oil and their fractions, gave different results.

There are several possible reasons for these differences. Firstly, the solvents used for solid-liquid extraction are not the same. Soumahoro *et al.*, (2020) used an ethanolwater mixture. Secondly, essential oil extraction methods are different. Hydro-distillation used by Soumahoro *et al.*, (2020) has drawbacks. Indeed, direct contact of plant material with extraction solvent causes chemical reactions, leading to changes in the final composition of the extract (Walton and Brown, 1999; Raaman, 2006).

The difference in composition of fractions from Eaq 1 and Eaq 2 could be explained by heat effect. Indeed, prolonged and powerful heating causes deterioration of certain plants and degradation of certain aromatic molecules. Water, acidity and temperature can also induce ester hydrolysis, leading to rearrangement, isomerization, racemization and/or oxidation (Bruneton, 1999). It is mainly to avoid these undesirable effects of heat that several research laboratories and have enabled the development of new extraction techniques for essential oil and flavors (Gavahian *et al.*, 2015; Mohammadhosseini *et al.*, 2015).

This study justifies the use of medicinal plant leaf decoctions in traditional treatment of certain infections. With the exception of polyterpenes and sterols, all the families of secondary metabolites sought were present in both aqueous extract without essential oil extraction and aqueous extract derived from residue of essential oil extraction. The latter showed best antibacterial activity. Under the effect of heat, any molecular rearrangements caused enhance biological properties.

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Author Contributions

Kouassi Roselin Cyrille Goly: Designed the model and wrote the manuscript, Original Draft Preparation and Writing; Zamble Bi Irie Abel Boli: Methodology, Original Draft Preparation and Writing; Konan Guy Sylvère N'Zi: Supply the strains tested and Writing; Yaya Soro: Conceived the original idea and Resources

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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