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Antibacterial and Antifungal Activity of the Essential Oil Extracted by Hydro-Distillation from *Artemisia annua* Grown in West-Cameroon

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Abstract: This study was carried out to assess the *in vitro* antimicrobial potential of the Essential Oil (EO) extracted by hydro-distillation from the variety of *A. annua* grown in West Cameroon. This evaluation was conducted by testing the microbial growth inhibition through agar diffusion, minimal inhibitory and minimal lethal concentrations. Tested microorganisms included bacteria isolates belonging to the following categories: *Staphylococcus aureus, Escherichia coli, Salmonella* Enteritidis, *Shigella flexneri, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae and Vibrio cholerae.* This activity was also tested on a dimorphic fungal species, *Candida albicans.* Data analysis revealed that the EO possessed an intrinsic antimicrobial activity that was potentiated by the solvent (DMSO). Inhibition zone diameters varied from 6 (*Pseudomonas aeruginosa* and *Shigella flexneri*) to 45 mm (*Vibrio cholerae*). It was also observed that *Vibrio cholerae* was susceptible to the lowest concentration of the essential oil used (0.3 mg/mL), while *Pseudomonas aeruginosa* was shown to tolerate the highest (80 mg/mL). Also, the minimal inhibitory and lethal concentrations were equal (MLC/MIC = 1), implying the absolute lethal property of the oil. This lethal potential on fungi, Gram-negative and Gram-positive bacteria makes of this plant an appropriate candidate for new conventional antimicrobial drug production and infectious disease prevention. Well exploited, it might be used to control the current epidemics of *Vibrio cholerae*-associated cholera in Cameroon. Additional studies should also be conducted to lay down reliable basis for comprehensive test interpretations that take into account correlations between these *in vitro* test results and the ones that would be obtained with conventional antimicrobials.

Keywords: Antimicrobial properties, *Artemisia annua*, essential oil

INTRODUCTION

Infectious Diseases (ID) have always been a major health concern in underprivileged communities worldwide, but not often regarded as a public health priority in these areas that are characterized by resource-limitation which is a serious handicap to research expected to facilitate disease prevention and case management (WHO, 2002). In these communities, a set of factors associate and contribute to the persisting poverty that contrasts with fast population growth (Nguendo Yongsi *et al.*, 2008). These factors include low purchasing power, low education level and inadequate use of antimicrobials in several domains like human and veterinary medicine, growth supplementation in animal husbandry, pesticides in crops production and other cryptic determinants yet to elucidate. The World Health Organization (WHO, 2002) observed that a great proportion of human, animal and plant diseases are due (or related) to the presence of microbes as consequence of poor sanitation. Most affected populations reside in tropical areas characterized by a high plant diversity and persistence of conducive factors (moist and temperature) for microbial growth and dissemination (Fotsing Kwetche, 2008; Nguendo Yongsi *et al*., 2008; Nguendo Yongsi, 2011; Tamatcho Kweyang *et al*., 2012). This high plant diversity represents a great opportunity for traditional medical practices. But this opportunity is rarely seized and exploited conveniently for the benefit of local populations. In connection with the low-income and other traditional beliefs, many people rely on these plants, however, as the first intention in disease management (Fotsing Kwetche *et al*., 2012). Another crucial global issue is microbial resistance that develops against available drugs in both privileged and underprivileged communities, making microbial drug-resistance a pandemic phenomenon. More and more, researchers focus their attention on the study of medicinal plant drug-potentials for new drug production (Mueller *et al*., 2000; Liu *et al*., 2001; Abad *et al*., 2012; Dehghani *et al*., 2012; Salih, 2012). One of these

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plants is *Artemisia annua* (*A. annua*), an annual herbaceous plant belonging to the family *Asteraceae*. Former investigations reported that *A. annua* could be used as antibacterial, antiseptic, carminative (facilitates the release of intestinal gas), digestive, febrifuge (reduces body temperature) and anti-malarial (Mueller *et al*., 2000; Liu *et al*., 2001; Li *et al*., 2011; Dehghani *et al*., 2012; Salih, 2012). But depending on the biotope in which this plant is grown and harvested, the chemical properties may vary considerably (Bhakuni *et al*., 2001; Chougouo Kengne, 2010). In an ongoing research program on infectious diseases and medicinal plants conducted by the Université des Montagnes, we tested the anti-bacterial and anti-fungal potential of the Essential Oil (EO) extracted by hydro-distillation from *A. annua* grown in West Cameroon on eight bacterial isolates from different species and a dimorphic fungal species. Evaluation parameters included: agar diffusion tests, Minimal Inhibitory Concentrations (MICs) and Minimal Lethal Concentration (MLC). The data recorded could help to prevent and combat some infectious diseases in the short run and guide production of conventional drugs in the long run.

MATERIALS AND METHODS

Local variety Essential oil characteristics and microorganisms: The essential oil extracted from *A. annua* grown in West Cameroon contains the following chemical compounds: camphor (major component), α-pinene, ß-pinene, 3-carene, α-terpinene, limonene, eucalyptol, artemisia ketone, copaene, camphene, caryophylene, menthol and α-terpineol (Chougouo Kengne, 2010). Microorganisms tested in this investigation included isolates belonging to the following categories: *Staphylococcus aureus* (a Gram-positive coccus), *Escherichia coli*, *Salmonella* Enteritidis, *Shigella flexneri*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Klebsiella pneumoniae* (Gram-negative rods) *and Candida albicans* (a dimorphic fungal species). These microorganisms were chosen based on their frequent implication in both nosocomial and community acquired infections on one hand and their ability to develop tolerance to antimicrobial agents on the other.

Test of bacterial growth inhibition through paper-disc-assisted agar diffusion: In the present research, we used the modified version of a former protocol (Hayes and Markovic, 2002). Bacterial isolates used belonged to known non-fastidious species. All strains were platted on a nutrient agar and incubated at 37°C for 24 h. From the resulting pure culture, a bacterial suspension equal to 0.5 McFarland was prepared and further diluted (1/100) to obtain the final density required for susceptibility tests by agar diffusion. A similar procedure was followed to assess the EO anti-fungal activity on a fresh culture obtained from Sabouraud agar. The microbial suspension was used to lawn a Mueller Hinton (MH) or a Sabouraud agar, (BioMérieux), (for bacteria or fungi, respectively) in 90 mm diameter Petri dishes. Three paper discs (6 mm diameter each) deposited on the preparation were then inoculated as indicated in Table 1.

Table 1: Paper disc chemical composition

	Element added to paper discs		
Disc number	EO (15 µL)	EO(15 µL) + 10% DMSO	SDW +10% DMSO
1	+	-	-
2	-	+	-
3*	-	-	+

EO: Essential Oil; SDW: Sterile distilled water, DMSO: Dimethylsulfoxyde; +: was deposited on the disc; -: was not deposited on the disc; *: negative control

After an hour of incubation at room temperature for all preparations, bacterial and fungal cultures were, respectively, put at 37°C and 30°C, overnight. Upon completion of this incubation time, anti-microbial activity was evaluated by measuring the growth inhibition zone diameter around each disc in a monolayer confluent growth. Each test was repeated three times and the results obtained were expressed in millimeter. Interpretations were conducted as previously done in Hayes and Markovic (2002). When the inhibition zone was equal to or larger than 15 mm, the tested organism was regarded as susceptible.

Determination of the Minimal Inhibitory Concentration (MIC): To test the MIC in the present survey, we used the macro-dilution technique performed in liquid medium (Hayes and Markovic, 2002). In a glass test tube, 200 µL of the EO were added to 2.3 mL of a mixture (0.01% (v/v)) of Mueller Hinton broth and Tween 80 (Cooper). From this original solution, a serial dilution was performed to have solutions with EO concentrations ranging from 80 to 0.3 mg/mL. To each one, 13 µL of bacterial suspension (0.5 McFarland/100) were added. The set was allowed to incubate aerobically at 37°C (or 30°C) for 24 h. When incubation has completed, all tubes were centrifuged at 5000 rpm for five minutes. The MIC was determined from the first test tube in which no deposit was obtained upon centrifugation. To assess and attest reproducibility, this experiment was also conducted three times.

Determination of the Minimal Bactericidal and Fungicidal Concentrations (MBC and MFC): The solutions from which no deposit was obtained after centrifugation were used to determine the MBC and MFC (Hayes and Markovic, 2002). Briefly, after homogenization, a loop (≈8-10 µL) of the suspension was lawn on MH (or Sabouraud) agar. This culture was incubated aerobically at 37°C (or 30°C) overnight. The

MBC/MFC of the oil was inferred from the culture medium in which no visible microbial growth was recorded upon revelation. Like the former tests and for the same reason, this experiment was repeated three times.

Data recorded were expressed diagrammatically and as tables, with Excel.

RESULTS

Microbial growth inhibition: The inhibitory zone diameter recorded with the agar diffusion of the essential oil, with and without addition of DMSO (dissolved and non-dissolved, respectively) are presented in Table 2.

This table indicates primarily that without the DMSO, the diameters varied from 6±00 to 45±00 mm. The largest diameter was observed with *V. cholerae* while the smallest were recorded with *P. aeruginosa* and *S. flexneri.* The values obtained from the test on *E. coli* and *K. pneumoniae* on one hand; and *S.* Enteritidis and *P. mirabilis* on the other were similar.

After addition of DMSO, the overall range remained between 6 ± 00 and 45 ± 00 mm. However, the diameter values increased significantly for some tested organisms (*S. aureus, C. albicans);* while a relative increase was obtained with the others (*P. mirabilis, S.* Enteritidis, *K. pneumoniae*). With addition of DMSO, the diameters of inhibition remained the same for *P. aeruginosa, S. flexneri* and *V. cholerae*.

With or without DMSO, the standard deviations associated attested closeness of the diameter values recorded individually in each case, then mean values accuracy.

Table 2: Inhibition diameters with and without DMSO

	Inhibition diameter±SD (mm)	
Bacterial/Fungus	EO without DMSO	EO with DMSO
Staphylococcus aureus	28±00	38±0.03
Vibrio cholerae	45±00	45±00
Escherichia coli	21±0.01	24±0.01
Salmonella Enteritidis	12±0.005	14±0.03
Klebsiella pneumoniae	20±0.04	22±0 .01
Proteus mirabilis	11±00	14±0.03
Shigella flexneri	6±00	6±00
Pseudomonas aeruginosa	6±00	6±00
Candida albicans	30±0.01	36±00

EO = Essential Oil; DMSO = Dimethylsulfoxyde; SD = Standard Deviation

Minimal Inhibitory Concentration *versus* Minimal Lethal Concentration (MIC *versus* MLC): Overall, concentrations in essential oil ranged from 0.3 to 80 mg/mL. Data obtained from minimal inhibitory and lethal concentraions for the tested microorganisms were combined and presented diagrammatically (Fig. 1).

This figure shows that, except for *P. aeruginosa*, the minimal inhibitory and lethal (bactericidal and fungicidal) concentrations were equal (MLC/MIC = 1) for all microorganisms. No concentrations in the range prepared could inhibit the growth of *P. aeruginosa* (MIC> 80 mg/mL). *V. cholera*e was the most susceptible isolate (MIC and MBC <0.3 mg/mL) while the highest MIC and MBC were obtained with *S. flexneri.* For the other, the MIC and MLC values were lower than or equal to 20 mg/mL.

DISCUSSION

This study aimed at assessing the antimicrobial (antibacterial and antifungal) potential of the essential

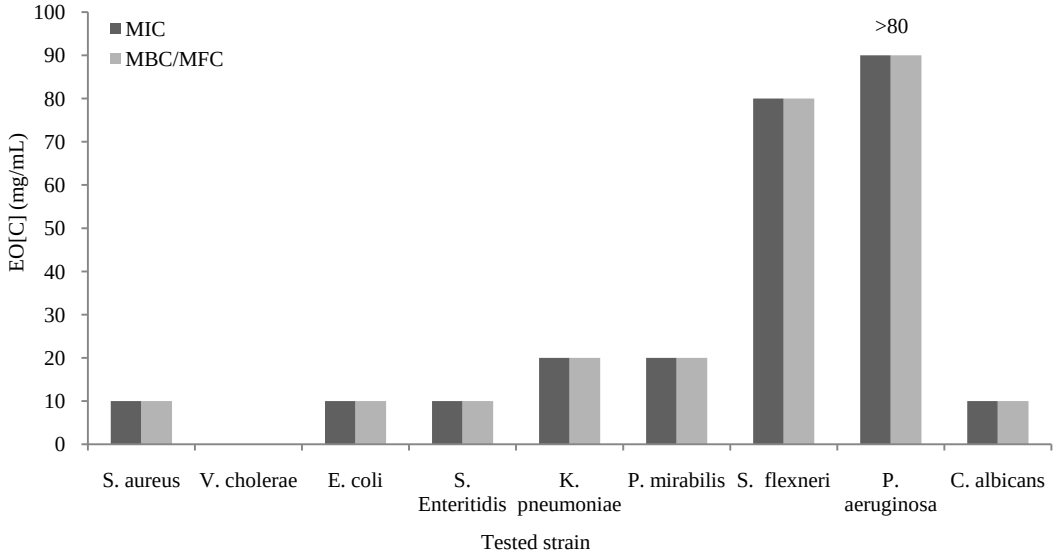

Fig. 1: Minimal inhibitory and lethal concentraion for various microorganisms

oil extracted from the variety of *A. annua* cultivated in West Cameroon. The results revealed inhibition of growth with some tested isolates. Similar findings were previously reported with the EO from plants grown under other climatic conditions (Dehghani *et al*., 2012; Salih, 2012). From data analyses made of growth inhibition diameters, it can be observed that the intrinsic inhibitory activity of the EO is not dependent upon solvent (DMSO) addition (as, no antimicrobial activity was obtained with the negative control). Increased inhibition observed in the presence of the solvent could be associated with the fact that the EO is more soluble, therefore, can easily diffuse in the medium, allowing greater contact with the tested microorganisms. This solvent effect could clearly be appreciated with microorganisms from which the test gave intermediate, rather than those from which extreme values were obtained.

The absolute bactericidal potential (MLC/MIC = 1) of the oil was observed in the present survey. This may be associated with the presence of such alcohols as menthol, α- terpineol and eucalyptol (Hammer, 2003; Chougouo Kengne, 2010). Hammer (2003) reported that alcohols in general are active against bacteria and that; their bactericidal property is more accentuated.

Out of the seven Gram-negative and one Gram-positive bacterial isolates tested in the current survey, the highest activity recorded was obtained with *V. cholerae* (inhibition diameter equal to 45 mm) while the least susceptible isolates were *P. aeruginosa* and *S. flexneri* (paper disc diameter in both cases). The *V. cholerae* isolate used in this study belonged to the O1-serogroup. Members of this serogroup are the ones incriminated in the current epidemics of cholera in Cameroon. In fact, cholera due to O1-antigen-positive *V. cholerae* isolates is endemic in Cameroon since 1973 (Guévart *et al*., 2006). Formerly restricted to the coastal (littoral) areas, cholera has recently spread to six out of the ten regions of the country in a decade (Guévart *et al*., 2006; Tamatcho Kweyang *et al*., 2012). It is likely that the use of this oil becomes a good alternative in preventing disease and managing cases (Lutgen, 2009; Ahmed *et al*., 2009). This would be of high interest in developing countries in the tropics where many factors combine: poor hygiene and sanitation accompanied by low-income and high plant diversity (favored by the appropriate climate). For this to be beneficial, however, community education should be improved on the issue. It is important that another study is conducted in animal model to know if raw material extract can alleviate pains, prevent severe disease and *V. cholerae* dissemination just like wound healing (lots of skin infections are caused by *S. aureus*). Previous studies reported tolerance of *P. aeruginosa* to the EO extracted from another variety of *A. annua* grown in Iran (Verdian-Rizi *et al*., 2008). Reasons for this tolerance are yet to be addressed, although *Pseudomonas* spp. is known to host large arrays of genetic determinants encoding resistance against several natural and conventional antimicrobials (Ullah *et al*., 2012). The antibacterial activity observed with *A. annua* EO appeared not to be Gram-dependent, unlike many conventional molecules.

Previous studies conducted in France (Juteau *et al*., 2002), Iran (Verdian-Rizi *et al*., 2008) and China (Li *et al*., 2011) assessed the antibacterial activity of *A. annua* on *S. aureus* and *E. coli*. While in France the isolates used grew in the presence of the oil (then resistant), they were strongly inhibited in China, as observed during these works in Cameroon. Though chemical characteristics of the EO were not given special emphasis as far as its activity on bacteria was concerned in France and China, it is known that they are influenced by local features like culture conditions, harvest, drying and storage, for instance (Abad *et al.*, 2012). Accordingly, antibacterial properties of the EO from *A. annua* grown in West Cameroon and China may have similar characteristics. In Cameroon (Chougouo Kengne, 2010) and China (Li *et al*., 2011), this EO was richer in limoneme, camphor and beta-pinene than in France (Juteau *et al*., 2002). From these findings, however, it cannot be ruled out that these components are responsible for the differential antibacterial properties of the EO specimens for a set of reasons: only a few characteristics have been studied in Cameroon, unlike in China and France, bacterial isolates used were not from reference strains in any of these studies (therefore, not expected to have identical properties at all points of view), the techniques used were slightly different, just to name a few. Further, complex interactions (known or cryptic) between different components of all chemicals may greatly influence the properties observed.

The present studies also reveal good activity on *C. albicans*. Similar results were reported in France (Juteau *et al*., 2002) and in Iran (Verdian-Rizi *et al*., 2008). In a previous investigation (Emira *et al*., 2010), susceptibility of *C. albicans* was also reported to EOs obtained from *Eucalyptus globulus* and *Maleuca alternifolia* grown in Tunisia and France, respectively. In these cases, the major chemical in the oil specimens was alpha-terpineol. In China this oil was also shown to inhibit the growth of other fungal species than *C. albicans*. Though not identified as the major component, this alcohol was also identified in the oil from Cameroon. For the same reasons as above, it cannot be ruled out, however, that the antifungal activity of the EO is due to this chemical.

When the MIC and MLC were assessed, data analysis revealed that no oil concentration in the range prepared could inhibit the growth of *P. aeruginosa;* while the lowest one was potent enough to inhibit *V. cholerae*. These findings are consistent with those obtained in disc-diffusion as discussed above; both

highlighting the fact that *P. aeruginosa* is the least susceptible while *V. cholerae* is the most. Brought together, they indicate that *A. annua* might be used to control the current epidemics of cholera in Cameroon and that, this control may not require a lot of resources (lethal concentration is very low).

All MICs were also equal to the MLCs (MLC/MIC = 1), implying that the EO used in the present study possesses a direct bactericidal/fungicidal activity on the tested microorganisms; reinforcing the needs for further researches aiming at regarding *A. annua* as a convenient candidate for conventional drugs against infectious diseases.

CONCLUSION

The present study conducted to evaluate the antimicrobial activity of the essential oil extracted from *A. annua* grown in West Cameroon revealed that it was active against most isolates tested. It was also noticed that the MIC and MLC (antibacterial and antifungal) values were equal in all cases, except for *P. aeruginosa* where no growth inhibition was obtained at any oil concentrations within the range of dilutions. Further studies focusing comprehensive test interpretations that take into account correlations between these *in vitro* results and the ones that would be recorded with conventional antimicrobials should be conducted; in order to use the lethal potential on fungi, Gram-negative and Gram-positive bacteria and make of this plant an appropriate candidate for new conventional antimicrobial drugs.

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