

(RESEARCH ARTICLE)



Antimicrobial properties of *Clausena anisata* (Rutaceae) essential oil from Bafou area against the pathogenic fungus *Candida albicans* and antioxidant activity

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Abstract

The aim of the present work was to determine the antimicrobial and antioxidant activities of *Clausena anisata* essential oil. The extraction of essential oils was performed by the hydro distillation method with a Clevenger apparatus. The determination of essential oils chemical composition was conducted by gas chromatography and gas chromatography coupled with mass spectrometry. Then the antimicrobial activity was assessed using disk diffusion method and micro titration. In order to determine the antioxidant activity 2 methods were carried out: the free radical scavenging assay was performed using the DPPH⁺ radical (2,2-diphenyl-1-picrylhydrazyl) and the method with ABTS radical reagents (acid2, 2'-azynobis-[3-ethylbenzothiazoline-6 sulfonic acid]). The yield of extraction of the essential oil was 0.77%. The study of its chemical composition revealed E- anethol (70.77%), methyl iso eugenol (13.85) as major compounds. The anti-radical DPPH test revealed a SC₅₀ of 1g/L; 0.265g/L respectively for DPPH and ABTS tests. The results also highlighted a potent antimicrobial property. An antimicrobial screening study revealed a susceptibility of *Candida albicans* isolates at a concentration of 8000 ppm. This activity was characterized by minimum inhibitory concentrations of 2000 ppm for all the tested pathogenic yeasts Sab1, Sab2, Sab3, Sab250, Sab250₁, Sab256, Sab259 and Sab290. The essential oil minimum inhibitory concentration (MIC) was lower than the MIC of fluconazole. The same observations were resulted with the fungicidal effect. These data can be used as a starting point for subsequent studies to produce antifungal and antioxidant drugs to face the challenge of the antimicrobial resistance and oxidative stress.

Keywords: *Clausena anisate*; Essential oil; Antimicrobial; DPPH; ABTS

1. Introduction

Fungal infections constitute a serious public health issue leading to approximately 1.5 million deaths among human beings. The mortality rate caused by fungal infections is far greater than the one caused by malaria and is similar to the mortality rates due to tuberculosis and HIV-AIDS [1]. In addition, the impact of these infections negatively affects the worldwide economy causing billions of dollars losses due to the direct or indirect management of the disease consequences [2]. Moreover, they are generally opportunistic infections. The microorganisms responsible of the infection can move from the commensal state to a pathogenic form especially in people with a weak or compromised

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immune system such as HIV immunocompromised, cancer, elderly and newborn patients [3]. *Candida* species are the most common fungal pathogens in humans, causing superficial and systemic infections which are candidiasis [4]. Some treatments including azoles, polyenes, echinocandins are mainly used to treat fungal infections. However, limitations in the current available classes of antifungal drugs, antimicrobial resistances, toxicity and high cost represent major challenges in the antifungal therapy [5]. All this underlines the urgency of the search for new therapeutic agents that are effective, low cost, low toxicity with no or less resistances. The World Health Organization (WHO) recognized the importance of traditional medicine using medicinal plants and reported that 80% of the world's population depends on traditional medicine for primary health – care [6]. On the same way, some studies were carried out on *Clausena anisata* (rutaceae) highlighting the antimicrobial potential of the ketone extracts isolated from leaves, the antifungal activity of *C. anisata* EO against *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus niger* and *Flavus moniliforme* [7]. *C. anisata* as a traditional plant used in Cameroon was also active against many other pathogenic microorganisms with inhibitory concentrations up to 3.91 µg/mL [8].

Moreover, the use of synthetic antioxidant molecules leads to potential toxicological risks. This is the case for butyl-hydroxyanisole BHA, butyl-hydroxytoluene BHT, tert-butylhydroquinone TBHQ and so on, which are suspected of having carcinogenic effects [9]. In order to face that issue, new plant sources containing natural antioxidants (carvacrol, eugenol, tocopherol, thymol, etc.) were sought by manufacturers in order to preserve the health of consumers by counteracting oxidative stress without increasing carcinogenic risks. Also, as part of the research and valorization of bioactive substances such as natural substances with antioxidant activity which are of interest in the field of bio pharmacology; several research approaches were conducted in the field of natural aromatic plants. Nowadays, researches showed that aromatic plant extracts can have interesting antioxidant properties to the point of considering replacing or reducing the use of synthetic antioxidants. According to previous experiments, *Citrus aurantifolia* EO exhibited an antioxidant potential with promising IC₅₀ values [10]. The mentioned challenges in the field of antifungals as well as in the search for natural antioxidants raised the proposition of the investigation on the antifungal and antioxidant potentials of *C. anisata* to serve as a starting point for future researches concerning antifungal and antioxidant drugs development. The objective of this work is to determine the antimicrobial properties of *C. anisata* (Rutaceae) essential oil from Bafou area against *C. albicans* isolates and evaluate its antioxidant activity.

2. Material and methods

2.1. Biological material and essential oils extraction

The aromatic plant *C. anisata* was harvested on July. A sample was identified at the National Herbarium of Cameroon (NHC) under the reference number 2711/SRFK. The fungal material was constituted of 08 *C. albicans* isolates.

The fresh leaves were collected and the aromatic material was treated and submitted to hydrodistillation during 5 hours to obtain essential oils. The yield of extraction was calculated.

$$\text{Yield (\%)} = ((\text{total mass of essential oils}) / \text{total mass of the vegetal material}) \times 100$$

2.2. Chemical composition of the extracted essential oils

2.2.1. Gas chromatography coupled with flame ionisation detector (GC/FID) analysis

Essential oils were analyzed on a Varian CP-3380 apparatus equipped with a flame ionization detector (FID) with a DB1 (100% dimethylpolysiloxane) fitted with a fused silica capillary column (30 m × 0.25 mm ; film thickness 0.25 mm) and Supelcowax 10 (polyethylene glycol) fused capillary column (30 m × 0.25 mm ; film thickness 0.25 mm); temperature program 50—2008 C at 58 C/min, injector temperature 2208 C, detector temperature 2508 C, carrier gas N₂ at a flow rate of 0.5 mL.min⁻¹. Two microliters of diluted samples (10/100, v/v, in methylene chloride) were injected manually in a split mode (1/100). The percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. The linear retention index of the components was determined relatively to the retention times of a series of n-alkanes (C₉—C₂₀) [11].

2.2.2. Gas chromatography coupled with Mass spectrometry (GC/MS) analysis

The GC/MS analysis was performed using a Hewlett Packard apparatus equipped with a HP1 fused silica column (30 m × 0.25 mm; film thickness 0.25 mm) and interfaced with a quadrupole detector (Model 5970). Column temperature was programmed from 508 C to 2008 C at 58 C/min; injector temperature was 2208 C. Helium was used as carrier gas at a flow rate of 0.6 mL.min⁻¹, the mass spectrometer was operated at 70 eV. Two microliters of diluted samples (10/100, v/v, in methylene chloride) were injected manually in the split mode (1/100). The identification of individual compounds

was based on the comparison of their relative retention times and their mass spectra of peaks with those obtained from authentic samples on libraries and published data [11].

2.3. Assessment of the antimicrobial activity

The antifungal susceptibility of isolates was determined using the disk diffusion method in a solid medium [12]. A volume of 48 μL of essential oils was diluted into 2952 μL of Dimethylsulfoxide (DMSO). The standard Sabouraud Dextrose agar medium was used as the culture and test medium. The inoculum solution (0.5 McFarland) from a culture of 24 hours was cultured on the medium poured in a petri dish. Then, small sterile paper disks of 6 mm were placed on the solid medium. A volume of 20 μL of the essential oil dilution was put on the disks for each pathogenic microorganism. All the petri dishes were incubated at 37°C during 24 hours. The expression of the results was obtained by measuring the inhibitory zones. The inhibition diameters were reported and compared to the reference diagrams for the classification of susceptible and resistant microbial isolates.

Then, the minimum inhibitory concentrations [13] were determined using a serial microdilution assay on 96 microplates. A total a ten decreasing concentrations of EO solutions were tested (8000-31.25ppm). A volume of 100 μL Sabouraud Dextrose Broth was poured in the microplates, the first line A-H contained 180 μL , and 20 μL of essential oils, in order to produce a concentration of 16000 ppm before adding the inoculum solution. Ten serial dilutions were performed and a volume of 100 μL of inoculum at 0.5 Mcfarland was added. The plates were incubated at 37°C during 18 hours. The assays were performed in triplicate on 8 *C. albicans* isolates. The MICs were considered as the lowest concentrations showing no visible growth.

2.4. Determination of the antioxidant activities

2.4.1. DPPH (2,2-diphenyl-2-picrilhydrazyl) anti-radical activity test

The free radical scavenging assay was performed was carried out as described by [14]. The DPPH solution was prepared by dissolving 20 mg of DPPH in 500 mL of 95° ethanol. It was stored away from light before use. Then different ranges of essential oil concentrations and an ascorbic acid test solution from a stock of 8 mg/mL were prepared (24 mg of essential oil in 3 mL of methanol and 400mg of ascorbic acid in 5ml of distilled water + 45ml of methanol. Each solution of essential oils as well as ascorbic acid (reference antioxidant) was set in 6 test tubes (C1 to C6). Negative control tube (C0) was also prepared. So for the dosage, a volume of 300 μL of essential oil solution and ascorbic acid were introduced into 2 mL of an ethanolic solution of DPPH. The negative control (C0) consisted of 2 ml of DPPH and 200 μL of methanol. After 60 min of incubation in the dark at room temperature, the absorbance was measured with a spectrophotometer at 517 nm. The assays were performed in triplicates.

The scavenging percentage (SC %) was calculated according to the following formula:

$$\text{SC \%}) = \frac{A_{ref} - A_{(test)}}{A_{ref}} \times 100$$

Aref= Absorbance at t = 60 min of the negative control (DPPH solution + methanol)

Atest = Absorbance at t = 60 min of the DPPH solution containing the free radical scavenger.

2.4.2. Anti-radical activity ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) test

The anti-radical ABTS activity was determined according to the main protocol stages as previously described [15]. The ABTS cation radical is generated by mixing in equal volume a solution of 4.9 mM of KMnO_4 (m=16.28mg and v=21ml) and a stock solution of ABTS at 7 mM (m=80.85mg and v= 21ml), everything was stored away from light and at room temperature for 15 hours before use. The solution obtained was diluted 20 times with distilled water. Thus for the dosage, to 2 mL of this freshly prepared solution were added 70 μL of essential oil solutions and 50 μL of ascorbic acid solution prepared at the same concentration range as for the previous antiradical test. After incubation at room temperature and in the dark for 30 min, the optical density reading was taken with a spectrophotometer at 734 nm for each series of analyses. Ascorbic acid was used as the positive control. Assays were performed in triplicate. The anti-radical parameters; SC50 (scavenging percentage for 50% radicals inhibition, EC50 (EO Effective Concentration to inhibit 50% of radicals) and PA (Antiradical Power) were calculated.

2.5. Statistical analysis

The experiments were performed in triplicate. The data were entered into an Excel sheet and presented in the form of tables, graphs and histograms. The results were statistically analyzed by ANOVA and least sensitive difference with Fisher test at a step probability factor of 5%.

3. Results

3.1. Essential oil extraction

The physical features of essential oils were shown in the following table.

Table 1 Physical characteristics of the extracted essential oils from fresh leaves

Characteristics	Description
Color	Fair yellow
Aspect	Oily
Flavor	Anise flavor

The obtained EO were fair yellow with an extraction yield of 0.77%. They were collected and kept in dark containers at 4°C to prevent them from oxidation.

3.2. Chemical characterization of EOs

The results of GC—MS analyses were reported in Table 2. The identified compounds were revealed according to the elution sequence on DB-1 column. A number of 22 secondary metabolites were identified in the sample. The major compounds were E- anethole (70.77 %) and Methyl iso eugenol (13,85 %). However, α -terpinol, cinamyl alcohol and β -caryophyllène were found in traces.

Table 2 Chemical composition of essential oil

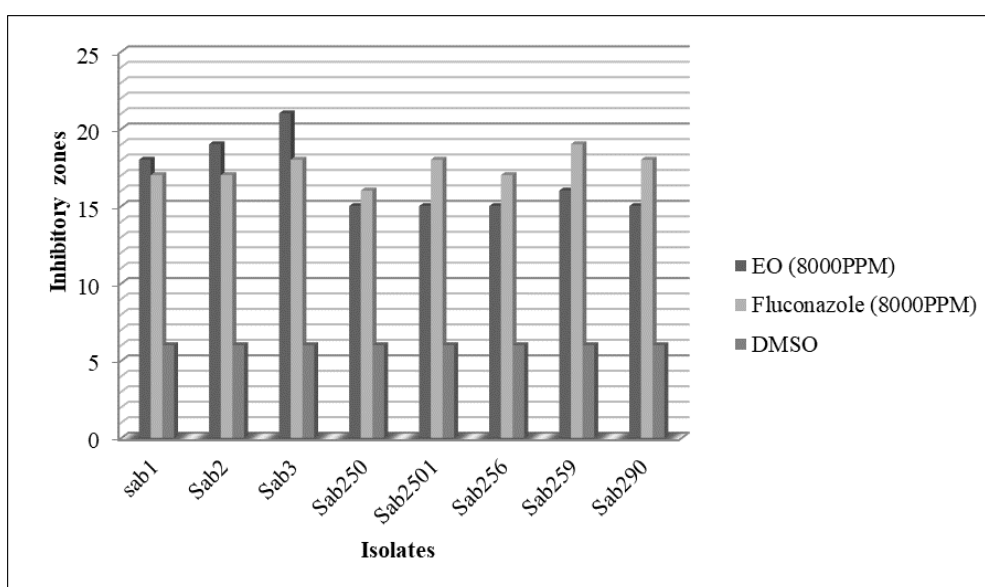
N°	RT	KI	Identified compounds	Percentage (%)
2	10.55	933	α -pinene	0.26
3	12.62	971	sabinene	0.77
4	12.27	976	β -pinene	0.26
5	12.64	986	myrcene	2.82
6	14.19	1021	p-cymene	0.52
7	14.41	1025	limonène	0.77
8	15.12	1041	E- β -ocimene	0.26
9	15.79	1055	γ -terpinene	3.33
10	17.15	1084	terpinolene	0.25
11	21.52	1171	α -terpinol	tr
12	22.56	1191	Estragole	4.10
13	25.12	1242	para anisaldehyde	0.25
14	25.49	1250	Z- anethole	0.25
15	27.52	1291	E- anethole	70.77
16	28.19	1305	Cinamyl alcohol	tr
17	32.18	1387	Cinamyl acetate	0.52

18	33.39	1413	Methyl eugenol	0.52
19	34.55	1438	β -caryophyllene	tr
20	34.92	1447	isoeugenol	0.25
21	36.12	1473	γ -gurjunène	0.50
22	36.78	1487	Methyl iso eugenol	13.85

Legend : RT : Retention time ; KI : Kovats Index ; tr : traces

3.3. Antimicrobial activities-Antifungal screening results

The screening study revealed that the inhibition zones generated by testing *C.anisata* EO at a concentration of 8000 ppm were very closed to those of fluconazole at the same tested concentration against 8 pathogenic isolates. *C. albicans* Sab3, Sab2 and Sab1 isolates were the most susceptible with inhibition zones of 18 mm, 19 mm and 21 mm respectively. At the same testing concentration on the same fungal pathogens, fluconazole exhibited inhibitory diameters of 17 mm, 17 mm and 18 mm respectively. The results were illustrated by figure 1 below.



Legend : Sab1.....290 : *C. albicans* isolates ; DMSO : Dimethyl sulfoxide

Figure 1 Inhibitory diameter zones of *C.anisata* EO against *C. albicans* isolates

Therefore, *C.anisata* essential oil has an antifungal activity closed to the tested reference antifungal fluconazole. The inhibitory diameters measured were recorded from 21 to 15 mm. *C.anisata* EO demonstrated an antifungal activity against the different strains of *C.albicans* used for the study.

3.4. Minimum inhibitory Concentrations (MIC)

The results of the MIC determination were presented in table 3. The MICs values ranged from 2000 to 4000 ppm. The tested EO exhibited a MIC of 2000 ppm on Sab3, Sab2501, Sab250, Sab256, Sab259, Sab290, Sab1 and Sab2. Whereas Fluconazole has shown a minimum inhibitory concentration of 4500 ppm against all the pathogenic yeasts.

The fungicidal activity of the EO was evaluated from subcultures after 72 hours from the previous wells where no growth was observed. After three days of incubation no growth was observed for each of the pathogenic yeasts of *C.albicans*. The assays were carried out in triplicate. These findings highlighted that MFCs were identical to MICs in the studied case.

Table 3 Minimum Inhibitory Concentrations values of tested samples

Tested concentrations (ppm)									
Isolats de <i>C.albicans</i>	8000	4000	2000	1000	500	250	125	62.5	31.25
Sab1	-	-	-	+	+	+	+	+	+
Sab2	-	-	-	+	+	+	+	+	+
Sab3	-	-	-	+	+	+	+	+	+
Sab250	-	-	-	+	+	+	+	+	+
Sab250 ₁	-	-	-	+	+	+	+	+	+
Sab256	-	-	-	+	+	+	+	+	+
Sab259	-	-	-	+	+	+	+	+	+
Sab290	-	-	-	+	+	+	+	+	+
Fluconazole	-	-	+	+	+	+	+	+	+

Legend : Sab1....290 : *C. albicans* isolates ; - : Inhibition ; + : Croissance

3.5. DPPH test Results

The Optical Densities (OD) read at 517nm were used to calculate the scavenging percentages of each concentration of the essential oil. Ascorbic acid was used to as the antioxidant reference. The final concentrations of essential oil and ascorbic acid in each test tube were used to plot the % SC curves in order to determine the SC50, EC50 and PA.

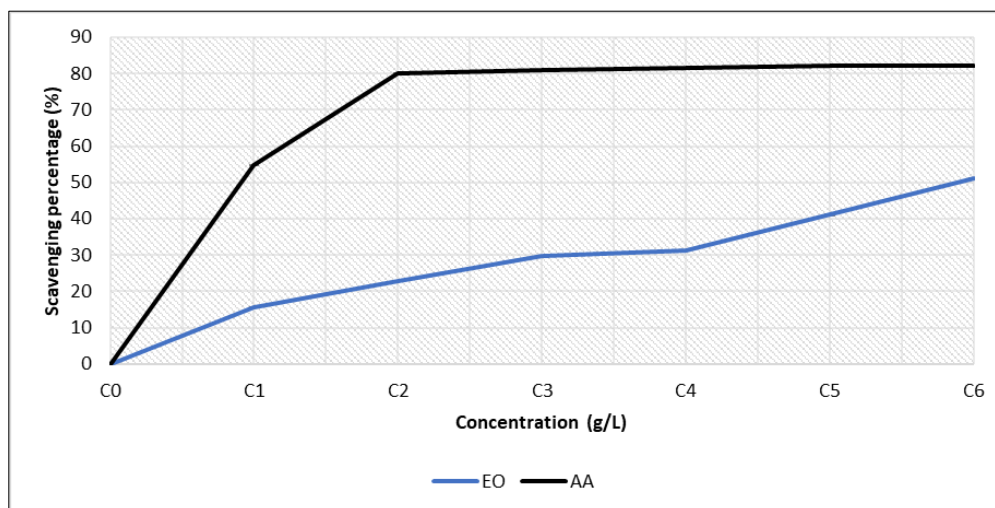


Figure 2 Variation of scavenging percentage of the free radical DPPH• according to the EO and AA concentrations

From this curve, SC50 values of the essential oil (EO) and ascorbic acid (AA) were brought out. These SC50 values represented the needed concentrations of the anti-radical useful to trap 50% of free radicals (DPPH). The analysis of figure 2 shown that the tested agents had dose-dependent activity. It was also found that the EO exhibited an anti-radical activity. From the graph SC50 were plotted to obtain the essential oil the Effective concentration EC50. It appears that the *C. anisata* EO extracted from fresh leaves had a SC50 of 1g/L. Ascorbic acid had the best SC50 value (0.04g/l). This allowed to calculate the effective concentration (EC50) and the anti-radical power (PA).

3.6. ABTS test results

After reading with a spectrophotometer, the ODs obtained allowed the calculation of the scavenging percentages of each concentration of EO and ascorbic acid. The final concentrations of EO and ascorbic acid in each test tube were also calculated in order to plot the % SC curves according to these concentrations.

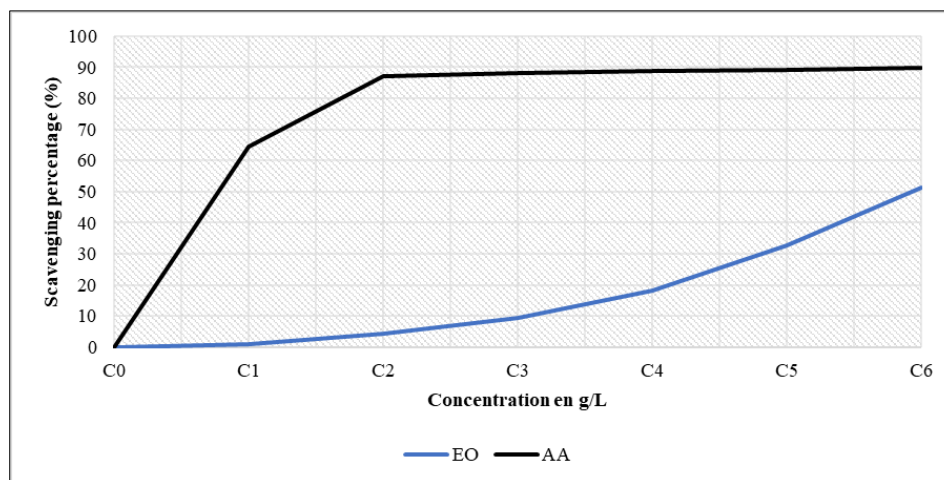


Figure 3 Variation of scavenging percentage of the free radical ABTS• according to the EO and AA concentrations

From the above curve, EO SC₅₀ were deduced. SC₅₀ is the concentration of the anti-radical useful to trap 50% ABTS•. The analysis of this figure 3 shown that the tested agents had a dose-dependent activity. The results revealed that *C. anisata* EO exhibited free radical scavenging activity of SC₅₀=0.265g/L whereas the reference Ascorbic acid under the same conditions was 0.01g/L. This was confirmed by the statistical analysis which revealed a significant difference ($p < 0.05$ ($p = 0.0001$)) among EO and the reference scavenging percentage values.

4. Discussion

The EO extraction yield obtained (0.77%) was higher than that previously obtained [7] from fresh leaves of *C. anisata* harvested in the same area in Ngaoundéré (0.21%). However, the extraction of the fresh leaves of *C. anisata* from Benin produced a yield of 1.62% [16]. This could be explained by the fact that the essential oil extraction yield varies from one region to another. In addition, this variation could be due to the harvest season, the time of harvest, the time of extraction and extraction conditions [17].

The Chemical analysis revealed that the essential oil obtained from the fresh leaves of *C. anisata* from Bafou was mainly rich in E-anethole (70.77%), Methyl iso eugenol (13.85%) and Estragole (4.10%) which were the major compounds. The data slightly differ from those obtained years before. Njonkep's results (unpublished data) reported for EO leaves of *C. anisata* collected in the Bafou site exhibited trans-anethole (69.3%) as the major secondary metabolite. The work reported that leaves of *C. anisata* EO from Mount Bamboutos produced myrtenyl acetate (93.1%) as the main compound without the presence of estragole. Likewise, the study was carried out by other scientists showed that the essential oil of *C. anisata* leaves from Ngaoundéré did not contain estragole. The major compounds were rather E-ocimene (15.2%), terpinene (11.4%) and D-germacrene (10.9%) [7]. Experiments performed in Nigeria, were roughly close to the ones of the present study [18]. On the same way, the chemical composition of the sample harvested in Bafou one year later in December 2016 appeared to be (E)-anethole (64.6%) and (E)-methyl isoeugenol (16.1%) as the major compounds [19]. These last results are similar to those obtained in that study. The differences recorded for the previous experiments regarding the EO chemical composition, could be explained by the fact that the composition of a plant could qualitatively and even quantitatively vary depending on the geographical site [19].

The antimicrobial activity of essential oils as well as their mode of action are directly influenced by the nature and proportion of their constituents which enter into their composition. The major compounds are often responsible for the observed antimicrobial activity [20]. The chemical composition of the essential oil of *C. anisata*, used in this study, is dominated by the presence of aromatic compounds such as E-anethol which is a derivative of phenylpropanoids, and represents 70.77% of the total oil. In fact, propanoids and its derivatives are molecules with a potent antimicrobial activity due to the presence the methoxy group in its structure together with the length of the alkyl chain, which could be responsible of the antimicrobial activity against *C. albicans* [21]. The inhibition diameters obtained are slightly greater than those produced by fluconazole. This antifungal activity may also be affected to the chemical complexity of the oil in term of the composition. Synergistic interactions between intra-compounds inside a same essential oil could be observed and therefore, enhance the antifungal activity [22]. The antimicrobial activity of the tested essential oil is mainly due to its chemical profile. It should be noted that the essence is characterized by the presence of E-anethol (70.77%) and Methyl iso eugenol (13.85%) or related compounds are known for their antimicrobial efficacy even used

alone without combination. Trans-anethol have shown a dose-dependent antifungal activity at concentrations range from 0.29 μ L/mL-0.8 μ L/mL, targeting mitochondrial membrane and DNA [23]. This explains the strong inhibitory activity of the essence against all the *C. albicans* strains (Sab3, Sab250, Sab2501, Sab256, Sab259, Sab290, Sab 1 and Sab2) and a high sensitivity to the oil.

Indeed, given the complexity of its chemical composition and the fragility of its constituents, the mode of action of an essential oil is probably quite complex and difficult to understand from a molecular point of view. Moreover, until now, no detailed mechanism has been given. It is therefore very likely that each constituent of the oil has its own mechanism of action as mentioned above with the case of trans-anethole. Essential oils have a very broad spectrum of action since they effectively inhibit the growth of bacteria, yeast as well as that of mold. Some derivatives of Eugenol were able to inhibit the growth of the mold *Botrytis cinerea* [24] confirming its broad antifungal activity spectrum. The alkyl chain of hydrocarbon skeleton maybe of main importance in the antimicrobial action of the components of these essential oils [21]. Previous antimicrobial research works were instigated in 2011 with a sample also collected from Cameroon. The obtained *C. anisata* EO revealed MICs of 5mg/mL against a diversity of filamentous pathogenic fungi [7]. In addition, secondary metabolites isolated from *C. anisata* using acetone, methanol, petroleum ether and chloroform were tested against pathogenic fungi and expressed an antifungal potency characterized by MIC values of 0.02-10 mg/mL [25, 26].

The obtained results concerning the DPPH test are almost similar to those obtained during experiments performed in Benin. The same method was used and SC₅₀ obtained was 1.67g/L from the fresh leaves of *C. anisata* EO [27]. Moreover, in South Africa, the same researches led to a SC₅₀ of 0.31g/L and 0.29g/L respectively for the aqueous and dichloromethane extracts of *C. anisata* leaves [28].

Concerning the antioxidant potential; the ABTS assay revealed a potent antioxidant activity when compare to those obtained through previous studies performed in South Africa highlighting SC₅₀ values of 0.26 g/L and 0.23 g/L for the aqueous and the dichloromethane extract of *C. anisata* dry leaves respectively [28]. However, *C. anisata* acetone extracts from dry leaves exhibited a SC₅₀ value of 64.08 mg/L [29] ; which is greatly different from the results in this study. Since many years ago natural medicines using traditional plants through their secondary metabolites were used to treat several ailments [30]. The results determined in the present study expressed the expanding approaches that could be used to valorize the secondary metabolites as antioxidant found in essential oils.

5. Conclusion

The aim of this work was to determine the antifungal and antioxidant activity of *C. anisata* essential oil. Thus, the results obtained showed that the essential oil of *C. anisata* extracted from its fresh leaves can be used as a starting point to develop alternative anticandidiasis and antioxidant drugs.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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