



Chemical composition, in-vitro antibacterial and antioxidant activities of *Syzygium aromaticum* essential oil

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Abstract

The aims of this study were to investigate the chemical composition, in-vitro antioxidant and antibacterial activities of *Syzygium aromaticum* essential oil. The essential oil was obtained by hydrodistillation. The GC–MS/GC–FID was used for determination of essential oil composition. The antioxidant activity was determined by DPPH radical scavenging assay and reducing power. The antimicrobial activity was evaluated by the broth microdilution method against six enteropathogens bacteria isolated from diarrhea of young calves. The results of the study revealed an average yield of the essential oil of $11.6\% \pm 0.97$ (w/w). About 65 components were identified and quantified. Eugenol was the major compound of the studied essential oil (78.72%) followed by β -caryophyllene (8.82%) and eugenyl acetate (8.74%). The antioxidant activity by DPPH assay showed IC₅₀ value of $4.82 \pm 0.06 \times 10^{-2}$ μ g/mL, while the reducing power assay have resulted an EC₅₀ value of $3.47 \pm 0.2 \times 10^{-2}$ μ g/mL. The results showed that the essential oil exhibited an MIC at 1.36 mg/mL to 2.72 mg/ml and MBC at 5.45 mg/mL to 10.9 mg/mL against all tested bacteria. The present investigation revealed that *Syzygium aromaticum* essential oil exhibited a good in-vitro antioxidant and moderate in-vitro antibacterial activities.

Keywords *Syzygium aromaticum* · Essential oil · Antimicrobial activity · Antioxidant activity · Enteropathogens bacteria

Introduction

Since last decades, natural products and their compounds have been the most productive source for new drug development [1]. Cloves (*Syzygium aromaticum*, *syn.* *Eugenia aromaticum* or *Eugenia caryophyllata*) are the aromatic dried flower buds [2]. It belongs to the genus *Eugenia* (family Myrtaceae). Commonly known as clove. It is broadly used in cooking, pharmacy, perfumery and cosmetics [3].

Syzygium aromaticum is commonly used in food preparation, as an anti-cancer agent and as a traditional remedy for asthma, digestive system disorder, dental disorders, respiratory disorders, headaches and sore throats in Asian countries [4]. Additionally, in traditional medicine, clove is widely used for treatment of dyspepsia, gastritis and

diarrhea. Furthermore as antipyretic, aphrodisiac, appetizer, expectorant, antiemetic, anxiolytic, myorelaxant, analgesic, decongestant, anti-inflammatory and hypnotic [3]. Besides the reported antimicrobial, antifungal, antiviral, antioxidant and insecticidal properties, the essential oil of *Syzygium aromaticum* shows anti-inflammatory, cytotoxic and anesthetic activities [5].

Neonatal calf diarrhea (NCD) is an important cause of severe economic losses in newborn calves worldwide in the livestock industry. These serious economic losses resulting from morbidity, poor growth rates, treatment costs, and mortality among affected young calves [6–8]. *Enterotoxigenic Escherichia coli*, *Rotavirus*, *Coronavirus* and *Cryptosporidium parvum* are the four most important enteropathogens causing NCD worldwide [6, 7].

Bacterial antimicrobial resistance is an emerging and serious public health concern especially in veterinary medicine, due to the possible transfer of resistance genes between animal and human strains [9]. This is the consequence of the use of antimicrobials in animals [10]. Pereira et al. [11] reported that a common practice in many dairies is to use sub-therapeutic levels of antimicrobials, such as tetracycline and neomycin, added to milk or milk replacers for disease

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prophylaxis and growth promotion, and to improve feed efficiency. Multidrug-resistant (MDR) *Enterobacteriaceae* are becoming a major worldwide concern in veterinary medicine [12, 13]. A high rate (68.75%) of MDR of *E. coli* isolated from neonatal diarrhea in calves less than 30 days old has been reported by [14].

The aims of the present study were to assess the chemical composition, antioxidant and the antibacterial activities of *Syzygium aromaticum* essential oil against several enteropathogens bacteria isolated from diarrhea of young calves.

Materials and methods

Extraction of essential oil

Clove flower buds (*S. aromaticum*) were purchased from local market in Tiaret (Algeria). Clove flower buds was crushed using mortar and pestle. The crushed clove bud was stored in dark container for future use. The essential oil of clove flower buds was extracted by hydro-distillation for 1 h 30' by mixing 20 g of crushed clove bud in 500 ml of distilled water. The obtained oil was collected and dried over anhydrous sodium sulfate and stored in amber vials with screw cap in a refrigerator at 4 °C prior to analysis. The percentage yield of *Syzygium aromaticum* essential oil was calculated using the following formula.

$$\text{Yield of essential oil} = \frac{\text{essential oils weight(g)}}{\text{sample weight(g)}} \times 100$$

Analysis of essential oil

The essential oil from flower buds of *Syzygium aromaticum* was analysed by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC/MS) realized by Pyrenessences Analysis. Briefly, the oil was analyzed using a Hewlett Packard 5973, with HP INNOWAX polar column (60 m × 0.25 mm × 0.25 μm). One microliter of essential oil diluted in ethanol (10%) was injected and analysed. Helium was the carrier gas with a flow rate of 30 psi/FID, 23 psi/MS. Column temperature was initially kept at 60 °C for 6 min and then gradually increased to 250 °C at 2 °C/min rate and finally held for 10 min at 250 °C. The temperature of the injector was fixed to 250 °C and the one of the detectors (FID) to 270 °C. The compounds were identified by a combined search of retention time and mass spectra (NKS library, 75,000 spectra). The percentages are calculated from the peak areas given by the GC/ FID, without the use of correction factor.

Antimicrobial study

Microorganisms

The antimicrobial activity of *S. aromaticum* essential oil was evaluated against seven strains of Gram-negative bacteria (*Escherichia coli* F5, *Escherichia coli* CS31A, *Kluyvera* sp., *Klebsiella* sp., *Salmonella* sp. and *Serratia* sp.) isolated from neonatal calves diarrhea.

Preparation of inoculum

Prior to the experiment, the strains were inoculated onto the surface of Mac Conkey agar media, the inoculum suspensions were obtained by taking five colonies from 24 h cultures. The colonies were suspended in 5 mL of sterile saline (0.85% NaCl) and shaken for 15 s. The density was adjusted to the turbidity of a 0.5 McFarland Standard (equivalent to $1-5 \times 10^8$ cfu/mL).

MIC and MBC determination of *Syzygium aromaticum* essential oil

The estimation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were measured by the broth micro-dilution method [15]. The essential oil was dissolved in sterilized physiological saline solution 0.85% (w/v) supplemented with Tween 20 (Sigma) at final concentration of 10% (v/v). Serial doubling dilutions of the oils were prepared in a 96-well microtiter plate ranged from 10.9 to 0.34 mg/mL. The inoculum was added to all wells and the plates were incubated at 37 °C for 24 h. The bacterial growth was visualized by adding 20 μL of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) aqueous solution [16]. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the oils that inhibited visible growth (red-colored pellet at the bottom of the wells after the addition of TTC), while the minimum bactericidal concentration (MBC) was defined as the lowest concentration that killed 99.9% of bacterial cells. To determine MBC, broth was taken from each well without visible growth and inoculated on Mueller Hinton agar for 24 h at 37 °C. Each experiment was repeated three times.

Antibiotic susceptibility test

Antimicrobial susceptibility testing of bacteria was carried out by the disk diffusion method using commercial disks, according to the recommendations of committee on standardization of susceptibility testing in veterinary medicine [17, 18]. The following antimicrobial agents, loaded on the

disks, were tested: amoxicillin + clavulanic acid (20/10 µg), ampicillin (10 µg), gentamicin (10 µg), tetracycline (10 µg), colistin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), ofloxacin (5 µg) and cefotaxime (30 µg). Susceptibility of strains isolates to commonly used antibiotic agents was measured and interpreted based on the recommendation of committee on standardization of susceptibility testing in veterinary medicine [17, 18].

Antioxidant activity

Reducing power

The Fe³⁺ reducing power of *Syzygium aromaticum* essential oil was determined by the method of Yen and Duh [19] with some modifications. Essential oil was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated for 20 min at 50 °C. After incubation, 2.5 mL of trichloroacetic acid (10%) was added to the mixture followed by a centrifugation at 3000 rpm for 10 min. The upper layer (1 mL) was mixed with 1 mL of distilled water and 0.5 mL of ferric chloride (0.1%). The absorbance of the obtained solution was measured at 700 nm. A higher absorbance indicates a higher reducing power. Reducing power assay of the sample was expressed by EC50 value. The EC50 value was the effective concentration at which the absorbance was 0.5 and was obtained by interpolation from regression analysis. All tests were carried out in triplicate. Quercetin and ascorbic acid were used as positive controls.

DPPH radical-scavenging assay

The DPPH radical scavenging assay was determined according to the method described previously by Tien et al. [20]. The scavenging rate on DPPH radicals was calculated according to the formula: Scavenging rate (%) = 100 × (A_{blank} - A_{sample})/A_{blank}, where A_{blank} represents the absorbance of the control reaction (containing all reagents except the tested compound), and A_{sample} represents the absorbance of the tested compound.

The antiradical activity was expressed as IC50 (µg/mL), which is defined as the extract concentration necessary to scavenge 50% DPPH free radicals and is obtained by interpolation from regression analysis. The determination was carried out in triplicate, and the results were expressed as mean values ± standard deviation (SD). Gallic acid, quercetin and ascorbic acid were used as positive controls.

Statistical analysis

The assays were performed in triplicate and the results were expressed as the mean values with standard

deviations (SD). The significant differences were obtained by a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test (p < 0.05). These statistical analyses were calculated using R software (version 3.3.0/ 2016-05-03).

Results and discussion

Yield and chemical composition

The *Syzygium aromaticum* essential oil was extracted with yield of 11.6% ± 0.7. This result is in agreement with those of Guan et al. [21] and Nana et al. [22] who obtained 11.5% and 10.54%, respectively. Although, these results are different from those acquired by several study [23–27] with a yield varying from 0.18 to 7.6%.

Yield difference can be influenced by harvest season and geographical origin [22], extraction technique [21]. However, Guen et al. [21] incriminates the particle size of ground cloves on the yield (The extraction yield increases by decreasing the particle size of the comminuted cloves).

Sixty-five signal peaks related to separate components were obtained by gas chromatography (GC–FID) and gas chromatography–mass spectrometry (GC/MS) in *S. aromaticum* essential oil, where compounds were shown in Table 1. The main constituents were eugenol (78.72%), β-caryophyllene (8.82%) and eugenyl acetate (8.74%).

Several studies indicate that eugenol, β-caryophyllene and eugenyl acetate are the major compounds of the essential oil of *S. aromaticum* with varying percentages [24, 26, 28–35].

Likewise, Barakat [36] reported that major compounds of *S. aromaticum* the essential essential oil were eugenol (80.19%), eugenyl acetate (7.91%), caryophyllene (3.79%), furan, tetrahydro-3-methyl (2.26%) and 2-propanone, methylhydrazone (1.54%).

Nevertheless, other studies have reported a difference in the majority compounds, namely the study of Lee et al. [4] who have shown that eugenol, β-caryophyllene, 2-propanone, methylhydrazone, cyclopentane, methyl, furan, tetrahydro-3-methyl and α-caryophyllene are the major compounds of *S. aromaticum* essential oil. While, Sokamte et al. [27] reported that eugenol, δ-Cadinene and β-elemene are the major compounds of this essential oil.

Previous investigations have demonstrated that the chemical composition of *S. aromaticum* essential oil can be influenced by the condition of the environment, the genotype, the geographical origin, the period of harvest, the place of drying, the temperature and the duration of drying and the method of extraction [4, 32, 27].

Table 1 Chemical composition of *Syzygium aromaticum* essential oil

Peak	RT (min)	Compounds	%
1	7.2	Ethanol	0.28
2	10.5	α -Pinene	0.01
3	14.7	β -Pinene	0.01
4	17.0	Δ 3-Carene	0.01
5	18.9	2-Heptanone	0.03
6	20.0	Limonene	0.03
7	20.8	1.8-Cineole	0.04
8	22.1	Cis- β -ocimene	0.01
9	23.3	Trans- β -ocimene	0.01
10	24.0	Acetate de 2-heptyle	0.04
11	27.0	2-Methyl-6-methylene-1.7-octadien-3-one	0.01
12	29.2	6-Methyl-5-hepten-2-one	0.01
13	32.8	2-Nonanone	0.03
14	37.6	α -Cubebene	0.01
15	40.1	α -Copaene	0.01
16	41.9	β -bourbonene	0.02
17	42.9	Linalol	0.01
18	45.6	ϵ -Cadinene	0.01
19	46.8	2-Undecanone	0.01
20	47.1	Terpinene-4-ol	0.02
21	47.4	β -Caryophyllene	8.82
22	48.4	Benzoate de methyle	0.01
23	50.2	Pulegone	0.01
24	50.3	Acetophenone	0.02
25	51.0	Zonarene	0.01
26	51.2	Benzoate d'ethyle	0.02
27	51.4	Acetate de dihydrocarvyle	0.04
28	51.8	α -Humulene	0.95
29	52.8	γ -Muuroleone	0.01
30	52.9	α -Terpineol	0.04
31	53.0	Acetate de terpenyle	0.02
32	54.2	Germacrene D	0.04
33	54.4	Cubenene isomere	0.02
34	54.8	Acetate de benzyle	0.03
35	54.9	α -Muuroleone	0.03
36	55.2	α -Selinene	0.02
37	55.7	Carvone	0.15
38	55.8	α -Farnesene	0.12
39	56.8	δ -Cadinene	0.11
40	57.2	γ -Cadinene	0.02
41	57.4	Alcool terpenique	0.02
42	58.0	Salicylate de methyle	0.10
43	58.5	Cadina-1.4-diene	0.02
44	59.1	α -Amorphene	0.01
45	61.4	Calamenene	0.02
46	66.5	Jasmone	0.03
47	67.8	Composé aromatique	0.01
48	69.9	Oxyde d'isocaryophyllene	0.02
49	70.4	Oxyde de caryophyllene	0.38
50	71.0	Methyleugenol	0.01

Table 1 (continued)

Peak	RT (min)	Compounds	%
51	73.4	Epoxy-6.7-humulene	0.05
52	73.8	Caryophyllenol	0.01
53	74.3	Cubenol	0.01
54	78.2	Sesquiterpenol	0.01
55	79.1	Eugenol	78.72
56	82.3	α -Cadinol	0.01
57	83.3	Acetate d'eugenyle	8.74
58	85.0	Epoxyde sesquiterpenique	0.02
59	85.6	Caryophylla-3.7-dien-6-ol	0.09
60	86.6	Chavicol	0.14
61	87.3	Epoxyde sesquiterpenique	0.04
62	87.5	Isoeugenol	0.02
63	89.1	Epoxyde sesquiterpenique	0.06
64	95.8	2. 3. 4-trimethoxyacetophenone	0.03
65	100.7	Benzoate de benzyle	0.03
		Total	99.81

Bold values indicate the majority compounds

Antioxidant activity

In this study, reducing power and DPPH radical scavenging capacity has been used to evaluate antioxidant activity of *S. aromaticum* essential oil. The reducing power was used to investigate antioxidant activity that is based on the capacity of antioxidants to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Table 2 show the EC_{50} value of reducing power of clove essential oil. The samples were evaluated for their radical scavenging capacity using DPPH assay, which is based on the scavenging of the stable DPPH \cdot by an antioxidant. Table 2 summarized the EC_{50} values of DPPH scavenging activity of clove essential oil.

The EC_{50} value for *S. aromaticum* essential oil was $3.47 \pm 0.2 \times 10^{-2}$ $\mu\text{g}/\text{mL}$ in comparison to 21.22 ± 1.66 $\mu\text{g}/\text{mL}$ and 13.98 ± 1.99 $\mu\text{g}/\text{mL}$ for the standard compound quercetin and ascorbic acid, respectively. However, The IC_{50} value for

Table 2 Antioxidant activity of *Syzygium aromaticum* essential oil in reducing power and DPPH assays

	Reducing power (EC_{50}) $\mu\text{g}/\text{mL}$	DPPH (IC_{50}) $\mu\text{g}/\text{mL}$
Quercetin	21.22 ± 1.66^c	$12.02 \pm 1.3^{a,b}$
Gallic acid	–	5.39 ± 0.41^b
Ascorbic acid	13.98 ± 1.99^b	6.19 ± 0.97^b
<i>Syzygium aromaticum</i> essential oil	$3.47 \pm 0.2 \times 10^{-2a}$	$4.82 \pm 0.06 \times 10^{-2a}$

Each value in the table is represented as mean \pm SD (n = 03). Means not sharing the same letter are significantly different (LSD) at $p < 0.05$ probability level in each column

S. aromaticum essential was $4.82 \pm 0.06 \times 10^{-2}$ µg/mL in comparison to the standard compound.

The IC₅₀ values higher than those obtained in our study were mentioned by Chaieb et al. [14] (0.2 µg/mL), Saeed and Shahwar [26] (4.5 µg/mL), Sokamte et al. [27] (23.17 µg/mL), Mahboubi and Mahboubi [28] (26 µg/mL), Singh et al. [11] (80 µg/mL), Zhang [37] (396.3 µg/ml) and Viuda-Martos et al. [38] (380 µg/mL). Singh et al. [11], Chaieb et al. [14] and Viuda-Martos et al. [38] showed that the essential oil IC₅₀ was lower than standards used, which is in agreement with our study.

In the present study, the EC₅₀ value for *S. aromaticum* essential oil was higher than that reported by Zhang [37] with an EC₅₀ of 683.4 µg/mL. Viuda-Martos et al. [38] and Saeed and Shahwar [26], have shown the highest capacity of clove essential oil, at different concentrations analyzed, to reduce iron.

Kennouche et al. [30] attributes this surprising antioxidant activity to high concentrations of clove oil in eugenol and eugenyl acetate. Additionally, Sokamte et al. [27] explained this high antioxidant activity by the synergistic interactions between eugenol and the other minority components present in this essential oil.

Three main mechanisms of action of eugenol may explain this interesting antiradical effectiveness: donation of hydrogen followed by the delocalization of the group substituted at the para-position; dimerization between two phenoxy radicals and complexation of DPPH[·] with an aryl radical [27].

Antimicrobial activities

Regarding the antibiotic susceptibility, all tested strains were resistant to ampicillin, amoxicillin + clavulanic acid, tetracycline, trimethoprim/sulfamethoxazole and ofloxacin (Table 3). The antimicrobial activity of *S. aromaticum* essential oil against Gram-negative bacteria associated with

neonatal calves diarrhoea is shown in Table 4. The MIC obtained in this study ranged from 1.36 to 2.72 mg/mL. While the MBCs varied from 5.45 to 10.9 mg/mL.

In our study, the MIC of the essential oils was determined by the broth micro-dilution method. *S. aromaticum* essential oil exhibited good in vitro antibacterial activity towards the clinical isolates of neonatal calves diarrhea. Ayoola et al. [23] have reported a low MIC (0.23 mg/mL) has been necessary for stopping the growth of *E. coli* ATCC35218. Likewise, Alitonou et al. [24] showed a lower value of MIC of *S. aromaticum* essential oil against *E. coli* ATCC 25922, with concentration of 0.2 mg/mL. However, Prabuseenivasan et al. [39] reported a higher concentration (> 1.6 mg/mL) for inhibits the growth of *E. coli*. Nevertheless, Ayoola et al. [23] cited a MIC of 1.6 mg/mL against an isolate of *E. coli*. On the other hand Naveed et al. [40] mentioned a higher concentration (5.4 ± 1.08 mg/mL) against *E. coli* SS1. Previous studies have reported MICs of 0.2% (w/v) against *E. coli*, *S. enteritidis* and *S. typhimurium* [31].

In the present study the *S. aromaticum* essential oil exhibited a MBC varied from 5.45 to 10.9 mg/mL. A lower MBC was mentioned by Alitonou et al. [24] with 3.12 mg/mL against *E. coli* ATCC 25922.

Table 4 Antimicrobial activity of *Syzygium aromaticum* essential oil

Bacteria	<i>Syzygium aromaticum</i> essential oil (mg/mL)	
	MIC	MBC
<i>Serratia</i> sp.	2.72	10.9
<i>Salmonella</i> sp.	2.72	5.45
<i>Kluyvera</i> sp.	1.36	5.45
<i>Klebsiella</i> sp.	1.36	10.9
<i>E. coli</i> F5	1.36	5.45
<i>E. coli</i> CS31A	1.36	10.9

Table 3 Antimicrobial susceptibility of strains

Antibiotic	A	B	C	D	E	F
Ampicillin (10 µg)	R	R	R	R	R	R
Amoxicillin + clavulanic acid (20/10 µg)	R	R	R	R	R	R
Gentamicin (10 µg)	S	R	R	R	R	S
Tetracycline (10 µg)	R	R	R	R	R	R
Colistin (10 µg)	S	R	S	S	R	R
Trimethoprim/sulfamethoxazole (1.25/23.75 µg)	R	R	R	R	R	R
Ofloxacin (5 µg)	R	R	R	R	R	R
Cifotaxime (30 µg)	S	R	R	S	S	R

A: *E. coli* F5, B: *E. coli* CS31A, C: *Klebsiella* sp., D: *Kluyvera* sp., E: *Salmonella* sp., F: *Serratia* sp.
R resistant, S sensible

Nazzaro et al. [41] reported that “eugenol acts on bacteria by altering the cell membrane, affecting the transport of ions and ATP, and by changing the fatty acid profile of different bacteria”. It also acts against various bacterial enzymes, including ATPase, histidine carboxylase. Moreover, eugenol demonstrated the disintegrating capability in the membrane [42]. This alteration results from the denaturation of the proteins by eugenol and the reaction of this compound with the cell membrane phospholipids changing their permeability [21] and the increase of this causes death of the organism [42].

Conclusions

The results of our study showed that the *S. aromaticum* essential oil is a rich source of eugenol. This essential oil exhibited a good in-vitro antioxidant and moderate in-vitro antibacterial activities and could be an alternative for the treatment of diarrhea in calves caused by the tested strains. These bioactivities are mainly due to the high content of eugenol in this specie. Nevertheless, additional studies will be needed to justify the potential use of this oil as an antibacterial agent against bacteria causing neonatal calves’ diarrhea.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest in this study.

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