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Research Article
Chemical Composition and Antibacterial Activity of *Cinnamomum aromaticum* Essential Oil Against Four Enteropathogenic Bacteria Associated with Neonatal Calve's Diarrhea

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Abstract

Objective: The aim of this study was to investigate the chemical composition and the *in vitro* antibacterial activity of *Cinnamomum aromaticum* essential oil against four strains of enteropathogenic bacteria isolated from neonatal calve's diarrhea namely *Escherichia coli* F5, *Escherichia coli* F17, *Kluyvera* spp. and *Klebsiella* spp. **Methodology:** The essential oil was extracted by hydrodistillation process. The composition was determined by Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Flame Ionization Detector (GC-FID). The agar incorporation method was used to determine the antimicrobial activity of *Cinnamomum aromaticum* essential oil against four strains of enteropathogenic bacteria isolated from neonatal calve's diarrhea including *Escherichia coli* F5, *Escherichia coli* F17, *Kluyvera* spp. and *Klebsiella* spp. **Results:** The results of the study revealed an average yield of the essential oil of 1.46±0.05% (w/w). About 89 components were identified and quantified. E-cinnamaldehyde was the major compound of the studied essential oil (94.67%). The results showed that the tested essential oil exhibited an antibacterial activity against all tested bacteria at a Minimum Inhibitory Concentration (MIC) of 0.625 μL mL^{-1}. **Conclusion:** The present investigation revealed that *C. aromaticum* essential oil is potentially good source of antibacterial agents and could be used as against the tested strains causing diarrhea in calves.

Key words: *Cinnamon aromaticum*, essential oil, chamical composition, antibacterial activity, minimal inhibitor concentration

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Calf diarrhea is a major cause of economic loss with high morbidity and mortality in the cattle industry worldwide[1]. The incidence of diarrhea in calves less than 1 month ranges between 15-20% and the greatest risk occurs especially during the first 2 weeks of life[2]. Several enteropathogens were recovered from neonatal calf with diarrhea, their relative prevalence varies geographically[3], but the most pathogens commonly incriminated in neonatal calf scours include viral (*Rotavirus* and *Coronavirus*), protozoal (*Cryptosporidium parvum*) and bacterial pathogens (enterotoxigenic *Escherichia coli* K99 and *Salmonella* spp.)[4]. To compensate this significant economic loss, various and large amounts of antimicrobial drugs are used in calves feed as a preventive and curative purposes[5]. Unfortunately, the randomly used treatment without selecting appropriate antimicrobial compound resulted in the emergence of resistant calf pathogens and commensals, which becomes a serious public health problem throughout the world[6]. Akam *et al.*[7] reported the resistance of *E. coli* F5+ to tetracycline, ticarcilline, ampicilline in Algerian cattle. Furthermore, De Verdier *et al.*[8] in Swedish showed that 61% of *E. coli* tested for antimicrobial susceptibility were resistant to one or more substances and 28% were multi-resistant.

Gram-negative bacteria often gain their resistance through the acquisition of a resistance gene from a shared gene pool with the aid of plasmids[9], resulting in the targeting of the dissemination of plasmids. This is one of the most used strategies by bacteria to circumvent antibiotic resistance at the molecular level[10]. Therefore, therapeutic control of multidrug resistant bacteria has a major concern in the area of global public health.

Essential oils, particularly, those from cinnamon, peppermint, tea tree, lavender and marjoram have been well investigated for their general antimicrobial properties against several pathogenic microorganisms[10].

The genus *Cinnamomum* (family: Lauraceae) contains more than 300 evergreen aromatic trees and shrubs. Four species have great economic importance for their multiple culinary uses as common spices including *Cinnamon zeylanicum* Blume (a synonym of *Cinnamon verum* J. Presl, known as Sri Lanka cinnamon), *Cinnamon loureiroi* Nees (known as Vietnamese cinnamon), *Cinnamon burmanni* (Nees and T. Nees) Blume (known as Indonesian cinnamon) and *Cinnamon aromaticum* Nees (a synonym of *Cinnamon cassia* (L.) J. Presl, known as Chinese cinnamon)[11].

Cinnamon has a long history of use as preservative and medicinal use in the East[12]. It has a reputation as an effective cure for colds[13]. In ayurvedic medicine it has been used as antiemetic, anti-diarrheal, anti-flatulent and stimulant agent[11].

The aim of the present study was to investigate the chemical composition of the essential oil of *Cinnamon aromaticum* and its antibacterial activity against four strains of bacteria isolated from neonatal calve's diarrhea.

MATERIALS AND METHODS

Extraction of essential oil: The bark of *Cinnamomum aromaticum* was purchased from a specialized store in Tlemcen (Algeria). The essential oil was extracted by hydrodistillation process by mixing 50 g of crushed *Cinnamomum aromaticum* in 500 mL of distilled water for 2 h and 30 min. The extracted essential oil was dried by anhydrous sodium sulphate and stored in sealed vials at 4°C before antibacterial activity testing, Gas Chromatography-Mass Spectrometric (GC-MS) and Gas Chromatography/Flame Ionization Detector (CG/FID) analysis. The average yield of the extracted essential oil was 1.46±0.05% (w/w). The percentage yield of cinnamon essential oil was calculated using the following formula:

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

CG/SM and GC/FID analysis: The GC/MS and GC/FID analysis was performed by Sarl Pyrenessences Analysis (Belcaire, France). Briefly, the cinnamon oil was analyzed using a Hewlett Packard 5973, with HP INNOWAX polar column (60 m×0.25 mm×0.25 μm). One microliter essential oil solution diluted in ethanol 10% was injected and analyzed. Helium was the carrier gas with a flow rate of 30 psi/FID; 23 psi/MS. The temperature was programmed at 60°C for 6 min and then increased by 2°C min^{-1} up to 250°C for 10 min. The compounds were identified by a combined search of retention time and mass spectra (NKS library, 75,000 spectra). The percentages were calculated from the peak areas given by the GC/ FID, without the use of correction factor.

Antimicrobial study

Microorganisms: The antimicrobial activity of the essential oil of *Cinnamomum aromaticum* was evaluated against four strains of Gram-negative bacteria (*Escherichia coli* F5, *Escherichia coli* F17, *Kluyvera* spp. and *Klebsiella* spp.). The microbial strains used in this study were isolated from neonatal calve's diarrhea.

Preparation of inoculum: Prior to the experiment, bacterial strains were inoculated onto the surface of MacConkey 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 sec. The density was adjusted to the turbidity of a 0.5 McFarland Standard (equivalent to 1-5×10^8 CFU mL^{-1}).

Antibiotic susceptibility test: Susceptibility to a panel of antimicrobial agents was determined by the standardized disc diffusion assay on Mueller-Hinton agar with commercial antimicrobial susceptibility discs according to the standardization of antimicrobial susceptibility testing in the veterinary medicine at the national level according to WHO recommendations[14,15]. The tested antibiotics and their corresponding disc concentrations were as follows: Amoxicillin+acid clavulanic (20/10 µg), ampicillin (10 µg), gentamicin (10 µg), tetracycline (10 µg), colistin (10 µg), trimethoprime/sulfamethoxazole (1.25/23.75 µg), ofloxacine (5 µg) and cifotaxime (30 µg). The prepared plates were incubated at 37°C for 24-48 h. The zone of inhibition was recorded and data were interpreted using the standardization of antimicrobial susceptibility testing in the veterinary medicine at the national level according to WHO recommendations[14,15].

Minimum Inhibitory Concentration (MIC) measurement: Screening of essential oil for antibacterial activity was achieved by the incorporation method as described by Amarti *et al.*[16], with slight modifications. The essential oil was dissolved in tween 20, which is prepared in sterile distilled water (1/9 v/v). A serial dilution of cinnamon essential oil was prepared with tween 20 (1/10, 1/20, 1/40, 1/80, 1/160 and 1/320). Each test tube contained 4.5 mL of Mueller Hinton agar medium to which were added aseptically 0.5 mL of the prepared dilutions to obtain a final concentrations of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 v/v. The tubes were thoroughly agitated to disperse the essential oil in the culture medium before pouring in to petri plates. The control contained only the culture medium and the tween 20. The plates were inoculated and incubated at 37°C for 24 h. The Minimum Inhibitory Concentration (MIC) was determined by identifying the plates with the lowest concentration of essential oil on which the strain did not grow. Tests were repeated in triplicate. All MIC values are expressed in v/v.

RESULTS

Chemical composition of essential oil: The results concerning the chemical analysis of the essential oil identified by GC-FID were given in Table 1. In general, the composition was relatively homogeneous. Eighty nine compounds were identified, representing 99.99% of total components. E-cinnamaldehyde (94.67%) was the major component followed by coumarin (0.88%) and cinnamyl acetate (0.74%) (Table 1).

Table 1: Composition of the essential oil of cinnamon

Peak	RT (min)	Components	%
1	5.3	Acetone	0.01
2	6.3	Acetate d'ethyle	0.01
3	7.2	Ethanol	0.25
4	10.6	α-pinene	0.03
5	12.7	Camphene	0.03
6	13.3	Hexanal	0.01
7	15.7	O-xylene	0.01
8	20.1	Limonene	0.03
9	20.8	1,8-cineole	0.10
10	23.1	γ-terpinene	0.01
11	23.9	Styrene	0.01
12	24.8	P-cymene	0.01
13	25.6	Terpinolene	0.01
14	35.7	Ethyl hexanol	0.01
15	36.6	Acide acetique	0.02
16	40.0	α-campholene aldehyde	0.01
17	40.1	α-copaene	0.03
18	42.0	Benzaldehyde	0.29
19	42.9	Linalol	0.03
20	44.9	α, cis-bergamotene	0.01
21	45.3	Pinocarvone	0.01
22	45.6	Fenchol	0.02
23	45.9	Acetate de bornyle	0.14
24	46.4	β-elemene	0.01
25	46.7	Hydrate de camphene	0.01
26	47.0	Terpinene-4-ol	0.10
27	47.2	β-caryophyllene	0.01
28	49.2	Methylene propenyl benzene (MW = 130)	0.01
29	49.8	Benzeneacetaldehyde	0.22
30	49.3	P-methoxy styrene	0.04
31	50.3	Acetophenone	0.02
32	50.8	2-methyl benzofurane	0.01
33	51.1	Isoborneol	0.01
34	51.2	δ-terpineol	0.01
35	51.3	Estragole	0.01
36	51.8	α-humulene	0.01
37	52.0	Aldehyde salicylique	0.02
38	52.7	γ-muurolene	0.01
39	52.8	α-terpineol	0.03
40	53.2	Borneol	0.14
41	54.3	Compose aromatique (MW = 138)	0.01
42	54.9	α-muurolene	0.01
43	55.2	α-selinene	0.01
44	55.7	Carvone	0.02
45	56.6	Dimethyl oxa bicyclohexane (MW = 112)	0.01
46	56.8	δ-cadinene	0.01
47	57.0	Compose (MW = 132)	0.02
48	57.2	γ-cadinene	0.01
49	58.2	Benzene propanal	0.70
50	59.6	Hydroxy phenyl ethanone	0.01
51	60.0	2,4-decadienal	0.03

Table 1: Continue

Peak	RT (min)	Components	%
52	60.9	Trans-carveol	0.01
53	61.3	Geraniol	0.01
54	61.4	Calamenene	0.01
55	61.6	P-cymene-8-ol	0.01
56	62.3	2-hydroxycineole	0.01
57	62.7	4-phenyl-2-butanone	0.01
58	63.3	Alcool benzylique	0.01
59	64.9	Propionate d'ethylphenyle	0.09
60	65.0	Z-cinnamaldehyde	0.01
61	65.2	Alcool phenylethylique	0.01
62	74.1	E-cinnamaldehyde	94.67
63	74.2	Epi-cubenol	0.01
64	74.5	Cubenol	0.03
65	75.7	Sesquiterpenol	0.01
66	77.4	Cinnamate d'ethyle	0.04
67	78.3	Acetate de cinnamyle	0.74
68	78.9	Eugenol	0.01
69	79.3	T-cadinol	0.01
70	80.1	α-muurolol	0.04
71	80.6	Cadinol Isomere	0.03
72	80.7	Carvacrol	0.01
73	82.0	Sesquiterpenol	0.01
74	82.3	α-cadinol	0.02
75	83.5	Sesquiterpenol	0.02
76	84.4	Alcool cinnamique	0.36
77	85.0	Epoxyde Sesquiterpenique	0.01
78	86.5	Sesquiterpenol	0.02
79	86.7	Chavicol	0.01
80	87.3	Epoxyde sesquiterpenique	0.04
81	89.1	Epoxyde sesquiterpenique	0.03
82	92.2	Trans-o-methoxy-cinnamaldehyde	0.05
83	93.4	Coumarine	0.88
84	96.4	3-methoxycinnamaldehyde	0.01
85	99.6	Compose aromatique	0.05
86	100.8	Benzoate de benzyle	0.01
87	106.1	α-phellandrene dimere (MW = 268)	0.03
88	107.3	Compose aromatique	0.03
89	108.9	α-phellandrene dimere (MW = 268)	0.03
		Total	99.99

Table 2: Antibiotic susceptibility of tested strains

Antibiotic	A	B	C	D
Ampicillin (10 µg)	R	R	R	R
Amoxicillin+acid clavulanic (20/10 µg)	R	R	R	R
Gentamicin (10 µg)	S	S	S	S
Tetracycline (10 µg)	R	I	R	R
Colistin (10 µg)	S	S	S	S
Trimethoprime/sulfamethoxazole (1.25/23.75 µg)	R	S	R	R
Ofloxacine (5 µg)	S	S	R	R
Cifotaxime (30 µg)	S	S	S	S

R: Resistant, S: Sensitive, I: Intermediate, A: *Escherichia coli* F17, B: *E. coli* F5, C: *Kluyvera* spp., D: *Klebsiella* spp.

Antibacterial activity

Antibiotic susceptibility: Regarding the antibiotic susceptibility, all tested strains were susceptible to cifotaxime, colistin and gentamicin. However, three strains were resistant to trimethoprime/sulfamethoxazole and two strains were resistant to ofloxacine (Table 2).

Table 3: Antibacterial activity of essential oil of cinnamon

	Dilution (v/v)						
Bacteria	1/100	1/200	1/400	1/800	1/1600	1/3200	Control
E. coli F17	-	-	-	-	-	+	+
E. coli F5	-	-	-	-	-	+	+
Kluyvera spp.	-	-	-	-	-	+	+
Klebsiella spp.	-	-	-	-	-	+	+

-: Inhibition, +: Development

Antimicrobial activity of essential oil (determination of MIC): Table 3 showed the results of the antibacterial activity of the essential oil of *C. aromaticum.* This essential oil exerted strong antibacterial activity. Concentration of 1/1600 v/v was sufficient to inhibit the growth of *Escherichia coli* F17, *Escherichia coli* F5, *Kluyvera* spp. and *Klebsiella* spp.

DISCUSSION

In the present study, the average yield of cinnamon essential oil was 1.46±0.05% (w/w). A similar result was obtained by Kasim *et al.*[17] with an average yield of 1.82%. Whereas the levels of the essential oil of *Cinnamomum cassia* Bark, from Guangxi and Guangdong Provinces (South China), ranged between 1.69±0.67 to 3.21±0.12% (w/w)[18]. Similarly, higher yields were recovered by Geng *et al.*[19] (2.7-3.3% (w/w)), Li *et al.*[20] (2.38%) and Huang *et al.*[21] (2.76% (v/w)). Likewise, Jeyaratnam *et al.*[22] obtained also a high yield (2.55% (w/w)) of essential oil from *C. cassia* by using microwave-assisted hydrodistillation.

Previous studies have shown that the branch bark fraction tended to yield more essential oil compared to the entire branch indicating that selecting the bark based on the tree growth stages as well as separating the stem bark into top, center and lower sections within a tree can significantly improve the extraction efficiency of essential oils[23].

The results of GC/FID were given in Table 1. It can be seen that trans-cinnamaldehyde was the major constituent of cinnamon bark essential oil (94.67%) followed by coumarin (0.88%) and cinnamyle acetate (0.74%). Several studies have mentioned that trans-cinnamaldehyde was the most abundant component in the essential oil *C. aromaticum*[18,21,24-27], however, the authors recorded different concentrations. For example, the rates of trans-cinnamaldehyde in the essential oil of *C. aromaticum* analyzed by CG/MS were 97.7, 92.2, 76.9, 72.23, 68.52 and 49.33% as reported by Singh *et al.*[24], Giordani *et al.*[25], Liu *et al.*[26], Poaty *et al.*[27], Deng *et al.*[18] and Huang *et al.*[21], respectively. While Chang *et al.*[28] reported that cis-2-methoxycinnamic acid and cinnamaldehyde were the major compounds of the essential oil of *C. aromaticum*

analyzed by CG/MS (43.06 and 42.37%, respectively). Adinew[29] mentioned that 2-propanol, 3-phenyl was the major constituent of cinnamon bark essential oil (analyzed by GC-MS and FT-IR) growing in South West of Ethiopia.

The chemical components of cinnamon oil have been extensively studied in the literature. Bruneton[30] found that the main component of the cinnamon (*Cinnamomum aromaticum*) bark oil was E-cinnamaldehyde and the content was 90%. It is reported that this essential oil contained little eugenol. Similar results were found in this study (94.67% of E-cinnamaldéhyde and 0.01% of eugenol).

Different extraction processes and assay methods could have contributed to differences in cinnamaldehyde levels of cinnamon essential oils[31]. Moreover, the geographical position, cultivation, variety of cinnamon, harvesting time and extraction method affect the effective yield and composition of the essential oil[29,32]. Medicinal plants have long been, over the years, played a key role in the human health and contributed to the development of modern therapeutic drugs[33,34]. In addition, plant essential oils and extracts have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine and natural therapies. Therefore, it is necessary to investigate scientifically these plants used in traditional medicine.

Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens[25]. In this study, the essential oil of cinnamon showed a strong activity against selected bacterial strains. This study showed a MIC at 0.625 μL mL^{-1} against 4 strains causing neonatal diarrhea in young calves. Lu *et al.*[35] reported similar results, with a MIC ranging between 0.1-0.4 μL mL^{-1}. Several studies showed that the essential oil of cinnamon exhibited strong and consistent inhibitory effects against various pathogens by Matan *et al.*[36] and Prabuseenivasan *et al.*[37].

Huang *et al.*[21] reported higher values of MIC of essential oils of *C. cassia* against two Gram-negative bacteria, namely *S. typhimurium* and *E. coli*, with concentration of 10 mg mL^{-1}.

Moreover, Mith *et al.*[38] showed a MIC of essential oils of *C. cassia* with 0.5/1 μL mL^{-1} and 0.25/0.25 μL mL^{-1} against *E. coli* O157:H7 ATCC 35150 and *E. coli* O157:H7 S0575, respectively.

Chao *et al.*[39] tested the antimicrobial activity of 45 essential oils on bacteria, fungi and yeast and found that cinnamon bark oil exhibited a high and broad-spectrum antimicrobial property. This strong antibacterial property of the essential oil of cinnamon is mainly due to its constituents, including cinnamaldehyde, which particularly displayed strong antibacterial activity compared to the other components[24,40]. In addition, Unlu *et al.*[41] reported that cinnamaldehyde is a main component of the essential oils of cinnamon, this component was effective against some Gram positive and Gram-negative bacteria. Furthermore, Nazzaro *et al.*[42] reported that cinnamaldehyde has at least three mechanisms of action against bacteria. At low concentrations, it inhibits enzymes involved in cytokine interactions or other less important cell functions and at higher concentrations, it acts as an ATPase inhibitor. At a lethal concentration, cinnamaldehyde perturbs the membrane. Some studies have reported conflicting information's on the membrane-perturbing activity of cinnamaldheyde. For example, a sub-lethal concentration of the molecule does not affect the integrity of the membrane in *E. coli* but can inhibit the growth and bioluminescence of the microorganism *Photobacterium leiognathi*; this suggests that cinnamaldehyde gains access to the periplasm and perhaps also to the cytoplasm. Cinnamaldehyde is indeed capable of altering the lipid profile of the microbial cell membrane.

CONCLUSION

The present study revealed that *Cinnamon aromaticum* essential oil is potentially good source of antibacterial agents and could be an alternative for the treatment of diarrhea in calves caused by the tested strains. Further *in vivo* investigations and clinical trials are needed to justify the potential use of this oil as an antibacterial agent against bacteria causing neonatal calve's diarrhea.

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