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Comparative study on the phytochemical composition and antibacterial activity of the essential oil of *Diphasia klaineana* stems at pre-flowering and fruit set

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ABSTRACT

The phytochemical composition and antibacterial activity of the essential oils of *Diphasia klaineana* at different developmental stages (pre-flowering and fruit set) were reported. The essential oils were extracted by continuous hydro distillation and their antibacterial activities were tested against *Staphyloccocus aureus* ATCC 25923. The yield of oil (w/w %) of stems in different stages was in the order: pre-flowering (0.21 %) > fruit set (0.07 %). GC and GC/MS were analyzed the essential oils composition. In total, 35 and 34 constituents were identified and quantified in the mentioned samples, respectively. Monoterpene hydrocarbons were the main group of compounds in the stems during pre-flowering (42.40 %) and fruit set (56.15 %). Major compounds at pre-flowering were *β*-elemol, sabinene, guaiol and terpinen-4-ol. The antibacterial effect of essential oils was estimated by the disk diffusion method using Müller–Hinton agar and the measurement of diameters of inhibition zones. The bioassay results showed some variations between the two tested oils in their inhibitory activity against the tested bacteria at 10 µL. The essential oils from *Diphasia klaineana* stems at pre-flowering exhibited potent antibacterial activity against *Staphyloccocus aureus* ATCC 25923, with a minimum inhibitory concentration (MIC) value of 25 mg/mL, while the stems essential oil at fruit set had no activity.

Keywords: *Diphasia klaineana*, Essential oils, Development stage, Phytochemical composition, Antibacterial activity, *Staphylococcus aureus*.

INTRODUCTION

Medicinal and aromatic plants are widespread across the African continent and have been used in the African people's traditional culture of health care for centuries. They constitute the major component of the practice of African traditional medicine and these have become very important in the development of health systems in Africa [1].

The essential oils (EOs), a mixture of compounds of the terpenoids class isolated from aromatic plants, have been of such a great interest during the last decades for exhibiting broad biological properties; among them, antibacterial, antiparasitic, antifungal, and antiviral properties have been reported [2].

The variation of essential oils composition can change from plant to plant even in the same species. These changes in the chemical profile and biological properties of different organs of medicinal and aromatic plants are associated with many factors such ecological conditions, phonological, harvest time, and genetic difference postharvest treatment, extraction methods, and conservation conditions [3,4].

Some studies have previously reported the influences of the seasons on the yield and chemical composition of essential oils from various plant species and showed great variations in the concentration of essential oil components [5–7].

The Ivorian flora is characterized by a large diversity of aromatic and medicinal plants. *Diphasia klaineana* is a tree up a height of 16 m which belongs to Rutaceae and commonly known as "iolo pubescent" in French. This plant is widely distributed in the forest understorey from Côte d'Ivoire to Ghana [8]. Total decoction of *Diphasia klaineana* is traditionally used in steam baths for the treatment of many respiratory disorders such as sinusitis. However, there are little reports on the phytochemical and biological activity of different parts of *Diphasia klaineana*. Our previous studies have described the chemical composition of the essential oils *D. klaineana* organs (fruit and leaves) and its biological properties [9–11].

The aim of this study is to evaluate the effect of season and growth stage (pre-flowering and fruit set) on the phytochemical composition and the antibacterial activities of the essential oils of *D. klaineana* stems, in order to determine the best growth stage at which this species possesses the highest content of beneficial phytochemicals and most potent antibacterial capacity.

MATERIALS AND METHODS

Plant material

Stems of *Diphasia Klaineana* were collected during the pre-flowering and fruit set, around the Denguélé region, from Fengolo, Odienné, North-west of Côte d'Ivoire, in June and September. The plant was identified at the Centre National Floristique of Université Felix Houphouët-Boigny (Abidjan, Côte d'Ivoire). A voucher specimen was deposited in our laboratory for future references. The stems of the plant were kept in an enclosure under permanent air conditioning at 18 °C for 3 days before the hydro distillation process.

Bacterial strains

Bacterial strains used for the evaluation of the antibacterial activity were obtained from the American type culture collection (ATCC). The Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) was used in the antibacterial assays. Strains were stored at 4 °C and grew on nutrient agar 24 h prior to any assay. Mueller-Hinton (MH) was used for the antibiotic susceptibility testing.

Extraction of essential oils

500 g of the fresh plant material was ground and hydro distilled for 9 h housing a Clevenger-type apparatus. The oily phase above the aqueous distillate was taken out and dried over anhydrous sodium sulfate. The extracted essential oils were placed in dark vials, covered with aluminum foil and stored at 4 ° C until further analysis The essential oils from the stems harvested at the pre-flowering is coded EO_{PF} and that of the fruit set is EO_{FS}.

GG and GC-MS analysis

The analysis of essential oil was performed using a Perkin Elmer auto system XL Gas equipped with a Rtx-1 Column nonpolar phase (60 m × 0.22 mm, coating thickness 0.25 µm) and a Perkin Elmer TurboMass mass detector. The mass selective detector was operated in electron-impact (EI) mode with ionization voltage 70 eV. GC conditions were the same as described above. The retention indices were calculated, for all volatile contents using a homologous series of n-alkanes C_8-C_{22}. The essential oil contents were identified by comparing their GC retention indices, mass spectra with publish data and National Institute of Standards and Technology mass spectra library data, provided by the software of GC-MS system. The components of essential oils are reported as a relative percentage of the total oil by peak area [12,13].

Antibacterial activity assay

The essential oil was tested for the antimicrobial activity by the agar-well diffusion method, using 100 µL of suspension of tested microorganisms, containing 2 × 10^8 CFU/mL for bacteria. Mueller-Hinton agar was sterilized in a vial by autoclaving at 125-140 ° C and was dispensed (15 ml) into sterilized Petri dishes with a diameter of 9 cm. Six wells were drilled in the inoculated medium using a sterile pasteur pipette (6 mm). Wells, every 6 mm, were cut through the agar using a sterile cork borer and the agar was removed leaving empty wells, which were filled with 50 µL of each essential oil (essential oil at pre-flowering or at fruit set), the positive control (gentamycin) or the negative control (solvent). The Petri dishes were incubated at 37 °C, for 24 h. The diameters of the inhibition zones (mm) were measured including the diameter of the wells. All tests were performed in triplicate [14,15].

Determination of minimal inhibitory and minimal bactericidal concentrations

The minimal inhibitory concentration (MIC) of essential oils of *D. klaineana* was determined using the macro dilution broth method. All tests were performed in Müller-Hinton broth (MHB). The investigated oils were dissolved in Tween 80 and then diluted until the highest concentration. Serial doubling dilutions of oils were prepared in a 96-well micropipette over the range of (50 to 0.39 mg/mL). Overnight broth cultures of each strain were prepared and the final concentration in each was adjusted to 5 × 10^5 CFU/mL for bacteria. The bacteria were incubated for 24 h at 37 °C. The MIC is defined through the lowest concentration of the essential oil that inhibited visible microorganism growth. Microorganism growth was indicated by turbidity. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as ≥ 99.9% [16].

RESULTS AND DISCUSSION

Essential oil content in various stages of growth

The essential oil yields (%, w/w) were studied in the stems of *Diphasia klaineana* and the results showed that the yields of the stems harvested at the pre-flowering (EO_{PF}) and that of the fruit set (EO_{FS}) were 0.21 %, 0.07 %, respectively (Figure 1). The oils EO_{PF} and EO_{FS} were yellow in colour. *Koné et al.* studied the essential oils of leaves of *Diphasia klaineana* before and during flowering, and concluded that the yield of the leaves essential oil during flowering stage (1.53 %) was low compared to the yield obtained before flowering (1.65 %) [9]. *Mirjalili et al.* indicated that the yield of oil (w/w %) of *Levisticum officinale* Koch at different developmental stages was in the order: flower (0.1 %) < ripened fruit (0.6 %) < green mature fruit (1.0 %) < immature fruit (1.5 %) [17]. In another research, the unripe galbuli *Juniperus excelsa* M. Bieb was characterized by a lower essential oil content than the ripe samples [18]. The variations in essential oil content of *Diphasia klaineana* could be related to physiological differentiation occurred during the phenological cycle. These results suggest that *Diphasia klaineana* fruits may posit a metabolic effect on growth and photosynthesis, resulting in lower essential oil yield [4].

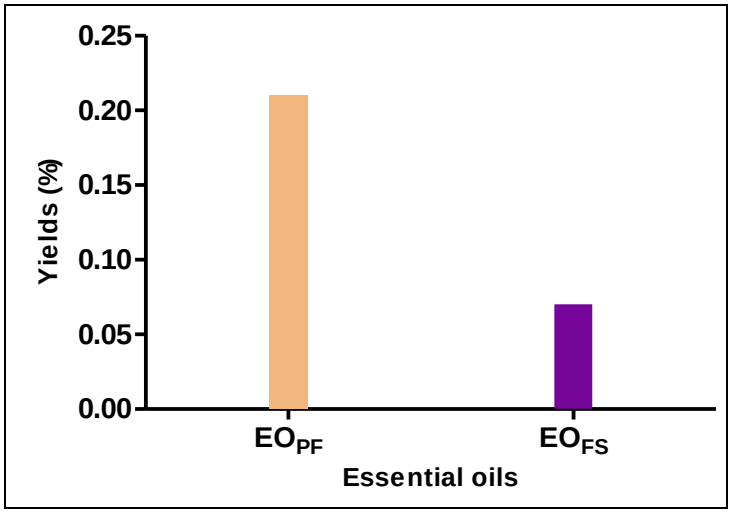

Figure 1: Yields of essential oils from stems during pre-flowering (EO_{PF}) and fruit set (EO_{FS}) of *D. klaineana*

Essential oils analysis

The results of the essential oils analysis are listed in Table 1 together with the retention indices of the identified compounds, where all the constituents are arranged in order of their elution on the Rtx-1 Column nonpolar phase. In total 35 and 34 constituents, respectively, were identified and quantified in the studied samples representing 89.64% (EO_{PF}) and 87.67 % (EO_{FS}) of the total oil, respectively. A comparison among the composition of the essential oils from *Diphasia klaineana* stems before flowering (EO_{PF}) and during fruit setting (EO_{FS}) were revealed both quantitative and qualitative differences. The GC and GC-MS analyses showed that the distribution of saturated hydrocarbons of the oil from flower was remarkably different from that of the oils at the fruiting stage. The results (Figure 2) revealed that the monoterpenes of EO_{PF} (42.40 %) were present in higher amount than EO_{FS} (31.52 %). However, the sesquiterpenes from EO_{FS} (56.15 %) were more abundant in EO_{PF} (47.24 %). The major constituent of the essential oils of *D. klaineana* stems was β-elemol (34.00 %) before flowering, and it was found that this compound increased during fruit set (41.22 %). On the contrary, the sabinene constituted 17.96 % of the essential oil of the harvest before flowering but decreased with the fruit setting. Like these two molecules, the content of the compounds varies during the two harvest periods. Some compounds content such as α-thujene (EO_{PF}: 0.36%; EO_{FS}: 0.21%), α-pinene (EO_{PF}: 0.94%; EO_{FS}: 0.44%), camphene (EO_{PF} : 0.20%; EO_{FS}: 0.10%), sabinene (EO_{PF}: 17.96%; EO_{FS}: 8.40%), β-pinene (EO_{PF}: 0.56%; EO_{FS}: 0.32%), myrcene (EO_{PF}: 2.96%; EO_{FS}: 2.11%), p-cymene (EO_{PF}: 2.29%; EO_{FS}: 0.65%), limonene (EO_{PF}: 3.77%; EO_{FS}: 2.45%), α-copaene (EO_{PF}: 0.03%; EO_{FS}: Abs%), γ-muurolene (EO_{PF}: 0.05%; EO_{FS}: 0 , 04%), β-selinene (EO_{PF}: 0.44%; EO_{FS}: 0.25%), α-murolene (EO_{PF}: 0.35%; EO_{FS}: 0.30%), α- (E, E) Farnesene (EO_{PF}: 0.07%; EO_{FS}: 0.04%) and γ-épi eudesmol (EO_{PF}: 0.13%; EO_{FS}: 0.12%), decreased from pre-flowering (EO_{PF}) to fruit setting (EO_{FS}) as shown in Figure 3.

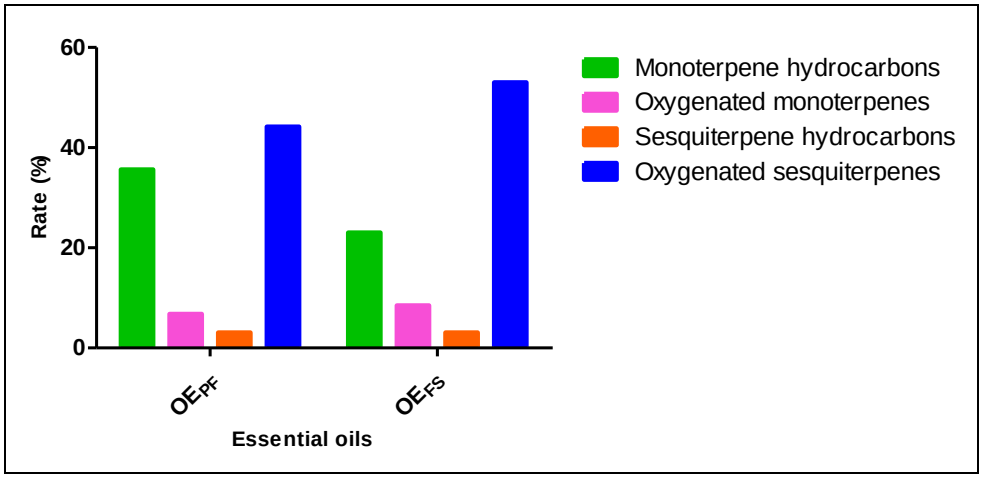

Figure 2: Variation in the chemical composition of *D. klaineana* essential oil at pre-flowering and fruit set

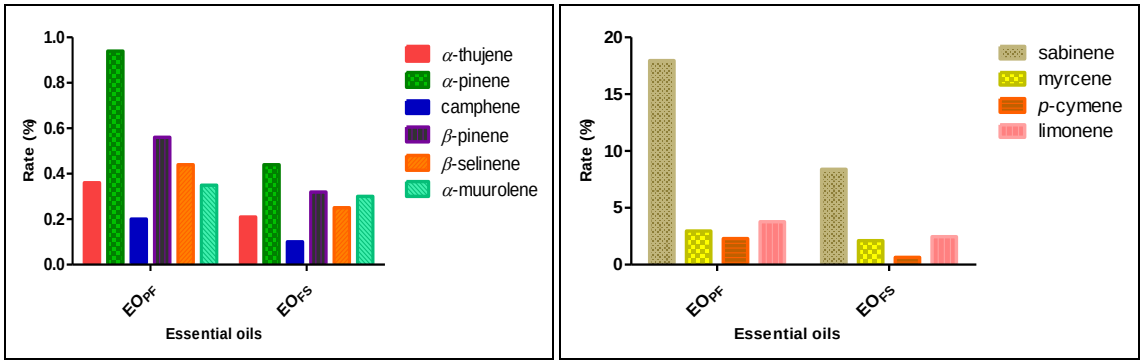

Figure 3: Fall in the levels of some compounds in essential oil from *D. klaineana* stems at pre-flowering (OE_{PF}) to fruit set (OE_{FS})

However, others being at their lowest content at pre-flowering (September) reach their maximum at fruit set (June). These are α-terpinene (EO_{PF}: 0.35%; EO_{FS}: 0.85%), (E) -β-ocimene (EO_{PF}: 3.13%; EO_{FS}: 3.47%), γ-terpinene (EO_{PF}: 0.98%; EO_{FS}: 1.61%), trans-sabinene hydrate (EO_{PF}: 0.21%; EO_{FS}: 0.34%), terpinolene (EO_{PF}: 0.71 %; EO_{FS}: 0.99%), linalool (EO_{PF}: 0.44%; EO_{FS}: 0.71%), terpinen-4-ol (EO_{PF}: 5.30%; EO_{FS}: 6, 24%), α-terpineol (EO_{PF}: 0.25%; EO_{FS}: 0.32%), bornyl acetate (EO_{PF}: 0.15%; EO_{FS}: 0.21%), neryl acetate (EO_{PF}: 0.07%; EO_{FS}: 0.11%), geranyl acetate (EO_{PF}: 0.12%; EO_{FS}: 0.19%), methyl eugenol (EO_{PF}: 0.10%; EO_{FS}: 0.17%), (E)-caryophyllene (EO_{PF}: 0.47%; EO_{FS}: 0.53%), α-humulene (EO_{PF}: 0.32%; EO_{FS}: 0.36%) , (E)–methyl isoeugenol (EO_{PF}: 0.14%; EO_{FS}: 0.20%), germacrene D (EO_{PF}: 0.79%; EO_{FS}: 0.99%), δ-cadinene (EO_{PF}: 0.33%; EO_{FS}: 0.34%), β-elemol (EO_{PF}: 34.00%; EO_{FS}: 41.22%), guaiol (EO_{PF}: 7.26%; EO_{FS}: 8 , 51%), γ-eudesmol (EO_{PF}: 2.79%; EO_{FS}: 3.20%) (Figure 4).

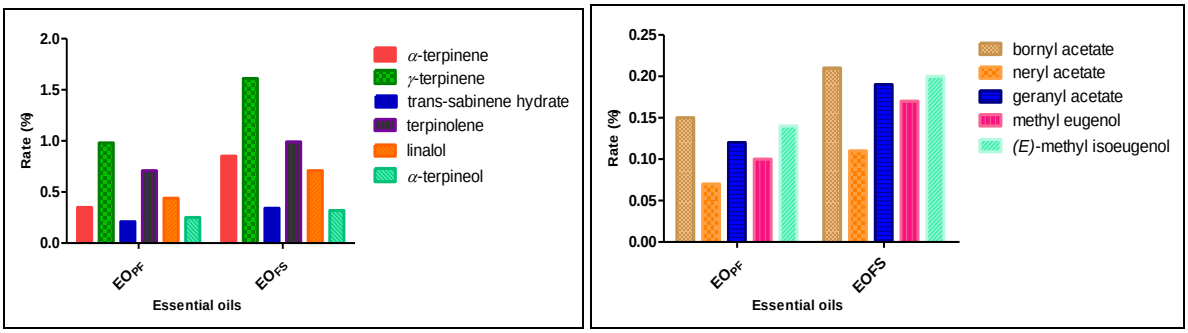

Figure 4: Increased levels of some compounds in essential oil from *D. klaineana* stems at pre-flowering (OE_{PF}) to fruit set (OE_{FS})

Table 1: Essential oils composition of *D. klaineana* stems at pre-flowering and fruit set

No	RI (non-polar)	RI (polar)	Compounds	% Pre-flowring (OE_{PF})	% Fruit set (OE_{FS})
1	922	IRPOL	α-Thujene	0.36	0.21
2	930	1023	α-Pinene	0.94	0.44
3	943	1020	Camphene	0.20	0.10
4	965	1069	Sabinene	17.96	8.40
5	970	1126	β-Pinene	0.56	0.32
6	980	1114	Myrcene	2.96	2.11
7	1008	1164	α-Terpinene	0.35	0.85
8	1011	1184	p-Cymene	2.29	0.65
9	1021	1269	Limonene	3.77	2.45
10	1024	1204	(Z)-β-Ocimene	1.41	1.43
11	1035	1236	(E)-β-Ocimene	3.13	3.47
12	1048	1253	γ-Terpinène	0.98	1.61
13	1053	1248	Trans-Sabinene hydrate	0.21	0.34
14	1078	1464	Terpinolene	0.71	0.99
15	1083	1286	Linalool	0.44	0.71
16	1161	1548	Terpinen-4-ol	5.30	6.24
17	1171	1603	α-Terpineol	0.25	0.32
18	1267	1697	Bornyl acetate	0.15	0.21
19	1340	1576	Neryl acetate	0.07	0.11

20	1357	1723	Geranyl acetate	0.12	0.19
21	1366	1753	Methyl eugenol	0.10	0.17
22	1372	2010	α-Copaene	0.03	-
23	1386	1486	β-Elemene	0.28	0.29
24	1415	1581	(E)-Caryophyllene	0.47	0.53
25	1448	1590	α-Humulene	0.32	0.36
26	1457	1668	(E)-Methyl isoeugenol	0.14	0.20
27	1466	2168	γ-Muurolene	0.05	0.04
28	1474	1681	Germacrene D	0.79	0.99
29	1479	1707	β-Selinene	0.44	0.25
30	1488	1717	α-Muurolene	0.35	0.30
31	1513	1716	δ-Cadinene	0.33	0.34
32	1535	1756	β-Elemol	34.00	41.22
33	1584	2081	Guaiol	7.26	8.51
34	1606	2089	γ-Epi-Eudesmol	0.13	0.12
35	1616	2101	γ-Eudesmol	2.79	3.20
			Total	89.64	87.67

Thus, fruit setting affected the chemical composition of the essential oils of *Diphasia klaineana* stems. The influence of the harvest period on the composition of essential oils in the leaves of *D klaineana* had been studied by *Koné et al.* (2019) [9]. In addition, other researchers have carried out the same type of study on other plants. *Daghbouche et al*, analysed the effect of phenological stages on essential oil composition of *Cytisus triflorus* L'Her. Monoterpene hydrocarbons were detected with a lower content vegetative and fruiting stages compared to flowering. In contrast, aldehydes were noted at higher content at vegetative stage. This study highlighted difference in content and composition of essential oil in *Cytisus triflorus* L'Her during growth stages. Flowering was the interesting stage for harvesting and with more specific composition in essential oil [19]. *Hazrati et al*, showed that the highest essential oils of *Stachys schtschegleevii* Sosn yield was observed in the flowering stage (0.25 %) and significant variations were detected in EO compounds during various stages of growth. They concluded that the flowering stage is the best harvesting time for *S. schtschegleevii* plants to produce essential oils for both food and pharmaceutical purposes [20].

Antibacterial activity

The antibacterial activity against *Staphylococcus aureus* ATCC 25923 of the essential oil of *D. klaineana* stems before flowering (OE_{PF}) and during fruit set (OE_{FS}) by evaluating the presence of inhibition zone, MIC and MBC values. The results indicated in Table 2 and in the paper represent the net zone of inhibition including the diameter (6 mm) of the wells. Biological activity was affected to essential oil as follows: not sensitive (diameter of the inhibition zone ≤ 8.0 mm), moderately sensitive (8.0 < diameter of the inhibition zone < 14.0 mm), sensitive (14.0 < diameter of the inhibition zone < 20.0 mm), and extremely sensitive (diameter of the inhibition zone ≥ 20.0 mm) [21]. As shown in Table 2, the diameters of the inhibition zones of the studied essential oils ranged from 6 to 14 mm with the highest inhibition zone value observed for OE_{PF} (14 mm) and the smallest zone value observed for OE_{FS} (6 mm). *Staphylococcus aureus* has now been recognized as a facultative intracellular pathogen, with the intracellular lifestyle playing a crucial role during recurrent infections. Intracellular bacteria have been demonstrated from various chronic conditions [22]. The bacteriostatic and bactericidal effectiveness of the essential oils at the pre-flowering and fruit set estimated by MIC and MBC, respectively, are shown in Table 2. According to Marmonier's method [23], a substance is bactericidal if MBC/MIC ≤ 4, and bacteriostatic if MBC/MIC> 4. Against *Staphylococcus aureus* ATCC 25923, EO_{PF} gave MIC at 25 mg/mL and MBC at 50 mg/mL. However, no effect was observed with EO_{FS}. In view of these values, essential oil of *D. klaineana* stems is bactericidal before flowering (MBC/MIC=2 for EO_{PF}) while during fruit set this oil had no antibacterial activity.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils from *D. klaineana* stems at pre-flowering and fruit set

Bacterial strains	Essential oils	Inhibition zones (mm)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Interpretation
Staphylococcus aureus ATCC 25923	EO_{PF}	14	25	50	2	Bactericidal
	EO_{FS}	6	00	00	00	No effect

The most active essential oil from *D. klaineana* stems (OE_{PF}) were characterize by a high content of monoterpene hydrocarbons (35.62 %). This is consistent with previous studies showing that the EOs of *R. officinalis* which showed predominance in monoterpene hydrocarbons (63 %) was highly active against *Staphylococcus aureus* strains [24]. The essential oil from *Citrus aurantium* L zest, essentially consisting of 85.22% limonene (hydrocarbon monoterpenes) was also active on *S. aureus* [25].

CONCLUSION

Our study on *Diphasia klaineana* demonstrates a high variation in the composition of essential oils and their antibacterial activity at pre-flowering and fruit set. Thus, the stems of *Diphasia klaineana* showd a higher yield of essential oil at pre-flowering, rich in *β*-elemol, sabinene, guaiol, terpinen-4-ol with a high antibacterial activity against *S. aureus* ATCC 25923. Regarding the essential oil obtained at fruit set, its yield was lower. It was marked by an increase in the content of *β*-elemol, guaiol, terpinen-4-ol and a decrease in sabinene. The essential oil at fruit set had no antibacterial effect against *S. aureus* ATCC 25923. However, further research is needed to evaluate the effectiveness of *Diphasia klaineana* on other types of bacteria to establish their utility as natural antimicrobial agents in health care.

Conflict of Interest

None declared.

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