PLOS ONE

RESEARCH ARTICLE

Phytochemical profiling and seasonal variation of essential oils of three *Callistemon species* cultivated in Egypt

Haidy A. Gad[1☯], **Iriny M. Ayoub**[1☯], **Michael Wink**[2]*

1 Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt, **2** Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, INF, Heidelberg, Germany

☯ These authors contributed equally to this work.
* wink@uni-heidelberg.de

Citation: Gad HA, Ayoub IM, Wink M (2019) Phytochemical profiling and seasonal variation of essential oils of three *Callistemon species* cultivated in Egypt. PLoS ONE 14(7): e0219571. https://doi.org/10.1371/journal.pone.0219571

Editor: Edy de Brito, Embrapa Agroindústria Tropical, BRAZIL

Received: March 4, 2019

Accepted: June 26, 2019

Published: July 11, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: Iriny M. Ayoub is thankful to the Science and Technology Development Fund in Egypt (STDF, project ID 25448) for funding her postdoctoral fellowship in Germany.

Competing interests: The authors have declared that no competing interests exist.

Abstract

The genus *Callistemon* comprises evergreen shrubs or small trees, widely cultivated as ornamentals and for essential oil production. *Callistemon* is well-recognized in folk medicine for its anti-cough, anti-bronchitis, and insecticidal activities. In the current study, we profiled the essential oil composition of the leaves of *C. citrinus*, *C. rigidus* and *C. viminalis* (Myrtaceae) collected during different seasons by GLC-MS coupled to multivariate data analysis. Antioxidant, anti-inflammatory and anti-proliferative activities of *Callistemon* essential oils were evaluated. A total of 29 compounds were tentatively identified. Oxygenated monoterpenes dominated in essential oils, where eucalyptol represented the major constituent in the three *Callistemon* species in all seasons. Multivariate data analysis including Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to discriminate between different *Callistemon* species in each season and to investigate any correlation between the metabolic profile of each species within different seasons. As expected, PCA plot could discriminate the three *Callistemon* species in the four seasons. The dendrogram from HCA confirmed the results of PCA as it showed the same segregation pattern regarding the discrimination of different *Callistemon* species. *C. viminalis* showed more pronounced antioxidant activity than *C. citrinus*, exhibiting IC_{50} values of 1.40 mg/mL and 1.77 mg/mL, respectively. Meanwhile, *C. rigidus* showed very weak antioxidant activity. All oils showed membrane stabilization activity in hypotonic solution induced haemolysis assay, where *C. viminalis* showed potent membrane stabilizing activity exhibiting IC_{50} value of 25.6 μg/mL comparable to that of the standard drug, indomethacin (17.02 μg/mL). Nevertheless, Callistemon essential oils were not cytotoxic in HCT-116 and Hela human cancer cell lines.

Introduction

The genus *Callistemon* (Myrtaceae) consists of evergreen shrubs or small trees. It is widely used for essential oil production, and as ornamental, wind-breaking and degraded-land

reclamation plants [1]. *Callistemon* originates from Australia, but is cultivated and introduced to most parts of the world [2]. *Callistemon* species possess attractive lanceolate leaves and characteristic 'bottlebrush' shaped flower spikes with prominent red stamens, thus, commonly named Bottlebrush [3]. The most widely cultivated member of this genus is *C. citrinus*, commonly known as Crimson or Lemon Bottlebrush [2]. *C. viminalis* is a small tree or shrub characterized by pendulous foliage and thus, was given the name Weeping Bottlebrush [2]. *C. rigidus*, commonly called Stiff or Erect Bottlebrush, is a stiff and upright shrub with red flower spikes [4].

Callistemon species produce essential oils. In folk medicine, *Callistemon* is well recognized for its insecticidal, as well as its anti-cough and anti-bronchitis activities [5]. The essential oils possess antimicrobial, antifungal, insecticidal, anthelmintic, antioxidant, anti-inflammatory and anti-nociceptive activities [5–8]. In China, *Callistemon* species, *mainly C. viminalis* is used in Traditional Chinese Medicine for treating hemorrhoids [9]. 1,8-Cineole figured as the predominant constituent reported in *Callistemon* essential oils. Other reported secondary metabolites include α-pinene, β-pinene, limonene, linalool, myrcene, and menthyl acetate [3]. The biological activity of an essential oil could not be ascribed to a single component but rather to the synergistic effects of the phytocomplex. This chemical complexity contributes to its biological activity, since each constituent of the phytocomplex is involved in the overall activity or may modulate the effects of the other constituents [10].

The main goal of this study is phytochemical profiling of essential oils from three *Callistemon* species (*C. citrinus*, *C. rigidus and C. viminalis*) in different seasons (spring, summer, autumn and winter) by GLC/MS. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied as pattern recognition techniques to discriminate between species and seasons. Moreover, essential oils were assessed for their antioxidant, anti-inflammatory and anti-proliferative activities.

Materials and methods

Plant material

Leaves of *Callistemon citrinus*, *C. rigidus and C. viminalis* (Myrtaceae) were collected in four different seasons from a private botanical garden in Giza, Egypt. Plants were kindly identified by Mrs. Trease Labib, Plant Taxonomy Consultant at the Ministry of Agriculture and former director of Orman Botanical Garden, Giza, Egypt. Voucher specimens were deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University (PHG-P-CC; PHG-P-CR; and PHG-P-CV).

Isolation of the essential oil

The essential oils from fresh leaves (100 g) of *Callistemon citrinus*, *C. rigidus and C. viminalis* were obtained by hydro-distillation for 4 h using a Clevenger-type apparatus. The oil was collected, dried over anhydrous sodium sulfate, weighed and kept in sealed vials at 4˚C for further analyses. The yield in % (w/w) was determined in triplicate, based on the initial plant weight.

GLC/MS analysis

Mass spectra were recorded using a Shimadzu GCMS-QP2010 (Kyoto, Japan) equipped with a split–splitless injector. Separation was achieved using an Rtx-5MS fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) (Restek, USA). The initial column temperature was kept at 45˚C for 2 min and programmed to 300˚C at a rate of 5˚C/min, and kept constant at 300˚C for 5 min. Injector temperature was 250˚C. Helium was used as a carrier gas with a

flow rate of 1.41 mL/min. Mass spectra were recorded applying the following conditions: Ion source temperature, 200˚C; ionization voltage, 70 eV; (equipment current) filament emission current, 60 mA. Split mode injection of diluted samples (1% v/v) was applied with split ratio 1: 15.

Identification of essential oil components

Essential oil components were tentatively identified by comparison of their mass spectra and retention indices with those listed in NIST Mass Spectral Library, 2011; Wiley Registry of Mass Spectral Data 8th edition; and data reported in literature [11–13].

Chemometric analysis

GLC/MS phytochemical profiling was subjected to chemometric analysis. Principal Component Analysis (PCA) comprises a first step in data analysis in order to provide an overview of all observations and samples to identify and evaluate groupings, trends and strong outliers [14, 15]. Hierarchal Cluster Analysis (HCA) was then applied to allow clustering of different *Callistemon* species. The clustering patterns was constructed by applying the complete linkage method used for group building; this representation is more efficient when the distance between clusters is computed by Euclidean method [14, 15]. For PCA and HCA, Unscrambler X 10.4 from CAMO (Computer Aided Modeling, AS, Norway) was employed.

Antioxidant activity

Antioxidant activity of *Callistemon* essential oils was investigated using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay [16]. An aliquot (40 μL) of various concentrations of the essential oils (0.04–5.12 mg/mL) was added to 0.004% w/v DPPH in methanol (3 mL). Absorbance was recorded immediately against a blank using a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with the data being recorded at 1 min intervals until the absorbance was stabilized (16 min). Ascorbic acid was used as a reference compound. All experiments were performed in triplicates. The inhibition percentage (I%) was calculated according to the following equation:

$$\text{Inhibition}\% = [(A_{blank} - A_{sample})/A_{blank}] \text{ x } 100$$

Where A_{blank} = Absorbance of the blank (non-reduced DPPH) at t = 0 min and A_{sample} = absorbance of the test sample at t = 16 min.

Anti-inflammatory activity

Membrane stabilization assay was used to assess the *in vitro* anti-inflammatory activity of *Callistemon* essential oils using hypotonic solution-induced erythrocyte hemolysis described by Shinde *et al*. [17].

Preparation of erythrocyte suspension. Whole blood was collected from rats via cardiac puncture under ether anesthesia into heparinized tubes. Blood was washed three times with 0.9% saline. The volume of saline was measured and reconstituted with isotonic buffer solution (pH 7.4) composed of 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) as 40% v/v suspension. The blood was then centrifuged at 3000 rpm for 10 minutes [17].

Hypotonic solution-induced hemolysis. Membrane stabilization activity of the essential oils was assessed using hypotonic solution-induced erythrocyte hemolysis [17]. Briefly, 0.5 mL of stock erythrocyte (RBCs) suspension was mixed with 5 mL of hypotonic NaCl solution (50 mM) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the tested essential oil

at a concentration of 7.81–1000 μg/mL. The control sample was composed of 0.5 mL of RBCs mixed with 5 mL hypotonic-buffered saline solution alone. Mixtures were incubated at room temperature for 10 min, then centrifuged at 3000 rpm for 10 min. Indomethacin was used as a reference standard. In 96 well plates, the absorbance (O.D.) of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis or membrane stabilization percentage was calculated according to the method described by Shinde et al. [17]. The IC_{50} value was defined as the concentration of the sample that inhibited 50% erythrocyte hemolysis under the assay conditions.

$$\%\text{Inhibition of Hemolysis (Or\%Membrane Stabilization\%)} = 100 \text{ x } (OD_1 - OD_2/OD_1)$$

Where,

OD_1 is the optical density of the hypotonic-buffered saline solution alone

OD_2 is the optical density of the test sample in hypotonic solution.

Anti-proliferative activity. The anti-proliferative activity of the essential oils was assessed against HCT-116 and Hela human cancer cell lines using MTT assay [13, 18]. Exponentially growing cells were seeded at a density of 10×10^4 cells/well (HCT-116) and 15 $x10^4$ cells/well (Hela) in 96-well plates. Stock solutions of essential oils in dimethyl sulfoxide (DMSO) were prepared. Essential oils were subjected to two-fold serial dilutions in the respective media where the maximal concentration of DMSO did not exceed 1%. Doxorubicin was used as a positive control. Cells were treated with 100 μL of the tested essential oils at concentrations ranging from 0.002–1.0 mg/mL Cells were incubated for 24 h at 37˚C. Afterwards, 0.5 mg/mL of MTT was added, and the plates were incubated for additional 4 h. The formazan crystals produced by viable cells were dissolved in DMSO (100 μl) and subsequently shaken for 10 min at room temperature. The absorbance was measured at 570 nm using a Tecan Safire II (Crailsheim, Germany) spectrophotometric plate reader. The percentage cell viability was calculated using the following formula: % cell viability = (OD of treated cells / OD of control cells) x 100.

Data analysis

All experiments were carried out in triplicate. IC_{50} value was determined as the concentration that resulted in 50% reduction in cell viability or inhibition of biological activity. IC_{50} values were calculated using a four parameter logistic curve using SigmaPlot 14.0, SYSTAT Software (CA, USA). Data were presented as mean ± standard deviation.

Results and discussion

GLC–MS analysis of the essential oils from different *Callistemon* species

Hydrodistillation of the fresh leaves of *C. citrinus*, *C. rigidus*, and C. *viminalis* yielded 0.43%, 0.84% and 0.41% w/w pale yellow essential oil, respectively. The identified components, their retention time, retention indices and percentages (average of three replicates for each species) for different seasons are summarized in Table 1. GLC/MS profiles of three *Callistemon* species collected during different seasons are displayed in supplementary material (S1–S4 Figs).

Twenty-nine components were tentatively identified in the three *Callistemon* species. The results in Table 1 demonstrate that oxygenated monoterpenes followed by monoterpenes are the major oil components of the three species accounting for (61.38% - 94.42%) and (4.70% - 30.37%) of the total identified components, respectively. Meanwhile, sesquiterpenes and other classes were present in low abundance.

The major secondary metabolite of the three essential oils in different *Callistemon* species was eucalyptol (syn. 1,8-cineole) ranging from (71.27% - 81.70%), (69.15% -81.70%), (64.63% -

Table 1. Chemical profile of the essential oils of *Callistemon citrinus* (CC), *C. rigidus* (CR) and *C. viminalis* (CV) in four different seasons.

Peak no.	RT	Compound	RI_{exp} [a]	RI_{lit} [b]	Spring			Summer			Autumn			Winter		
					CC	CR	CV	CC	CR	CV	CC	CR	CV	CC	CR	CV
1	7.48	α-Thujene	920	923	0.04	0.02	0.09	0.03	0.67	0.04	0.26	0.02	0.07	0.05	0.01	0.94
2	7.68	α-Pinene	927	927	1.11	18.64	11.6	1.23	12.21	12.15	10.7	8.72	8.38	2.09	9.18	20.75
3	8.12	Camphene	943	943	0	0.06	0	0	0.02	0.02	0.01	0.02	0.02	0	0	0.02
4	8.9	β-Thujene	971	971	0.27	0	0.09	0.02	0	0	0	0	0	0.02	0	0
5	8.98	β-Pinene	974	974	0.8	1.21	1.13	0.86	0.65	1.09	0.58	0.67	1.36	1.42	0.51	0.73
6	9.44	β-Myrcene	991	991	1.19	0	1.06	1.36	0.30	0.25	0.17	0.02	0.70	1.76	0	0.05
7	9.73	2-Carene	1001	1001	0	0	0	0	0.03	0	0	0	0	0	0	0.07
8	9.83	Pseudo limonene	1004	1003	0.13	0.18	0.26	0.09	3.34	0.08	0.4	0.1	0	0.13	0.08	0
9	9.80	α-Phellandrene	1003	1003	0	0	0	0	0	0	0	0	0	0	0	0.87
10	9.98	3-carene	1009	1009	0	0	0	0	0	0	0	0	0	0	0	0.08
11	10.24	α-Terpinene	1017	1017	0.15	0	0.13	0.12	0.16	0	0	0	0	0.08	0	0.28
12	10.52	o-Cymene	1026	1026	0.10	0.42	0.28	0.15	3.67	0.47	1.78	0.25	0.96	0.31	0.25	5.7
13	10.54	D-Limonene	1025	1031	0	0	0	0	0	0	5.41	0	0	0	0	0
14	10.8	Eucalyptol	1035	1035	81.63	71.27	79.17	81.70	69.15	79.13	64.63	79.39	76.57	70.77	80	55.69
15	10.91	trans-β-Ocimene	1039	1040	0	0	0	0	0.05	0	0	0	0	0	0	0
16	11.24	cis-β-Ocimene	1049	1047	0.05	0.05	0	0.02	0.23	0	0	0	0	0	0	0.03
17	11.57	γ-Terpinene	1060	1060	0.65	0.5	0.76	0.62	0.76	0.46	0.2	0.21	0.61	0.72	0.15	0.71
18	12.5	Terpinolene	1090	1090	0.24	0.26	0.18	0.17	0.39	0.07	0.19	0.22	0.11	0.16	0.06	0.1
19	12.91	Linalool	1103	1103	7.61	0	0	7.78	0.34	0.18	0	0	0.36	13.80	0.17	0.76
20	13.33	Fenchol	1117	1117	0	0.05	0	0	0	0.03	0	0	0	0	0.05	0
21	14.13	trans-Pinocarveol	1142	1142	0	0.11	0	0	0	0.03	0.09	0.10	0.08	0	0.16	0.21
22	15.32	4-Terpinenol	1180	1180	1.17	0.39	0.69	1.40	0.63	0.72	0.28	0.55	1.41	1.61	0.42	1.01
23	15.74	α-Terpineol	1194	1193	2.84	4.59	2.13	3.353	6.14	3.63	10.27	5.72	4.82	4.27	5.69	3.4
24	17.6	Nerol	1258	1251	0.2	0.06	0.34	0.17	0.05	0.19	0	0.07	0.78	0.31	0.04	0.15
25	20.6	Eugenol	1363	1362	0.05	0	0	0.07	0	0	0	0	0	0.23	0	0
26	20.72	exo-2-Hydroxycineole acetate	1367	1367	0	0	0	0	0	0	0	0	0	0.10	0.09	0
27	21.23	Neryl acetate	1385	1385	0.09	0	0.11	0.11	0.11	0.06	0	0	0.24	0.13	0	0.04
28	22.34	*β*-Caryophyllene	1427	1427	0.14	0	0.15	0.03	0	0.03	0	0	0.17	0.07	0	0
29	26.42	*Spathulenol*	1586	1582	0	0	0	0	0	0	0	0	0	0.06	0.06	1.58
		Monoterpene hydrocarbons			4.76	21.30	15.62	4.70	22.51	14.67	19.72	10.26	12.25	6.78	10.26	30.37
		Oxygenated Monoterpene			93.47	76.47	82.35	94.42	76.48	83.92	75.29	85.84	84.04	90.76	86.62	61.38
		Sesquiterpene hydrocarbons			0.14	0	0.15	0.03	0	0.03	0	0	0.17	0.07	0	0
		Oxygenated Sesquiterpene			0	0	0	0	0	0	0	0	0	0.06	0.06	1.58
		Others			0.15	0	0.11	0	0	0	0	0	0.24	0.47	0.09	0.04
		Total identified			98.52	97.77	98.23	99.35	97.8	98.68	95.02	96.10	96.7	98.15	97.04	93.37

Compounds listed in order of their elution on RTX-5 GC column. Identification, was based on comparison of the compounds′ mass spectral data (MS) and retention indices (RI) with those of NIST Mass Spectral Library (2011), Wiley Registry of Mass Spectral Data 8th edition and literature.

[a] Retention index determined experimentally on RTX-5 column relative to n-alkane series (C8–C28)

[b] Published retention indices.

https://doi.org/10.1371/journal.pone.0219571.t001

79.39%) and (55.69% - 80%) in spring, summer, autumn and winter, respectively, with the highest variation in summer and spring. Essential oils of the three *Callistemon* species in four seasons showed α-terpineol and α-pinene as major components in addition to eucalyptol. α-Thujene, β-pinene, O-cymene, γ-terpinene, terpinolene and 4-terpinenol were detected as common constituents in all *Callistemon* species.

Our results were consistent with previous reports on the essential oils obtained from the leaves of *Callistemon* species from different geographical regions. A study conducted on thirty Australian *Callistemon* species reported that 1,8-cineole was the major component (45–80%) of the majority of leaf essential oils. Other identified compounds include α-pinene (2–40%), limonene (2–9%) and α-terpineol (1–13%) [19]. Essential oil from the leaves of *C. lanceolatus* (syn. *C. citrinus*) from the north-eastern region of India exhibited 1,8 cineole (58.3%) as a major constituent followed by α-pinene, α-phellandrene, limonene and α-terpineol [20]. Likewise, the oil of *C. citrinus* from Reunion was found to be rich in 1,8-cineole (68.0%) followed by α-pinene and α-terpineol [21]. These results matched to a great extent the oil from the lower Himalayan region except for α-terpineol which was present at a lower percentage [22]. Essential oils from Brazil, were characterized by a high content of 1,8-cineole (77.0% and 65.0%) for *C. citrinus* and *C. viminalis*, respectively [23]. Essential oils from *C. viminalis* leaves were reported to possess 1,8-cineole (47.9%–82.0%) as the predominant constituent [24]. Similarly, oils of *C. citrinus* and *C. rigidus* from Cameroon were dominated by 1,8-cineole (73.8% and 79.1%, respectively) [25].

However, essential oils from *C. citrinus* leaves from Western Himalayas revealed high content of *α*-pinene (32.3%) followed by limonene (13.1%) and *α*-terpineol (14.6%), whereas, 1,8-cineole was only 9.8% of the leaf oil which controverted with previous studies from other geographical regions [26]. Thus, remarkable qualitative and quantitative variations in essential oil composition could be traced among plants collected in different geographical regions and /or seasons which necessitates construction of a simple and efficient chemometric model that could discriminate closely related species collected in different seasons.

Discrimination of different *Callistemon* species by chemometric analyses

Different bar charts were constructed for the major identified components of *Callistemon* essential oils. As shown in Fig 1, bar charts exhibited quantitative and qualitative differences regarding the metabolic profile of each species in each studied season. There are very close correlations between different *Callistemon* species in different seasons, as all samples showed eucalyptol as the major metabolite. Metabolic profiling (29 components, Table 1) were subjected to both PCA and HCA to reveal the chemical variability, and the inter-relationships between the oils in each season and among different species.

PCA explained 99% and 100% of the variance of the data in spring and summer seasons, respectively, as shown in Fig 2A & 2B. The three species were significantly discriminated from each other, and each species was located in a different quadrant away from the other species. Loading plots showed that the main discriminating markers were eucalyptol, α-pinene and linalool. However, regarding autumn and winter season, PCA described about 100% of data discrepancy as presented in Fig 3A & 3B, where each species was completely segregated from each other. In addition to eucalyptol, α-pinene, and linalool, the loading plot showed that one main discriminating metabolic maker was α-terpineol, which highly influenced the segregation between the samples in winter season. However, for autumn both eucalyptol and α-terpineol were recognized as a marker for separation between different species.

Additionally, HCA was applied as unsupervised pattern recognition method in order to confirm results obtained by PCA. The dendrograms obtained for different seasons, displayed in Fig 4, revealed segregation of different *Callistemon* species into three main clusters endorsing the results of PCA. HCA dendrograms revealed the near distance of *C. viminalis* and C. *rigidus* in spring, summer and autumn as presented in Fig 4A, 4B & 4C, respectively. On the other side, regarding winter season, HCA showed nearness of *C. citrinus*, *C. rigidus* in relation to *C. viminalis*.

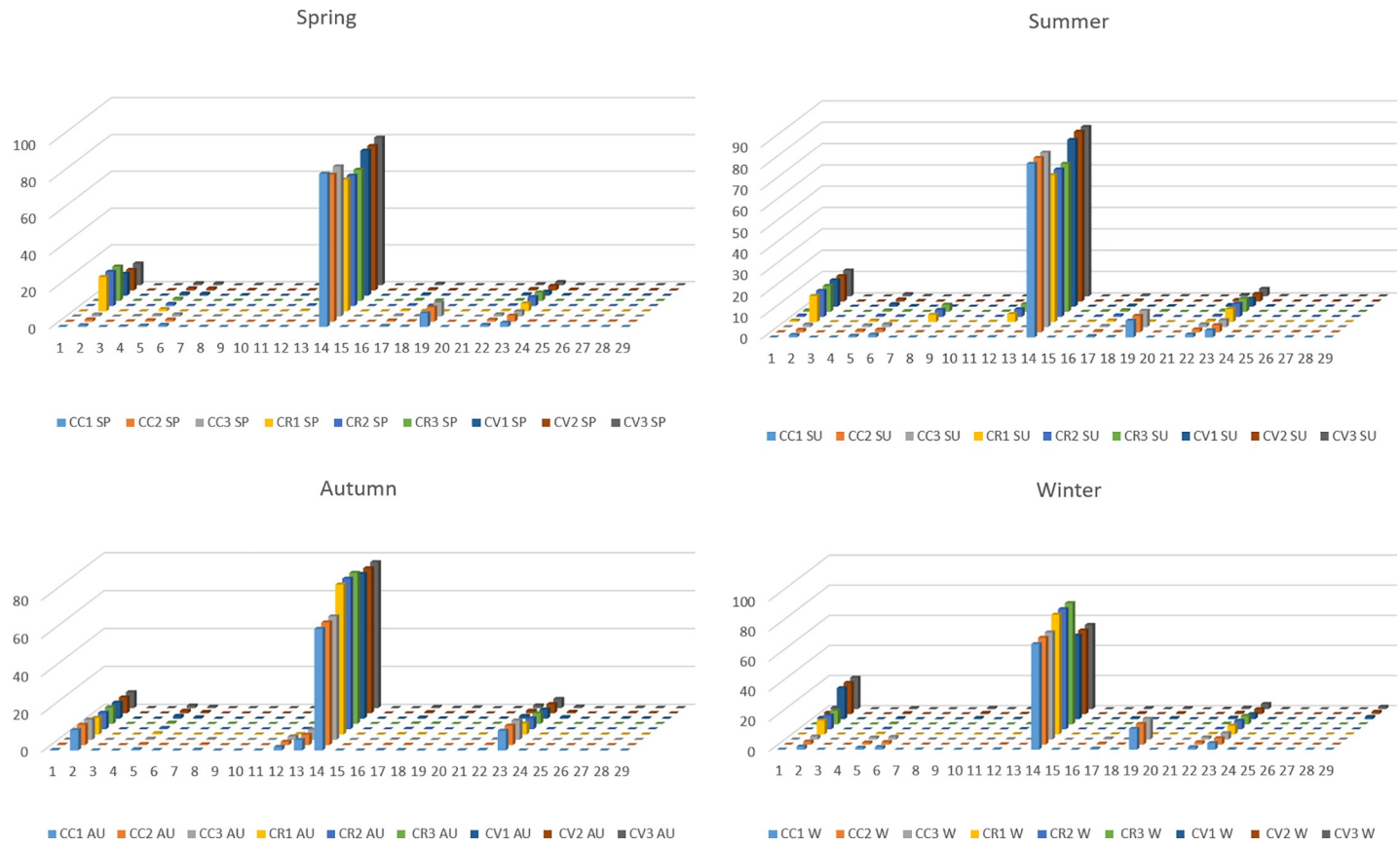

Fig 1. Bar charts of the main identified components of *Callistemon* species in spring, summer, autumn and winter.

https://doi.org/10.1371/journal.pone.0219571.g001

In an attempt to find the relationship between the phytochemical profile of each *Callistemon* species in different seasons, PCA was applied as shown in Fig 5A, 5B & 5C. Regarding *C. citrinus* and *C. viminalis*, PCA score plot demonstrated the discrepancy in the chemical composition of the essential oils collected in each season where they were completely segregated from each other with eucalyptol, α-pinene and α-terpineol as major metabolites with the highest impact on the separation of *C. citrinus*. In addition to eucalyptol, α-pinene, β-myrcene exhibited an influence on the segregation of *C. viminalis* in different seasons. For *C. rigidus*, a substantial difference was observed between essential oils constituents in spring and summer that are distanced from each other, with regard to that of autumn and winter that are closely related. From the loading plot, it was found that *O*-cymene and pseudolimonene were the main markers responsible for the segregation of *C. rigidus* in summer, however α-pinene discriminates the species in spring.

HCA results for *C. citrinus*, *C. rigidus* and *C. viminalis* in different seasons are illustrated in Fig 6A, 6B & 6C. The resulting dendrograms displayed the same pattern as all showed three main clusters. Regarding *C. citrinus* and *C. viminalis*, the dendrograms revealed the close distance between spring and summer, as they are grouped in the same cluster. On the contrary, *C. rigidus*, indicated a close association between winter and autumn.

The essential oil composition of all *Callistemon* species in different seasons exhibited common major constituents as eucalyptol, α-terpineol and α-pinene, which make their discrimination a major obstacle. By applying metabolomics fingerprinting in combination with chemometric analysis, such as PCA and HCA, this problem could be solved and was helpful to

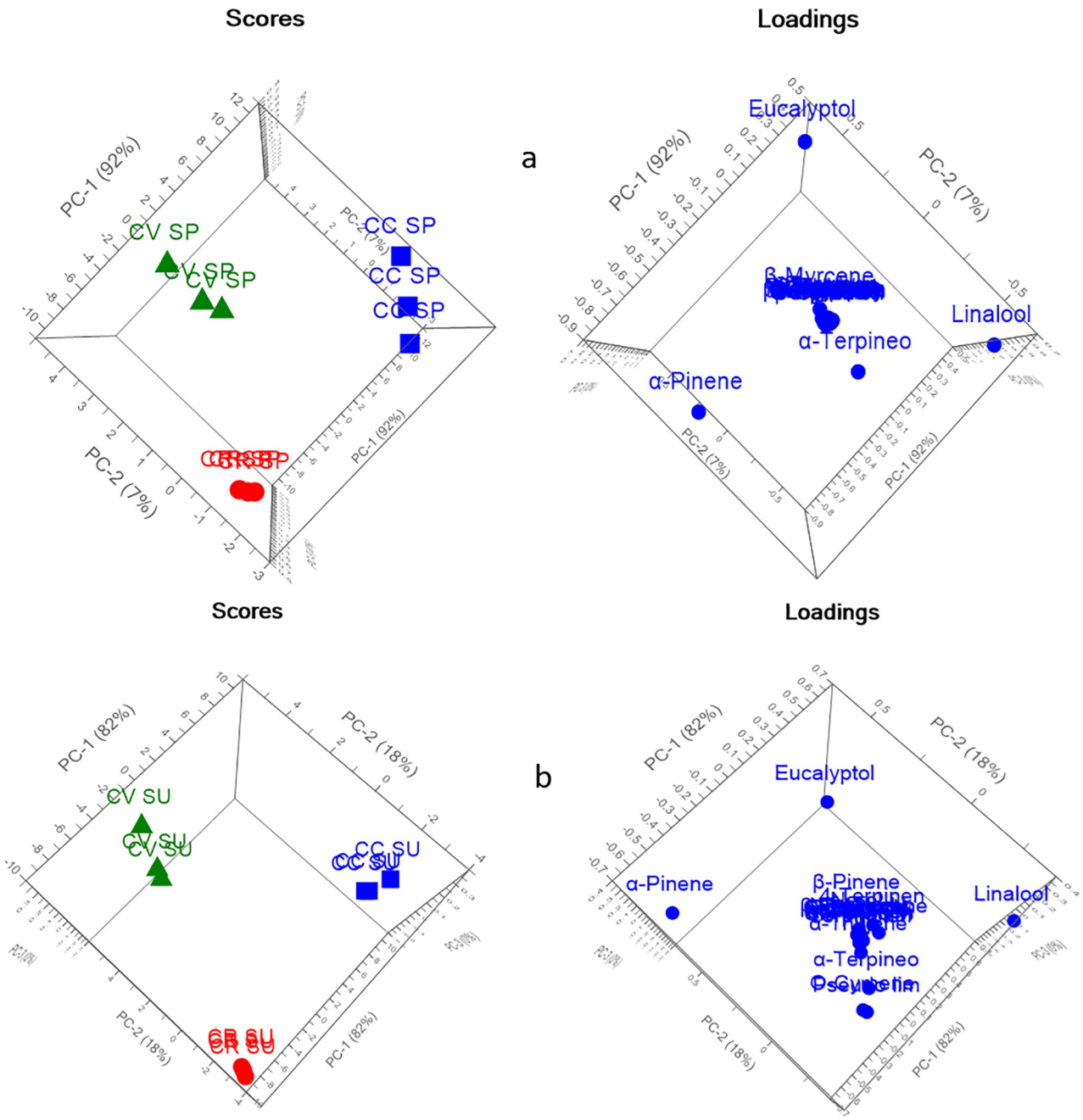

Fig 2. PCA score and loading plots of different *Callistemon* species (a) spring and (b) summer.

https://doi.org/10.1371/journal.pone.0219571.g002

identify the plants as it does not only rely on major components, but takes into consideration all metabolic profiling [27].

Biological activity

Antioxidant activity. Essential oils have attracted attention for the plethora of bioactivities they possess. *Callistemon* essential oils were assessed for DPPH radical scavenging capacity. Antioxidant activity was presented herein as the concentration of essential oil that resulted

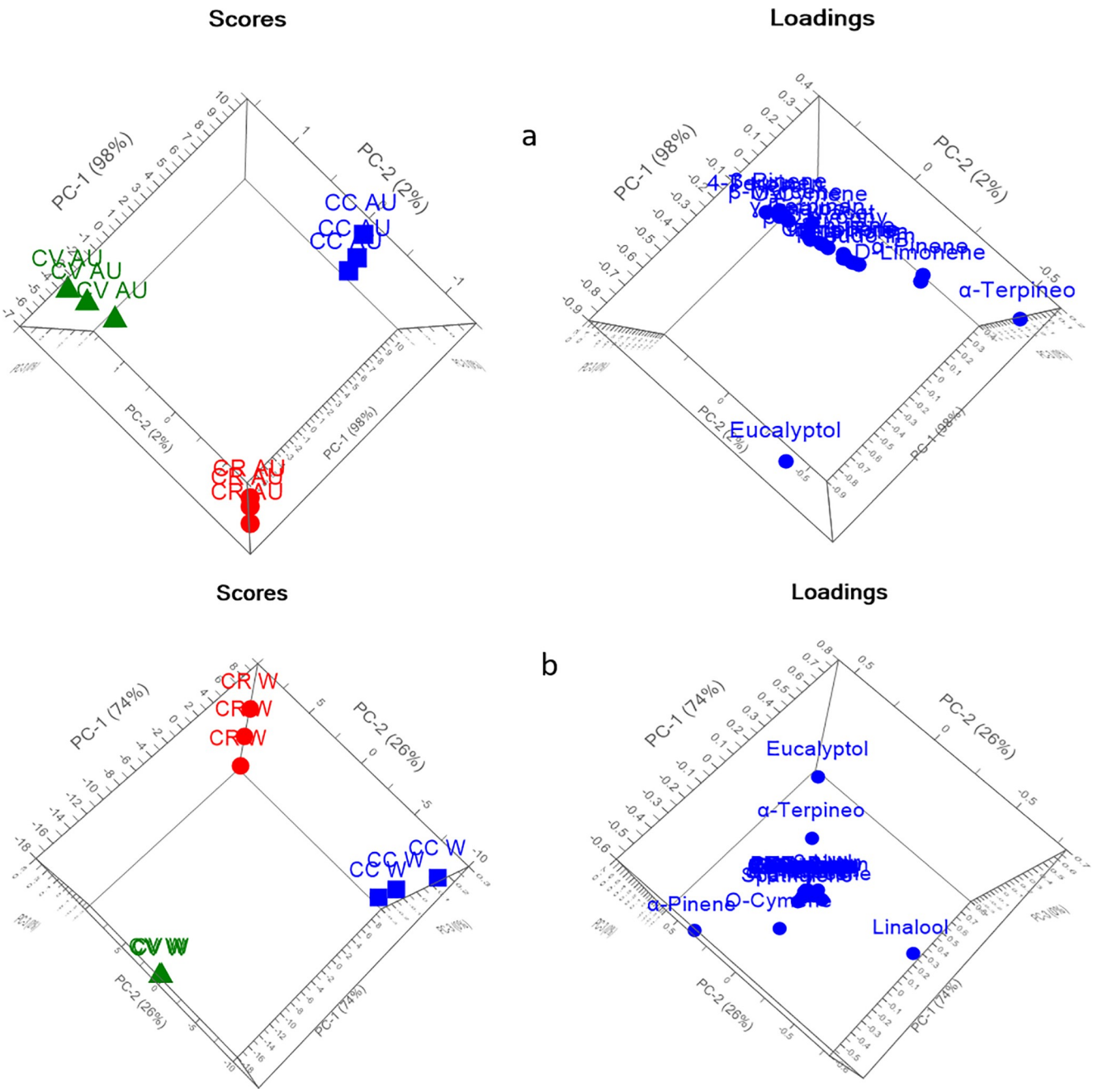

Fig 3. PCA score and loading plots of different *Callistemon* species (a) autumn and (b) winter.

https://doi.org/10.1371/journal.pone.0219571.g003

in 50% free radical inhibition (IC_{50}). *C. viminalis* showed more pronounced antioxidant activity than *C. citrinus*, exhibiting IC50 values of 1.40 mg/mL and 1.77 mg/mL, respectively. Nevertheless, *C. rigidus* showed very weak antioxidant activity with IC_{50} above the tested concentration range. Meanwhile, ascorbic acid exhibited IC_{50} value of 14.2 μg/ml. Results were in agreement with previous studies. Essential oil from the leaves of *C. citrinus* showed free radical scavenging activity with IC_{50} value of 4.02 mg/mL [28]. In another study, a pronounced free radical inhibitory activity (91.1 ± 0.3%) at a concentration of 1 mg/mL was observed for *C. citrinus* leaf essential oil, comparable to 0.1 mg/mL gallic acid (95.7 ± 2%) [29].

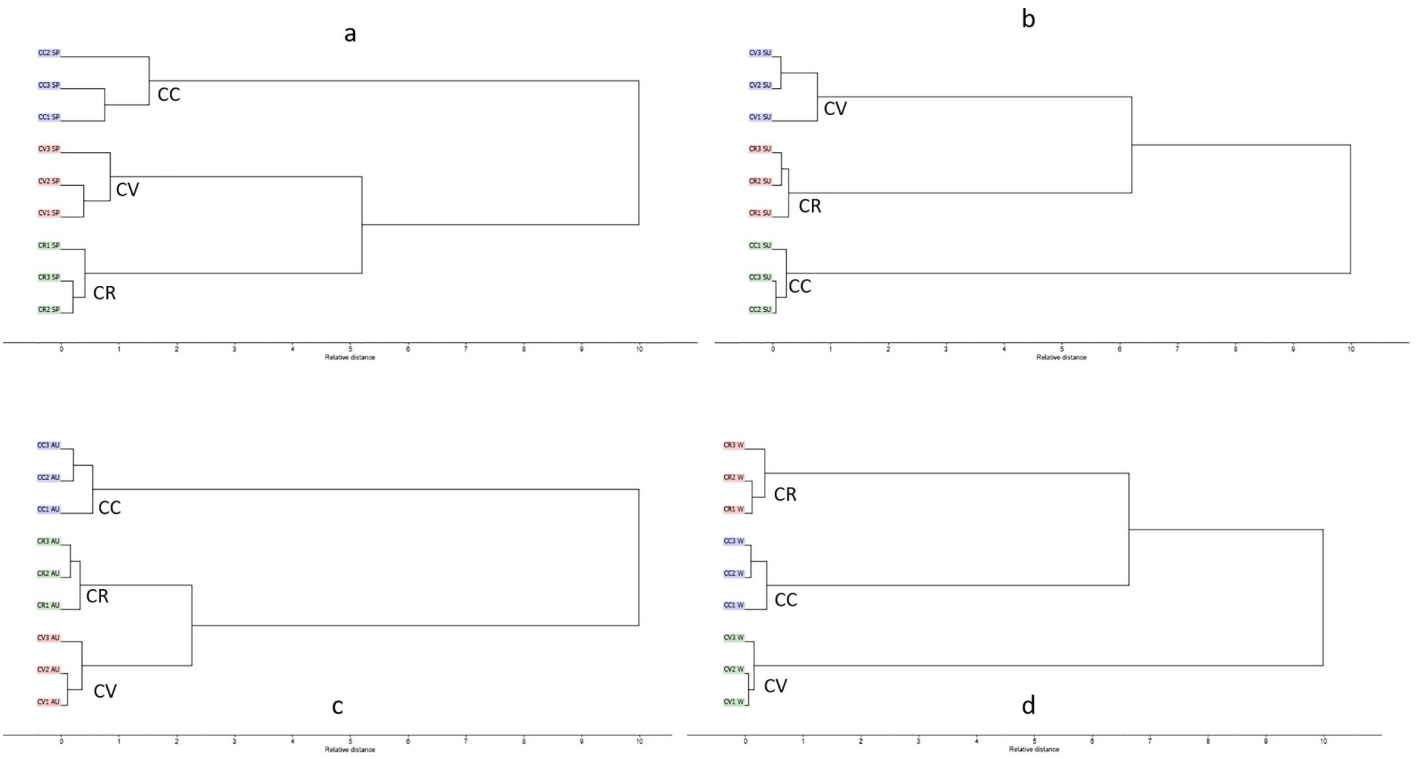

Fig 4. HCA of different *Callistemon* species (a) spring (b) summer (c) autumn (d) winter.

https://doi.org/10.1371/journal.pone.0219571.g004

Anti-inflammatory activity. Inflammation is a normal defensive response to tissue injury or infection, functioning to combat invaders to remove damaged or dead host cells [30]. Erythrocytes membrane stabilization assay is considered a common tool to screen for anti-inflammatory candidates [17]. In this study, *Callistemon* essential oils showed inhibitory activity to the hemolysis of erythrocytes induced by hypotonic solution. *C. viminalis* showed potent membrane stabilizing activity exhibiting IC_{50} value of 25.6 μg/mL. Results were comparable to Indomethacin (IC_{50} 17.02 μg/mL). Moreover, *C. citrinus* showed moderate activity with IC_{50} value of 39.9 μg/mL. Meanwhile, *C. rigidus* displayed weak activity with IC_{50} value of 217.1 μg/mL.

The erythrocyte membrane is analogous to the lysosomal membrane. Thus, its stabilization serves as a parameter to assess the ability to stabilize the lysosomal membrane [17]. Stabilization of the lysosomal membrane is necessary to limit the inflammatory response through inhibition of the release of lysosomal constituents of activated neutrophils such as proteases and bactericidal enzymes. Exposure of erythrocytes to a hypotonic medium results in membrane lysis [31]. A possible mechanism for membrane stabilization activity of *Callistemon* essential oil observed herein could be attributed to the ability of essential oil constituents to integrate into cellular membranes, increasing the surface area to volume ratio of the cells that might be brought about by expansion of the membrane or shrinkage of the cell, as well as an interaction with membrane proteins [17]. Numerous terpenoids have been previously reported to possess anti-inflammatory activity. 1,8-cineole, was reported to inhibit the production of leukotrienes (LTB4) and PGE2 [32]. Furthermore, α-terpineol was reported to inhibit histamine release and reduce the production of inflammatory mediators [33]. Ahmed et al. reported that *C. citrinus* chloroform fraction exhibited membrane stabilizing anti-inflammatory potentials in

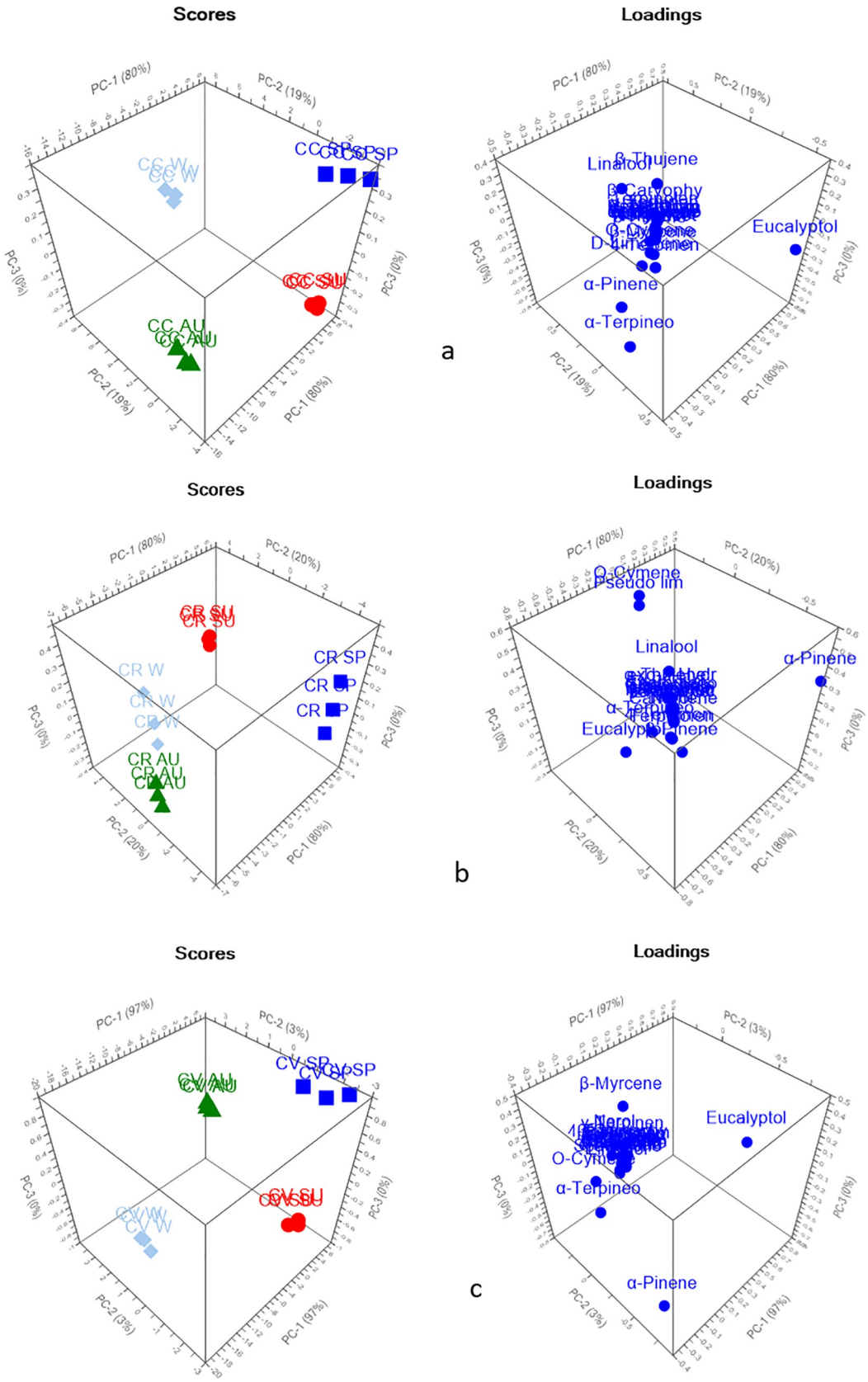
Scores
Loadings
PC-1 (80%)
PC-2 (19%)
PC-3 (0%)
CC SP
CC W
CC SU
CC AU
Linalool
β-Thujene
Eucalyptol
α-Pinene
α-Terpineo
a
Scores
Loadings
PC-1 (80%)
PC-2 (20%)
PC-3 (0%)
CR SU
CR SP
CR W
CR AU
O-Cymene
Pseudo lim
Linalool
α-Pinene
Eucalyptol
b
Scores
Loadings
PC-1 (97%)
PC-2 (3%)
PC-3 (0%)
CV SP
CV AU
CV SU
CV W
β-Myrcene
Eucalyptol
α-Terpineo
α-Pinene
c

Fig 5. PCA score and loading plots of different *Callistemon* species (a) *C. citrinus* (b) *C. rigidus* (c) *C. viminalis* in all seasons.

https://doi.org/10.1371/journal.pone.0219571.g005

hypotonic solution-induced hemolysis, results were comparable to acetylsalicylic acid, the standard drug [31].

Anti-proliferative Activity. The anti-proliferative activity of essential oils has been widely explored and numerous studies are now available in literature [10]. In this study, the anti-proliferative activity of *Callistemon* essential oils was assessed on HCT-116 and Hela human cancer cell lines using MTT assay. The three essential oils showed no cytotoxic activity. *C. citrinus* essential oil exhibited an IC_{50} value of 0.60 mg/mL on HCT-116 cancer cell line, whereas, IC_{50} values of 0.85 mg/mL and 0.51 mg/mL were recorded for *C. rigidus* and *C. viminalis*, respectively. Doxorubicin exhibited IC_{50} value of 4.62 μM on the aforementioned cancer cell line. On the other hand, the IC_{50} values recorded for *C. citrinus*, *C. rigidus* and *C. viminalis* on Hela human cancer cell line were 2.427, 3.428 and 1.928 mg/mL, respectively. These IC_{50} values indicate that the essential oils are not cytotoxic.

Previous studies conducted by Kumar *et al.* showed that *C. citrinus* leaf essential oil was not cytotoxic to rat glioma (C-6), human colon cancer (Colo-205), human cervical cancer (SiHa) and human peripheral blood mononuclear cells (PBMCs) at concentrations up to 100 μg/mL [26]. In the same context, essential oils obtained from *C. viminalis* leaves were not cytotoxic to melanoma cells (HT144) at concentration (200 μg/mL) where only 40% reduction in percentage cell viability was observed [34].

Conclusion

The phytochemical profiling of the essential oils from three *Callistemon* species by GLC/MS showed that oxygenated monoterpenes represent the major class of the oil components with eucalyptol as the major secondary metabolite. The chemical profiles show high qualitative and quantitative similarities between species. Different chemometric analysis techniques were

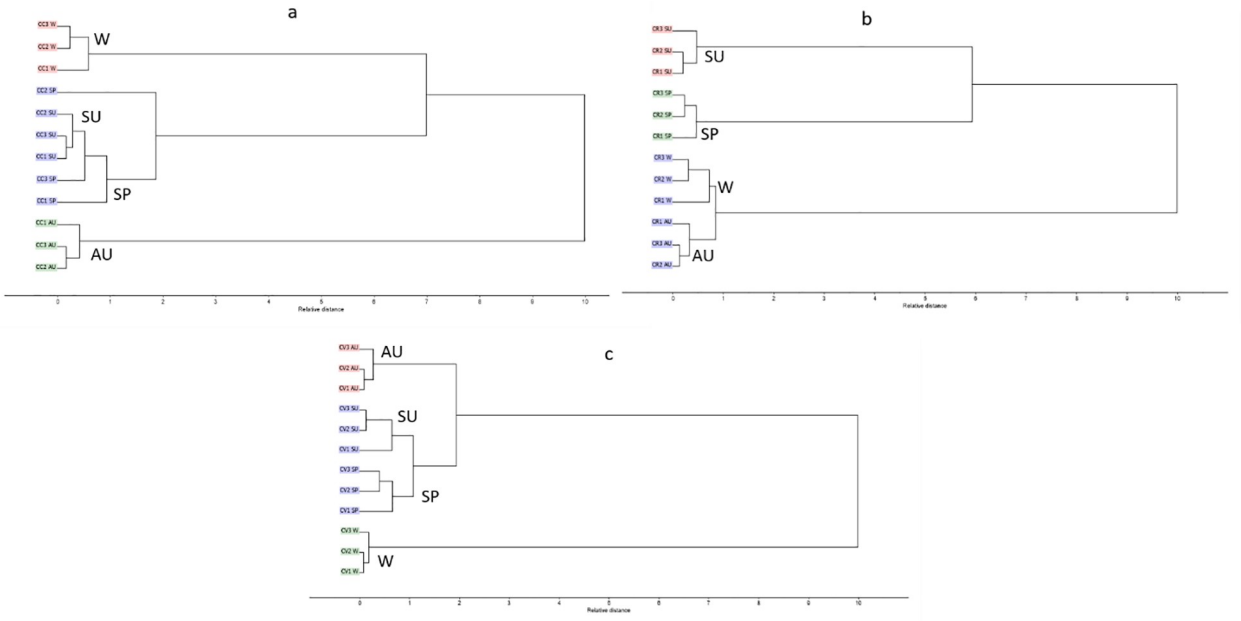

Fig 6. HCA of different *Callistemon* species (a) *C. citrinus* (b) *C. rigidus* (c) *C. viminalis* in all seasons.

https://doi.org/10.1371/journal.pone.0219571.g006

effectively applied as a discriminatory tool to differentiate between three *Callistemon* species, in each season, and within the same species in different seasons. *C. viminalis* essential oil exhibited pronounced membrane stabilization activity, which was equivalent to that of the standard drug, indomethacin. The three essential oils showed no cytotoxic activity against tested cancer cell lines. Future studies should be implemented to unravel other potential bioactivities of *Callistemon* essential oils.

Supporting information

S1 Fig. GLC/MS chromatograms of the essential oils of *C. citrinus* (CC), *C. viminalis* (CV) and *C. rigidus* (CR) collected in spring.
(TIF)

S2 Fig. GLC/MS chromatograms of the essential oils of *C. citrinus* (CC), *C. viminalis* (CV) and *C. rigidus* (CR) collected in summer.
(TIF)

S3 Fig. GLC/MS chromatograms of the essential oils of *C. citrinus* (CC), *C. viminalis* (CV) and *C. rigidus* (CR) collected in autumn.
(TIF)

S4 Fig. GLC/MS chromatograms of the essential oils of a *C. citrinus* (CC), *C. viminalis* (CV) and *C. rigidus* (CR) collected in winter.
(TIF)

Acknowledgments

Iriny M. Ayoub is thankful to the Science and Technology Development Fund in Egypt (STDF, project ID 25448) for funding the postdoctoral fellowship in Germany, where she performed the anti-proliferative activity against HCT116 and Hela cancer cell lines.

Author Contributions

Conceptualization: Haidy A. Gad, Iriny M. Ayoub, Michael Wink.

Data curation: Haidy A. Gad, Iriny M. Ayoub.

Funding acquisition: Iriny M. Ayoub.

Investigation: Haidy A. Gad, Iriny M. Ayoub.

Methodology: Haidy A. Gad, Iriny M. Ayoub.

Project administration: Iriny M. Ayoub.

Resources: Michael Wink.

Supervision: Michael Wink.

Writing – original draft: Haidy A. Gad, Iriny M. Ayoub.

Writing – review & editing: Haidy A. Gad, Iriny M. Ayoub, Michael Wink.

References

1. Zandi-Sohani N, Hojjati M, Carbonell-Barrachina AA. Volatile Composition of the Essential Oil of *Callistemon citrinus* Leaves from Iran. Journal of Essential Oil Bearing Plants. 2012; 15(5):703–7. https://doi.org/10.1080/0972060X.2012.10644109

2. Lumely P, Spencer R. Callistemon: In flora of New South Wales; Harden GJ. New South Wales University Press: Sydney, Australia. 1991:168–73.
3. Oyedeji OO, Lawal OA, Shode FO, Oyedeji AO. Chemical composition and antibacterial activity of the essential oils of *Callistemon citrinus* and *Callistemon viminalis* from South Africa. Molecules. 2009; 14 (6):1990–8. https://doi.org/10.3390/molecules14061990 PMID: 19513000
4. Pierre DYS, Okechukwu EC, Nchiwan NE. Larvicidal and phytochemical properties of *Callistemon rigidus* R. Br.(Myrtaceae) leaf solvent extracts against three vector mosquitoes. Journal of vector borne diseases. 2014; 51(3):216. PMID: 25253215
5. Goyal PK, Jain R, Jain S, Sharma A. A Review on biological and phytochemical investigation of plant genus *Callistimon*. Asian Pacific Journal of Tropical Biomedicine. 2012; 2(3):S1906–S9.
6. Sudhakar M, Rao CV, Rao AL, Ramesh A, Srinivas N, Raju D, et al. Antinociceptive and anti-inflammatory effects of the standardized oil of Indian *Callistemon lanceolatus* leaves in experimental animals. East and Central African Journal of Pharmaceutical Sciences. 2004; 7(1):10–5.
7. Kamal A, Fareeda A. Phytochemistry and Pharmacology of *Callistemon viminalis* (Myrtaceae): A Review. The Natural Products Journal. 2017; 7(3):166–75. http://dx.doi.org/10.2174/2210315507666161216100323.
8. Zubair M, Hassan S, Rizwan K, Rasool N, Riaz M, Zia-Ul-Haq M, et al. Antioxidant Potential and Oil Composition of *Callistemon viminalis* Leaves. The Scientific World Journal. 2013; 2013:8. https://doi.org/10.1155/2013/489071 PMID: 23818824
9. Ji T. Traditional Chinese medicine pills for treating hemorrhoid. CN 101352524 A. 2009;20090128.
10. Russo R, Corasaniti MT, Bagetta G, Morrone LA. Exploitation of Cytotoxicity of Some Essential Oils for Translation in Cancer Therapy. Evidence-Based Complementary and Alternative Medicine. 2015; 2015:9. https://doi.org/10.1155/2015/397821 PMID: 25722735
11. Adams RP. Identification of essential oil components by gas chromatography/mass spectorscopy: Allured Publishing Corporation; 2007.
12. Ayoub IM, Youssef FS, El-Shazly M, Ashour ML, Singab AN, Wink M. Volatile constituents of *Dietes bicolor* (Iridaceae) and their antimicrobial activity. Z Naturforsch C. 2015; 70(7–8):217–25. Epub 2015/09/15. https://doi.org/10.1515/znc-2015-0164 PMID: 26368045.
13. Elkady WM, Ayoub IM. Chemical profiling and antiproliferative effect of essential oils of two *Araucaria* species cultivated in Egypt. Industrial Crops and Products. 2018; 118:188–95. https://doi.org/10.1016/j.indcrop.2018.03.051.
14. Brereton RG. Chemometrics: applications of mathematics and statistics to laboratory systems: Ellis Horwood Ltd; 1990.
15. Brereton RG. Applied chemometrics for scientists: John Wiley & Sons; 2007.
16. Yen GC, Duh PD. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. Journal of agricultural and food chemistry. 1994; 42(3):629–32.
17. Shinde U, Phadke A, Nair A, Mungantiwar A, Dikshit V, Saraf M. Membrane stabilizing activity—a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. Fitoterapia. 1999; 70(3):251–7.
18. Ashour ML, El-Readi M, Youns M, Mulyaningsih S, Sporer F, Efferth T, et al. Chemical composition and biological activity of the essential oil obtained from *Bupleurum marginatum* (Apiaceae). Journal of Pharmacy and Pharmacology. 2009; 61(8):1079–87. https://doi.org/10.1211/jpp/61.08.0012 PMID: 19703352
19. Brophy JJ, Goldsack RJ, Forster PI, Craven LA, Lepschi BJ. The Leaf Essential Oils of the Australian Members of the Genus *Callistemon* (Myrtaceae). Journal of Essential Oil Research. 1998; 10(6):595–606. https://doi.org/10.1080/10412905.1998.9700986
20. Sharma RK, Kotoky R, Bhattacharyya PR. Volatile oil from the leaves of *Callistemon lanceolatus* DC grown in north-eastern India. Flavour and fragrance journal. 2006; 21(2):239–40.
21. Chane-Ming J, Vera RR, Fraisse DJ. Chemical composition of essential oil of *Callistemon citrinus* (Curtis) Skeel from Reunion. Journal of Essential Oil Research. 1998; 10(4):429–31.
22. Srivastava S, Ahmad A, Jain N, Aggarwal K, Syamasunder K. Essential oil composition of *Callistemon citrinus* leaves from the lower region of Himalayas. Journal of Essential oil research. 2001; 13(5):359–61.
23. Silva CJ, Barbosa LC, Demuner AJ, Montanari RM, Pinheiro AL, Dias I, et al. Chemical composition and antibacterial activities from the essential oils of Myrtaceae species planted in Brazil. Química Nova. 2010; 33(1):104–8.
24. Salem MZM, El-Hefny M, Nasser RA, Ali HM, El-Shanhorey NA, Elansary HO. Medicinal and biological values of *Callistemon viminalis* extracts: History, current situation and prospects. Asian Pacific journal of tropical medicine. 2017; 10(3):229–37. https://doi.org/10.1016/j.apjtm.2017.03.015 PMID: 28442106

25. Jazet PM, Tatsadjieu LN, Ndongson BD, Kuate J, Zollo PHA, Menut C. Correlation between chemical composition and antifungal properties of essential oils of *Callistemon rigidus* and *Callistemon citrinus* of Cameroon against *Phaeoramularia angolensis*.Journal of medicinal plants research. 2009; 3(1):009–15.

26. Kumar D, Sukapaka M, Babu GK, Padwad Y. Chemical composition and in vitro cytotoxicity of essential oils from leaves and flowers of Callistemon Citrinus from western himalayas. PloS one. 2015; 10(8): e0133823. https://doi.org/10.1371/journal.pone.0133823 PMID: 26308916

27. Gad HA, El-Ahmady SH, Abou-Shoer MI, Al-Azizi MM. Application of Chemometrics in Authentication of Herbal Medicines: A Review. Phytochemical analysis: PCA. 2012; 24(1):1–24. Epub 2012/06/09. https://doi.org/10.1002/pca.2378 PMID: 22678654.

28. Shukla R, Singh P, Prakash B, Dubey N. Antifungal, aflatoxin inhibition and antioxidant activity of Callistemon lanceolatus (Sm.) Sweet essential oil and its major component 1, 8-cineole against fungal isolates from chickpea seeds. Food Control. 2012; 25(1):27–33.

29. Abdelhady MI, Aly HAH. Antioxidant antimicrobial activities of *Callistemon comboynensis* essential oil. Free Radicals and Antioxidants. 2012; 2(1):37–41. https://doi.org/10.5530/ax.2012.2.8.

30. Dhifi W, Bellili S, Jazi S, Bahloul N, Mnif W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. Medicines. 2016; 3(4):25. https://doi.org/10.3390/medicines3040025 PMID: 28930135

31. Ahmed F, Rahman MS. Preliminary assessment of free radical scavenging, thrombolytic and membrane stabilizing capabilities of organic fractions of *Callistemon citrinus* (Curtis.) skeels leaves. BMC complementary and alternative medicine. 2016; 16:247–. https://doi.org/10.1186/s12906-016-1239-1 PMID: 27460997.

32. Andrade L, de Sousa D. A review on anti-inflammatory activity of monoterpenes. Molecules. 2013; 18 (1):1227–54. https://doi.org/10.3390/molecules18011227 PMID: 23334570

33. Carson CF, Hammer KA, Riley TV. *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. Clinical microbiology reviews. 2006; 19(1):50–62. https://doi.org/10.1128/CMR.19.1.50-62.2006 PMID: 16418522.

34. de Oliveira CM, das Graças Cardoso M, Ionta M, Soares MG, de Andrade Santiago J, da Silva GÁF, et al. Chemical Characterization and in vitro Antitumor Activity of the Essential Oils from the Leaves and Flowers of Callistemon viminalis. American Journal of Plant Sciences. 2015; 6(16):2664.