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Variations of the chemical composition and bioactivity of essential oils from leaves and stems of *Liquidambar styraciflua* (Altingiaceae)

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Abstract

Objectives This study aimed to evaluate the variations of the chemical composition and bioactivity of essential oils of *Liquidambar styraciflua* L. (Altingiaceae) collected in different seasons.

Methods The oils were analysed by GLC/FID and GLC/MS. The antioxidant activity was investigated by diphenylpicrylhydrazyl (DPPH) and superoxide anion radical scavenging assays and the deoxyribose degradation assay. Inhibition of both 5-lipoxygenase (5-LOX) and prostaglandin E2 (PGE2) production in hepatic cancer (HepG-2) cells were used to assess the anti-inflammatory activity. The cytotoxic activity was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Key findings Altogether, 64 volatile secondary metabolites were identified. The major components of the leaf oil were d-limonene, α-pinene and β-pinene, and of the stem oil were germacrine D, α-cadinol, d-limonene, α-pinene, and β-pinene. Leaf and stem oils collected in spring could reduce DPPH• (IC50 = 3.17 and 2.19 mg/ml) and prevent the degradation of the deoxyribose sugar (IC50 = 17.55 and 14.29 μg/ml). The stem oil exhibited a higher inhibition of both 5-LOX and PGE2 than the leaf oil. The cytotoxic activity of leaf and stem oils was low in cancer cell lines (IC50 = 136.27 and 119.78 μg/ml in cervical cancer (HeLa) cells).

Conclusions Essential oils of *L. styraciflua* exhibited an interesting anti-inflammatory activity with low cytotoxicity, supporting its traditional use to treat inflammation.

Introduction

Aromatic plants which produce essential oils have been widely used since centuries for either medicinal or cosmetic purposes. The family Altingiaceae represents a small family with only two genera (*Liquidambar* and *Altingia*) which were formerly included in the family Hamamelidaceae. These taxa are distributed mainly in Southeast Asia and America,[1] but as ornamental plants that have been cultivated in many countries around the world. Plants of this family are well embedded in both traditional Chinese and Ayurvedic medicines. Different *Liquidambar* and *Altingia* extracts or resins termed storax have been used for treatment of inflammation, stomach pain, bronchitis, enlarged liver and amenorrhoea. Externally, they are employed as an antiseptic against skin diseases, such as eczema, scabies, and leucoderma.[2,3]

The genus *Liquidambar* is represented by four species in the temperate zone of the northern hemisphere. *L. styraciflua* L., commonly known as American sweet gum or red gum, is native to eastern North America and widely cultivated in many areas around the world while *L. orientalis* Mill. (Oriental sweet gum) is distributed in Asia Minor. *L. formosana* Hance (Chinese sweet gum) and *L. acalycina* H.T. Chang (Chang's sweet gum) occur in China, India and Taiwan.[4,5]

Most of the biological activity studies focused on the bactericidal,[6] fungicidal,[7] nematicidal,[8] and antiviral activity[9] of *Liquidambar orientalis* extracts and storax. In addition, storax exhibited a rapid sedative and anticonvulsant activity when used intranasally.[10] Moreover, leaf and

fruit extracts of *L. formosana*, and their isolated secondary metabolites, showed very promising antioxidant and hepatoprotective activity.[11,12]

However, an anti-inflammatory activity of either storax or essential oils of *Liquidambar styraciflua* has not been reported, although they are widely used either internally or topically to treat many symptoms related to inflammation. Furthermore, when storax is burned for ceremonial purposes in olibanum, it produces a strong aromatic smell.[13]

The chemical composition of the volatile components of *Liquidambar* is incompletely known. Earlier studies of the leaf oil from *L. styraciflua* and storax identified terpinen-4-ol, α-pinene, sabinene, and γ-terpinene as the main components.[14–17] The main components of *L. orientalis* oil were similar; in addition, viridiflorene and germacrene D were detected in high concentrations.[18] However, to our knowledge, nothing was reported on the chemical composition of the stem essential oil, and only one report is found regarding the chemical composition of the leaves oil of *L. styraciflua*. Moreover, nothing could be traced regarding the seasonal variation in the chemical compositions of both stems and leaves essentials oil of *L. styraciflua*. The anti-inflammatory activity of both oils has also not been investigated yet. Hence, in this communication, we report the chemical variation in the composition profiles of both *L. styraciflua* oils cultivated in Egypt during the four seasons. Moreover, an in-vitro assessment of cytotoxicity and anti-inflammatory activity was also conducted by evaluating the antioxidant effect of the oils and their inhibitory activity on both 5-lipoxygenase and cyclooxygenase enzymes. In an effort to better understand the relevant biological activity of some known medicinal plants that have not explored yet, this study was conducted.

Materials and Methods

Plant materials

The aerial parts of *L. styraciflua* were collected in January (winter), April (spring), July (summer), and October (autumn) 2009 from the Alzohreya Botanical Garden (Cairo, Egypt) by one of the authors (HE). The plant was kindly authenticated by Agric. Eng. Therese Labib (Herbarium Section, Orman Garden, Giza, Egypt) and Dr Mohamed El Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Dokki, Giza, Egypt). Voucher specimens of the plant material are deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University (PHE.1217–1224).

Chemicals

Cell culture media, supplements, deuterated solvents, and dimethyl sulfoxide (DMSO) were purchased from Roth (Karlsruhe, Germany) and Greiner Labortechnik (Frickenhausen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin (≥98%), phenazine methosulfate (PMS), diphenylpicrylhydrazyl (DPPH), ascorbic acid (vitamin C), 2-deoxy-ribose (2-DR), nitro blue tetrazolium (NBT), norhydroguaretic acid (NDGA), sodium linoleate, lipopolysaccharide (LPS), quercetin and indomethacin were obtained from Sigma (Sigma Aldrich GmbH, Sternheim, Germany) and Gibco (Invitrogen; Karlsruhe, Germany). Other chemicals, including EDTA, ferric chloride ($FeCl_3$) and trichloroacetic acid, came from AppliChem (Darmstadt, Germany), while thiobarbituric acid was purchased from Fluka (Buchs, Switzerland).

Essential oil isolation

The essential oils were obtained by hydrodistillation for 6 h (500 g each from fresh leaves and stems of *L. styraciflua*) using a Clevenger-type apparatus. The oils were dried over anhydrous sodium sulfate and kept in sealed vials at −30°C for further analyses. The yields were calculated in triplicate based on the fresh weight (w/w).

GLC/FID analysis

The GLC analyses were carried out on a Varian 3400 (Varian GmbH, Darmstadt, Germany) equipped with OV-5 fused bonded column (30 m × 0.25 mm × 0.25 μm) (Ohio Valley, Ohio, USA) and FID detector. Conditions: carrier gas was helium (2 ml/min); initial temperature 45°C, 2 min isothermal, 300°C, 4°C/min 300°C, then 20 min isothermal. Detector and injector temperatures were 300 and 250°C, respectively. The split ratio was 1 : 20. PeakSimple 2000 chromatography data system (SRI Instruments, California, USA) was used for recording and integrating the chromatograms. Average areas under the peaks of three independent chromatographic runs were used to calculate the abundance of each component (total area = 100%).

GLC/MS analysis

The analyses were carried out on Hewlett-Packard gas chromatograph (GC 5890 II, Hewlett-Packard GmbH, Bad Homburg, Germany) equipped with the same column and conditions which were mentioned for GLC/FID. The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany). The injector temperature was 250°C. Helium carrier gas flow rate was 2 ml/min. All the mass spectra were recorded with the following condition: filament emission current, 100 mA; electron energy, 70 eV; ion source, 175°C; diluted samples (0.5% V/V) were injected with split mode (split ratio, 1 : 15). Compounds were identified by compari-

son of their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), and the literature.[19]

Cytotoxicity

Cell culture

HeLa (cervical cancer), MCF-7 (breast cancer), and HepG-2 (hepatic cancer) cell lines were maintained in Dulbecco's modified Eagle's medium complete media supplemented with 1% L-glutamine 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in addition to 10 mM non-essential amino acids in case of HeLa cell lines. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Sensitivity to drugs was determined in triplicate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay.[20] The samples and doxorubicin (positive control) were dissolved in dimethylsulfoxide (DMSO). The essential oils were diluted in the medium in final concentrations ranging from 0.1 to 1000 μg/ml in 96-well plates. Wells containing the solvent as a negative control and wells without the solvent were included in the experiment. Cells (2×10^4 cells/well of exponentially growing cells of each individual HeLa, MCF-7, and HepG-2 cell lines) were seeded in a 96-well plate (Greiner Labortechnik). The cells were cultivated for 24 h and incubated with various concentrations of samples at 37°C for 24 h and then with 0.5 mg/ml MTT for 4 h. The formed formazan crystals were dissolved in 100 μL DMSO. The absorbance was detected at 570 nm with a Tecan Safire II Reader (Tecan GmbH, Crailsheim, Germany). The cell viability rate (%) of three independent experiments was calculated as the follows:

$$\text{Cell viability \%} = \frac{\text{Mean of OD of sample treated cells}}{\text{OD of untreated control cells}}\%$$

$$\text{Cell viability rate (\%)} = (\text{OD of treated cells}/\text{OD of control cells}) \times 100\%$$

Antioxidant activity

Radical scavenging activity

The radical scavenging activity of the essential oils was evaluated using diphenylpicrylhydrazyl (DPPH•).[21] Equal volumes of sample solutions containing 0.01–1 mg/ml of the oils and 0.2 mM methanolic solution of DPPH• were mixed and the absorbance was measured against a blank at 517 nm using a Tecan® Safire II Reader (Tecan GmbH) after incubation in the dark for 30 min at room temperature compared with DPPH• control after background subtraction. Ascorbic acid was used as a positive control. The percentage inhibition was calculated from three different experiments using the following equation:

$$\text{Inhibition \%} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}}\%$$

Where, the inhibition % = radical scavenging activity; OD = absorption at 517 nm, and control = non-reduced DPPH.

Desoxyribose degradation assay

The ability of the essential oils to prevent the degradation of the desoxyribose was determined.[22,23] A mixture of freshly prepared 28 mM 2-desoxy-2-ribose (2-DR) in phosphate buffer pH 7.1, 1.04 mM EDTA and 200 μM $FeCl_3$, 1.0 mM H_2O_2 and 1.0 mM ascorbic acid was mixed with an equal volume of various essential oil concentrations (0.002–6 mg/ml of the oils) and kept at 37°C for 1 h. A mixture of thiobarbituric acid and 2.8% trichloroacetic acid, (1 : 1 v/v) was added to the reaction mixture and incubated at 100°C for 20 min. Absorbance was measured at 532 nm against blank using a Tecan® Safire II Reader (Tecan GmbH). Quercetin was used as a positive control. Inhibition of desoxyribose degradation was measured in triplicate at 532 nm as above.

Superoxide scavenging activity

The superoxide anion scavenging activity was determined according to Ewing and Janero (1995). Superoxide anion radicals were generated via the univalent oxidation of reduced phenazine methosulfate (PMS) by nicotinamide adenine dinucleotide (NADH). Nitro blue tetrazolium (NBT) is converted in this process to a stable formazan product.[24] Briefly, 25 μl of different concentrations of oils were pipetted into 200 μl freshly prepared 0.1 mM EDTA, 62 μM NBT and 98 μM NADH in 50 mM phosphate buffer (pH 7.4). The reaction initiated by the addition of 25 μl freshly prepared 33 μM PMS in 50 mM phosphate buffer containing 0.1 mM EDTA. The total volume of control reaction mixture was 250 μl (50 mM phosphate buffer containing 0.1 mM EDTA, 50 μM NBT, 78 μM NADH, and 3.3 μM PSM). NBT reduction was measured at 560 nm after 5 min using a Tecan® Safire II Reader (Tecan GmbH). The inhibition of superoxide scavenging activity was calculated as above.

Anti-inflammatory activity

Prostaglandin E2 assay

The effect of the samples on prostaglandin E2 (PGE2) production was assessed using the Monoclonal EIA Kit

(514010; Cayman Chemicals, Michigan, USA) in HepG-2 cells.[25] HepG-2 cells (2×10^4 cells/well) were seeded in 96 microwell plates and incubated for 24 h to allow them to attach. Cells were treated with 1 μg/ml lipopolysaccharide (LPS) in combination with a 20 μg/ml of the oil and indomethacin (a prostaglandin synthase inhibitor) and were incubated at 37°C in 5% CO_2 for 24 h. Culture supernatants were collected and centrifuged. The level of PGE2 in the supernatant of the culture medium was measured using the ELISA kit according to the manufacturer's instructions. Inhibition of the prostaglandin E2 level was normalized relative to the untreated control.

5-Lipoxygenase inhibition

Inhibition of soybean lipoxygenase (which has been used in place of human lipoxygenases) by the oils was determined spectrophotometrically after Baylac and Racine (2003) with minor modifications.[26] One ml of 0.1 M phosphate buffer pH 9.0 containing 10 μl enzyme (7.9 U/ml) and 20 μl of 10 different concentrations of the essential oils (20–420 μg/ml oils) were incubated at room temperature for 10 min. The reaction was initiated by adding 25 μl of 62.5 μM sodium linoleate and the reaction kinetics were monitored at 234 nm at 10 s intervals using Biochrom Ultrospec II spectrophotometer (LKB-Biochrom, Cambridge, UK). The initial reaction rates were determined from the slope of the straight line portion of the curve, and inhibition of the enzyme activity was calculated from three independent experiments by comparison with the control (ethanol). Norhydroguaretic acid (NDGA) was used as a positive control (IC50 0.24 μg/ml).

Statistical analysis

All experiments were carried out three times unless mentioned otherwise. Continuous variables were presented as mean ± S.E. The IC50 was determined as the concentration of a sample which resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC50 values were calculated using a four-parameter logistic curve (SigmaPlot 11.0, Systat Software, Inc., Richmond, California, USA), and all the data were statistically evaluated using Student's *t*-test or the Kruskal–Wallis test (GraphPad Prism 5.01, GraphPad Software, Inc., San Diego, California, USA) followed by Dunn's post-hoc multiple comparison test when the significance value is <0.05 using the same significance level. The criterion for statistical significance was taken as $P < 0.05$. Correlations were calculated using Spearman's coefficient (r) assuming that the data were sampled from Gaussian populations.

Results and Discussion

Chemical composition

Based on the GLC analyses, altogether 64 volatile components were identified in the essential oils from leaves and stems of *L. styraciflua* (Table 1). In the leaf oil (LO), a total of 58 compounds was identified representing 95.46, 93.49, 94.45, and 97.57% of the total detected compounds in spring, summer, autumn and winter, respectively. A similar number of compounds were identified from the stem oil (SO) representing 99.77, 97.60, 98.35, and 97.14% in the same season pattern, respectively. The leaf oil contained a higher percentage of non-oxygenated hydrocarbons (82.39–85.46%) and lower amounts of oxygenated hydrocarbons (10.38–12.11%) as compared with the stem oil (70.41–74.57%) and (22.99–26.69%), respectively (Table 1).

Major differences between stems and leaves concern both α-/β-pinene, and *d*-limonene. These components constitute ~60% of the leaf oil during the spring; whereas this percentage dropped to almost to 32% in the stem oil. Interestingly, sesquiterpenes, β-caryophyllene, germacrene D, and α-/δ-cadinol showed the opposite trend: The main sesquiterpene hydrocarbons constitute ~25.5% of the stem oil compared with ~10% in the leaf oil during summer. A cluster analysis of the identified compounds in both oils at different seasons based on their chemical structure and nature is presented in a gradual color scale in Figure 1.

However, this analysis did not reveal any correlation or variation between the chemical compositions of the essential oil of the same organ within the different seasons. The correlations between the percentages of the identified components in each season were analysed using non-parametric correlation (Spearman's coefficient (r)). The correlation coefficients (r) for individual compounds in the different season of the same plant organ were higher (r value ~ 0.9) than the correlation between different organs (leaves and stems) (r value ~ 0.6). As a result, it can be concluded that yields and quality of the essential oils depend mainly on the organ and not the season (Table 1). Many studies have indicated that the essential oil composition is highly influenced by many genetic and environmental factors including climatic, seasonal, geographical and geological factors.[27,28] However, based on the statistical analysis (Table 1) of our results in evaluating the effect of seasonal effect, which is only applicable here, it was shown that the chemical profiles of the essential oils obtained from fresh leaves and stems of *Liquidamber styraciflua* L. are qualitatively similar in composition and qualitatively different in the four seasons. This is may be due to the insignificant change of the temperature in the temperate and subtropical regions such as Egypt. In this context, it is noticed that the monoterpene hydrocarbons are usually found in the highest concentration during

Table 1 The chemical composition, physical characters and yields of the essential oils of *Liquidambar styraciflua* leaves and stems in four seasons

Compound name	RI		% of total oil[a] (leaf)				% of total oil[a] (stem)				Method of identification
	Rep.	Calc.	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
n-Octane	800	800	0.70	0.68	0.55	0.71	0.41	0.49	0.47	0.41	MS, RI, AT
3-Hexen-1-ol	856	851	0.04	0.06	0.04	0.05	Tr	0.09	tr	0.10	MS, RI
n-Hexanol	864	864	–	–	–	–	Tr	0.08	0.02	tr	MS, RI
n-Nonane	900	898	0.04	0.05	0.04	0.05	0.15	0.34	0.19	0.14	MS, RI
Tricycline	921	921	0.03	0.07	0.04	0.05	Tr	tr	tr	tr	MS, RI
α-Thujene	926	926	0.45	0.49	0.38	0.44	0.18	0.14	0.48	0.16	MS, RI, AT
α-Pinene	934	934	27.95	26.17	27.82	27.35	14.19	11.08	16.01	11.77	MS, RI, AT
Camphene	948	948	0.76	0.73	0.98	0.82	0.95	0.59	1.82	0.78	MS, RI, AT
Sabinene	973	973	0.79	0.75	0.64	0.75	0.33	0.39	0.31	0.28	MS, RI
β-Pinene	977	977	11.20	10.06	11.25	10.70	5.34	5.58	8.58	4.36	MS, RI, AT
β-Myrcene	990	990	3.34	3.97	3.46	4.21	3.06	2.38	2.68	1.98	MS, RI, AT
p-Mentha-1(7),8-diene	996	996	0.26	0.25	0.23	0.20	–	–	–	–	MS, RI
α-Phellandrene	1002	1003	–	–	–	–	0.37	0.21	tr	0.14	MS, RI, AT
α-Terpinene	1017	1017	0.25	0.24	0.22	0.24	0.31	0.13	tr	0.11	MS, RI, AT
o-Cymene	1023	1023	0.09	0.09	0.10	1.62	0.26	0.32	tr	0.28	MS, RI
d-Limonene	1031	1031	22.34	21.69	20.71	21.07	12.89	12.77	11.20	12.59	MS, RI, AT
β *cis*-Ocimene	1043	1044	0.08	0.09	0.08	0.15	–	–	–	–	MS, RI
β *trans*-Ocimene	1046	1049	–	–	–	–	0.25	0.19	tr	0.03	MS, RI
γ-Terpinene	1062	1061	0.45	0.48	0.39	0.12	0.86	0.61	0.37	0.76	MS, RI
α-Terpinolene	1086	1088	0.44	0.49	0.41	0.18	0.78	0.29	0.19	0.57	MS, RI
n-Nonal	1103	1103	0.18	0.17	0.18	0.37	0.63	0.59	0.29	0.59	MS, RI
β-Fenchol	1118	1117	0.41	0.45	0.39	0.22	0.81	0.77	0.78	0.76	MS, RI
trans 4-Isopropyl-1-methyl-2-cyclohexen-1-ol	1119	1021	0.04	0.09	0.07	0.11	–	–	–	–	MS, RI
1-Terpinenol	1133	1133	0.02	tr	0.02	tr	–	–	–	–	MS, RI
trans-Pinocarveol	1141	1141	0.25	0.28	0.25	0.18	0.51	0.39	0.29	0.44	MS, RI
trans-Verbenol	1144	1147	0.03	tr	0.03	0.08	0.09	0.16	tr	tr	MS, RI
Pinocarvone	1160	1162	0.22	0.14	0.31	0.22	0.21	0.08	0.26	0.19	MS, RI
Borneol	1165	1168	0.22	0.22	0.21	0.62	0.23	0.28	0.31	0.20	MS, RI, AT
Terpin-4-ol	1182	1179	1.95	2.14	1.88	1.82	2.28	2.47	2.69	1.85	MS, RI, AT
cis 3-Hexenyl butyrate	1184	1182	0.05	tr	0.03	tr	–	–	–	–	MS, RI
p-Cymen-8-ol	1186	1184	–	–	–	–	Tr	0.16	tr	tr	MS, RI
α-Terpineol	1188	1192	1.86	1.71	1.66	1.90	4.25	4.51	4.62	3.91	MS, RI, AT
Myrtenol	1194	1197	–	–	–	–	Tr	tr	tr	0.03	MS, RI, AT
cis-Piperitol	1195	1200	tr	tr	tr	tr	Tr	0.06	tr	0.05	MS, RI
Verbenone	1204	1206	0.04	0.04	0.03	0.03	0.13	0.49	0.24	0.27	MS, RI, AT
trans-Carveol	1215	1219	0.04	0.04	0.03	0.05	0.17	0.39	0.15	0.21	MS, RI
Carvone	1239	1237	0.04	0.04	0.04	0.05	0.11	0.17	0.02	0.09	MS, RI, AT
δ-Elemene	1344	1344	0.05	0.05	0.05	0.04	2.11	1.61	2.46	2.23	MS, RI
Eugenol	1356	1357	0.04	0.11	0.09	tr	1.97	1.53	0.89	1.01	MS, RI, AT
α-Copaene	1379	1385	0.34	0.21	0.29	0.31	0.26	0.24	0.28	0.32	MS, RI
Isolongifolene	1389	1388	0.02	tr	tr	tr	0.09	0.15	0.22	0.25	MS, RI
β-Bourbonene	1390	1393	0.10	0.10	0.10	0.09	0.55	1.01	1.73	2.05	MS, RI
β-Elemene	1398	1397	0.24	0.23	0.24	0.23	0.59	0.52	0.71	1.11	MS, RI

Table 1 Continued

Compound name	RI		% of total oil[a] (leaf)				% of total oil[a] (stem)				Method of identification
	Rep.	Calc.	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	
β-Caryophyllene	1428	1428	2.56	2.51	2.68	3.10	5.43	5.52	5.44	6.89	MS, RI, AT
β-Gurjunene	1433	1435	0.04	0.02	0.02	0.03	0.59	0.71	0.73	1.37	MS, RI
α –Amorphene	1453	1450	0.11	0.07	0.09	0.06	Tr	0.07	tr	tr	MS, RI
α-Humulene	1459	1460	1.50	1.72	1.67	1.32	3.89	3.91	3.69	4.89	MS, RI, AT
allo-Aromadendrene	1466	1466	0.66	0.75	0.64	0.67	1.97	2.24	1.88	2.51	MS, RI
γ-Muurolene	1478	1479	0.17	0.12	0.11	0.17	0.91	0.93	0.98	1.84	MS, RI
Germacrene D	1484	1487	4.80	4.91	4.86	4.58	9.89	10.91	6.69	8.73	MS, RI, AT
Epizonarene	1499	1498	0.68	0.72	0.68	0.72	–	–	–	–	MS, RI
α-Muurolene	1500	1498	–	–	–	–	0.39	0.28	0.35	0.35	MS, RI
γ-Cadinene	1513	1511	2.11	2.22	2.32	2.99	3.02	3.13	3.09	2.98	MS, RI
δ-Cadinene	1530	1533	2.42	2.37	2.45	2.42	3.99	4.17	3.88	4.17	MS, RI
α-Cadinene	1534	1542	0.08	0.09	0.11	0.07	Tr	tr	tr	0.04	MS, RI
α-Calacorene	1544	1553	tr	tr	tr	tr	Tr	tr	tr	0.06	MS, RI
β-Caryophyllene oxide	1564	1564	0.12	0.40	0.36	tr	0.38	0.41	0.35	0.47	MS, RI, AT
Spathulenol	1577	1580	0.05	0.05	0.06	tr	Tr	tr	tr	tr	MS, RI
4-Hydroxy germacrene D	1577	1587	tr	tr	tr	tr	0.45	0.42	0.53	0.34	MS, RI
Isoaromadendrene oxide	1582	1593	0.35	0.38	0.40	0.39	0.20	0.17	tr	0.37	MS, RI
Veridiflorol	1601	1603	0.16	0.25	0.21	0.21	tr	tr	tr	tr	MS, RI
Cubenol	1638	1638	1.65	1.69	1.64	1.55	3.18	3.22	3.11	3.01	MS, RI
δ-Cadinol	1652	1651	1.24	1.45	1.44	2.02	4.88	4.91	4.22	4.32	MS, RI
α-Cadinol	1665	1664	1.38	1.39	1.47	2.24	5.28	5.34	5.01	4.78	MS, RI
Monoterpene hydrocarbons			4.78	5.16	5.16	6.41	13.34	13.47	12.34	12.11	
Sesquiterpene hydrocarbons			4.78	5.16	5.16	6.41	13.34	13.47	12.34	12.11	
Aliphatic hydrocarbons			9.56	10.32	10.32	12.82	26.68	26.94	24.68	24.22	
Total hydrocarbons			19.12	20.64	20.64	25.64	74.01	53.88	49.36	48.44	
Oxygenated monoterpenes			38.24	41.28	4.85	51.28	8.79	107.76	98.72	8.00	
Oxygenated sesquiterpenes			76.48	82.56	46.13	102.56	136.16	215.52	197.44	104.88	
Other oxygenated components			152.96	165.12	92.2692.26	205.12	272.320	431.04	394.880	209.760	
Total Oxygenated components			305.92	330.24	184.7892	410.24	544.64	862.08	789.768	419.52	
Total identified compounds			95.46	93.49	94.45	97.57	99.77	97.60	98.35	97.14	
Color			Yellow	Yellow	Yellow	Yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	
Odor			Aromatic	Aromatic	Aromatic	Aromatic	Slightly aromatic	Slightly aromatic	Slightly aromatic	Slightly aromatic	
Specific gravity (25 °C)			0.8831	0.8873	0.8843	0.8902	0.9061	0.9072	0.9047	0.9025	
Yield (%v/w weight fresh plant)			0.55	0.52	0.31	0.27	0.49	0.40	0.25	0.24	

[a]The composition (in %) is an average of three analyses. MS, identification based on mass spectral data; RI, identification based on comparison of published Kovats retention indices; AT, identification based on co chromatography with authentic samples.

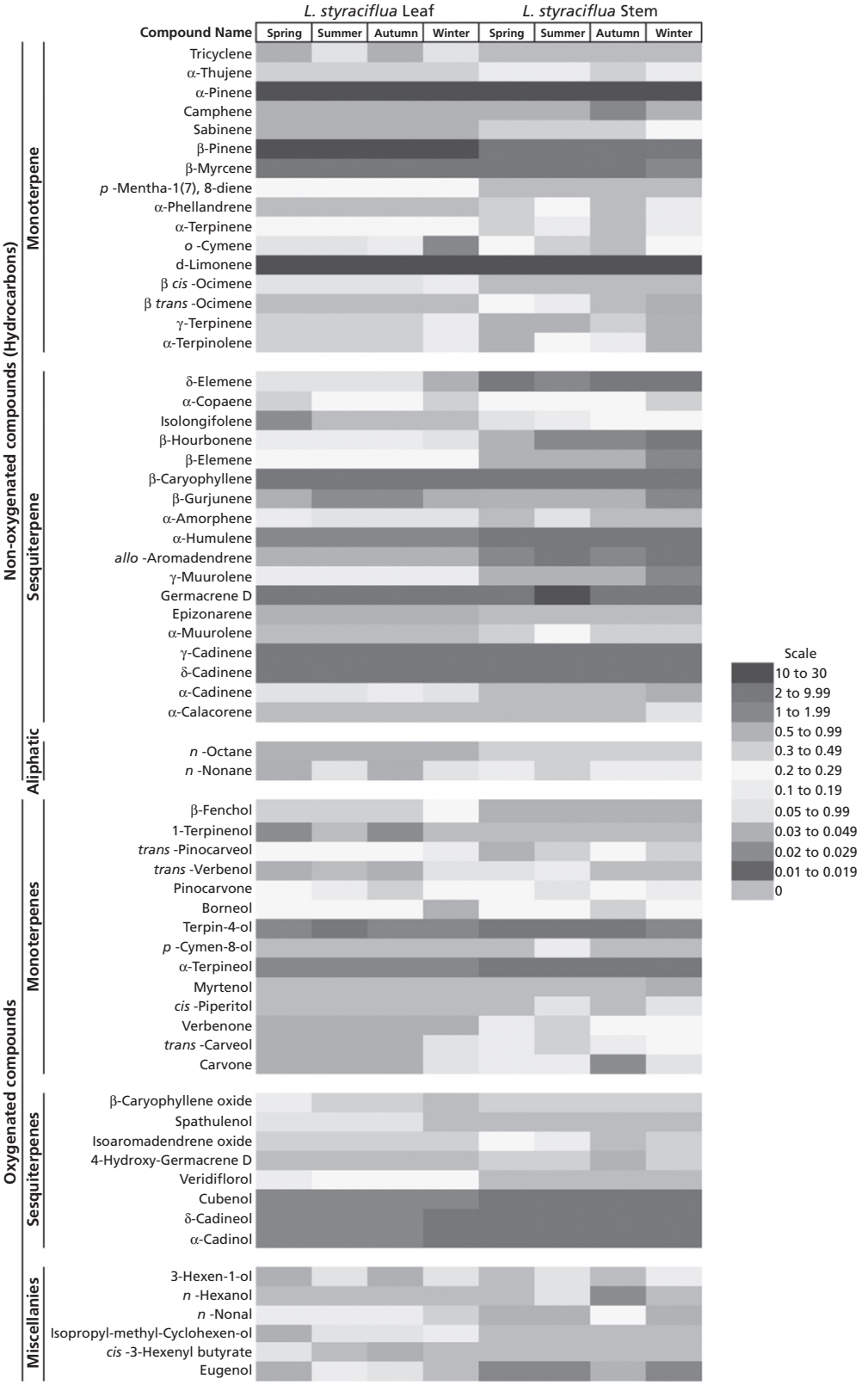

Figure 1 Clustering assignment of the chemical components of the essential oils of *L. styraciflua* (stems and leaves) depending on their quantity; a gradual scaling from dark red (high %) to dark green (low %) was used.

the spring. However, this percentage decreases by changing the season leading to the addition of more building blocks and formation of other sesquiterpenes or oxygenated hydrocarbons. This variation can be explained in relation with the biosynthetic point of view.[29]

As compared with Australian leaf oil,[14] Egyptian plants show a higher yield (0.1% w/w vs ~0.24 – 0.48% w/w). Whereas Australian leaf oil is composed mainly of terpinen-4-ol (30%) and sabinene (13%) the Egyptian oil was richer in hydrocarbons, mainly mono- and sesquiterpenes, but showed a lower amount of oxygenated compounds. These observed differences may be attributed to genetic variability or different environmental conditions.

Moreover, the composition of the widely known *Liquidambar oriantalis* showed that the most prominent components of its aerial part essential oil are terpinen-4-ol (≈34%), γ-terpinene (≈14%) and α-terinene (≈8%), which indicate the monoterpenes hydrocarbons are the main components of the genus *Liquidambar*.[18]

Biological activity

Cytotoxicity

The safety of the phytopharmaceuticals for humans is critical especially when they are applied orally. As a pilot study, the cell growth inhibition of HeLa, MCF-7 and HepG-2 cell lines by leaf and stem oils is represented in Figure 2. The representative oils did not exhibit any substantial cytotoxicity as compared with the cytotoxic positive control doxorubicin (Figure 2). However, the stem oil was slightly more cytotoxic ($P < 0.001$) than the leaf oil. In HeLa cells IC50 values were 119.78 and 136.27 μg/ml, respectively. However, MCF-7 cells were more resistant to essential oils than the other cell lines.

Generally, the cytotoxicity of the essential oils is mediated by the lipophilic terpenoids which probably influence the integrity of the biomembranes and membrane proteins. These components can dissolve in the bilayer of biomembranes thereby disturbing membrane permeability

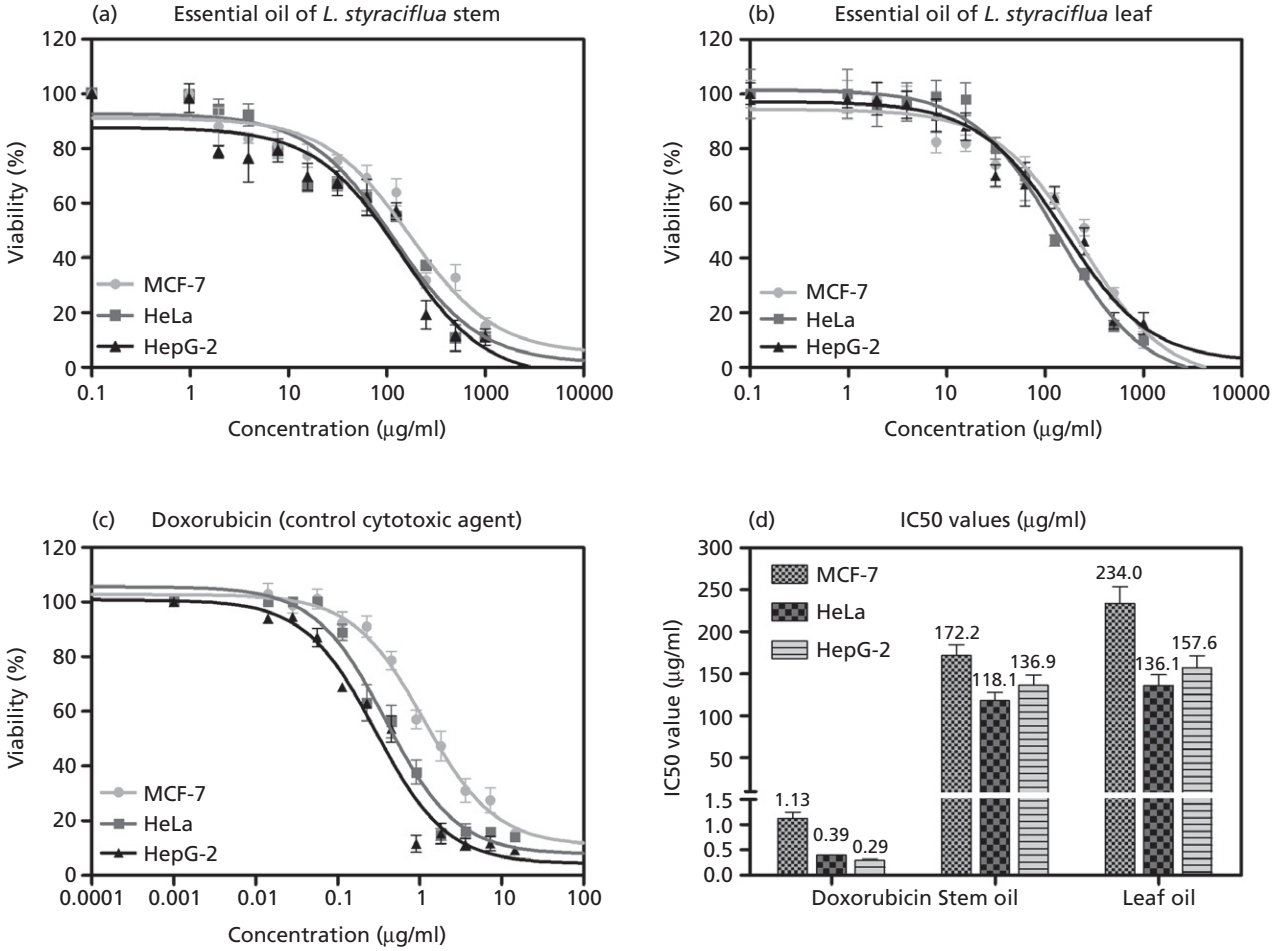

Figure 2 Dose dependent cytotoxicity and IC50 values for essential oils from *L. styraciflua* stems and leaves in MCF-7, HeLa, and HepG-2 cell lines. Doxorubicin used as a cytotoxic reference drug. The data shown are means ± SD obtained from three independent experiments.

and the close interaction between membrane lipids and proteins. As a consequence, the conformation of membrane proteins, such as ion channels and transporters is changed, which usually leads to a loss of function.[30] The cytotoxicity seen in our experiments might be attributed to some specific components of the oils. Some of these, such as the more reactive β-caryophyllene, β-caryophyllene oxide, and α-humulene, showed a potent cytotoxic activity against different cell lines, including HeLa cells (IC50 values 3.86 and 2.70 μg/ml).[31,32] Also D-limonene, which exhibited a potent *in vivo* antitumor activity against rodent mammary, liver, lung, stomach, and skin cancers, was present in our samples and could contribute to their cytotoxicity.[33]

Antioxidant and anti-inflammatory activity

Oxidative stress caused by reactive oxygen species (ROS) plays a crucial role for a variety of human health disorders such as cardiovascular disease, cancer, neurodegenerative diseases (stroke, Parkinson's and Alzheimer's disease) and ageing.[34,35]

The inflammatory response of the body involves the liberation of inflammatory mediators like histamine, prostaglandins and leukotrienes from mast cells, formation of cytokines (mainly TNF-α, IL-6) by macrophages followed by the liberation ROS including superoxide (O_2•) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and hydroxyl ($OH^•$) radicals. The free radicals derived from nitrogen are nitric oxide ($NO^•$) and peroxynitrite anion ($ONOO^•$).[34,36] Thus, a reduction of the production of inflammatory mediators by inhibiting the activities of 5-lipoxygenase or cyclooxygenase in addition to a direct scavenging of ROS by antioxidants are considered as promising therapeutic targets.

In a first set of experiments, the antioxidant activity of the essential oils was evaluated using a range of *in vitro*

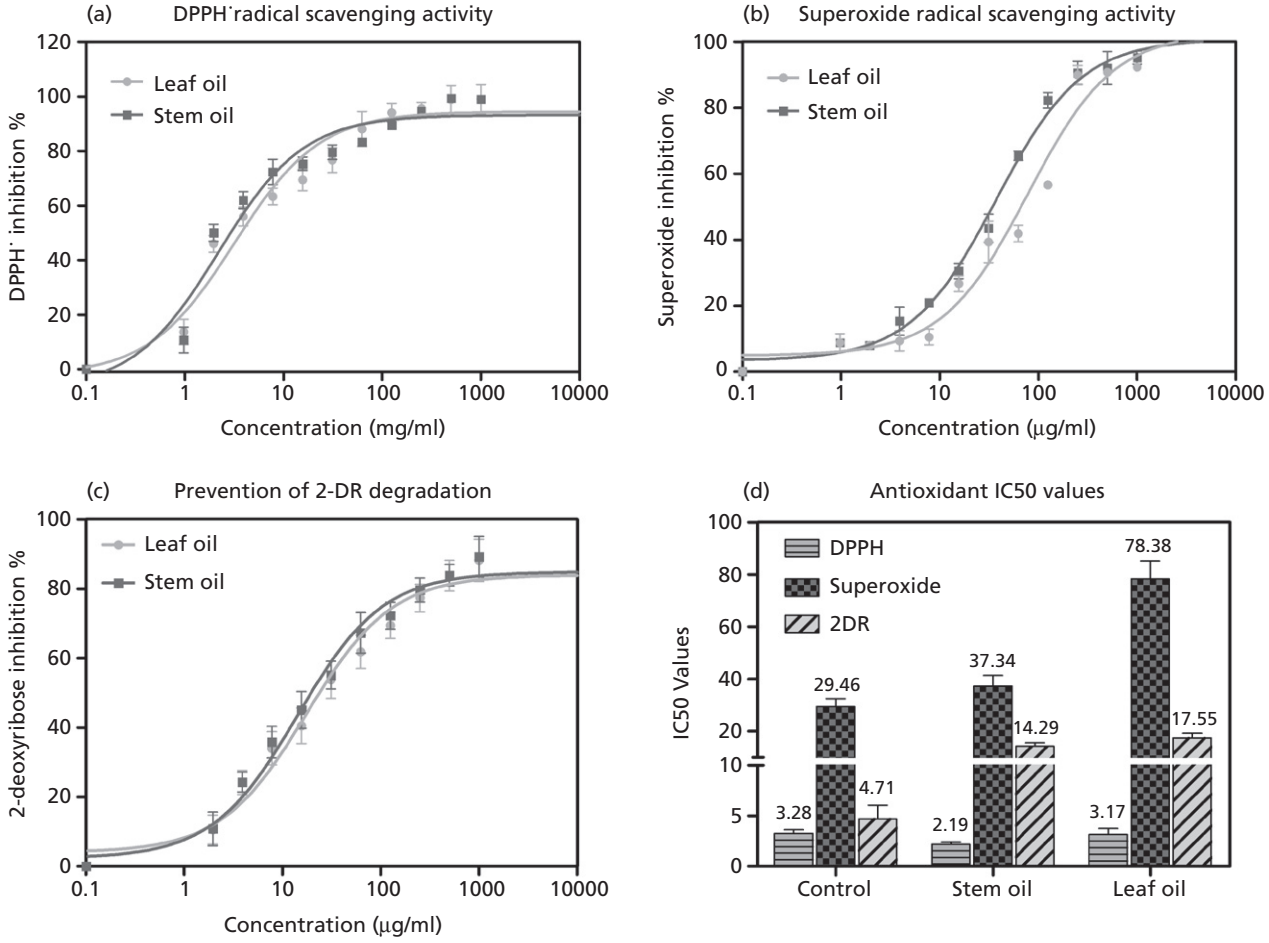

Figure 3 The inhibitory effects of the essential oils from *L. styraciflua* stems and leaves in on DPPH (a), superoxide radical (b), and 2-deoxyribose (c). The data represented as IC50 values (μg/ml except in DPPH assay with mg/ml) and the ascorbic acid (μg/ml) was used as a positive control for DPPH and superoxide and quercetin for 2DR (d). Data are expressed as the mean ± SD of three individual experiments.

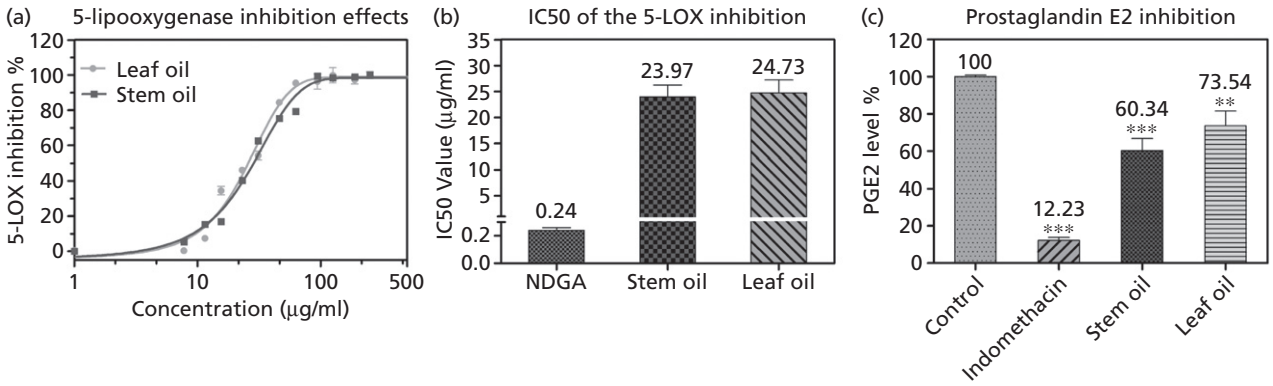

Figure 4 The effects of the essential oils from *L. styraciflua* (stem and leaf) on the formation of inflammatory mediators. Dose-dependence of 5-LOX inhibition (a), and IC50 values (µg/ml) of both oils in comparison to NDGA as a positive control (b). The inhibitory effect of both oils on PGE2 production in LPS stimulated HepG-2 cells compared with indomethacin effect (c). Data are expressed as the mean ± SD of three individual experiments.

experiments such as the DPPH, 2-deoxyribose, and superoxide dismutase assays (Figure 3A–3C). Compared with the antioxidant activity of essential oils from other sources,[25,37–39] both spring-collected essential oils exhibited some antioxidant activity. In the DPPH scavenging activity assay, the stem oil (IC50 2.19 mg/ml) was slightly more active than the leaf oil (IC50 3.17 mg/ml) but very low as compared with vitamin C (as a positive control). However, the effects on ($OH^{\cdot}$) radicals and ($O_2^{\cdot}$) anion were more pronounced. The IC50 values for the 2-deoxyribose test were 17.55 and 14.29 µg/ml, respectively, and 37.34 and 78.38 µg/ml for the superoxide dismutase test (Figure 3). The presence of ~25% oxygenated metabolites in the stem oil might be responsible for the observed antioxidant activity. However, absence of the phenolic constituents in both oils, which normally are essential for a potent antioxidant activity in the $DPPH^{\cdot}$ assay, can explain the inactivity of the oils in the DPPH assay.

In a second set of pilot experiments, the anti-inflammatory activity was assessed by measuring the inhibition of 5-Lipoxygenase (5-LOX) activity and PGE2 production in LPS stimulated hepatic cells. Figure 4 shows the dose-dependent inhibitory effects (0.1–250 µg/ml) of the oils on 5-LOX activity. There was no significant difference between stem and leaf oils; both oils exerted similar activity with IC50 values around 24 µg/ml. Figure 4B shows that stimulation of HepG-2 cells with LPS (1 µg/ml) for 24 h induced PGE2 production (set to 100%). The cells treated with oils showed a significant inhibition of PGE2 release ($P < 0.01$). A dose of 25 µg/ml of stem and leaf oil inhibited the PGE2 production by 39.66 and 26.46%, respectively. The presence of β-caryophyllene oxide, even in small concentrations, may be responsible for the inhibition of 5-LOX,[40] while β-caryophyllene and α-humulene are known inhibitors of cyclooxygenase-2.[41]

Conclusions

We assume that the essential oils of *L. styraciflua* are able to exhibit an interesting anti-inflammatory activity in addition to their known antiseptic properties. The *in vitro* data would support the traditional use of storax to treatment inflammation and infections. Because of their low cytotoxicity, storax and the essential oils remain useful phytotherapeutics as already evidenced by the experiences of traditional medicine.

Declarations

Conflict of interest

The authors declare that they have no competing interests.

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