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RESEARCH ARTICLE

Exploring new applications of tulip tree (*Liriodendron tulipifera* L.): leaf essential oil as apoptotic agent for human glioblastoma

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Abstract

Liriodendron tulipifera L. (Magnoliaceae), also known as "tulip tree," is a hardwood plant native to North America, cultivated all over the world and used on an industrial level, especially for its fine wood and to make honey. It has also been traditionally exploited for its antimalarial properties. However, our knowledge about the bioactivity of its essential oil remains patchy. In this research, we focused on the biological evaluation of the volatile fractions obtained from different parts of the plant which are normally discharged by industry, including leaves, flowers, and fruits. For the purpose, the essential oils were obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC-MS). Then, they were evaluated as radical scavenging, antioxidant, antimicrobial, and antiproliferative agents by using DPPH, ABTS, FRAP, disk diffusion, and MTT methods, respectively. The significant toxicity exhibited on human tumor cells, namely A375 malignant melanoma, HCT116 colon carcinoma, MDA-MB 231 breast adenocarcinoma, and T98G glioblastoma multiforme cell lines, prompted us to study the mechanism of action by acridine orange/ethidium bromide double staining and caspase 3 assays. Our findings shed light on the potential applications of tulip tree derivatives as anticancer drugs.

Keywords Antimicrobial activity · Antioxidant potential · Essential oil composition · Sesquiterpenes · Cytotoxicity · Caspase-3 · Apoptosis

Introduction

Plants represent a huge reservoir of compounds with various biological activities, showing a great potential for a wide number of real-world applications, including extremely diverse research fields, such as antimicrobial drug design, biopesticide development, and functional food optimization, just to cite three major examples of timely interest worldwide (Banumathi et al. 2017; Benelli and Pavela 2018a, b; Benelli 2018; Pavela et al. 2019). Among them, essential oils are widely studied for their high-efficacy and eco-friendly features, which have been successfully exploited for a number of practical uses (Dudai et al. 1999; Isman 2006; Miresmailli and Isman 2014; Pavela and Benelli 2016; Chellappandian et al. 2018; Ngahang Kamte et al. 2018). Essential oils are liquid mixtures of volatile, low-molecular weight, and hydrophobic compounds obtained from botanical raw materials through the classical processes of steam distillation, hydrodistillation, and cold pressing, and composed of monoterpenoids, sesquiterpenoids, aromatics, and aliphatic compounds (Benelli et al. 2018a). They can also be obtained from byproducts after crop manufacturing (Benelli et al. 2018b; Fiorini et al. 2019).

The tulip tree (*Liriodendron tulipifera* L.) is a hardwood plant, 25–30 m tall and 1 m in diameter, belonging to the Magnoliaceae family and native to North America (Keeler 1902). The hallmark of this basal angiosperm is its showy, fragrant, tulip-like flowers appearing in June–July (Fig. 1). They are composed of yellow-greenish petals and a receptacle with a central yellow spot containing nectars (Pignatti 1982). The leaves are large, glabrous, deciduous, palmate, and becoming yellow in Fall. The pine cone–like fruit is composed

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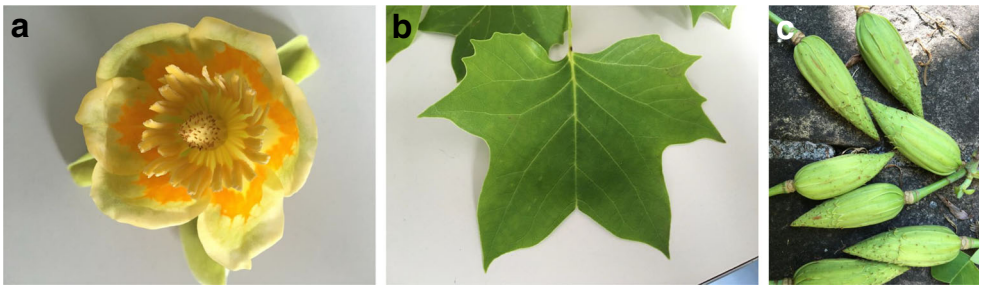

Fig. 1 Tulip-like flower (**a**), palmate leaf (**b**), and cone-like fruits (**c**) of *Liriodendron tulipifera*

of numerous winged seeds (samaras) falling in Autumn or next Spring after drying (Pignatti 1982).

Tulip tree is cultivated in Europe since the Middle Ages. It is rather appreciated for its fine wood which is resistant to parasites and is used in nautical and paper industries, to make pianos and in carpentry (Byrne and Nagle 1997). This tree is however mostly used as an ornamental plant in landscape architecture (Pickett et al. 1996). One old specimen of *L. tulipifera* cultivated in Lecco (North Italy), with 52 m of height, is considered as the tallest tree currently grown in Italy. Owing to the large production of nectars, tulip tree is also used to make honey (Zhou et al. 2016).

The tulip tree bark has been traditionally used by First Nation people of North America as tonic and febrifuge and to treat malaria (Kang et al. 2014). Interestingly, tulip tree was used as a substitute of *Cinchona* bark as a source of antimalarial drugs by American settlers and, later, by the US army during the World War II (Thacher 1967; Spencer et al. 1947). Previous phytochemical studies conducted on different parts of tulip tree (e.g., bark and leaves) put in evidence the presence of three main groups of secondary metabolites, namely aporphine alkaloids, germacranolide sesquiterpene lactones, and lignans (Li et al. 2013; Chiu et al. 2012; Graziose et al. 2011; Kang et al. 2014). These constituents have shown interesting biological properties, such as antioxidant, antimicrobial, cytotoxic, and antiplasmodial (Li et al. 2013a, b; Chiu et al. 2012; Hsu and Chen 1991; Graziose et al. 2011; Kang et al. 2014).

Regarding the volatile fraction of tulip tree, only a few studies have been produced so far. Miller et al. (2009) studied the leaf essential oil chemical variability at variance with the phenological stage. They found sesquiterpene hydrocarbons as the most abundant volatile components in spring and monoterpene hydrocarbons in summer and autumn. This oil proved to be effective against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and inhibited the growth of Hs 578T human tumor cells (Miller et al. 2009). On the other hand, flowers and fruits were poorly investigated, probably for difficulty in their collection.

Continuing our research line focusing on disclosing new biological properties of unexplored medicinal and aromatic plants (Petrelli et al. 2017; Nabavi et al. 2018; Iannarelli et al. 2018; Quassinti et al. 2014; Woguem et al. 2014), in the present study, after analyzing the chemical composition of the essential oils obtained from leaves, flowers, and fruits of the tulip tree grown in Italy, we assayed them as radical scavenging, antioxidant, antimicrobial and antiproliferative agents by using DPPH, ABTS, FRAP, disk diffusion, and MTT methods, respectively. The mechanism of action on tumor cells was further elucidated by acridine orange/ethidium bromide double staining and caspase 3 assays. Our findings shed light on the potential applications of tulip tree derivatives as anticancer drugs.

Materials and methods

Plant material

Leaves, flowers, and immature pine cone-like fruits of *L. tulipifera* were collected in May–June 2016 from an old specimen, about 160–170 years old, cultivated in the Botanical garden "C. Cortini" of the University of Camerino, Camerino, Italy (N 43°08′ 05.31″, 13° 04′ 08.81″, 648 m a.s.l.). For leaves, collection was performed during flowering (May) and fruiting (June) times in order to see possible variation according to the phenological cycle. The specimen authentication was performed by one of us (F. Maggi) using comparison with literature (Pignatti 1982). After identification, a voucher specimen was archived in the Herbarium Universitatis Camerinensis, School of Biosciences and Veterinary Medicine, University of Camerino, under the codex CAME 27744 and recorded in the botanical database anArchive (http://www.anarchive.it).

Hydrodistillation

Fresh leaves (1179 and 2000 g harvested in May and June, respectively), flowers (550 g), and fruits (1700 g), immediately after their collection, were hydrodistilled in a 10-L flask using a Clevenger-type device for 4 h. At the end, the oil was decanted, separated from the aqueous layer, and collected in dark vials equipped with PTFE silicon caps which were stored at – 20 °C before biological evaluation. The oils were of different color, i.e., brownish those from leaves, and pale yellow those from flowers and fruits. Their yield values (0.1, 0.3, 0.05, and 0.02%, respectively) were determined on a dry weight basis (w/w).

Gas chromatography-mass spectrometry analysis

Tulip tree essential oils were diluted 1:100 in *n*-hexane (Carlo Erba, Milan, Italy) and injected (2 μL) into a gas chromatograph Agilent 6890D equipped with a 5973 N single quadrupole mass spectrometer detector operating in electron impact (EI) mode (full scan, acquisition mass range: 29–400 m/z) with an ionization voltage of 70 eV. For separation of volatiles, an apolar capillary column HP-5MS (30-m length, 0.25 mm i.d., 0.1 μm ft., Folsom, CA) was thermostatted in an oven using the following ramp: 60 °C for 5 min, increase to 220 °C at 4 °C/min, then to 280 °C at 11 °C/min held for 15 min. A C8–C30 *n*-alkanes mixture was bought from Supelco (Bellefonte, CA) and used to calculate the temperature-programmed retention index (RI) of each peak which was compared with those contained in NIST17, ADAMS, and FFNSC2 libraries (Adams 2007; FFNSC2 2012; NIST17 2017). In addition, the computer matching of mass spectrum (MS) with those reported by WILEY275, ADAMS, FFNSC2, and NIST17 libraries was used for peak identification (Zorzetto et al. 2015). Finally, for 31 compounds (see Table 1), the identification was supported by comparison with analytical standards (Sigma-Aldrich, Milan, Italy). The MSD ChemStation software (Agilent, Version G1701DA D.01.00) was used to analyze chromatograms. For each sample, analyses were repeated three times and the mean peak area percentages were reported; they were obtained by peak area integration without using response factors.

Antioxidant assays

Stock solutions of essential oils or pure components (5 mg/mL) in ethanol were used for all assays. The radical scavenging activity was evaluated by the DPPH, ABTS, and FRAP assays as previously described (Casiglia et al. 2017). Microplate assays were used to evaluate the antioxidant activity and ferric reducing antioxidant power as previously reported (Casiglia et al. 2017), and in the all assays, the activity of tulip tree essential oils was compared with that of the positive control Trolox. Values were expressed as tocopherol-equivalent antioxidant capacity (mmol TE/g of product). Each experiment was repeated at least three times.

Antimicrobial activity

The different essential oils from *L. tulipifera* were tested against four bacterial strains and one yeast, namely *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 24433, respectively. Blood agar plates were used to culture bacterial strains overnight at 37 °C, while Sabouraud Dextrose Agar was used for *C. albicans*. Tests were conducted using the disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2011). Discs (6 mm in diameter) were spotted either with 10 μL of essential oil or pure reference compound in control discs. A calliper was used to measure the diameters (mm) of zone inhibition (including the 6-mm disc). Reference antimicrobials ciprofloxacin (for bacteria) and nystatin (for yeast) were used as quality positive controls. All tests were done at least in duplicate.

Cell culture and sample treatment

HCT116 human colon carcinoma, MDA-MB 231 human breast adenocarcinoma, and T98G human glioblastoma multiforme cell lines were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA); A375, human melanoma, was provided by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia; Italy). HCT116 cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640; Corning, Manassas, VA, USA) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin (Corning, Manassas, VA, USA), 2 mM L-glutamine (Corning, Manassas, VA, USA), and 10% of heat inactivated bovine fetal serum (HI-FBS) (Corning, Manassas, VA, USA). MDA-MB 231 and A375 cell lines have maintained in medium Dulbecco (DMEM; Corning, Manassas, VA, USA) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% of HI-FBS. T98G cell line was maintained in Minimum Essential Medium (MEM; Corning, Manassas, VA, USA) with 2 mM of L-glutamine, 0.1 mM of non-essential amino acids (PAA Laboratories; GE Healthcare Life Sciences, Chalfont, UK), 1 mM of sodium pyruvate (PAA Laboratories; GE Healthcare Life Sciences, Chalfont, UK), 100 IU/mL of penicillin G, 100 μg/mL streptomycin, and 10% of HI-FBS. All cell lines were kept in an incubator at 37 °C, in a humidified atmosphere with 5% CO_2. Cells were maintained in culture by detachment with tripsin/EDTA and diluted in fresh medium before reaching the cell confluence state (approximately 80% confluence). Exponentially, growing cells were plated at 2×10^4 cells/mL into 96-well microtiter tissue culture plates (Corning Incorporated, NY, USA) and incubated for 24 h before the addition of the essential oils or pure compounds. Essential oils or pure compounds were diluted in ethanol (the concentration of ethanol in the final culture medium was < 1 % which had no effect on the cell viability).

Cytotoxicity assay

The reduction of tetrazolium salt, 3- (4,5-dimetialtiazole-2yl) -2,5-diphenyltetrazole bromide (MTT; Sigma-Aldrich,) to formazan by mitochondrial succinate dehydrogenase, was used to evaluate the viability of the tumor cells as previously

Table 1 Chemical composition of leaf, flower, and fruit essential oils from *Liriodendron tulipifera*

No	Componenta	RI calcb	RI litc	Plant part (%)d				IDe
				Leaves (May)	Leaves (June)	Flowers	Fruits	
1	α-pinene	926	932		0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	Std,RI,MS
2	camphene	939	946	Tr[f]	0.3 ± 0.1	0.5 ± 0.1	0.9 ± 0.2	Std,RI,MS
3	sabinene	966	969			Tr		Std,RI,MS
4	β-pinene	969	974	Tr	Tr	0.1 ± 0.0	0.1 ± 0.0	Std,RI,MS
5	myrcene	989	988	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	1.3 ± 0.3	Std,RI,MS
6	α-phellandrene	1003	1002		Tr	Tr	Tr	Std,RI,MS
7	limonene	1025	1024	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	Std,RI,MS
8	1,8-cineole	1028	1026	Tr	Tr	Tr	0.1 ± 0.0	Std,RI,MS
9	(*Z*)-β-ocimene	1037	1032	8.5 ± 1.7	12.5 ± 2.1	14.2 ± 2.9	25.2 ± 3.5	Std,RI,MS
10	(*E*)-β-ocimene	1047	1044	6.8 ± 1.3	3.7 ± 0.9	6.5 ± 1.2	12.2 ± 2.0	Std,RI,MS
11	γ-terpinene	1055	1054	Tr	Tr	Tr	0.1 ± 0.0	Std,RI,MS
12	terpinolene	1085	1086	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.0	Std,RI,MS
13	linalool	1100	1095	0.3 ± 0.1	0.1 ± 0.0	Tr	1.2 ± 0.3	Std,RI,MS
14	*n*-nonanal	1105	1100	0.2 ± 0.0	Tr	Tr		RI,MS
15	*allo*-ocimene	1128	1128	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	Std,RI,MS
16	(*Z*)-myroxide	1134	1131	Tr			Tr	RI,MS
17	camphor	1139	1141	Tr	Tr	Tr	0.4 ± 0.1	Std,RI,MS
18	camphene hydrate	1142	1145	Tr			0.3 ± 0.0	RI,MS
19	borneol	1159	1165	0.7 ± 0.1	Tr		4.6 ± 0.9	Std,RI,MS
20	terpinen-4-ol	1173	1174	Tr			0.1 ± 0.0	Std,RI,MS
21	α-terpineol	1187	1186	0.3 ± 0.1	Tr		0.2 ± 0.0	Std,RI,MS
22	methyl chavicol	1196	1195	Tr	0.1 ± 0.0	Tr		RI,MS
23	geraniol	1256	1249	0.1 ± 0.0				Std,RI,MS
24	(2*E*)-decen-1-ol	1270	1268				0.3 ± 0.0	RI,MS
25	bornyl acetate	1281	1287	1.7 ± 0.4	1.4 ± 0.3	1.7 ± 0.4	7.8 ± 1.5	Std,RI,MS
26	δ-elemene	1332	1335	0.3 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.1 ± 0.0	RI,MS
27	α-cubebene	1344	1345	Tr	0.1 ± 0.0	Tr	Tr	RI,MS
28	cyclosativene	1358	1369	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	Tr	RI,MS
29	α-ylangene	1364	1373	Tr	0.1 ± 0.0	0.1 ± 0.0		RI,MS
30	α-copaene	1368	1374	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	Tr	Std,RI,MS
31	β-bourbonene	1376	1387		Tr	Tr		RI,MS
32	β-cubebene	1384	1387	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1		RI,MS
33	β-elemene	1387	1389	17.1 ± 3.4	16.4 ± 3.2	16.7 ± 3.1	7.9 ± 1.8	Std,RI,MS
34	methyl eugenol	1405	1403	0.5 ± 0.1	0.1 ± 0.0	Tr	0.4 ± 0.1	RI,MS
35	(*E*)-caryophyllene	1409	1417	0.8 ± 0.2	1.7 ± 0.4	1.6 ± 0.3	0.3 ± 0.1	Std,RI,MS
36	β-copaene	1420	1430	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	RI,MS
37	γ-elemene	1428	1434	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0		RI,MS
38	α-guaiene	1432	1437	0.1 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	Tr	RI,MS
39	α-humulene	1443	1452	0.4 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.2 ± 0.0	Std,RI,MS
40	(*E*)-β-farnesene	1457	1454	0.3 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	0.2 ± 0.0	Std,RI,MS
41	β-chamigrene	1468	1476	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	RI,MS
42	germacrene D	1474	1484	18.9 ± 3.7	22.3 ± 4.1	27.2 ± 4.9	6.6 ± 1.3	RI,MS
43	β-selinene	1477	1489	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	RI,MS
44	*trans*-muurola-4(14),5-diene	1482	1493	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	RI,MS
45	δ-selinene	1485	1492	0.2 ± 0.0				RI,MS
46	*epi*-cubebol	1487	1493				0.8 ± 0.2	RI,MS
47	byciclogermacrene	1488	1500	1.4 ± 0.3	4.1 ± 0.9	3.7 ± 0.7		RI,MS

Table 1 (continued)

No	Componenta	RI calcb	RI litc	Plant part (%)d				IDe
				Leaves (May)	Leaves (June)	Flowers	Fruits	
48	α-muurolene	1495	1500	1.2 ± 0.3				RI,MS
49	*unknown sesquiterpene*	1495	~~1501~~	5.5 ± 1.1	5.1 ± 0.9	4.8 ± 1.0	5.9 ± 1.2	RI,MS
50	α-bulnesene	1498	1509		0.2 ± 0.0	0.1 ± 0.0		RI,MS
51	*n*-pentadecane	1500	1500	0.1 ± 0.0				Std,RI,MS
52	δ-amorphene	1500	1511	0.1 ± 0.0	0.1 ± 0.0		Tr	RI,MS
53	(*E*,*E*)-α-farnesene	1507	1505		0.9 ± 0.2		0.3 ± 0.1	RI,MS
54	γ-cadinene	1507	1513	0.7 ± 0.2		0.4 ± 0.1		RI,MS
55	δ-cadinene	1517	1522	2.2 ± 0.5		1.3 ± 0.3	1.5 ± 0.3	RI,MS
56	*trans*-cadina-1,4-diene	1525	1533	Tr	Tr	Tr	Tr	RI,MS
57	α-cadinene	1530	1537	Tr	Tr	Tr		RI,MS
58	elemol	1541	1548	0.1 ± 0.0	0.1 ± 0.0		Tr	RI,MS
59	germacrene B	1546	1559	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	RI,MS
60	elemicin	1559	1555	Tr				RI,MS
61	(*E*)-nerolidol	1563	1561	3.2 ± 0.6	15.7 ± 3.2	10.5 ± 2.3	3.6 ± 0.8	RI,MS
62	germacrene D-4-ol	1566	1574				0.1 ± 0.0	RI,MS
63	viridiflorol	1581	1592		0.1 ± 0.0			Std,RI,MS
64	salvial-4(14)-en-1-one	1583	1594	Tr				RI,MS
65	1-epi-cubenol	1619	1627	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.2	RI,MS
66	*epi*-α-muurolol	1634	1640	1.1 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	1.5 ± 0.3	RI,MS
67	α-muurolol	1639	1644	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.9 ± 0.2	RI,MS
68	selin-11-en-4-alpha-ol	1644	1658	0.9 ± 0.2	0.5 ± 0.1		1.5 ± 0.3	RI,MS
69	α-cadinol	1646	1652	1.7 ± 0.4	0.8 ± 0.2	1.1 ± 0.3	1.7 ± 0.4	RI,MS
70	*unknown sesquiterpene*	1669		3.2 ± 0.7	Tr	Tr	0.3 ± 0.1	RI,MS
71	*unknown sesquiterpene*	1673		3.4 ± 0.6	Tr	Tr	0.3 ± 0.0	RI,MS
72	germacrone	1684	1693	Tr				Std,RI,MS
73	*n*-heptadecane	1700	1700	Tr				Std,RI,MS
74	(*2Z,6E*)-farnesol	1720	1722	0.2 ± 0.0				RI,MS
75	*unknown sesquiterpene*	1730		3.2 ± 0.6	Tr	0.1 ± 0.0	0.2 ± 0.0	RI,MS
76	*unknown sesquiterpene*	1758		2.5 ± 0.3	1.9 ± 0.4	1.8 ± 0.4	2.6 ± 0.5	RI,MS
77	(*2E,6E)*-farnesyl acetate	1842	1845	Tr	0.2 ± 0.0	0.1 ± 0.0	Tr	RI,MS
78	*n*-heneicosane	2100	2100	0.3 ± 0.1			0.1 ± 0.0	Std,RI,MS
79	*n*-tricosane	2300	2300	0.2 ± 0.0			0.2 ± 0.0	Std,RI,MS
	Total identified (%)			91.0	97.0	97.5	97.1	
	Oil yield (%, w/w)			0.1	0.3	0.05	0.2	
	Grouped compounds (%)							
	Monoterpene hydrocarbons			16.2	17.8	22.2	42.8	
	Oxygenated monoterpenes			2.3	1.6	1.7	15.1	
	Sesquiterpene hydrocarbons			50.5	57.1	59.5	24.0	
	Oxygenated sesquiterpenes			19.9	20.3	14.1	14.5	
	Others			1.2	0.2		0.6	

[a] Compounds are listed according to their elution from a HP-5MS (30 m × 0.25 mm, 0.1 μm) capillary column

[b] Retention index experimentally determined using a mixture of C8–C30 *n*-alkanes (Sigma-Aldrich)

[c] Retention index values taken from Adams (2007) and NIST17 (2017)

[d] Peak are percentages are means of three determinations ± SD

[e] Peak assignment: *Std* comparison with authentic standard; *MS* mass spectrum overlapping with those recorded in Wiley, Adams, and NIST 17 libraries; *RI* correspondence of the calculated index with those contained in Adams e NIST 17

[f] Tr, % < 0.1

described (Mosmann 1983). After 24 h from seeding the cells in culture plates, the essential oils and pure compounds were added to the medium in a concentration range of 0.78 to 200 μg/mL. Cisplatin was used as positive control in a concentration range of 0.05 to 50 μg/mL. After 72 h of incubation, 10 μL of the MTT (5 mg/mL in PBS solution) was put in each well and the plates were kept at 37 °C for 4 h in a humidified atmosphere with 5% CO_2. The intracellular reduction of tetrazolium salts by the enzyme succinate dehydrogenase causes the formation of blue formazan crystals at the bottom of the well. After incubation, the supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added per well. The plates were stirred for about 15 min in order to solubilize the crystals formed and allowed to read the absorbance at a wavelength of 540 nm with the Titertek Multiscan micro Elisa spectrophotometer (Labsystem Helsinki). Cell viability was calculated as a percentage ratio of the absorbance of the sample to the vehicle (ethanol). Each experiment was repeated thrice.

Acridine orange/ethidium bromide staining

Acridine orange/ethidium bromide (AO/EB) double staining assay was used to characterize apoptotic or necrotic morphological changes (Cotter and Martin 1996) of cells treated with different concentrations of essential oil. Two types of cells can be observed according to the fluorescence emission. Constant bright green nuclei with an organized structure are typical of viable cells, uniformly orange to red nuclei with a condensed structure characterize necrotic cells. In brief, T98G cells were plated in 24-wells plates at the concentration of 10^5 cell/mL. After 24 h for the seeding, cells were treated with 1.6, 3.2, and 6.4 μg/mL of essential oil from *L. tulipifera* leaves collected during flowering stage. After 2 and 24 h of incubation, cells were trypsinized, washed with PBS, and suspended at 10^6 cells/mL. A total of 25 μL of cell suspension were mixed with 1 μL of the dual fluorescent staining solution (100 μg/mL AO and 100 μg/mL EB in PBS) for 3 min. The cell suspension (10 μL) was placed on a glass slide and examined under an Olympus IX71 fluorescent inverted microscope. Images were recorded with an Olympus DP70 digital camera.

Determination of caspase-3 activity

Caspases-3 activation was determined by the colorimetric Caspase-3 Assay Kit (Sigma, St. Louis, MO, USA). A total of 10^6 cells were incubated for 2, 4, and 6 h with 6.4 μg/mL of essential oil from *L. tulipifera* leaves collected during flowering stage. The cells were washed with PBS and suspended in 100 μL of chilled cell lysis buffer for 20 min. After centrifugation, 15 μL of supernatant were collected and added into 96-well plates with 75 μL of final reaction buffer. Caspase-3 colorimetric substrate Ac-DEVDpNA (10 μL) was added to each well and plates were incubated at 37 °C. The colorimetric reaction was developed and measured at 405 nm with a Titertek Multiscan microElisa (Labsystems, Helsinki). Each sample was analyzed in triplicate.

Statistical analysis

Data represent the mean ± standard deviation (SD) of three independent determinations. One-way analysis of variance (ANOVA) was used to find significance of the differences between the mean values whether the p value was lower 0.05. IC_{50} values (the essential oil concentration able to reduce by 50% the viability in comparison to vehicle) were calculated by non-linear regression analysis using the software GraphPad Prism 5 (GraphPad Software, S. Diego, CA, USA).

Results and discussion

Essential oil compositions

The chemical profiles obtained from leaves, flowers, and fruits of tulip tree are reported in Fig. 1 Supporting Material, whereas the list of components identified are depicted in Table 1. A total of 70 volatile components were identified in the different parts of tulip tree, accounting for 91.0–97.5% of the total compositions. The oil samples from leaves and flowers were characterized by high amounts of sesquiterpene hydrocarbons (50.5–59.5%). Oxygenated sesquiterpenes (14.1–20.3%) and monoterpene hydrocarbons (16.2–22.2%) were the other main fractions of these oils. On the other hand, monoterpene hydrocarbons were the main fraction (42.8%) of the fruit oil, followed by sesquiterpene hydrocarbons (24.0%), oxygenated monoterpenes (15.1%), and oxygenated sesquiterpenes (14.5%).

In the leaf oils, the major compounds were germacrene D (18.9-22.3%), β-elemene (16.4–17.1%), (*E*)-nerolidol (3.2–15.7%), (*Z*)-β–ocimene (8.5–12.5%), and (*E*)-β–ocimene (3.7–6.8%). Other components occurring in percentages above 1% were bicyclogermacrene (1.4–4.1%), δ-cadinene (2.2%), bornyl acetate (1.4–1.7%), (*E*)-caryophyllene (0.8–1.7%), α-cadinol (0.8–1.7%), and *epi*-α-muurolol (0.7–1.1%). It is worth noting the presence of several sesquiterpenes of unknown structure (compounds no 49, 70, 71, 75, and 75 in Table 1), more concentrated in the leaf oil at flowering, with percentages ranging from 2.5 (compound no 76) to 5.5% (compound no 49). Their MS fragmentation patterns are reported in Fig. 2. Based on their MW (204, 216, 216, 218, and 220, respectively), we can assign compound no 49 to the class of sesquiterpene hydrocarbons and the remaining ones to that of oxygen-containing sesquiterpenes. The leaf oil obtained during flowering stage was characterized by a significant higher amount of these unknown sesquiterpenes (17.8 vs 7.1%) and lower content of (*Z*)-β-ocimene (8.5 vs

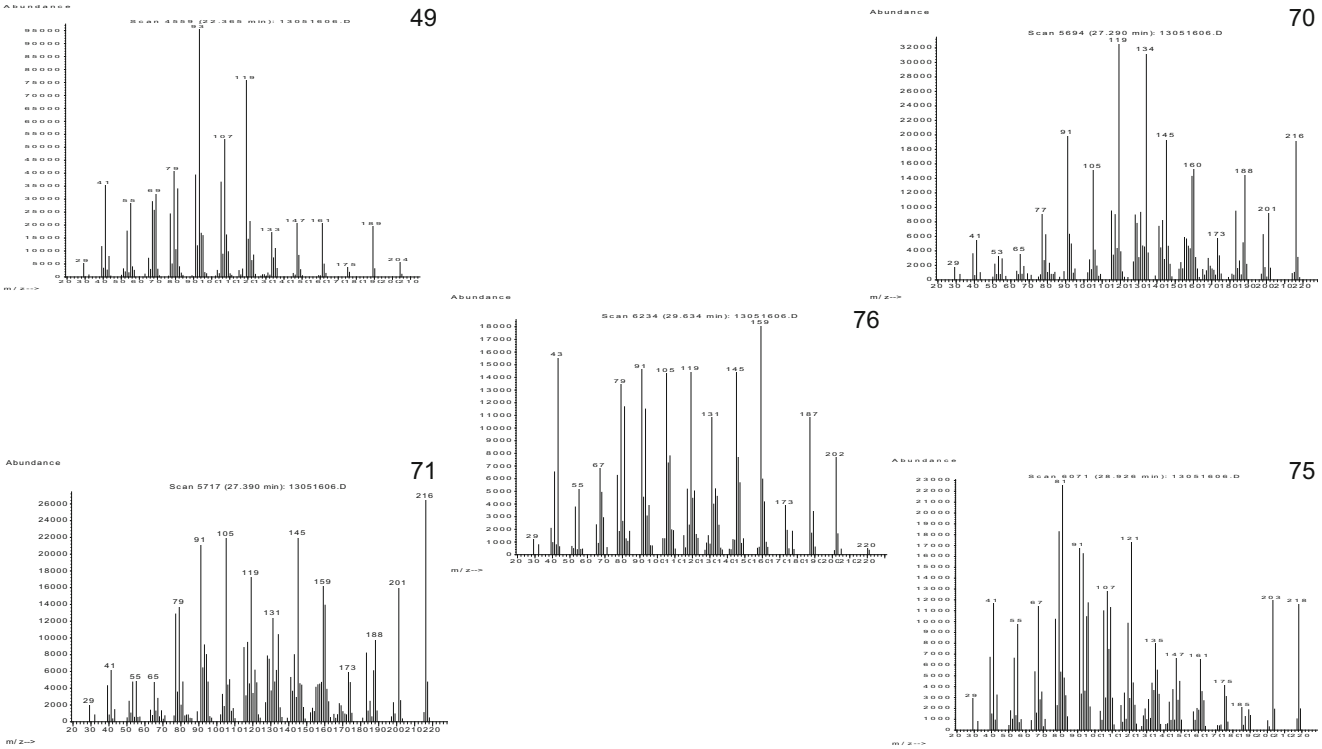

Fig. 2 MS fragmentation pattern of the unknown sesquiterpenes detected in *Liriodendron tulipifera* oil. Numbers refer to those reported in Table 1

12.5%) and (*E*)-nerolidol (3.2–15.7%) with respect to the oil obtained at fruiting stage.

Germacrene D (26.5%) and β-elemene (16.3%), (*Z*)-β–ocimene (13.9%), and (*E*)-nerolidol (10.3%) were the main components of the flower oil. Other noteworthy compounds were (*E*)-β–ocimene (6.3%), bicyclogermacrene (3.6%), (*E*)-caryophyllene (1.6%), bornyl acetate (1.6%), and δ-cadinene (1.3%). Within the group of unknown sesquiterpenes, compound no 49 was the most abundant one (4.8%) followed by compound 76 (1.8%). Taken together, they were significantly poorer than in leaf oil obtained at flowering (6.7 vs 17.8%) but in similar content to that found in leaf oil at fruiting (7.1%).

The oil obtained from the cone-like fruits was dominated by monoterpenoids like the two isomers of β–ocimene, namely (*Z*)-β–ocimene (25.4%) and (*E*)-β–ocimene (12.2%), and the oxygen-containing bornyl acetate (7.8%), whereas the sesquiterpenes like germacrene D (6.6%), β-elemene (8.0%), and (*E*)-nerolidol (3.7%) were poorer than in leaf and flower oils. Other noteworthy constituents were borneol (4.6%), α-cadinol (1.7%), selin-11-en-4-α-ol (1.5%), *epi*-α-muurolol (1.5%), and linalool (1.2%). Among the unknown sesquiterpenes, the hydrocarbon no 49 was the most abundant one (5.9%) followed by the oxygenated compound no 76 (2.6%). As a whole, they reached 9.3% that was higher than that of flower oil (6.7%) but lower than that of leaf oil obtained at flowering (17.8%).

To the best of our knowledge, the tulip tree essential oil was studied twice, by Miller et al. (2009) and Smith et al. (1988). The former analyzed the leaf essential oil obtained from two trees grown in Alabama, USA, in different months, i.e., from April to October. They found that the oil yield attained the highest value in May then decreased up to October. The monoterpenes (*Z*)-β–ocimene and (*E*)-β–ocimene and the sesquiterpenes β-elemene and germacrene D were the major constituents; however, their relative content changed during the year, with samples gathered in the first part of the season (e.g., April) being richer in germacrene D (42–44%) and β-elemene (18-23%) and those obtained later (May to October) containing higher amounts of (*Z*)-β–ocimene and (*E*)-β–ocimene (as a whole, 40–60%). Smith et al. (1988) analyzed the oil obtained from branches during the winter and found out that (*Z*)-β–ocimene was the major constituent, followed by α-pinene, β-pinene, myrcene, limonene, and bornyl acetate. Interestingly, they did not detect at all the (*E*)-isomer of β-ocimene.

Our results were quite consistent with those of Miller et al. (2009) and confirmed that tulip tree leaf oil chemical profile changes during the phenological cycle.

The two isomers of β-ocimene are very common in nature being the components of a plethora of plant essential oils, being particularly abundant in those from *Helosciadium nodiflorum* (L.) W.D.J. Koch (Afshar et al. 2017; Benelli et al. 2017; Maggi et al. 2019). β-ocimene owns a pleasant sweet, warm herbaceous odor and is used in perfumery (https://www.perfumerflavorist.com/fragrance/rawmaterials/synthetic/Ocimene-186183431.html).

This monoterpene plays a crucial role in the plant defense, being capable of inducing important responses after insect or

pathogen attacks (Kang et al. 2018). It was also found to display noteworthy fungicidal activity (Maggi et al. 2019).

β-Elemene is a sesquiterpene occurring in the rhizome oil from *Curcuma wenyujin*, an important traditional Chinese remedy used to treat cancer (Quassinti et al. 2013). Indeed, several *in vitro* studies showed that β-elemene exerts antiproliferative activity against many cancer cells. For instance, it inhibits the growth of glioblastoma (Zhu et al. 2011) non-small-cell lung cancer (NSCLC) (Wang et al. 2005) and cisplatin-resistant ovarian cancer cells (Li et al. 2005). Interstingly, β-elemene synergizes the activity of cisplatin against lung cancer cells by inducing apoptosis (Li et al. 2013a, b).

Germacrene D is the precursor of many plant sesquiterpenes (Casiglia et al. 2017) and has been considered as a useful compound for pest control (Zihare and Blumberga 2017). It also plays a role as plant pheromone (Nishii et al. 1997). This compound is endowed with important radical scavenging and antimicrobial activity and antiproliferative effects on tumor cell lines such as A375, MDA-MB 231, and HCT116 (Casiglia et al. 2017; Şahin et al. 2004). Germacrene D-rich essential oils are traditionally used for the treatment of skin disorders such as s itching, varicella, and measles (Setzer et al. 2006).

(*E*)-Nerolidol is a woody-smelling oxygenated sesquiterpene contained in many plant essential oils, flowers, and fruits (Dall'Acqua et al. 2017). It is used as a flavoring agent and in perfumery being recognized as a safe substance (GRAS). This compound showed important antimicrobial properties, being able to inhibit the biofilm formation in *Staphylococcus aureus* (Lee et al. 2014) and *Candida albicans* (Curvelo et al. 2014). *In vivo*, it was found as a potential antioxidant agent (Nogueira Neto et al. 2013). (*E*)-Nerolidol also showed anti-nociceptive and anti-inflammatory properties, being able to interact with the GABA receptors and to decrease TNF-α and IL-11β levels (Fonseca et al. 2016). In our previous study, this compound was found to be highly toxic to a panel of human tumor cells, showing IC_{50} values of 2.92, 4.13, and 5.76 μg/mL on A375, HCT116, and MDA-MB 231 cell lines, respectively (Dall'Acqua et al. 2017).

Antioxidant activity

Radical scavenging activity is of great importance for antioxidant defense and various methods are used to analyze the radical-trapping efficacy of antioxidants (Arnao 2000). The DPPH and ABTS methods are between the most used for this purpose. The antioxidant activity of the examined essential oils and some pure major components (Table 1), estimated by the two methods, is reported in Table 1 Supporting Material. From the results, essential oils and components as β-elemene and β-ocimene showed weak activity against the DPPH radical at the concentration used on the assays while high activity was shown towards the ABTS+ radical. The rank of antioxidant activity of the examined essential oils, estimated by the ABTS assay, was as follows: leaves (June) > leaves (May) > flowers ≈ fruits. In the ABTS assay, weak activity was shown by β-elemene, while for β-ocimene (IC_{50} = 166.0 μg/mL), moderate antioxidant activity was observed. From the data, higher antioxidant activity was reported for the two oils from leaves (June:IC_{50} = 37.35 μg/mL; May:IC_{50} = 81.99 μg/mL) that was about 11 and 25 times lower respect to Trolox used as reference. Leaf oils showed also an antioxidant activity of about 10 times (leaves obtained in June) or 5 times (leaves obtained in May) higher than those of flower and fruit oils. Table 1 Supporting Material shows the ferric reducing antioxidant power from which we can observe that only essential oil from flowers (TEAC = 14.44 μmol TE/g) showed ferric reducing capacity in terms of Trolox concentrations. As previously reported (Viuda-Martos et al. 2010; Amorati et al. 2013), the antioxidant effect of an essential oil is the result of a complex interaction between its components. Frequently, synergism between major and minor components occurs (Bakkali et al. 2008). From Table 1, probably the little differences in antioxidant activity observed for the leaf essential oils with respect to fruit and flower ones could be due to the presence of a major concentration of oxygenated sesquiterpenes that have been reported to have greater antioxidant properties (Ngo et al. 2017). Different works reported the antioxidant activity of identified compounds from *L. tulipifera*, screened for scavenging free radical activity assays with DPPH, ABTS, and FRAP assays. The results showed that these components have potential antioxidant capacity (Li et al. 2013a, b; Chiu 2012). Leaf essential oil seems to have high *in vitro* antioxidant potential, similar to that indicated in a previously work on essential oil from leaves of *L. tulipifera* by Yadav et al. (2015) and can be a source of natural antioxidants with can help to treat diseases related to oxidative stress.

Antimicrobial activity

The essential oils from all sources displayed a growth inhibitory activity against the selected microbial species (Table 2 Supporting Material). The range of inhibition zone diameters was between 7 to 14 mm but the activities were not evenly distributed among the different microbial species and by the different sources of the essential oil. On the average, *S. aureus* was the most sensitive strain while *P. aeruginosa* was the less susceptible and the essential oil from fruits was the most active overall. However, these differences were not statistically significant (Fisher exact test, probability significance level set at 0.5).

Our results extended the observations made by Miller and colleagues (Miller et al. 2009) that found the essential oil from *L. tulipifera* slightly active against three bacterial species, namely *B. subtilis*, *S. aureus*, and *E. coli*. Even if *B. subtilis* was not considered in our study, we extended the proof of activity against *E. faecalis* and *P. aeruginosa* among the

bacteria and *C. albicans* as reference pathogenic yeast. The latter in fact was uniformly susceptible to the essential oils irrespective of the part of the plant used as the source of material (inhibition zone diameter: 12 mm). Moreover, it is possible to mostly associate this activity to β-ocimene that is among the most represented components of the oil (Table 1). The pure compound was effectively inhibiting the yeast (Table 2 Supporting Material). On the contrary, β-ocimene alone was not effective against the bacterial species. Therefore, the antibacterial activity must be dependent upon other molecules composing the essential oil mixture.

MTT assay for cytotoxicity of essential oils

In order to study the biological activity of the essential oils from the various parts of *L. tulipifera* and that of β-elemene and β-ocimene used as two of the most representative components, a screening was initially performed for the evaluation of cytotoxic activity on human tumor cell lines A375 (melanoma), MDA-MB231 (breast adenocarcinoma), HCT116 (colon carcinoma), and T98G (glioblastoma multiforme). The results summarized in Table 2 were obtained after 72 h of incubation in the presence of the various samples tested in the concentration range between 0.78 and 200 μg/mL. Data analysis showed that tulip tree essential oils have a significant antiproliferative effect on all human tumor cell lines examined. In particular, the oil obtained from the leaves collected during the flowering stage was considerably more active than the leaf oil obtained at fruiting stage in all the tested cell lines, with a greater action against the A375, T98G, and MDA-MB 231, showing IC_{50} values of 3.22, 3.23, and 3.40 μg/mL, respectively (Fig. 3). The essential oil of leaves collected during flowering stage is altogether more active even than that of flowers and fruits; however, the major cytotoxic action is attributed to the flower oil on T98G, with an IC_{50} value of 2.5 μg/mL. This is an interesting result since, usually, the T98G cell line is quite resistant in cytotoxicity tests (Ornano et al. 2013; Mustafa et al. 2018; Brunetti et al. 2019).

The cytotoxic activity could be partly attributed to the main components of the *L. tulipifera* essential oils such as β-elemene (7.9–17.1%), germacrene D (6.6–27.2%), and (*E*)-nerolidol. In our experimental conditions, β-elemene exerted cytotoxic activity against all cell lines tested, with IC_{50} values ranging between 15.18 and 23.26 μg/mL. Germacrene D has been proved to be active against human hepatocellular carcinoma (Hep G2), human ductal carcinoma (Hs 578T), and human breast adenocarcinoma (MDA-MB 231 and MCF-7) (Setzer et al. 2006). Germacrene D-rich essential oil from *Kundmannia sicula* (L.) DC resulted active against MDA-MB 231, A375, and HCT116 (Casiglia et al. 2017). (*E*)-nerolidol exerted notable cytotoxicity on A375, MDA-MB 231, and HCT 116 cell lines, showing IC_{50} values of 2.92, 5.76, and 4.13 mg/mL, respectively (Dall'Acqua

Table 2 Cytotoxicity of leaf, flower, and fruit essential oils from *Liriodendron tulipifera*, expressed as IC_{50} values, on human tumor cell lines

Essential oils	Cell line (IC_{50} μg/mL)[a]			
	A375[b]	HCT116[c]	MDA-MB 231[d]	T98G[e]
Leaves (May)	3.22	6.11	3.40	3.23
95% C.I.[f]	2.94–3.52	5.37–6.96	3.18–3.63	3.07–3.54
Leaves (June)	16.34	16.83	13.37	8.49
95% C.I.	14.59–18.29	14.04–20.10	11.38–15.71	7.17–10.04
Flowers	12.83	6.78	7.55	2.50
95% C.I.	11.31–14.55	5.74–7.99	6.99–8.17	2.33–2.68
Fruits	8.31	12.41	7.57	7.17
95% C.I.	6.97–9.90	9.84–15.6	6.72–8.53	6.87–7.47
β-elemene	15.18	12.98	23.26	20.26
95% C.I.	13.60–16.93	11.11–15.16	19.48–27.77	17.77–23.09
β-ocimene	77.07	73.47	62.97	59.02
95% C.I.	69.38–85.60	65.31–82.64	42.11–94.15	48.56–71.74
Positive control				
Cisplatin	0.45	2.39	2.92	2.22
95% C.I.	0.32–0.51	2.00–2.81	2.20–3.11	2.02–2.45

[a] IC_{50} = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation)

[b] Human malignant melanoma cell line

[c] Human colon carcinoma cell line

[d] Human breast adenocarcinoma cell line

[e] Human glioblastoma multiforme cell line

[f] Confidence interval

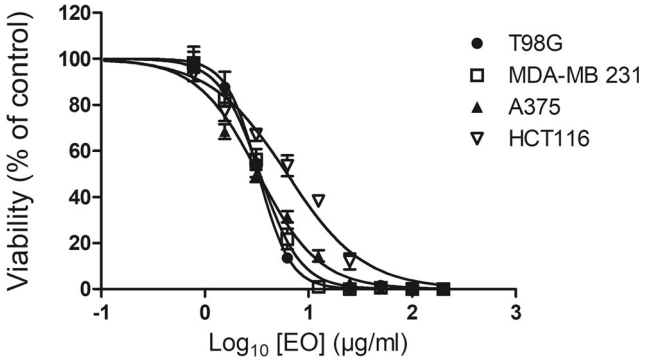

Fig. 3 Non-linear curve fitting for dose response curves to determine IC_{50} values of *Liriodendron tulipifera* essential oil from leaves collected at flowering stage on cell viability in T98G, MDA-MB 231, A375, and HCT116 cell lines. These cell lines were treated with various doses of essential oil (0.78–200 μg/mL) for 72 h and viability was determined by MTT assay. Values represent mean ± SD from triplicate experiments

et al. 2017). As regards other major volatile components of *L. tulipifera*, β-ocimene showed moderate cytotoxic activity against the four tumor cell lines (Table 2). However, it should be emphasized that the activity is the result of a combined effect of the various molecules making up the oil that act together with a synergistic effect. Moreover, the difference in cytotoxicity found in the leaf oils from the two harvesting times could be attributed to the presence of the unidentified sesquiterpenes (Fig. 2), which are present in concentrations ranging from 2.5 to 5.5 % in leaf oil collected during flowering, while in that obtaining during fruiting are present only in traces.

Apoptotic and necrotic effects of the *L. tulipifera* essential oil in T98G cells

We used the oil from the leaves in the analysis tests to highlight the type of mechanism at the base of the cytotoxicity of the *L. tulipifera* essential oil on the T98G cell line which resulted particularly sensitive to the oils (Table 2). Leaf oil–treated T98G cells were subjected to AO/EB staining to detect tumor cell apoptosis (Liu et al. 2015). Usually, AO will enter the nucleus and stains live cells as green color whereas EB will penetrate the nucleus of dead cells due to loss of membrane integrity and stains as red color. After a short period of incubation with compound, viable cells appeared as green fluorescence with highly organized nuclei. Necrotic cells fluoresced orange to red without chromatin fragmentation. As shown in Fig. 4 a, the cells treated for 2 h with different oil concentrations up to twice the IC_{50} values (i.e., 6.4 μg/mL) showed typical viable cells with green fluorescence staining suggesting that cytotoxic mechanism of essential oil cannot be due to necrosis processes. Extending the incubation to 24 h, orange fluorescence begins to appear in cells showing orange to red color with highly condensed or fragmented chromatin and apoptotic bodies forms that can be assimilated to the late apoptotic cells (Fig. 4b) (Ribble et al. 2005).

Effects of *L. tulipifera* essential oil on caspase-3

Caspases are cysteine proteinases playing as important mediators of apoptotic processes. Notably, caspase-3 activation is positively

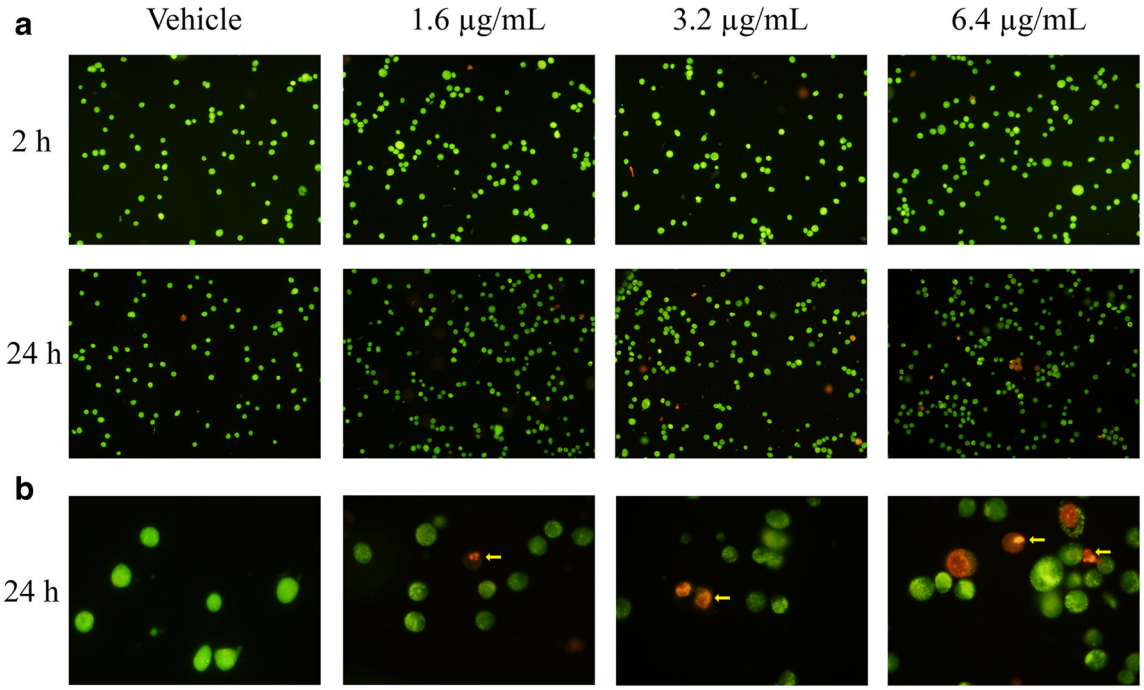

Fig. 4 Effects of *Liriodendron tulipifera* essential oil from leaves collected at flowering stage on T98G cell morphology. **a** Cells were exposed to 1.6, 3.2, and 6.4 μg/mL of essential oil for 2 and 24 h, stained with acridine orange and ethidium bromide, and observed by fluorescent microscopy. **b** T98G cell morphology at × 40 magnification. Viable cells show a fluorescent green color; late apoptotic cells show a red/orange nucleus with chromatin condensation (yellow arrows)

associated with induction of apoptosis of both intrinsic and extrinsic pathways (Porter and Jänicke 1999). Activation of caspase-3 was assessed using the colorimetric method to confirm the apoptosis induction by *L. tulipifera* leaf essential oil in T98G cells. As shown in Fig. 5, the essential oil induced caspase-3 activation when compared with control in a time-dependent manner. These data suggest the hypothesis that the leaf essential oil exerts cytotoxic activity triggering apoptotic processes on glioblastoma cell line T98G. Noteworthy, this oil can be of interest for the National Cancer Institute (NCI) as a candidate anticancer agent, since its IC_{50} is lower than 20 μg/mL (Boik 2001).

Glioblastoma is a lethal tumor of the central nervous system with high incidence (Louis et al. 2016). It is characterized by microvascular proliferation, marked necrosis, and resistance to conventional therapies (Arévalo et al. 2017). In this respect, the discovery of new therapeutic alternatives based on natural products has recently attracted the attention of many scientists (Kaur and Verma 2015).

Here we show that leaf essential oil exerts concentration-dependent cytotoxicity triggering apoptosis processes in T98G glioblastoma cell line. These data open the possibility to use the leaf essential oil as potential source for anticancer therapy or the use of the oil in synergism with traditional therapies. Of course, it must undergo a series of further studies to validate and approve its use in cancer.

Conclusions

Tulip tree is one of the most appreciated plants around the world for ornamental purposes and wood industry uses. Its exploitation produces a significant amount of residual material that can be valorized as source of valuable compounds with application in different fields. In this work, we showed that leaves of tulip tree are a source of bioactive essential oils which produce a significant apoptosis in drug-resistant T98G cells. Their effects are noteworthy, especially when compared with other plant essential oils, and encourage further investigations in order to support their use in chemotherapy.

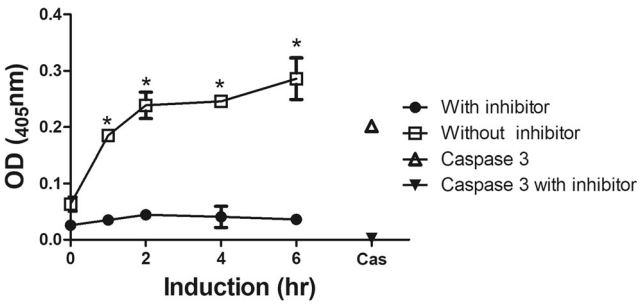

Fig. 5 Effect of *Liriodendron tulipifera* essential oil from leaves collected at flowering stage on the activity of caspase 3 in T98G cells after 1-, 2-, 4-, and 6-h exposition. Cas, caspase 3 positive control, 0.025 μg/mL. The data represent the mean ± SD from 2 independent experiments (with 3 parallel samples in each), statistical significance (ANOVA, $*p < 0.01$): vs. control (0 h)

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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