SUPPLEMENTARY MATERIAL

Antitumor effect of *Cymbopogon densiflorus* (Linneu) essential oil in bladder cancer cells

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Abstract: The aim of this study was to analyse the antitumor effect of the *Cymbopogon densiflorus* essential oil *in silico* and *in vitro* on bladder cancer cells RT4 and T24, with different *TP53* status. The oil was extracted by hydrodistillation and the gas chromatography coupled to the mass spectrometry was used for characterization. *In silico* analysis was carried out by Pass online software. Cytotoxicity, cell proliferation, cell cycle progression, apoptosis and wound healing assays were performed. Five major compounds were identified. I*n silico* analysis showed that major compounds present high potential for antitumor activities. The treatment with *C. densiflorus* essential oil reduced cell viability of bladder cancer cells. Only in wild-type cells, the increase of apoptosis rates and the decrease of cell migration were observed. In conclusion, the *C. densiflorus* essential oil presents antitumor effects on *TP53* wild-type and mutated bladder cancer cells, however the action mechanism is *TP53* status dependent.

Keywords: bladder cancer, *Cymbopogon densiflorus*, cytotoxicity, essential oil, *in silico* analyses.

Experimental section

Cell lines

The RT4 (low grade tumor with wild *TP53* gene) and T24 (high grade tumor with mutated *TP53* gene) cell lines from human bladder carcinoma were purchased from the Cell Bank of the Federal University of Rio de Janeiro. The MRC5 is a normal human fibroblast cell line derived from fetal lung and was kindly donated by Prof. Dr. Luiz Orlando Ladeira of the Department of Physics of the Institute of Exact Sciences of the Federal University of Minas Gerais. The cells were maintained in supplemented DMEM medium at 37 °C and 5% CO_2.

Plant specimens and essential oil extraction

C. densiflorus leaves were collected in Ouro Preto/MG, Brazil (20º23'51 "S; 43º30'40" W). An exsiccatum of this specimen was deposited at Herbarium Prof. José Badini – UFOP (OUPR 28122/SisGen AC902F3). The essential oil extraction was performed by a hydrodistillation process as mentioned before (Seibert et al. 2019a).

Chemical characterization of the essential oil

The chemical characterization of the essential oil was carried out by gas chromatography coupled to mass spectrometry (GC-MS) (Shimadzu, model QP2010) at the same conditions to the study performed by Seibert et al. (2019a). Hydrocarbons of 9 to 25 carbons were used as standards and the Kovats index (IK) of each compound was calculated. The identification of the substances was performed by the interpretation of the respective mass spectra and the comparison with mass spectra of the Wiley Mass Spectral Database, the data from the literature and the Kovats index (Adams 1989).

In silico analysis

The major compounds identified by GC-MS were analysed *in silico* by the Prediction Activity Spectrum of Substances (PASS) program. PASS online is a software designed to evaluate the overall biological potential of an organic molecule on the human organism, according to its

structural arrangement. The results of the PASS online analysis are provided as a probability that the single compound is active (Pa) and inactive (Pi) (www.pharmaexpert.ru/passonline) (Seibert et al. 2019b). Four antitumor effects and nine mechanisms of action related to the tumorigenesis process were analysed. The results were expressed by difference Pa-Pi and were classified as Pa-Pi < 0.2: low potential; Pa-Pi < 0.5: moderate potential; Pa-Pi ≥ 0.5: high potential.

Cytotoxicity and cell proliferation assays

For the cytotoxicity (immediately after treatment) and cell proliferation (24 hours after treatment) assays, cells were placed in 96-well plates ($1x10^4$ cells/well for T24 and MRC5 and $1.5x10^4$ cells/well for RT4). Afterwards cells were treated with different concentrations of *C. densiflorus* essential oil (5-10,000 µg/mL) for 24 hours. Untreated cells were used as control. After treatment, the cells were processed and analysed by MTT (Sigma Chemical, St. Louis, USA), according to manufacturer's instructions. For the cytotoxicity assay, cell viability was assessed immediately after the end of treatment. For the cell proliferation assay, the culture medium containing *C. densiflorus* essential oil was removed, the cells were washed and incubated with fresh culture medium for additional 24 hours (recovery time). Both tests were conducted in triplicate. The selectivity index (SI) of the *C. densiflorus* essential oil was calculated by the following equation:

$$SI = \frac{IC_{50} \text{ on MRC5 cells}}{IC_{50} \text{ on tumor cells}}$$

SI value above 2 indicates selectivity (Badisa et al. 2009).

Cell morphologic analysis

Possible morphological changes in cells after treatment were analysed according to Da Silva et al. (2012).

Cell cycle progression

For cell cycle analysis, 2×10^5 cells were placed in 12-well plates. After 24 hours, the cells were treated with *C. densiflorus* essential oil for 24 hours. After the treatment, the cells were detached and the sediment was fixed with 70% ethanol and maintained at -20 °C for 12 hours (Brassesco et al. 2012). Subsequently, the cells were washed with Hanks' solution,

resuspended in 200 µL of labeling solution (Galbraith et al. 1983), placed on ice and protected from light for 30 minutes. The percentages of cells in the G0/G1, S and G2/M phases were measured by flow cytometry (BD FACSCalibur) and analysed by FlowJo® software. Data from 30,000 cells were collected in each file and the experiments were conducted in triplicate.

Apoptosis assay

Apoptosis rates were evaluated by the FITC AnnexinV/Dead Cell Apoptosis kit (Invitrogen). 2×10^5 cells were placed in 12-well plates. After 24 hours, the cells were treated with *C. densiflorus* essential oil for 24 hours. After the treatment, the cells were collected and the labeling was carried out with propidium iodide and annexin V following the kit protocol. The percentages of unlabeled or labeled cells by propidium iodide and/or annexin V were measured by a flow cytometer (BD FACSCalibur) and analysed by FlowJo® software. Data from 50,000 cells were collected in each file and the experiments were conducted in triplicate.

Wound healing assay

To evaluate the effects on cell migration, 5×10^5 cells were placed in 12-well plates. After 24 hours, a straight scratch was made on the cell monolayer by a sterile 200 µL pipette tip. The cells were treated with 39 µg/mL of *C. densiflorus* essential oil for 24 hours. Untreated cells were used as control. Then, cell migration was observed and the images were captured by an inverted microscope. Quantification of cell motility was performed by the software ImageJ measuring the distance between the invading front of the cells (Gándara et al. 2014). T24 cells were analyzed immediately after the treatment and RT4 cells were analyzed 24 hours after the end of the treatment. The doubling time of RT4 cells (37 hours) (Masters et al. 1986) is greater than that of T24 cells (19 hours) (Bubeník et al. 1973). Thus, untreated RT4 cells need a longer time to close the slit than T24 cells.

Statistical analysis

Statistical analyses were performed by GraphPad Prism® 5. The data were analysed by one-way ANOVA, and *post hoc* analysis by Tukey's test. The results were considered statistically significant at $P < 0.05$.

Table S1: Chemical composition of the essential oil from *Cymbopogon densiflorus* leaves obtained by CG-MS.

Peak	Component	Classification	KI	EKI	% composition of essential oil of *Cymbopogon densiflorus*
1	-	-	-	927.3	0.04
2	Sylvestrene <iso->	HM	1008	1005.9	0.07
3	Terpinene < α->	HM	1017	1023.3	0.71
4	Limonene	HM	1029	1027.9	0.36
5	-	-	-	1059.6	0.03
6	-	-	-	1069.4	0.10
7	Cymenene <ρ->	HM	1091	1090.4	0.30
8	-	-	-	1096.6	0.07
9	-	-	-	1114.3	0.05
10	**Mentha-2,8-dien-1-ol <*trans*-ρ->**	OM	**1122**	**1121.8**	**12.91**
11	Limonene oxide <*cis*->	OM	1136	1124.1	0.06
12	**Mentha-2,8-dien-1-ol <*cis*-ρ->**	OM	**1137**	**1135.9**	**9.49**
13	Terpineol <*cis*-β->	OM	1144	1141.7	0.03
14	karahanaenone	OM	1159	1149.3	0.15
15	-	-	-	1154.6	0.46
16	Pentyl cyclohexa-1,3-diene <5->	HM	1160	1167.1	0.71
17	-	-	-	1181.3	0.05
18	**Mentha-1(7),8-dien-2-ol <*trans*-ρ->**	OM	**1189**	**1187.8**	**18.38**
19	-	-	-	1193.2	0.04
20	Dihydro carvone <trans->	OM	1200	1197.7	0.78
21	**Piperitol <*cis*->**	OM	**1196**	**1200.2**	**12.42**

22	Dihydro carvone <*trans*->	OM	1200	1204.0	0.26
23	Caranone <*cis*-4->	OM	1200	1026.5	5.75
24	-	-	-	1208.3	0.76
25	-	-	-	1212.0	0.83
26	Piperitol <*trans*->	OM	1208	1217.0	3.86
27	-	-	-	1219.0	2.22
28	-	-	-	1222.0	0.01
29	-	-	-	1225.0	0.18
30	**Mentha-1(7),8-dien-2-ol <*cis*-ρ->**	OM	**1230**	**1229.0**	**16.85**
31	Carveol <*cis*->	OM	1229	1232.0	0.55
32	Carvone	OM	1243	1243.0	4.99
33	-	-	-	1250.0	0.43
34	-	-	-	1268.0	0.08
35	-	-	-	1269.0	0.01
36	Perilla aldehyde	OM	1271	1274.0	0.65
37	-	-	-	1286.0	0.30
38	Cyclopent-2-em-1-one <2-pentyl->	OM	1289	1292.0	0.20
39	-	-	-	1302.0	0.04
40	-	-	-	1438.0	0.11
41	Isoamyl octanote	OS	1445	1447.0	0.66
42	Phenyl ethyl hexanoate	OS	1642	1639.6	0.22
43	-	-	-	1836.1	0.15
44	-	-	-	2129.0	2.51
45	-	-	-	2137.0	0.31
46	-	-	-	2153.0	0.09
47	-	-	-	2224.0	0.78
	Hydrocarbon monoterpene (HM)				**2.15**
	Oxygenated monoterpene (OM)				**87.33**
	Oxygenated sesquiterpene (OS)				**0.88**
	Total				**90.36**

KI: Kovats Index; EIK: Experimental Kovats Index. (-) Not identified. The major compounds are highlighted in bold.

Table S2: *In silico* analyses of the potential antitumor effects and mechanism of action of the essential oil of *Cymbopogon densiflorus* leaves using the online PASS program.

Biological activity	Values of Pa - Pi				
	A	**B**	**C**	**D**	**E**
Antineoplastic	0.722	0.722	0.916	0.916	-
Anticarcinogenic	-	-	0.531	0.531	-
Antimetastatic	-	-	0.590	0.590	0.551
Antimitotic	-	-	0.26	0.26	0.086
Mechanism of action					
Stimulant of caspase 8	0.562	0.562	0.589	0.589	0.328
Inhibitor of *MMP9* expression	0.545	0.545	0.554	0.554	0.543
TP53 expression enhancer	0.446	0.446	0.571	0.571	0.480
Inhibitor of *HIF1A* expression	0.385	0.385	0.252	0.252	0.468
Stimulant of caspase 3	0.334	0.334	0.375	0.375	0.328
Inhibitor of *JAK2* expression	0.303	0.303	0.377	0.377	0.560

Apoptosis agonist	0.296	0.296	0.695	0.695	0.416
Inhibitor of *BRAF* expression	0.222	0.222	-	-	-
Cytostatic	0.164	0.164	0.180	0.180	0.280

1 Pa: potential to be active; Pi: potential to be inactive.

2 Pa-Pi < 0,2: Low potential; Pa-Pi < 0.5: Moderate potential; Pa-Pi ≥ 0.5: High potential.

3 (A) *trans*-*p*-mentha-2,8-dien-1-ol; (B) *cis*-*p*-mentha-2,8-dien-1-ol; (C) *trans*-*p*-mentha-1(7),8-

4 dien-2-ol; (D) *cis*-*p*-mentha-1(7),8-dien-2-ol and (E) *cis*-piperitol.

5 (-) Not indicated or unsatisfactory.

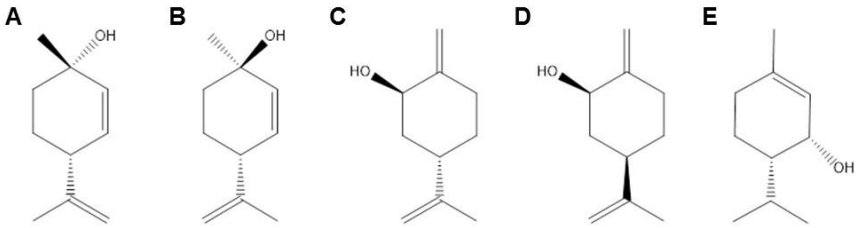

Figure S1: Chemical structures of the major compounds of the essential oil from *Cymbopogon densiflorus* leaves identified by GC-MS. A: *trans*-*p*-menta-2,8-dien-1-ol. B: *cis*-*p*-menta-2,8-dien-1-ol. C: *trans*-*p*-menta-1(7),8-dien-2-ol. D: *cis*-*p*-menta-1(7),8-dien-2-ol. E: *cis*-piperitol.

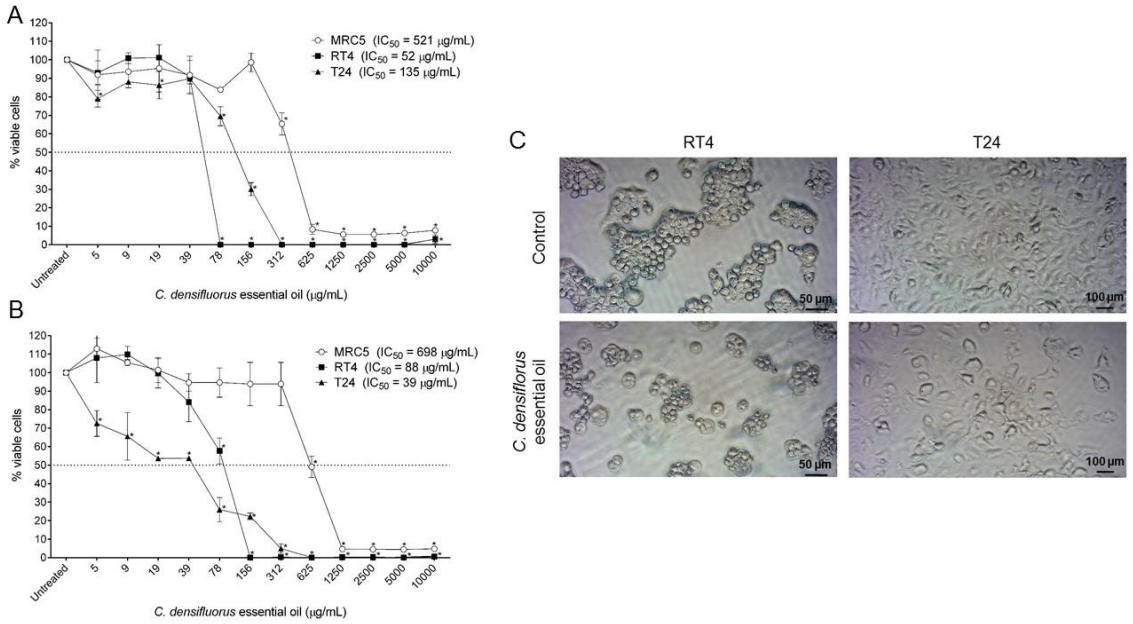

Figure S2: Percentages of viability in RT4, T24 and MRC5 cells immediately (A) and 24 hours (B) after treatment for 24 hours with the essential oil from *Cymbopogon densiflorus* leaves. * $p < 0.05$ compared with control. (C) Photomicrography of RT4 and T24 cells before and after 24-hour treatment with the essential oil from *Cymbopogon densiflorus* leaves (19 µg/mL). Phase-contrast microscope, x200.

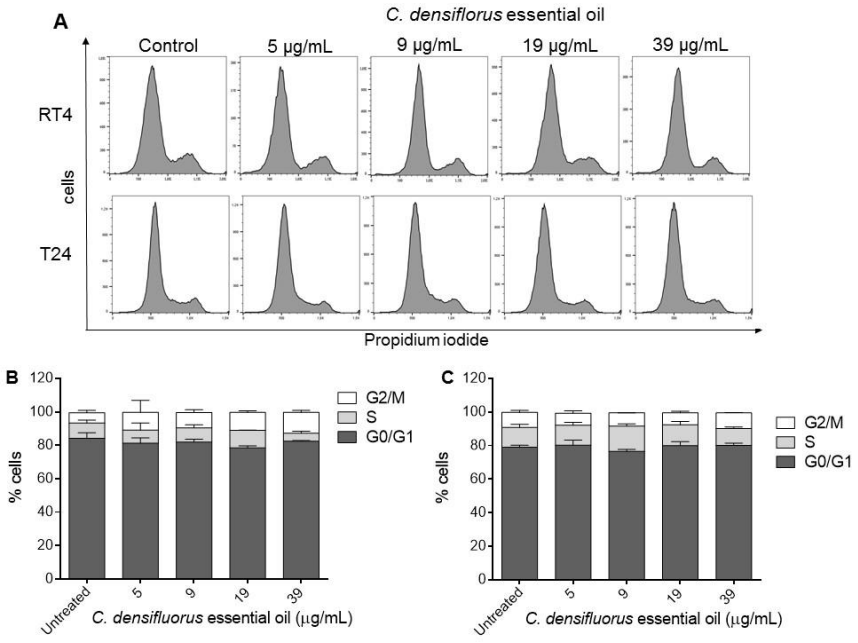

Figure S3: (A) Histograms representative of cell cycle kinetics after treatment for 24 hours with the essential oil from *Cymbopogon densiflorus* leaves. (B) Percentage of RT4 cells in each cell cycle phase. (C) Percentage of T24 cells in each cell cycle phase.

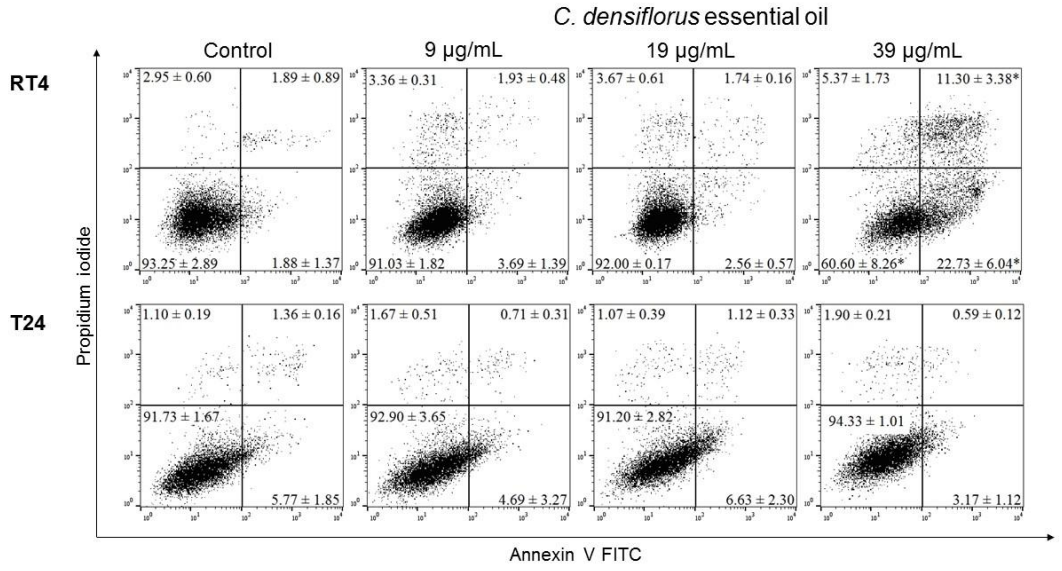

Figure S4: Apoptosis and necrosis rates in RT4 and T24 cells after treatment for 24 hours with the essential oil from *Cymbopogon densiflorus* leaves. * $p < 0.05$ compared with control.

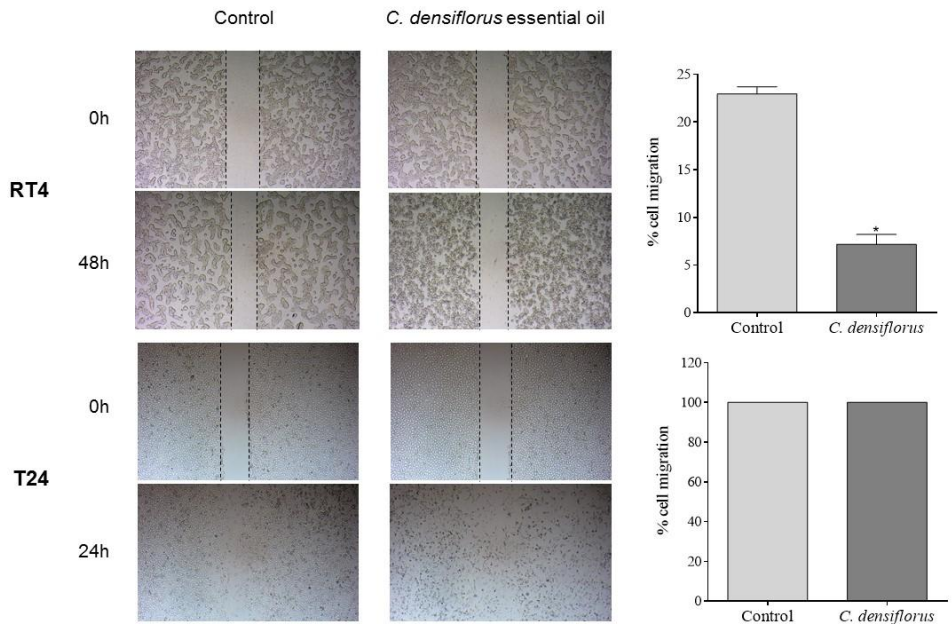

Figure S5: Cell migration of RT4 and T24 cells after treatment for 24 hours with 39 µg/mL of essential oil from *Cymbopogon densiflorus* leaves. * $p < 0.05$ compared with control.

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