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Original Article

Real time monitoring of cytotoxicity of *Callistemon citrinus* against Colo-205 cell line

Alim Hüseyin Dokumacı[1*], Peter Olutope Fayemi[2, 3], Mukerrem Betul Yerer[1]
[1]Department of Pharmacology, Erciyes University, Faculty of Pharmacy, Kayseri, 38039, Turkey
[2]Department of Food Engineering, Erciyes University, Kayseri, 38039, Turkey
[3]Department of Livestock and Pasture Science, University of Fort Hare, 5700 Alice, South Africa

ORCID IDs of the authors: A.H.D. 0000-0003-0035-1479; P.O.F. 0000-0002-2466-2223; M.B.Y. 0000-0002-4503-8032.

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ABSTRACT

Callistemon citrinus is a member of Myrtaceae family that thrives under different ecological conditions. The leaves, flowers, stem backs and roots of the plant contain various phytochemicals that are useful in folk medicine for different remedies such as antimicrobial, anti-nociceptive, fungicide and anti-inflammatory purposes. In this study, we investigated the cytotoxic effect of *Callistemon citrinus* leaf and flower methanolic extracts against human Colo-205 Cell Line using real time cell analyzer device for monitoring in time-dependent manner. To determine the mechanism of cytotoxicity of the extracts, Western blotting assay was used for measuring evocation of Akt pathway. Extracts were found to exert cytotoxic effect at a dose dependent manner. IC_{50} values of leaves and flowers extract were 6.49 µg/mL and 5.22 µg/mL, respectively. At the early stages of the experiment, Akt pathway was triggered at high extract concentrations. Although, high extract concentrations showed proliferative effect at early stages, this effect reversed after 5 and 8 h resulting in low cell viability. Findings from this study therefore showed that extracts of leaf and flower from *Callistemon citrinus* demonstrated cytotoxic effect against Colo-205 but seems not to be related Akt signaling pathway.

Keywords: Akt/p-Akt, *Callistemon citrinus*, colon cancer, xCELLigence

INTRODUCTION

Colon cancer is a one of the major causes of mortality and morbidity all over the World. According to a recent epidemiological report, intestinal cancers (colon and rectum) death rate was 13% of total cancer deaths from 172600 reported mortalities which was second order after the lung cancer (Malvezzi et al. 2015). Currently, therapeutic approaches for treating human colorectal include radiotherapy, chemotherapy and surgery. In addition to conventional treatment of colon carcinoma, there are a lot of alternative medicine in use from natural products. One of such natural products include phytochemicals from ornamental plants. *Callistemon citrinus* is one of the medicinal ornamental plants from Myrtaceae family (Brophy et al. 1998). Different parts of the plant contain alkaloids, polyphenols, flavonoids, tannins, steroids, aliphatic acids, monoterpenoids, triterpenoids, sesquiterpenes and several phytochemicals. The leaf oils of *C. citrinus* are known to have antimicrobial, antifungal, antinociceptive and anti-inflammatory activities (Sudhakar et al. 2004; Oyedeji et al. 2009). In folk medicine, various parts of this herb are used in making traditional pills for treating dysentery, cough, bronchitis, hemorrhoids and rheumatism (Paluri et al. 2012). In our previous study, the *in vitro* cytotoxic effects of leaf and flower extracts from *C.citrinus* has recently been reported against MCF-7 breast cancer cell line by our group (Fayemi et al. 2015).

Although, reports on bioactivities of Callistemon are available yet information on *in vitro* cytotoxicity of this plant is limited against colon cancer. *In vitro* cytotoxicity tests are prerequisites for describing intrinsic toxicodynamics of phytochemicals in any potential

Address for Correspondence :
Alim Hüseyin Dokumacı, e-mail: ahuseyindokumaci@gmail.com

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medicinal plant (Sutar et al. 2014). Therefore, monitoring cell viability and toxicity is crucial for preclinical screening of a new compound in order to ascertain its bioactivity in aiding cell proliferation or causing apoptosis. In this study however, cytotoxic effect of extracts from *Callistemon citrinus* species' flower (CF) and leaves (CL) against colon cancer cell line was investigated by monitoring its effects in a real time manner using Real Time xCELLigence system. In addition, we also investigated the mechanism of action in relation to Akt/phosphorylated Akt pathway. Because of the potential anti-apoptotic effects of plant materials, this pathway was selected which is activated by several natural products. Akt protein is one of the key proteins in Wnt signaling that evoked at the beginning of the apoptosis pathway (Koseoglu et al. 2007; Brahmachari 2012). Akt is Ser/Thr kinase which is also referred to as protein kinase B (PKB) in mammalian genomes have three Akt genes namely: Akt1 (Protein kinaseB α), Akt2 (Protein kinaseB β) and Akt3 (Protein kinaseB γ) and they are extensively expressed in various tissues. Akt1 is most abundantly expressed in the heart, lung, brain and colon. Akt2 is predominantly expressed in the skeletal muscle and embryonic brown fat and Akt3 is expressed most abundant in embryonic heart, kidney and also brain (Coffer et al. 1992; Altomare et al. 1995; Altomare et al. 1998; Brodbeck et al. 1999). Akt1, Akt2 and Akt3 genes are located on 14q32, 19q13.1-13.2 and 1q44 respectively (Staal et al. 1988; Cheng et al. 1992; Murthy et al. 2000). The phosphatidylinositol 3-kinase(PI3K)-Akt signaling pathway is evoked by many types of cell stimuli or toxic reagents and regulates fundamental cellular functions such as proliferation, transcription, translation, cell survival and growth (Datta et al. 1999; Vivanco et al. 2002).

The Akt/protein kinase B (PKB) kinase is an effector of phosphoinositide 3-kinase (PI3K) that plays important roles in the pathogenesis of human cancers (Blume-Jensen et al. 2001). Previous studies have reported that PI3K/Akt pathway plays a critical role in cell survival or apoptosis in various human cancer cells such as lung (Brognard et al. 2001), prostate (Kreisberg et al. 2004), pancreas (Grille et al. 2003), and breast carcinoma (Sun et al. 2001). Colon carcinoma is characterized by different morphological, genetic and cellular events. There are some occasion which causes to cancer formation such as over expression of Akt1 and its activation level (phosphorylated Akt) and site is link to development and progression of colon cancer (Roy et al. 2002). In order to activate Akt, it requires that the phosphorylation of Thr308 in the activation loop by the phosphoinositide-dependent kinase 1 (PDK1) and Ser473 within the carboxyl-terminal (Figure 1). Phosphorylation of Akt is promoted by phosphatidylinositol-3-OH kinase (PI3K) products, which subsequently facilitate transmembrane signaling by serving as membrane-localization molecule. It has been reported that phosphorylation of Akt was apparently detected at high level in normal colorectal cells mucosa. They also performed PI3K/Akt pathway related apoptosis mechanisms which are caspase 3, EGF (endothelial growth factor) and TNFα (Tumor necrosis factor α) (Itoh et al. 2002). To the best of our knowledge, cytotoxicity of *C. citrinus* on colon cancer cell line and its mechanism of action over Akt/p-Akt pathway was investigated. The aim of this study was investigate the dynamic monitoring of cytotoxic effect of CF and CL extract on Colo-205 cell line. In addition we existed if the Akt pathway was evoked by extracts for evaluating the mechanism of action.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO; Sigma Aldrich, Steinheim, Germany), Tris base (Sigma-Aldrich, Steinheim, Germany), Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Steinheim, Germany), Trypsin-EDTA (Sigma-Aldrich, Steinheim, Germany), Fetal Bovine Serum (FBS; Sigma-Aldrich, Steinheim, Germany) were used. Akt (Cell Signalling Technology (CST), 9272S, Leiden, Netherlands), Phospho-Akt (Ser473) antibody (CST 4060S, Leiden, Netherlands), β-Actin Antibody (CST 4967, Leiden, Netherlands) antibodies were used for western blotting.

Plant materials

Fresh leaves and flowers of *C. citrinus* were harvested, air dried and grounded into coarse powder. 40 g of plant material from leaves was diluted in 960 mL deionized water and 40 g of extract from flowers in a mixture of 480 mL methanol with 480 mL deionized water, and then distillated using Heidolph rotary evaporator (Hei-VAP HL/G3**;** Heidolph Instruments GmbH, Schwabach, Germany). The distillates from leaves and flowers were lyophilized for 24 h, then stored in plastic vials at -80°C until analysis. HPLC grade water (18.2MΩı-cm) was prepared using a Millipore Simplicity 185 Direct-Q water purification system (Millipore Corp., Bedford, USA).

Cell culture

Colo-205 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) The cells were grown in DMEM with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin and incubated in a humidified at-

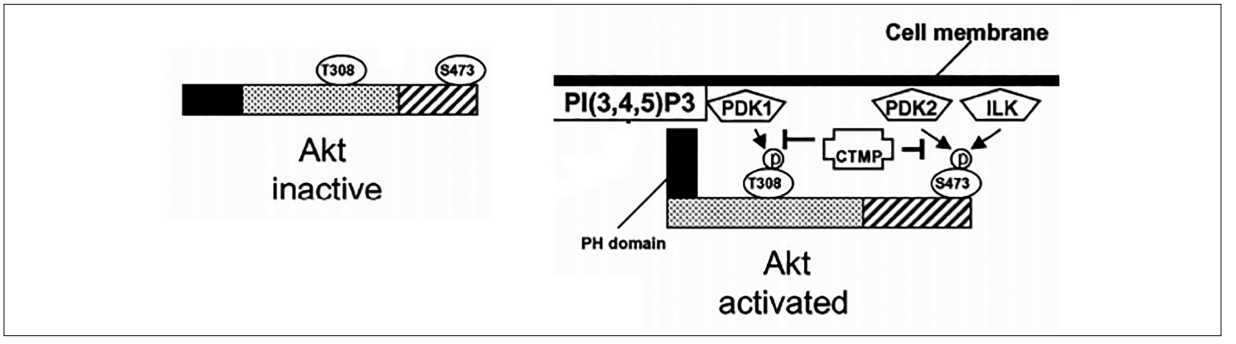

Figure 1. Akt phosphorylation sites and activating with cellular bioactive molecules

mosphere containing 5% CO_2 at 37°C. When the confluence nearly reached 80%, the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) solution and detached from the flasks with trypsin/EDTA. The cells were centrifuged with the Universal 320R (Hettich, Zentrifugen, 1406; Kirchlengern, Germany) at 1000rpm for 5 mins at 25°C, seeded on 6 wells plate and 96 wells E-plate for western blot and xcelligence analysis, respectively.

Western blotting

Western blot analysis was carried out using crude lysates of Colo-205 human colon cancer cells. Cells were treated with CL and CF extract at 400, 200, 100 and 50 µg/mL concentrations for 12 h both. Cells were lysed in commercial ripa lysis buffer (SC-24948; Santa cruz; CA, USA). The lysate was centrifuged at 4 °C for 30 min at 12000 rpm. The clear supernatant was collected and the total protein amount was determined by Lowry method. 30 µg protein lysates were resolved on 8% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Then electro-transferred onto polyvinylidenedifluoride (PVDF) membrane. After blocking with 5% non-fat milk in Tris-buffered saline (TBS, 0.1 M, pH 7.4). Membrane were incubated with primary antibodies anti-phospho-Akt (Ser473) (1:500 dilution, Cell Signaling Technology), anti- Akt (1:1000 dilution), anti-pAkt (1:1000 dilution), and anti-tubulin (1:2000 dilution). β-actin protein was assigned as a control for protein loading. After overnight incubation at 4 °C conditions, membranes were incubated with secondary antibody, HRP-conjugated goat/rabbit anti-IgG, for 1 h at room temperature. After each step blots were washed three times with Tween (0.2%)-Tris-buffer saline (TBST). Protein bands were detected by enhanced chemiluminescence method (ECL; Santa Cruz Biotechnology, CA, USA) on XO-MAT film. The blots were scanned and analyzed using ImageJ software.

Real Time Cell Analyzer (RTCA) system for cellular monitoring against Colo-205

The xCELLigence system was used for monitoring the real time effect of extracts from *C. citrinus* against Colo-205 cells following manufacturer's instructions (Ke et al. 2011a). The xCELLigence system has got four main units: RTCA analyzer, the RTCA SP station, the RTCA computer with integrated software and disposable E-plate 96 (Figure 2). The RTCA SP station was

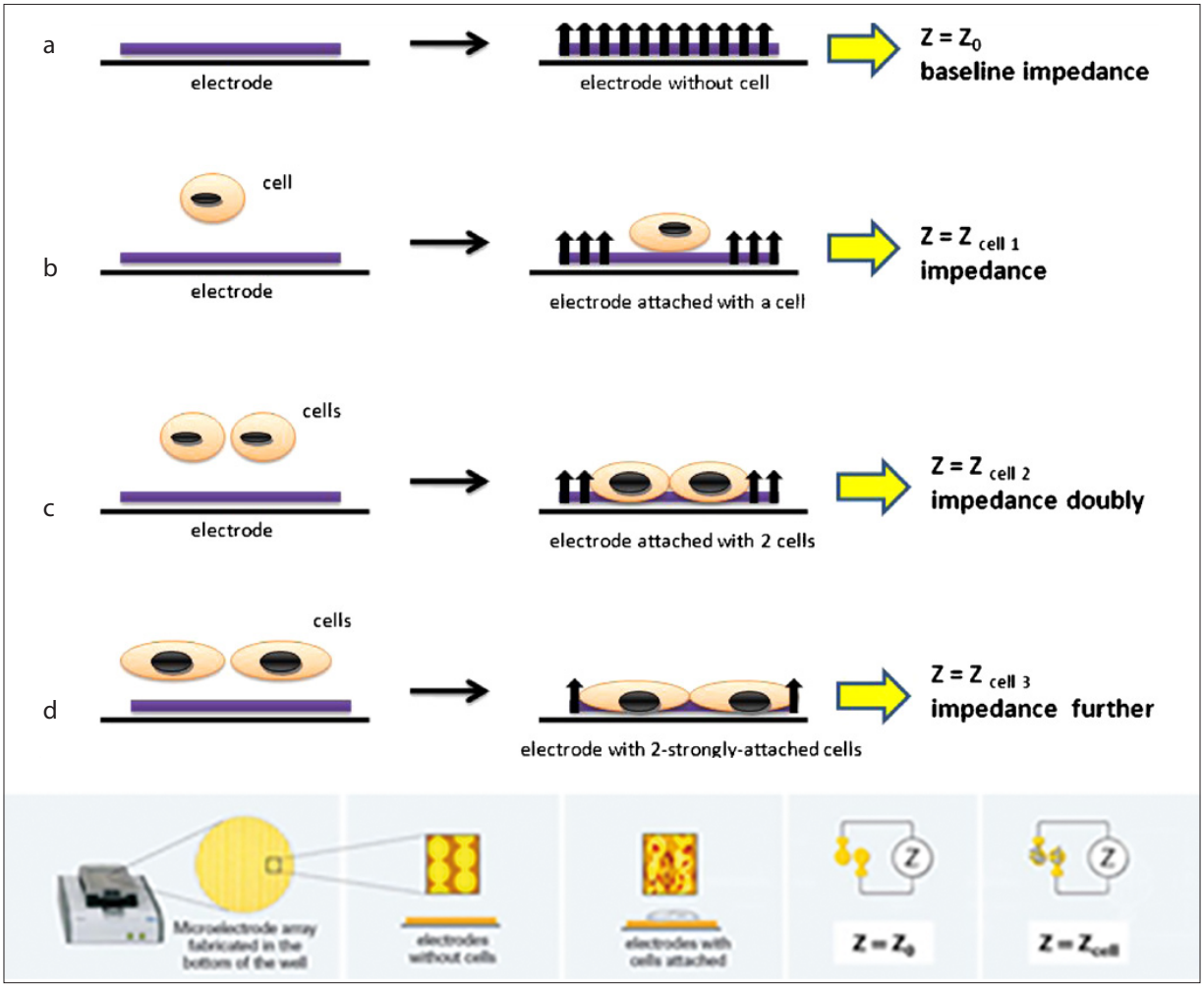

Figure 2. a-d. 96 well E- plate bottom includes golden electrodes which is sensitive to cell adhesion, proliferation and enlargement. Absence of cell (a) at the bottom of the well impedance will be 0 ($Z=Z_0$). Presence of adhesive cell can alter impedance (b) and more cells (c) increase this impedance. Also cell enlargement effects impedance (d), when cells enlarge without mitosis division, impedance alters and cell index increases.

placed inside incubator, while an analyzer and laptop computer with software are on the outside. E-plate 96 is a single use, disposable device used for performing cell-based assays on the RTCA SP instrument. However the E-plate 96 differs from standard 96-well microtiter plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells inside each well to measure alterations on impedance (Bird et al. 2009; Ke et al. 2011b). Electronic impedance alterations can be measured at least every 2 minutes to allow monitoring and detection of physiological changes on the cells. The impedance measured between electrodes in an individual well depends on electrode geometry, the number of the cells in the well and whether the cells are attached to the electrodes or not. In the presence of cells, cells attached to the electrode sensor surfaces and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance. Cell index is related with cell adhesion, cellular morphology alterations also cell detachment via cellular death. So we can evaluate the cytotoxic effect of any material with cell index alterations (Ke et al. 2011b).

Colo 205 cells were grown on cell culture flasks and after reaching approximately 80% confluence, the cells were detached and seeded inside E-plate and incubated for 30 min incubation at room temperature. E-plate 96 was placed into the cell culture incubator different cell numbers (50 000, 25 000, 12 500, 6250 and 3125) to assign optimal seeding concentration. We decided to seed 12500 cell/well for optimal seeding because of log growth phase achieved on this cell number. Cell proliferation, attachment and spreading were monitored every 15mins via the alterations impedance of E-plate wells.

Cytotoxicity assay using Xcelligence system

To determine the cytotoxic effect of CF and CL on Colo-205 cell line, RTCA (xcelligence) was used. A total of 12500 cells/well were seeded in the E-plate 96 wells and approximately 46h post-seeding when the cells were in the log growth phase, cells were treated with extracts and only medium control. Controls received medium and treated groups (CF and CL at 400, 200, 100, 50, 10, 1 µg/mL concentrations) were replicated 4-times and the experiments were run for 97 h. All calculations were done with the RTCA-integrated software of the xcelligence system. The RTCA software performs the curve-fitting of selected "sigmoidal dose-response equation" and calculated logarithmic half maximum effect of concentration [log (IC_{50})] values at a given time point based on log concentration producing 50% reduction of cell index (CI) value relative to the control CI value (100%). Cell index value represents the alteration of cell viability, cell attachment and cellular growth rate. Likewise, when the cell index data obtained during the experiment at the particular time points, dynamic monitoring of the cells' response can be elucidated.

Statistical analysis

For each group, data were derived from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis was used for all the data analysis. Standard deviation (SD) was calculated with the same method. Statistical significance between the collection methods were assessed as $p<0.05$ and was calculated using the same program. Results were presented as the mean±SD.

RESULTS

Effects of *Callistemon citrinus*'s leaves and flowers on cell viability

The results of IC_{50} values calculated for CL and CF using RTCA software were 6.49 µg/mL and 5.22 µg/mL at 24 h post treatment, respectively. At higher concentrations of 400, 200 and 100 µg/mL for CL extract, cell index increased for about 5 h after the treatment time. Shortly after the treatment, it was observed that cell index decreased and continued decreasing until the end of experiment. At lower concentrations of 10 and 1 µg/mL for CL (Figure 3), cell index profiles were parallel to control and did not affect cells after the treatment time. Interestingly, 50 µg/mL dose initially increased the cell index rapidly and continued the increment gradually but was observed to decrease at 85 h. 400 and 200 µg/mL concentrations of CF increased cell index (Figure 4) after the post treatment and continued this increment about 8 h different from the CL high dose concentrations, than cell index decreased consistently. 50 µg/mL dose firstly increased cell index as the high concentrations but cell index did not decrease after 5 h,

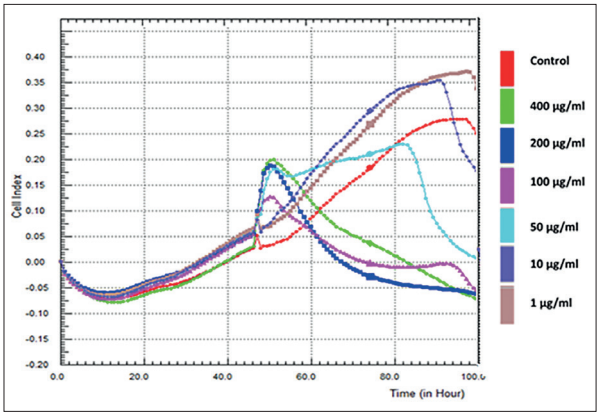

Figure 3. Cell index profiles of Colo 205 cell line treated with different concentrations of leaves extracts from *Callistemon citrinus*

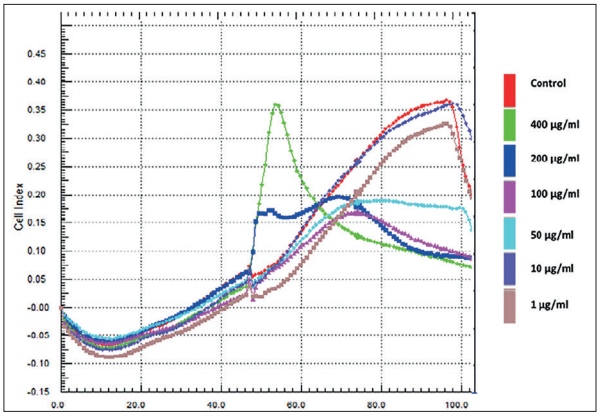

Figure 4. Cell index profiles of Colo 205 cell line treated with different concentrations of flower extracts from *Callistemon citrinus*

it showed cytostatic effect after 14 h post treatment, than it decreased cell index rapidly after about 32 h post treatment interestingly its cell response profile was similar CL. When we look at other concentrations (10, 1 µg/mL) there was no apparent difference in comparison with control (Figure 3).

Effects of *Callistemon citrinus*'s leaves and flowers on the Cell Index Alterations

All the statistical analysis were performed with two way ANOVA and groups were compared to control as presented in Table 1.The cell index alterations (CIA) of the groups that were treated with CL extract gave an idea about cell proliferation, adhesion and enlargement getting increase or decrease. Cell index alterations were calculated as difference at 6th, 12th, 24th and 48th time points compared to the first treatment point (time 0). High dose of 400, 200 and 100 µg/mL, respectively for CL extract, CIA levels decreased significantly ($p<0.05$) at 24th and 48th h in a time dependent manner (Figure 5). On the contrary, the CIA for at 50 µg/mL dose decreased but it was slower in comparison to high concentrations and only 48th h value was significant (Table 1). Control and low concentrations (10, 1 µg/mL) CIA increased depending on experimental time schedule. But only 10 µg/mL decreased CIA level at 48th h. It was an expected situation for control group because cell index of healthy cell increases due to proliferation and culture medium efficiency.

CF extract treated groups showed different CIA profiles comparing to CL. 400 and 200 µg/mL concentrations CIA was higher than control because of high cell index at 6 h but this values decreased and get lower than control at 24 h. 100 and 50 µg/mL concentrations CIA was increased slightly and consistently (Table 2). 10, 1 µg/mL concentrations were similar profiles to control (Figure 6).

Western blot results

The role of PI3K-Akt pathway in initiating cell survival and proliferation has been extensively reported in scientific literatures. Although from previous studies, a lot of substrates have been discovered for manipulating Akt activity and yet some questions are still waiting to be answered. In our study, Akt and pAkt protein expression levels were investigated to understand Akt activity. When we look at activated Akt level at 12th h for CL extracts, p-Akt levels were increased in higher concentrations particularly for 400, 200 and 100 µg/mL concentrations in a dose dependent manner but none of those increments were statistically significant ($p>0.05$). In comparison with the control, the value of p-Akt was low for 50 µg/mL dose for CL extract but did not have significant difference ($p>0.05$) (Figure 7). Results obtained from CF at 400, 200 and 100 µg/ml concentrations also increased p-Akt slightly but those increments were not significant ($p>0.05$) in comparison to the control. Akt protein expression were increased by 200 and 100 µg/mL concentrations of CL and 400

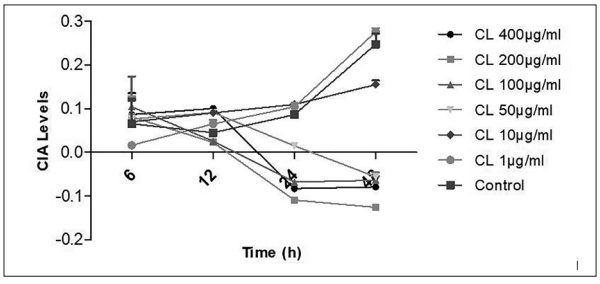

Figure 5. CIA of *Callistemon citrinus*' Leaves at 6th, 12th, 24th and 48th h compared to the first treatment time point. CIA values were given mean±SD.

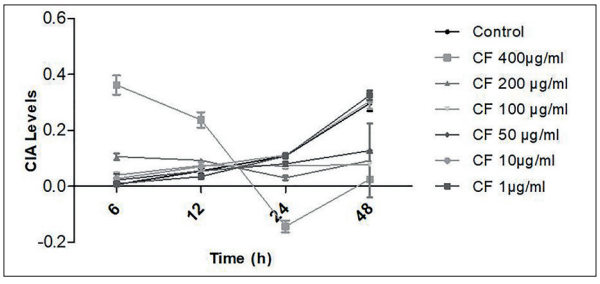

Figure 6. CIA of *Callistemon citrinus*' flowers at 6th, 12th, 24th and 48th h compared to the first treatment time point. CIA values were given mean±SD.

Table 1. CIA values of CL were analyzed comparing to control with two way ANOVA.

	6th h	12th h	24th h	48th h
CL 400 µg/mL vs Control	2.1±1.1	5.5±3.1 * p=0.0174	16.9±9.1 *** p=0.0001	32.7±17.7 *** p=0.0001
CL 200 µg/mL vs Control	1.6±0.8	2.2±1.2	19.5±10.6 *** p=0.0001	34.3±20.3 *** p=0.0001
CL 100 µg/mL vs Control	3.9±2.1	1.9±1.1	15.3±8.3 *** p=0.0001	31.1±16.9 *** p=0.0001
CL 50 µg/mL vs Control	1.1±0.5	4.6±2.5	7.09±3.8	30.3±16.5 *** p=0.0001
CL 10 µg/mL vs Control	0.4±0.2	4.5±2.4	2.3±1.2	9.2±5.1 *** p=0.0001
CL 1 µg/mL vs Control	4.9±2.6 * p=0.0456	2.1±1.1	1.4±0.9	2.8±1.5

statistically significance assigned $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Percent alterations±SD values given in the table.

Table 2. CIA values of CF were analyzed comparing to control with two way ANOVA.

	6th h	12th h	24th h	48th h
CF 400 µg/mL vs Control	35.6±7.45 *** p=0.0001	18.2±8.9 *** p=0,0001	25.1±1.2 *** p=0,0001	27.1±1.3 *** p=0,0001
CF 200 µg/mL vs Control	10.1±2.9 *** p=0.0001	3.8±1.8	7.7±0.3 ** p=0,0015	20.4±1.5 *** p=0,0001
CF 100 µg/mL vs Control	3.4±1.6	1.9±0.9	3.5±0.7	21.6±4.5 *** p=0,0001
CF 50 µg/mL vs Control	1.6±0.7	0.6±0.1	2.7±1.1	16.9±0.8 *** p=0,0001
CF 10 µg/mL vs Control	2.1±1	16.1±0.78	0.3±0.1	0.7±0.1
CF 1 µg/mL vs Control	0.3±0.1	2.1±0.2	0.2±0.1	2.8±0.6

Statistically significance assigned p<0.05 (*), p<0.01 (**), p<0.001 (***). Percent alterations±SD values given in the table.

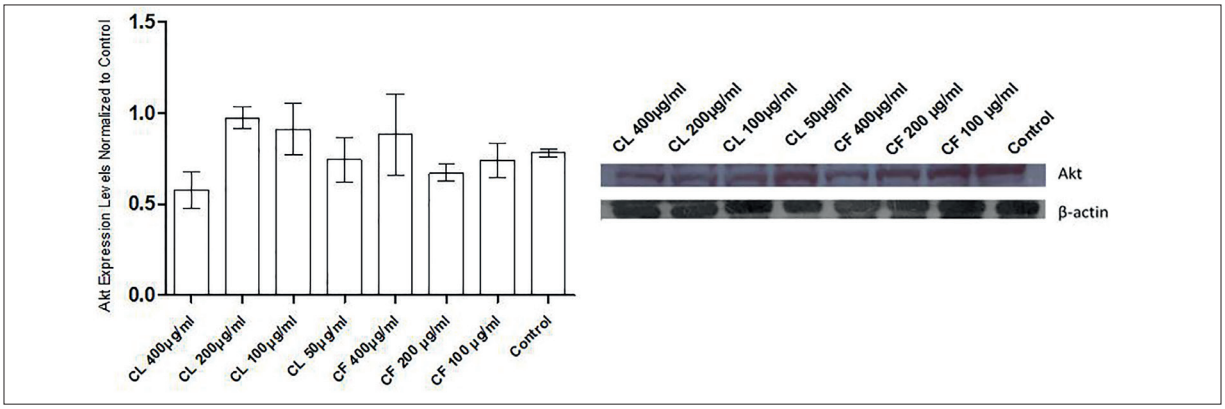

Figure 7. Expressed levels of Akt1 and β-actin by western blot analysis after 12 h treatment with flowers and leaves extract of *Callistemon citrinus*

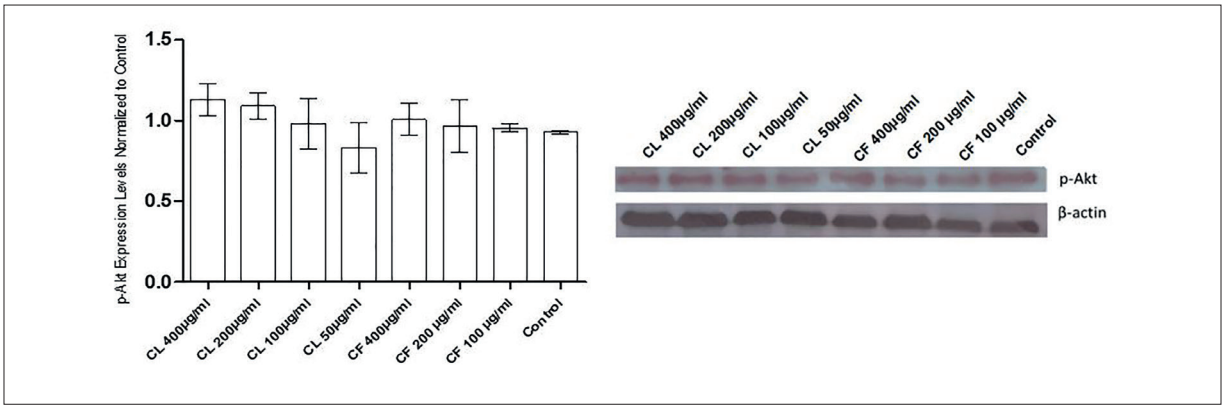

Figure 8. Expressed levels of p-Akt1 (Ser473) and β-actin by western blot analysis after 12 h treatment with flowers and leaves extract of *Callistemon citrinus*

µg/mL dose of CF. The other concentrations of CL and CF extracts decreased Akt comparing to control (Figure 8).

DISCUSSION

The aim of this study was to investigate the cytotoxic effects of CF and CL extracts on COLO-205 cell line.

Akt activity mainly plays a role in apoptosis pathway on cancer and normal physiological conditions. Therefore we investigated Akt and p-Akt levels for understanding if Akt pathway evoked or not evoked by CL and CF extracts. In this study we captured a statistically significant increment after 5 h of treatment for CL and 8 h of treatment for CF at the highest concentrations. In 2015 Kumar et al. investigated essential oils of this plant and they found that essential oils induced apoptosis on human lung cancer (A549 cell line) and rat glioma (C-6 cell line) cells. Also they reported growth inhibition effect of essential oils on Colo-205 cell line. Hydrodistillation product of *Callistemon citrinus*

contains essential oils which is response for its major effect (Kumar et al. 2015). However extraction method is so important for evaluating the effect of plant material. In addition to our results on Colo-205 cell line, this data give us an idea about extracts of CF and CL essential oils might be a hope for alternative or supplement cancer therapy in addition to conventional cancer therapy. Supporting this increase in cell proliferation, the Akt levels decreased and p-Akt levels increased at 400 µg/mL for CL, revealing that the Akt is phosphorylated triggered the cell proliferation at this dose. In addition, real time monitoring made it possible to catch relevant time points for the induction of cell proliferation. For the colon cancer cell line it was important for us to show when this cytotoxic effect starts and which dose would be the best dose for treatment. Although this increase in proliferation at high concentrations followed by a decrease, this dose was decided not to be relevant concentration for treatment, since it was triggering Akt and p-Akt pathway at the beginning. For the following concentrations 200, 100 and 50 µg/mL Akt/p-Akt both decreased reflecting this pathway inactivated at these concentrations. Our findings also supported real time monitoring results for CL extracts. For the CL extracts the IC_{50} level was 6.49 µg/mL. For the CF extracts, the real time monitoring results were approximately similar to CL results at all concentrations. CF extracts did not affect the Akt phosphorylation significantly, revealing that the action of mechanism might depend on another pathway of extract of CF. IC_{50} level for extract of CF were 5.22 µg/mL and the relevant treatment time should be at least 24 h to see its effect.

CONCLUSION

There is limited report on potential cytotoxic effect of *Callistemon citrinus* plant material. Compared to conventional endpoint cell-based assays, dynamic monitoring of cell response, such as cell adhesion, proliferation, and cell survival is one of the advantages of the xcelligence system to optimize the cell concentration for *in vitro* and *in vivo* assays and also allows both cell and assay conditions to be constantly obtained before and during the time of the experimentation.

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