Macedonian pharmaceutical bulletin, **73** (1) 11 - 16 (2027)
ISSN: 1857-8969
DOI: 10.33320/maced.pharm.bull.2027.73.01.002
Original scientific paper

Cytotoxicity and modulation of hydrogen peroxide-induced oxidative stress by *Rosmarinus officinalis* extract in differentiated SH-SY5Y neuroblastoma cells

Donika Hoxha[1], Dushko Shalabalija[1], Ljubica Mihailova[1], Filip Todorov[1], Gresa Hoxha Gashi[2], Gjoshe Stefkov[1]*

[1]*Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Majka Treza 47, 1000 Skopje, North Macedonia*
[2]*Independent researcher, Prishtina, Kosovo*

Received: June 2026; Accepted: August 2026

Abstract

Rosmarinus officinalis L. (rosemary) is a Lamiaceae species with a long history of traditional use for cognitive complaints, and its phenolic constituents are widely proposed as antioxidant agents for neurodegenerative disease. In this study a methanolic extract of *R. officinalis* leaves was characterised by LC-HRMS/MS, its cytotoxic profile was established in retinoic acid-differentiated SH-SY5Y neuroblastoma cells by the MTT assay, and its effect on hydrogen peroxide-induced intracellular reactive oxygen species was then measured by the DCFH-DA assay within the range shown to be non-cytotoxic. The extract was cytotoxic above 50 µg/mL, with viability falling from 84.7% of control at 50 µg/mL to 39.4% at 100 µg/mL, whereas the 5 to 20 µg/mL range left viability unaffected at both 24 and 48 h. Within that non-cytotoxic range the extract reduced the DCFH-DA signal in a concentration-dependent manner, from 26.65 to 39.34% at 10 µg/mL and from 36.07 to 57.63% at 20 µg/mL, with the signal building to a maximum at 90 min rather than declining. At 100 µg/mL and above the response inverted: fluorescence in treated cells exceeded that of the peroxide-only control, reaching −108.6% at 500 µg/mL. These findings indicate that the extract attenuates H_2O_2-associated intracellular oxidative stress within concentrations that were non-cytotoxic under the tested conditions, and that the same extract shifts to a net pro-oxidant reading once that threshold is crossed, so that the concentration range tested determines the direction of the result obtained.

Key words: *Rosmarinus officinalis*, cytotoxicity, oxidative stress, SH-SY5Y, reactive oxygen species, neuroprotection

Introduction

Oxidative stress is a well-established contributor to the pathogenesis of Alzheimer's disease and other neurodegenerative conditions, arising from an imbalance between reactive oxygen species (ROS) production and the capacity of neuronal antioxidant defences to neutralise them (Liu et al., 2017). Polyphenol-rich plant extracts, and Lamiaceae species in particular, are widely investigated as candidate sources of antioxidant agents capable of mitigating this oxidative burden. A recent comprehensive review of preclinical and clinical studies on rosemary confirmed that its neuroprotective effects span multiple mechanisms, including cholinesterase inhibition and modulation of oxidative and inflammatory pathways, with efficacy reported across H_2O_2-, scopolamine-, and amyloid-beta-induced models of neuronal injury, although the same review noted that clinical evidence remains comparatively limited (Oresanya and Orhan, 2024).

Rosmarinus officinalis L. is among the most extensively studied Lamiaceae species in this context. Its

*email: gost@ff.ukim.edu.mk

leaves are rich in the phenolic diterpenes carnosic acid and carnosol, whose radical-scavenging activity has been attributed to the ortho-dihydroxyl group at C11 and C12 and which exceed the antioxidant potency of the synthetic antioxidants BHT and BHA in several lipid systems (Birtic et al., 2015). Rosmarinic acid, present at lower concentrations, inhibits acetylcholinesterase and exerts cholinergic and neuroprotective effects (Andrade et al., 2018), and binds cupric ions so as to limit copper-catalysed ROS generation, with whole rosemary extract proving more effective than isolated rosmarinic acid in this respect, including in the presence of amyloid-beta (Kola et al., 2024). In the same cell model used here, a methanolic *R. officinalis* extract has been shown to suppress H_2O_2-induced cytotoxicity, mitochondrial membrane potential disruption and apoptotic markers in SH-SY5Y cells (Park et al., 2010), while carnosic acid acts through Nrf2-dependent induction of endogenous antioxidant defences in the same line (de Oliveira et al., 2018).

What is much less often established is the concentration range over which such measurements can be believed. A cell-based antioxidant readout taken at a concentration that is itself killing the cells reports the death of the culture as much as the chemistry of the extract, and plant extracts are known to interfere directly with colorimetric viability reagents, giving false-positive viability where the cells are in fact dead (Karakaş et al., 2017). The present study therefore reversed the usual order of work. The extract was first characterised chemically, its cytotoxic profile was then established across a broad concentration range, and only afterwards was its effect on H_2O_2-induced ROS measured, at concentrations the MTT data had shown to be non-cytotoxic. The results show that the extract reduces the DCFH-DA-derived oxidative signal below its cytotoxic threshold, that this reduction builds rather than decays over two hours, and that above the threshold the same extract yields a net pro-oxidant reading.

Material and methods

Plant material and extract preparation

Rosmarinus officinalis L. leaves (Rosmarini folium concisum) were obtained as a packaged herbal drug from Alkaloid AD Skopje, North Macedonia. The material was visually inspected for impurities and foreign matter, ground to a fine powder in a laboratory grinder, and kept at room temperature in dry, dark conditions until extraction. Because the material was supplied as a pharmacopoeial herbal drug of documented botanical identity, no voucher specimen was deposited. Extraction was carried out in two successive hot steps under continuous magnetic stirring: 10 g of powdered leaves were combined with 150 mL of analytical-grade methanol (100%) and heated with stirring for 2 h; the mixture was filtered and the residue re-extracted with 100 mL of fresh methanol under the same conditions for a further 2 h. The combined filtrates were concentrated to dryness on a rotary evaporator at 40 °C under reduced pressure, pre-frozen at −80 °C for 1 to 2 h and freeze-dried for 24 h. The dry extract was weighed gravimetrically, giving a yield of 26.8% (2.68 g from 10 g of plant material), and stored at −20 °C in the dark until use. For the cell-based assays the lyophilised extract was dissolved in dimethyl sulfoxide (DMSO; Carlo Erba, Italy) to a stock concentration of 10 mg/mL, from which working solutions were obtained by serial dilution in low-serum culture medium immediately prior to use.

Phytochemical characterisation of the extract

The polyphenolic profile of the extract was determined by LC-HRMS/MS on a Thermo Scientific Vanquish Core HPLC system interfaced with an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Separation was achieved on a Hypersil GOLD C18 column (50 × 2.1 mm, 1.9 µm) at 300 µL/min with an injection volume of 5 µL, using ultrapure water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B): 5% B for 1 min, a linear ramp to 95% B over minutes 1 to 10, isocratic at 95% B until minute 12, and a return to starting conditions by minute 15. Detection was in negative electrospray ionisation mode, with full scan spectra from m/z 100 to 1500 at 60,000 FWHM and data-dependent MS^2 spectra at 15,000 FWHM by collision-induced dissociation at 35% normalised collision energy. Compounds were identified from accurate mass, MS^2 fragmentation and retention data, and quantified against external calibration curves of ten authentic standards, each remaining compound being assigned the standard of its structural class. Analyses were performed in triplicate (n = 3) and concentrations are reported as mg per gram of extract dry weight. Because most compounds were quantified against a surrogate standard, the values are semi-quantitative and are best interpreted as relative abundances.

Cell culture and differentiation

SH-SY5Y human neuroblastoma cells were cultured in 1:1 (*v/v*) MEM/Ham's F-12 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mM L-glutamine at 37 °C under 5% CO_2. Cells were differentiated to a post-mitotic neuronal phenotype using a 10-day retinoic acid protocol (10 µM all-trans retinoic acid in low-serum medium), with medium renewal every three days, prior to use in cytotoxicity and oxidative stress assays.

MTT cytotoxicity assay

Differentiated cells were seeded in 96-well plates and allowed to adhere for 48 h, then treated with extract at 5, 10, 20, 50, 100, 200 or 500 µg/mL for 24 h, with the 5 to 20 µg/mL range also assessed at 48 h. Viability was assessed by MTT reduction: cells were incubated with MTT solution (5 mg/mL in PBS) for 3 h at 37 °C in the dark, formazan crystals were dissolved in DMSO, and

absorbance was read at 570 nm. Viability was expressed as a percentage of untreated control. Each condition was measured in four replicate wells (n = 4).

DCFH-DA intracellular ROS assay

Based on the cytotoxic threshold identified by the MTT assay, modulation of intracellular ROS was assessed only at concentrations confirmed to be non-cytotoxic (5, 10, and 20 μg/mL). Differentiated cells seeded in black 96-well plates were loaded with DCFH-DA in serum-free medium for 30–45 min at 37 °C in the dark, then washed. Extract working solutions and H_2O_2 (100 μM final) were added simultaneously, and fluorescence was recorded at 30, 45, 60, 90, and 120 min (excitation 485 nm, emission 528–535 nm). Two control conditions were included on every plate: wells receiving H_2O_2 without extract, which served as the positive control and defined the maximal oxidative signal, and wells receiving medium only, which defined the baseline signal of unchallenged cells. Antioxidant activity was calculated relative to the H_2O_2-only positive control, blank-corrected, using the formula: % antioxidant activity = [(F(H_2O_2) − F(sample)) / (F(H_2O_2) − F(untreated))] × 100. Each condition was measured in four replicate wells (n = 4).

Statistical analysis

Cell viability is expressed as a percentage of the untreated control and reported as the mean of four replicate wells, with SD given where available. The DCFH-DA results are reported as mean percentage inhibition (n = 4). The data are descriptive and no inferential statistical analysis was performed.

Results and discussion

Qualitative composition of the extract

The two-step methanolic extraction gave a yield of 26.8% (2.68 g from 10 g of dried leaves). LC-HRMS/MS screening of the extract identified rosmarinic acid, sagerinic acid, isorhamnetin hexoside, acacetin and a feruloylquinic acid isomer among the hydroxycinnamic acid and flavonoid fractions, together with the phenolic diterpenes carnosol and carnosic acid, two rosmadial isomers and rosmanol. The diterpenes were detected but not quantified, since no authentic standards for this class were available, so any attribution of the activities described below to the diterpene fraction is qualitative. The full quantitative phenolic characterisation of this and the other Lamiaceae extracts obtained under the same conditions will be reported separately.

Cytotoxicity in differentiated SH-SY5Y cells

The concentration range over which the extract is cytotoxic was established first. Over the 50 to 500 μg/mL range, viability of retinoic acid-differentiated SH-SY5Y cells fell from 84.7% of the untreated control at 50 μg/mL to 39.4% at 100 μg/mL and remained below 26% at 200 and 500 μg/mL (19.8% and 25.2% respectively; Table 1), so that viability crossed 50% between 50 and 100 μg/mL. Over the lower range subsequently used for the DCFH-DA experiment, the extract was inert: viability at 24 h was 102.1 ± 45.8% at 5 μg/mL, 85.9 ± 10.2% at 10 μg/mL and 95.5 ± 39.0% at 20 μg/mL. The same held after 48 h, so cytotoxicity did not accumulate with prolonged exposure at these concentrations. The 5 to 20 μg/mL range was therefore taken as the working range in which a cell-based antioxidant readout can be interpreted, and 100 μg/mL and above as a range in which cytotoxic and antioxidant effects cannot be separated.

Modulation of hydrogen peroxide-induced ROS at non-cytotoxic concentrations

Within the non-cytotoxic range, the cells were then challenged with hydrogen peroxide. At 5 μg/mL the extract produced no reduction of the DCFH-DA signal above the intra-assay baseline at any timepoint. At 10 μg/mL the reduction rose from 26.65% at 30 min to a maximum of 39.34% at 90 min, and at 20 μg/mL from 36.07% to 57.63% over the same interval, falling back only slightly by 120 min (Table 2, Fig 1). The effect was therefore both concentration-dependent and, within the range tested, threshold-like: a fourfold difference in concentration separated no measurable effect from a reduction of more than half the peroxide-induced signal.

The shape of the time course is worth noting. A finite pool of directly reacting antioxidant would be expected to act early and then be consumed, giving a signal that is maximal at the first reading and declines thereafter. The opposite was observed: at both active concentrations the reduction increased steadily over the first 90 minutes and was still close to its maximum at 120 min. Carnosic acid and carnosol, both detected in this extract though not quantified, are established direct scavengers (Birtic et al., 2015), and a directly reacting component is likely to contribute. A slower-acting contribution is also plausible,

Table 1. Cell viability (% of untreated control; n = 4) of differentiated SH-SY5Y cells treated with *Rosmarinus officinalis* extract at 50, 100, 200 and 500 μg/mL for 24 h (MTT assay)

Concentration (μg/mL)	50	100	200	500
Viability (% of control)	84.7	39.4	19.8	25.2

Table 2. Reduction of the H_2O_2-induced DCFH-DA signal (%, relative to the H_2O_2-only control) for *Rosmarinus officinalis* extract at 5, 10 and 20 µg/mL over 30–120 min in retinoic acid-differentiated SH-SY5Y cells. Values are mean (n = 4)

Conc. (µg/mL)	30 min	45 min	60 min	90 min	120 min
5	0.0*	0.0*	0.0*	0.0*	0.0*
10	26.65	31.71	36.65	39.34	36.92
20	36.07	43.83	52.09	57.63	55.05

*No activity detected above intra-assay baseline.

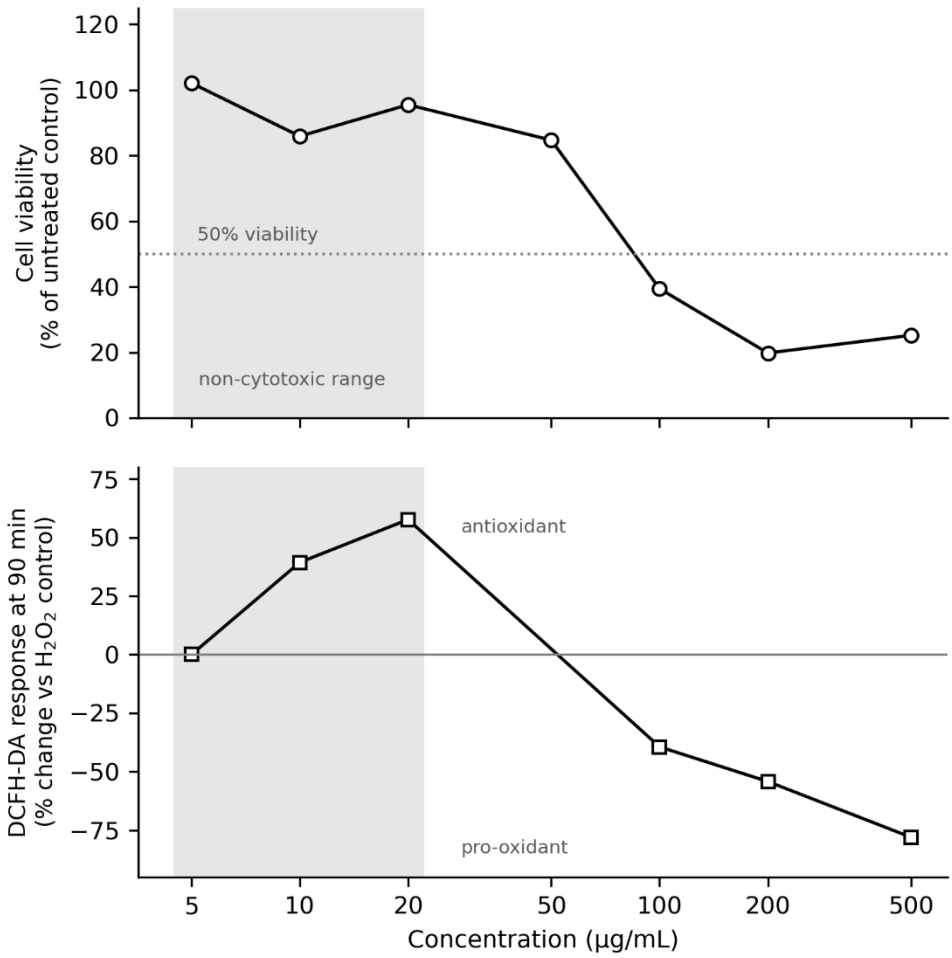

Fig. 1. Concentration dependence of the two responses in retinoic acid-differentiated SH-SY5Y cells treated with *Rosmarinus officinalis* extract. Upper panel, cell viability after 24 h as a percentage of the untreated control; the dotted line marks 50% viability. Lower panel, the DCFH-DA response at 90 min expressed as the percentage change relative to the H_2O_2-only control, positive values indicating attenuation and negative values a net pro-oxidant reading. The shaded band marks the non-cytotoxic range used for the DCFH-DA experiment. Values are mean (n = 4).

since carnosic acid pretreatment suppresses H_2O_2-induced mitochondrial bioenergetic disturbance and redox impairment in this same cell line through an Nrf2-dependent mechanism (de Oliveira et al., 2018), and induction of endogenous antioxidant enzymes operates on a slower timescale than chemical scavenging. Nrf2 activation and enzyme induction were not measured here, so this remains an interpretation of the time course rather than a demonstration of its mechanism.

When the same assay was run at 100, 200 and 500

μg/mL, the direction of the result reversed. DCF fluorescence in extract-treated cells exceeded that of the peroxide-only control at all three concentrations, so that the calculated antioxidant activity took negative values, a negative result denoting fluorescence above that of the control rather than below it: −5.7, −5.8 and −11.1% at 30 min and −54.7, −77.0 and −108.6% at 120 min for 100, 200 and 500 μg/mL respectively. At −108.6% the signal was more than twice that of the peroxide-only control. These are the same concentrations at which viability had fallen below 40%, so the readings combine any genuine pro-oxidant chemistry with the loss of cell integrity and should be read as the direction of the redox shift rather than as a quantitative pro-oxidant effect in living neurons. The practical point stands regardless of which of the two dominates: the concentration range chosen determines the sign of the result obtained from this extract.

Rosemary is among the most frequently cited plant antioxidants, and its activity is routinely attributed to carnosic acid and carnosol. The composition of the present extract complicates that attribution. Both diterpenes were detected, but so were rosmarinic and sagerinic acid and several flavonoids, and the diterpene fraction could not be quantified against an authentic standard. Rosmarinic acid is itself a strong candidate for the activity observed, given that it binds cupric ions and limits copper-catalysed ROS generation, and that whole rosemary extract outperforms isolated rosmarinic acid in that setting, which implies a contribution from other constituents as well (Kola et al., 2024). What the data support is that the extract as a whole lowers the peroxide-induced signal at low concentrations; which constituent does so is not resolved here.

The biphasic pattern observed here, antioxidant below the cytotoxic threshold and pro-oxidant above it, is the more consequential finding. It is consistent with the hormetic behaviour described for polyphenol-rich extracts, in which the same reducing capacity that scavenges radicals at low concentration generates reactive quinone intermediates in excess. It also means that a study reporting only high concentrations and a study reporting only low ones would describe the same extract in opposite terms, and that neither would be wrong about its own data. This is not a hypothetical concern: methanolic plant extracts have been shown to interfere with the MTT reagent itself, yielding false-positive viability at concentrations where the cells had in fact died (Karakaş et al., 2017), so a viability control that is not itself verified can place an antioxidant measurement in the wrong range without any of it being visible in the data.

These observations are consistent with earlier work in this cell line, in which *R. officinalis* extract suppressed H_2O_2-induced cytotoxicity, mitochondrial membrane potential disruption and apoptotic signalling (Park et al., 2010), and with the wider evidence consolidated for rosemary across preclinical oxidative and amyloidogenic injury models (Oresanya and Orhan, 2024). What the present data add is the boundary: the concentration above which those effects can no longer be measured in living neurons.

Conclusion

The present study shows that a methanolic *R. officinalis* extract attenuates H_2O_2-associated intracellular oxidative stress in retinoic acid-differentiated SH-SY5Y cells at 10 and 20 μg/mL, concentrations at which viability was unaffected at both 24 and 48 h, and that the reduction of the DCFH-DA signal builds to a maximum at 90 min rather than decaying. Above 50 μg/mL the extract is cytotoxic, and at 100 μg/mL and above the same assay returns a net pro-oxidant reading. The concentration range in which the extract can be described as an antioxidant is therefore narrow, and establishing that range before interpreting any cell-based readout is a prerequisite rather than a precaution. Further work with independent biological repeats, an extract-only probe control and a reference antioxidant is needed to place the size of the effect on a comparable scale.

Acknowledgement

The authors thank the Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, for access to laboratory facilities and instrumentation.

References

Andrade, J.M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C.P., Rijo, P., 2018. Rosmarinus officinalis L.: an update review of its phytochemistry and biological activity. Future Sci. OA 4, FSO283. https://doi.org/10.4155/fsoa-2017-0124

Birtic, S., Dussort, P., Pierre, F.X., Bily, A.C., Roller, M., 2015. Carnosic acid. Phytochemistry 115, 9–19. https://doi.org/10.1016/j.phytochem.2014.12.026

de Oliveira, M.R., da Costa Ferreira, G., Peres, A., Dal Bosco, S.M., 2018. Carnosic acid suppresses the H_2O_2-induced mitochondria-related bioenergetics disturbances and redox impairment in SH-SY5Y cells: role for Nrf2. Mol. Neurobiol. 55, 968–979. https://doi.org/10.1007/s12035-016-0372-7

Karakaş, D., Arı, F., Ulukaya, E., 2017. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. Turk. J. Biol. 41, 919–925. https://doi.org/10.3906/biy-1703-104

Kola, A., Vigni, G., Lamponi, S., Valensin, D., 2024. Protective contribution of rosmarinic acid in rosemary extract against copper-induced oxidative stress. Antioxidants 13, 1419. https://doi.org/10.3390/antiox13111419

Liu, Z., Zhou, T., Ziegler, A.C., Dimitrion, P., Zuo, L., 2017. Oxidative stress in neurodegenerative diseases: from molecular mechanisms to clinical applications. Oxid. Med. Cell. Longev. 2017, 2525967. https://doi.org/10.1155/2017/2525967

Oresanya, I.O., Orhan, I.E., 2024. Deciphering neuroprotective effect of Rosmarinus officinalis L. (syn. Salvia rosmarinus

Spenn.) through preclinical and clinical studies. Curr. Drug Targets 25, 330–352. https://doi.org/10.2174/0113894501255093240117092328

Park, S.E., Kim, S., Sapkota, K., Kim, S.J., 2010. Neuroprotective effect of Rosmarinus officinalis extract on human dopaminergic cell line, SH-SY5Y. Cell. Mol. Neurobiol. 30, 759–767. https://doi.org/10.1007/s10571-010-9502-3

Резиме

Испитување на цитотоксичноста и модулацијата на оксидативниот стрес предизвикан од водород пероксид при третман со екстракт од *Rosmarinus officinalis* во диференцирани SH-SY5Y клетки

Доника Хоџа[1], Душко Шалабалија[1], Љубица Михаилова[1], Филип Тодоров[1], Греса Хоџа Гаши[2], Ѓоше Стефков[1]*

[1]Фармацевтски факултет, Универзитет „Св. Кирил и Методиј", Мајка Тереза 47, 1000 Скопје, Северна Македонија
[2]Независен истражувач, Приштина, Косово

Клучни зборови: *Rosmarinus officinalis*, цитотоксичност, оксидативен стрес, SH-SY5Y, реактивни кислородни видови; невропротекција

Rosmarinus officinalis L. (рузмарин) е вид од фамилијата Lamiaceae со долга историја на традиционална употреба при когнитивни заболувања, а неговите фенолни компоненти широко се предлагаат како антиоксидативни агенси во невродегенеративните болести. Во ова истражување метанолниот екстракт од листовите на *R. officinalis* беше карактеризиран со LC-HRMS/MS, неговиот цитотоксичен профил беше утврден на диференцирани SH-SY5Y невробластомни клетки со помош MTT тестот, а потоа неговото дејство врз интрацелуларните реактивни кислородни видови предизвикани од водород пероксид беше измерено со DCFH-DA тестот, во опсегот кој претходно се покажа како безбеден за употреба врз клетките. Екстрактот беше цитотоксичен во концентрации над 50 μg/mL, при што вијабилноста опадна од 84,7% при 50 μg/mL на 39,4% при 100 μg/mL, додека опсегот од 5 до 20 μg/mL не влијаеше врз виталноста ниту по 24 ниту по 48 часа. Во тој нецитотоксичен опсег екстрактот го намали DCFH-DA сигналот на концентрациски зависен начин, од 26,65 до 39,34% при 10 μg/mL и од 36,07 до 57,63% при 20 μg/mL, при што сигналот растеше до максимум дури и после 90 минути. При концентрации повисоки од 100 μg/mL, одговорот се промени во спротивна насока: флуоресценцијата во третираните клетки ја надмина вредноста измерена кај контролата третирана само со H_2O_2, при што при концентрација од 500 μg/mL беше регистрирана вредност од −108,6%. Овие резултати укажуваат дека екстрактот го намалува интрацелуларниот оксидативен стрес предизвикан од H_2O_2 при концентрации кои, во условите на испитувањето, не покажуваат цитотоксичен ефект. Сепак, при надминување на одреден концентрациски праг, екстрактот преминува кон нето-прооксидативен ефект. Оттука, концентрацијата на екстрактот претставува значаен фактор кој ја определува насоката и интензитетот на измерениот биолошки одговор.