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Biological effect of natural chitosan nanoparticles with transformed roots extract of *Leonotis nepetifolia* (L.) R.Br. in an in vitro model

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ABSTRACT

Nanotechnology is a rapidly growing field of science that has a considerable impact on many branches of medicine. Nanoparticles are developing at a rapid pace due to their effect which is achieved by combining them with drugs as well as their increased importance in the drug delivery system. Synthesis of chitosan nanoparticles by ionic gelation is becoming increasingly important due to its low cost and environmentally friendly approach. This preliminary study was the first one to investigate the cytotoxic effect of chitosan nanoparticles with combination of *Leonotis nepetifolia* (L.) R.Br. transformed root extract against cervical cancer (HeLa), breast cancer (MCF-7), glioma cancer (MO59J) cell lines and normal human fibroblast (Hs68) cell line. Besides, a preliminary analysis of apoptosis induced by caspase 3/7, microscopic observations of morphology changes and apoptosis-related genes expression in cancer cells have been showed. According to MTT results, the synthesized chitosan nanoparticles in combination with *Leonotis nepetifolia* normal and transformed root extracts showed a stronger cytotoxic effect on all tested cancer lines (HeLa, MCF-7 and MO59J), compared with the nanoparticles and the extracts alone, with $IC_{50} = 0.608, 0.306, 0.112$ mg/mL, respectively. In contrast, no cytotoxic effect was shown against the human fibroblast (Hs68) line in the tested concentration range. Preliminary induction of apoptosis showed changes in cell morphology, genes expression and caspase 3/7 activation after treatment with chitosan nanoparticles in combination with normal and transformed from *Leonotis nepetifolia* root extracts. These discoveries have positive implications for further studies on development of nanoparticles in anti-cancer therapy. The key objective of this work is to test the anti-cancer potential of *L. nepetifolia* transformed root extract obtained by agrotransformation in combination with natural chitosan nanoparticles as a component capable of enhancing the effect of the extract against various in vitro models.

1. Introduction

Combination of biotechnology and nanotechnology seems to be a promising approach to improve treatment of diseases (Alghamdi et al., 2022; Kher and Kumar, 2022). Our hypothesis is to enhance the

biological effect of a biotechnology-derived plant extract by using natural nanoparticles. Currently, nanometer-sized particles are preferred. A natural polymer, called chitosan is used in one type of nanoparticles. Chitosan nanoparticles (ChNPs) in particular have been widely adopted in the medical field due to their cationic properties, electrostatic

Abbreviations: Bax, apoptosis regulator BAX; Bcl-2, apoptosis regulator BCL2; Casp-3, Caspase-3; Casp-8, Caspase-8; Casp-9, Caspase-9; ChNPs, chitosan nanoparticles; DAPI, 4',6-diamidino-2-phenylindole; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NPs, nanoparticles; NR, normal root; PBS, Phosphate-buffered saline; TP53, Tumor protein P53; TR, transformed root.

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interactions, biodegradability and biocompatibility. Numerous studies demonstrate a positive therapeutic effect of ChNPs combined with plant-derived compounds in in vitro and in vivo studies (Jiménez-Gómez and Cecilia, 2020; Mohammed, 2017). Moreover, ChNPs combined with pure plant-derived compounds may serve as a complement to synthetic drug treatment by improving uptake across cell membranes and reducing toxicity (Bonifácio et al., 2014). Therefore, an efficient phytochemical-nanoparticle system could be an interesting solution for providing low-cost therapy, especially if plant cultures are obtained in vitro and are a reservoir of important secondary metabolites. Highly productive cultures of primarily transformed roots may be obtained by being infected with the bacterium *Rhizobium rhizogenes*. These roots are rich in valuable phytochemicals that not only allow us to obtain higher quantities in a short period of time and at lower production costs, but also do not cause environmental degradation, which is extremely important from an ecological point of view (Gutierrez-Valdes et al., 2020).

Leonotis nepetifolia (L.) R.Br. (Lamiaceae), also known as "Lion's Ear", is a widely prevalent species in India and has been traditionally used for medicinal purposes in numerous countries across the world. This plant is a rich source of secondary metabolites belonging to various classes. Numerous studies report multidirectional biological effects, such as, anti-rheumatic, analgesic, anti-diabetic, antioxidant, anti-cancer, anti-inflammatory or supportive in difficult-to-heal wounds (de de Menezes Barbosa et al., 2018; Tidke et al., 2021). Our previous research showed that the *L. nepetifolia* transformed roots extract demonstrates a stronger cytotoxic effect in various cancer cell lines compared with non-transformed roots (Kowalczyk et al., 2021a).

However, there are few studies on nanoparticles with transformed root extracts. This one is the first on *L. nepetifolia* species. Therefore, the goal of this preliminary study was to examine the biological effect of ChNPs combined with extracts obtained from both normal (NR) and transformed (TR) roots of *L. nepetifolia* in in vitro investigations. The synthesis and characteristic of ChNPs are presented. Cytotoxic effects, morphological changes, activation of caspase 3/7 and apoptosis-related genes expression are evaluated. Fig. 1.

2. Materials and methods

2.1. Preparation of chitosan nanoparticles (ChNPs)

The following materials were used: chitosan (Primex, Chitoclear fg 95, average $M_w \sim 234.55$ kDa, DD=86.8%, 99.17 cP viscosity measured for 1% water solution in 20 °C, Iceland), acetic acid (Avantor, 80%, pure, Poland), Sodium tripolyphosphate pentabasic-TPP (Sigma-

Aldrich-Merck, purum p.a., $\geq 98.0\%$, Cat. No.: 72061), Polysorbate 80 – Tween 80 (Lach:ner, s.r.o, Czech Republic), sodium chloride (Chempur, pure, Poland), distilled water.

ChNPs were prepared based on the ionotropic gelation method developed by Calvo et al. (1997) and Porras-Gómez et al. (2018) with some modifications. Briefly, chitosan was dissolved at 0.5% w/w in 0.5% v/w acid acetic solution. TPP was dissolved in distilled water to obtain a 0.5% w/w concentration. Tween 80 was dissolved in distilled water to obtain a 1.25% w/w concentration (chitosan dry weight: Tween 80 wt ratio such as 1:1.12). 50 mL of Tween 80 solution (as a surfactant) was added into 100 mL of chitosan solution. The mixture was stirred vigorously at room temperature for 10 min and later 25 mL of the TPP solution was added drop-wise to the chitosan/Tween 80 solution and homogenized for 1 h. The final suspension was collected by centrifugation (12,000 rpm for 20 min) and then the sample was washed twice with saline solution and centrifuged under the same conditions.

2.2. Size analysis and characteristic of ChNPs

Mean diameter and polydispersity index (PDI) of the nanoparticles were analyzed by the Zetasizer Nano ZS (Malvern, UK) instrument. Measurements were made at 25 °C in water.

In order to determine the particle size and PDI values, 1 mL of sample was placed in the ZEN0040 cuvette (Malvern, UK), and the results were measured using the dynamic light scattering (DLS) technique. In order to analyze the influence of nanoparticle aggregation on the size and polydispersion, measurements were carried out for nanoparticles solution after 10 min preliminary sonification. Sample has been measured 3×11 times and the results were analyzed by Malvern Instruments Ltd. software.

2.3. ChNPs morphology

The surface structure and shape of the obtained ChNPs was analyzed using microphotography obtained by means of a scanning electron microscope-SEM (FEI, USA, Quanta 200 model, with the Q150R S vacuum sputtering machine) in 12000x and 100000x magnification.

2.4. Plant materials and extract preparation from NR and TR roots derived from *L. nepetifolia*

The study utilized two sets of extracts obtained from the roots of *Leonotis nepetifolia*, namely normal (NR) and transformed by *Rhizobium rhizogenes* (TR). Seeds from which in vitro plant cultures were initiated, were obtained from the Botanical Garden of the Technical University

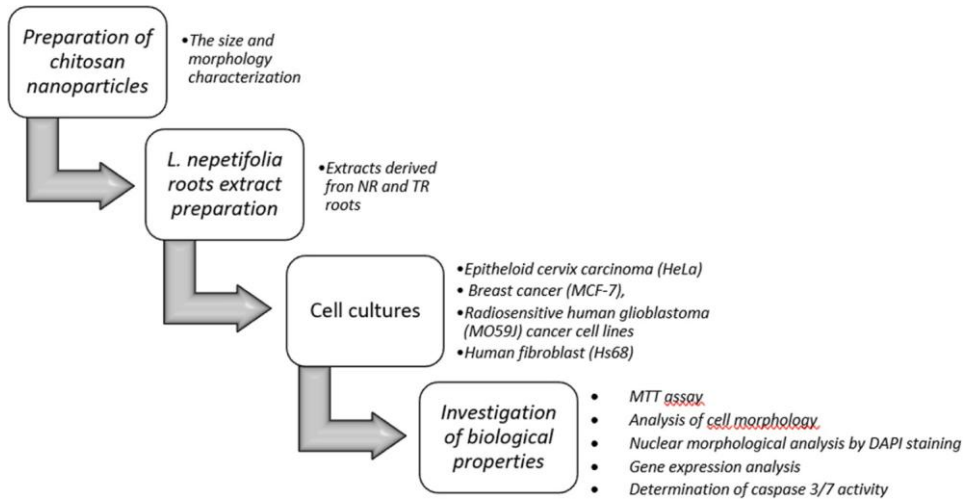


Fig. 1. Diagram summarising experimental procedures.

Braunschweig, Germany. The botanical identity of plants was confirmed by Przemysław Sitarek according to the International Plant Name Index (IPNI) using <http://powo.science.kew.org/>. The plant materials were lyophilized and ground into powder, followed by extraction with 80% (v/v) aqueous methanol. The extraction process involved the use of an ultrasonic bath at 35 °C for 15 min, after which the solvent was replaced and the extraction repeated twice more with 300 mL of the same solvent for 15 min. Additional details about the extraction method can be found in a previous study by Kowalczyk et al. (2021a) (see Supplementary material).

2.5. Preparation of chitosan nanoparticles in combination with NR or TR root extract from *L. nepetifolia*

A mixture of nanoparticles with NR or TR root extracts was obtained by incorporating the extract into the outer side of the nanoparticle matrix. An extract (NR or TR root) was added to the nanoparticles. The final concentrations of TR or NR root extracts as well as ChNPs concentrations were 2.5 mg/mL. After thorough mixing, the mixture was left for 30 min at room temperature. A series of dilutions in saline (the concentration range: 0–2.5 mg/mL), was then prepared from the above mixture for further biological studies.

2.6. Cell culture

Epitheloid cervix carcinoma (HeLa), breast cancer (MCF-7), radio-sensitive human glioblastoma (M059J) cancer cell lines and human fibroblast (Hs68) cell line was obtained from the American Type Culture (ATCC; Manassas, VA, USA). The cell lines were cultured in EMEM, DMEM-F-12 and DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Biowest, Nuaille, France). DMEM/F-12 was additionally supplemented with 1% (v/v) MEM Non-essential amino acids solution (Sigma Aldrich, Saint Louis, MO, USA). The cell cultures were maintained at 37 °C in a humidified incubator in 95% air and 50 mL/L CO₂.

2.7. MTT assay

Cell viability was determined by the MTT assay after treatment with increasing concentrations of ChNPs, NR extract, TR extract, NR+ ChNPs and TR + ChNPs for 24 h. This colorimetric assay is based on reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) to purple formazan crystals by metabolically active cells (van Meerloo et al., 2011). Approximately 10000 cells/well were seeded into 96-well plates for 16 h (overnight) before treated with ChNPs alone, ChNPs with NR root extract and ChNPs with TR root extract of *L. nepetifolia* and NR and TR root extracts alone within the range of concentrations (0–2.5 mg/mL). The cells were cultured for 24 h (37 °C, 5% CO₂). After 24 h of incubation, the serum-containing media was replaced with serum-free media 100 µL and 10 µL of MTT solution (5 mg/mL), followed by incubation at 37 °C for 4 h. Then the media was replaced with DMSO (100 µL) in each well, and the absorbance was determined at 570 nm using a Bio-Tek Synergy HT Microplate Reader. Data are expressed as a % of viability when compared with untreated cells considered as 100% of viability.

2.8. Observations of cell morphology

After treatments with ChNPs alone and in combination with TR root extract of *L. nepetifolia* cancer cells and at IC₅₀ concentrations were removed from a CO₂ incubator and observed under an inverted microscope (Leica DM IL LED Fluor; Leica Microsystems, Wetzlar, Germany) to assess cell morphology and each sample was observed at 200 magnification.

2.9. Nuclear morphological analysis by DAPI staining

The morphology of the cell nuclei was evaluated by fluorescence microscopy after DAPI staining. Hs68, M059J, MCF-7 and HeLa cancer cells with ChNPs alone and in combination with TR extract from *L. nepetifolia* were treated for 24 h. Next, cancer cells were washed with PBS (pH 7.4), fixed with ice-cold 70% ethanol and resuspended in DAPI, then incubated for 15 min at 37°C wrapped in aluminum foil. The cells were then washed with PBS and examined under the control of an Olympus fluorescence microscope (BX60, Olympus, Tokyo, Japan).

2.10. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

In order to evaluate mRNA expression levels of the main apoptotic genes *BCL2*, *BAX*, *TP53*, *CASP3*, *CASP8* and *CASP9*, one-step RT-qPCR was performed. The total RNA from control and treated cells was extracted using Total RNA Mini (A&A Biotechnology, Gdansk, Poland) following the manufacturer's protocol. Relative gene expression was performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with TranScriba 1step PCR Mix Probe (A&A Biotechnology) and TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): Hs00608023_m1 (*BCL2*), Hs00180269_m1 (*BAX*), Hs00153349_m1 (*TP53*), Hs00234387_m1 (*CASP3*), Hs01018151_m1 (*CASP8*) and Hs00962278_m1 (*CASP9*). 18 S rRNA (Hs99999901_s1) was served as an endogenous control. The RT-qPCR protocol consisted of reverse transcription performed at 50 °C for 10 min and initial denaturation carried out at 95 °C for 3 min. Subsequently, 40 cycles of denaturation at 95 °C for 30 s and 60 °C annealing/extension for 1 min were performed. The final volume of each reaction was 10 µL. Obtained data were analyzed with CFX Maestro Software 2.3 for Windows PC (Bio-Rad). The relative expression (fold change) of target mRNA was measured using the 2^{-ΔΔCT} methodology (Schmittgen and Livak, 2008).

2.11. Determination of caspase 3/7 activity

Briefly, like in our previous studies (Sitarek et al., 2022), also in this study. we assessed caspase-3/7 activity using CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen™). This assay provides a fluorogenic substrate for activated caspases 3 and/or 7 and consists of four-amino acid peptide (DEVD), conjugated to nucleic acid binding dye (nonfluorescent in live cells). If caspases 3 and/or 7 are active (in apoptotic cells), the substrate is cleaved and a fluorogenic response will be emitted (Timmer and Salvesen, 2007). After IC₅₀ treatment with and without NR and TR extracts/ChNPs, cells were labeled with caspase-3/7 Green Detection Reagent. Caspase activity was determined after 30 min of incubation at 37 °C in the dark with 5 µM Green Detection Reagent by measuring fluorescence intensity of the cells (the excitation wavelength = 502 nm and the emission wavelength = 530 nm) using a Bio-Tek Synergy HT microplate reader. Each sample was run in triplicate.

2.12. Statistical analysis

All the data were analyzed using Prism v. 5.00 for Windows (GraphPadSoftware, Inc., San Diego, CA, USA). For all the measurements, one-way ANOVA followed by Tukey's test was used to assess the statistical significance between groups. $p \leq 0.05$ was considered to be statistically significant.

3. Results and discussion

At present, a very interesting topic in the field of nanomedicine is nanoparticles (NPs). NPs can be categorized into various classes depending on their characteristic, shape or size. There are numerous types of nanoparticles, including, but not limited to carbon-based NPs,

metal NPs, ceramics NPs, semiconductor NPs, lipid-based NPs, and polymeric NPs and they are used as potential drug carriers (Bommakanti et al., 2022; De Jong and Borm, 2008; Rizvi and Saleh, 2018). Studies show that they can enhance the effects of drugs (Khiev et al., 2021; Mitchell et al., 2021; Patra et al., 2018). Furthermore, their distinctive benefits such as drug protection from degradation, targeted drug delivery, and reduced toxicity and side effects make them a promising alternative to conventional dosage forms, particularly in the treatment of various diseases, including cancer. The purpose of our preliminary study is to test for the first time the cytotoxic effects of ChNPs in combination with plant extracts from *in vitro* cultures of *Leonotis nepetifolia* transformed roots in various cancer cell lines. Natural polymers that are used to prepare nanoparticles include heparin, dextran, albumin, gelatin, alginate or chitosan. Due to its ideal properties, chitosan as a natural polymer has become an object of intensive research. Chitosan-based nanoparticles are mainly characterized by biocompatibility, biodegradability, non-toxicity, cost-effective, and environmentally friendly. Most nanoparticles are insoluble in water, while chitosan (e.g. in the form of acetic or lactic acid salt) is one of the most studied water-soluble polymers (Bashir et al., 2022; Bommakanti et al., 2022; Mohammed et al., 2017; Nitta and Numata, 2013). Chitosan, a linear polysaccharide, is derived from the deacetylation of chitin, a structural component found in the exoskeleton of crustaceans. It is regarded as the second most abundant polysaccharide after cellulose, along with chitin (Elieh-Ali-Komi and Hamblin, 2016). ChNPs have the capability of being utilized as a drug delivery system to target cancer therapy and can be administered via oral or intravenous means to host cells (Herdiana et al., 2021; Mohammed et al., 2017). Numerous studies show that ChNPs alone or in combination with plant-derived material (extracts and pure compounds) or drugs (chemotherapeutics) can work more effectively in cancer therapy by enhancing the therapeutic effect. A natural bioactive compound derived from plants may serve as a promising chemopreventive agent, offering enhanced efficacy and fewer side effects for patients (Antunes et al., 2021; Wei et al., 2019). The degradation of ChNPs is a critical factor that affects the pharmacokinetic profile of ChNPs. This is due to factors such as sustained effects, subcellular size, and the biocompatibility of biodegradation products with human tissues (Mahapatro and Singh, 2011).

In addition, the induction of apoptosis by caspase 3/7 activity and the observation of morphological changes of cells after treatment of the same extracts with nanoparticles will be preliminarily checked.

3.1. Characteristic of nanoparticles

ChNPs were first described in 1994 by Ohya et al. (2014) and since then several methods have been used to synthesize nanoparticles. Currently, five methods are used to obtain ChNPs: ionic gelation,

polyelectrolyte complex method, microemulsion method, emulsification solvent diffusion method, and coprecipitation method (Loo et al., 2022). The most common method of obtaining nanoparticles is ionotropic gelation (Divya and Jisha, 2017). For research purposes, ChNPs were produced based on the ion gelation method developed by Calvo et al. (1997) and then refined by Porras-Gómez et al. (2018) as a result of the reaction between positively charged amino groups of chitosan and negatively charged TPP groups. The method was additionally modified by adding a surfactant - Tween 80 to the chitosan acetate solution. Sukmawati et al. (2018) proved that the addition of Tween 80 during the production process by ion gelation allows to obtain the desired size of nanoparticles (nano-scale). It has also been proven that increasing the concentration of Tween 80 allows for obtaining smaller ChNPs. Additionally, ultrasound was used to form nanoparticles. It has been shown that the use of sonication methods leads to a reduction in the mean diameter of the particles and their polydispersity, but they did not affect the zeta potential (Tang et al., 2003). The shape and morphology of ChNPs were observed by scanning electron microscopy (SEM; Fig. 2). The shape and morphology of ChNPs observed by scanning electron microscopy as shown in Fig. 2 were almost spherical and uniform in shape.

The hydrodynamic diameter in water and the corresponding polydispersity index (PDI) of the nanoparticles were observed by the Dynamic Light Scattering (DLS) method, and the results are presented in Table 1.

The average diameter of tested nanoparticles in the sample was 414.7 nm. A high PDI polydispersity index implies the presence of nanoparticles of various sizes. The ChNPs fraction was observed with an average diameter of 40.4; 70.7; 231.3; 307.7 and 679.9 nm. The initial sonification of the tested sample allows to obtain optimized nanoparticle size and polydispersion, which suggests formation of small nanoparticle agglomerates in the aqueous environment. The most numerous fractions of nanoparticles are ChNPs with an average diameter of 70.7 and 679.9 nm, as shown in Fig. 3.

3.2. Phytochemical analysis

Our previous study of the tested root extracts from *L. nepetifolia* revealed the presence of phenols and flavonoids such as: catechol,

Table 1

Particle size and polydispersity index of synthesized ChNPs (with initial sonification for 10 min).

Sample	Sonification (min)	Size (nm)	PDI
ChNPs	10 min	414.7	0.505

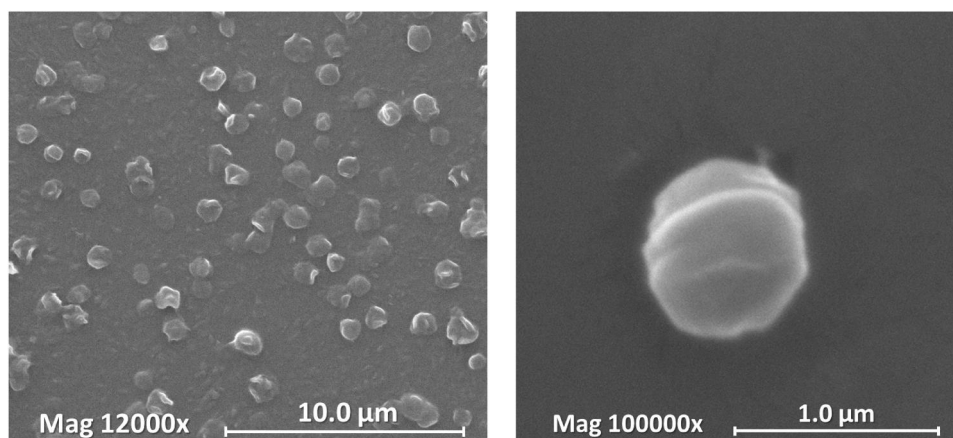


Fig. 2. SEM pictures of ChNPs at 12000x and 100000x magnification.

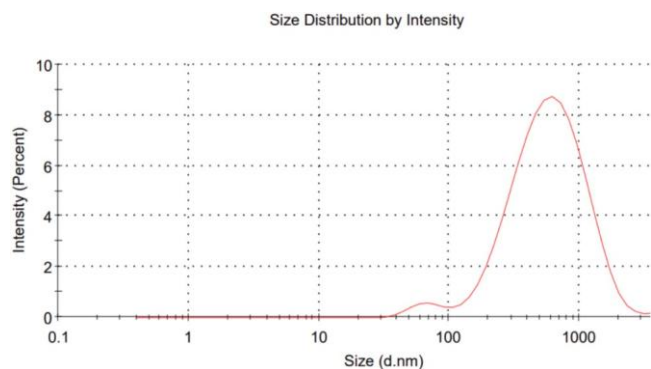


Fig. 3. Dependence of the intensity on the size of nanoparticles in tested ChNPs sample.

hydroquinone, chlorogenic acid, gallic acid, α -resorcinic acid, protocatechuic acid, m-coumaric acid, o-coumaric acid, (+)-catechin, 4-hydroxybenzoic acid, gentisic acid, coumarin, rutin, ellagic acid, hesperidin, p-coumaric acid, rosmarinic acid, sinapic acid with different contents for NR and TR extracts. Some of the most dominant compounds were (+)-catechin (5.46 and 6.80 mg/g DW), p-coumaric acid (2.54 and 4.90 mg/g DW), m-coumaric acid (1.50 and 2.04 mg/g DW) and rosmarinic acid (1.84 and 2.64 mg/g DW) for NR and TR root extracts, respectively. Numerous studies confirm that *Rhizobium rhizogenes* transformation can not only be a source of higher accumulation of secondary metabolites but also the extracts obtained in this way showed more potent biological properties. Our study also confirms these data

and the TR extract obtained by this way also showed better properties in an in vitro model (Kowalczyk et al., 2021a).

3.3. Cytotoxic effect of chitosan nanoparticles in combination with NR and TR root extracts from *L. nepetifolia*

The MTT assay is a dependable, sensitive, and quantitative colorimetric method used to assess cell viability. This assay relies on the capacity of the cellular enzyme mitochondrial dehydrogenase in living cells to reduce the yellow, water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble deep blue/purple formazan product (Buranaamnuay, 2021). The MTT cytotoxicity test after treatment with ChNPs and extracts or the combination of extracts with ChNPs was carried out on different cell lines (Fig. 4). The results showed no cytotoxic effect after treatment with NR and TR extracts from *L. nepetifolia* roots and ChNPs in the tested concentration range on human fibroblast (Hs68). In turn, for the three tested cancer cell lines (M059J, MCF-7 and HeLa) different cytotoxicity were demonstrated (Fig. 4). The most sensitive to the effects of both NR and TR extracts with combination of ChNPs was the glioblastoma cell line (M059J) with inhibitory concentration values $IC_{50} = 0.176$ and 0.112 mg/mL, but stronger cytotoxic effect was observed for the combination of ChNPs and TR extract. A similar effect has been shown for the breast cancer cell line (MCF-7). The combination of ChNPs /NR or TR extracts generated higher cytotoxicity than single extracts with $IC_{50} = 0.434$ and 0.306 mg/mL (Fig. 4). The HeLa cancer cell line was the least sensitive to both ChNPs and extracts. In the tested concentration range (0–2.5 mg/mL), the IC_{50} was not achieved for both ChNPs and the tested NR and TR root extracts from *L. nepetifolia*. The combination of

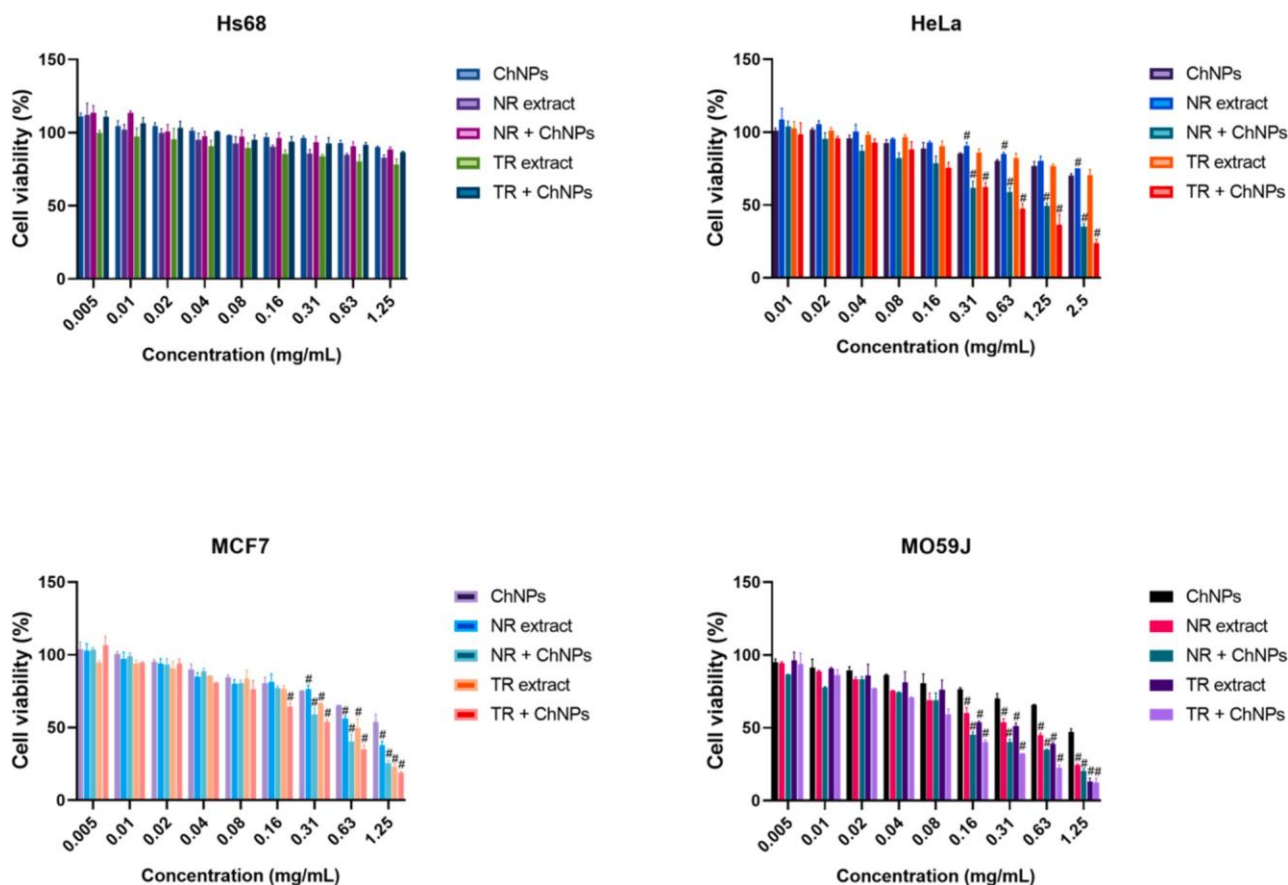


Fig. 4. Effects of ChNPs, NR extract, TR extract, NR+ ChNPs and TR + ChNPs on the viability of Hs68, HeLa, MCF-7 and M059J cells, as determined by the MTT assay. Values represent the means \pm SD as percentage (%) of control values. Statistical analysis was performed by one way analysis of variance with all pairwise multiple comparison procedures done by Dunnet's test; $^{\#}p < 0.05$ vs. ChNPs.

extracts with ChNPs enhanced the cytotoxic effect and allowed to generate the IC₅₀ concentration (1.01 and 0.608 mg/mL, respectively). In each case, the enhancement of the cytotoxic effect for all tested cancer cell lines was demonstrated in the combination of ChNPs with extracts, while the TR root extract with ChNPs was more toxic (Fig. 4).

In research conducted by Asiri et al. (2019), it was found that various forms of ChNPs exhibited a dose-dependent reduction in the proliferation of colon cancer (HCT-116) cells. At a lower dose of 2 mg/mL, no noteworthy impact on cancer cell survival was observed, whereas higher doses of 4 mg/mL, 6 mg/mL, and 8 mg/mL resulted in significant cell death. In turn, Qi et al. (2005a), (2007) showed that ChNPs exhibit a cytotoxic effect against liver (BEL7402), gastric (BGC823) and colon (Colo320) cancer cell lines with IC₅₀ = 15, IC₅₀ = 21 and IC₅₀ = 28 µg/mL, respectively, while showing no cytotoxic effect on normal human liver (L-02) cells in the range of concentrations tested (0–100 µg/mL). The aforementioned authors also observed that ChNPs significantly impeded cell proliferation in gastric cancer cells (MGC803), displaying an IC₅₀ value of 5.3 µg/mL after 48 h of treatment (Qi et al., 2005b). Conversely, Loufty et al. (2017) and Yao et al. (2013) illustrated the cytotoxic impact of ChNPs on the liver cancer cell line (HepG2), resulting in IC₅₀ values of 239 µg/mL and 36 µg/mL, respectively. The examples presented here are consistent with our results and confirm the cytotoxic effect of ChNPs in various cancer cell lines with no cytotoxic effect against normal cell lines. The literature reports that ChNPs acquire a positive charge due to the presence of active amine groups in their composition. The potential toxicity of these cationic nanoparticles may be attributed to their electrostatic interaction with the negatively charged cell membrane. Additionally, their small size may enable them to infiltrate the cell membrane, ultimately causing cell death (Kumar et al., 2004; Rabea et al., 2003).

In the second stage of the study, we tested whether the combination of ChNPs with extracts from normal (NR) and transformed (TR) roots of *L. nepetifolia* could enhance the cytotoxic effect in HeLa, MCF-7 and M059J cancer cell lines. Our study showed that the combination of ChNPs with the extracts significantly enhances the cytotoxic effect in the tested cancer lines. This effect may be attributed to the presence of nanoparticles, which facilitate improved delivery of these extracts through cell membranes. The IC₅₀ was 0.608, 0.306, 0.112 mg/mL for the HeLa, MCF-7 and M059J cancer cell lines, respectively. It is noteworthy that for the HeLa cancer cell line, the IC₅₀ was achieved only after the *L. nepetifolia* root extract was combined with ChNPs in the tested concentration range. This treatment confirms the effectiveness of the combination, manifesting with enhanced action of these agents (Fig. 4). Our study is consistent with the literature and confirms the enhancing effect of such a combination compared with extracts or nanoparticles alone. Rajivgandhi et al. (2020) demonstrated that ChNPs enhanced the cytotoxic effect of *Morinda citrifolia* (L.) essential oil against A549 cells, achieving 54% inhibition at 40 µg/mL. Similarly, Amalina et al. (2021) found that chitosan-*Citrus aurantium* (L.) peel extract nanoparticles (NPs-CA) had a particle size-dependent cytotoxic effect against MDA-MB-231 breast cancer cells, with IC₅₀ values of 83 µg/mL and 320 µg/mL. Fadholly et al., (2019, 2020) showed a stronger cytotoxic effect of *Annona squamosa* (L.) leaf extract-loaded ChNPs against human colon cancer (WiDr) and cervical cancer (HeLa) cells, with IC₅₀ values of 292.39 µg/mL and 344.48 µg/mL, respectively. Sujima Anbu et al. (2016) also exhibited dose-dependent cytotoxicity against the cervical SiHa cancer cell line with ChNPs containing *Gymnema sylvestris* (R.Br) leaves extract and ChNPs with *Cinnamomum zeylanicum* (J.Presl) bark extract, with IC₅₀ values of 87.75 µg/mL and 90.35 µg/mL, respectively. Similarly, Proboningrat et al. (2019) demonstrated that chitosan-based *Pinus merkusii* (Jung, et de Vriese) bark extract nanoparticles (Nano-PMBE) were cytotoxic to cervical cancer cell line (HeLa) with an IC₅₀ of 384.10 µg/mL. In other studies, Sahyon and Al-Harbi (2020) demonstrated cytotoxic effects of ChNPs with *Phoenix dactylifera* (L.) extract against breast (MCF-7), colon (CaCo3) and cervical (Hela) cancer cell lines with IC₅₀ = 8.16, 17.67 and

7.27 µg/mL, respectively. Hesami et al. (2022) reported dose-dependent effects on a breast cancer cell line (MCF-7) with IC₅₀ values for *Chelidonium majus* (L.) essential oil root ChNPs and *Chelidonium majus* (L.) essential oil leaf ChNPs of 77.6 and 41.5 µg/mL, respectively. Alipanah et al. (2022) found that ChNPs containing *Zataria multiflora* (Boiss.) essential oil exhibited cytotoxic effects against human melanoma (A-375) and breast cancer cell lines (MCF-7 and MDA-MB-468) with IC₅₀ values of 32, 46, and 105 µg/mL, respectively. On the other hand, Shahiwala et al. (2019) demonstrated that an extract from the *Indigofera intricata* (Boiss.) possessed the lowest breast cancer cell viability (MCF-7) of 45.21% at the highest dose of 250 mg/mL. In contrast, a 500-fold reduction from 250 mg to 0.5 mg of extract concentration required to achieve the same proliferative activity of cancer cells for the extract with ChNPs was observed. Finally, Soltani et al. (2022) showed that ChNPs with *Boswellia sacra* (Flueck.) essential oil exhibited selective cytotoxicity on HepG2 cells with IC₅₀ = 62.94 µg/mL. The examples cited in the discussion clearly show the enhancement of the cytotoxic effect after the combination of nanoparticles with plant extracts in an in vitro model of various cancer cell lines. In addition, our study showed for the first time a cytotoxic effect for *L. nepetifolia* root extract on HeLa, MCF-7 and M059J cancer cell lines when combined with ChNPs, with a stronger effect observed for the transformed extract (TR), which may be due to the fact of the higher content of active compounds in the tested extract.

3.4. Cell morphology by inverted microscope and after staining with DAPI after TR treatment with *L. nepetifolia* root extract in combination with chitosan nanoparticles

Due to the better activity of the TR extract in cytotoxic study, it was used for the analysis morphological observations. Microscopic observation after treatment with ChNPs and TR root extract from *L. nepetifolia* and chitosan nanoparticle-essentials combinations after 24 h of exposure showed morphological changes in cancer cells (HeLa, MCF-7 and M059J). Fig. 5 shows that HeLa, MCF-7 and M059J cells treated with ChNPs and TR root extract and nanoparticles/extracts combinations at IC₅₀ concentrations changed into round shapes compared with untreated cells (Fig. 5). No significant changes in morphology were demonstrated in normal Hs68 cells after treatment with the test compounds (Fig. 5).

To further characterize cell death induced by TR root extract from *L. nepetifolia* in combination with ChNPs, nucleus morphology was determined by staining with the DNA-binding dye DAPI, in the presence and absence of extract and ChNPs. DAPI is a nuclear stain that is observed as blue fluorescence when excited by fluorescence microscopy. Due to the better activity of the TR extract in cytotoxic study, it was used for the analysis with the DAPI dye. In our study, DAPI staining revealed nuclear morphological changes and irregular shape, at IC₅₀ concentrations in HeLa, MCF-7 and M059J cells treated with TR root extract from *L. nepetifolia* and ChNPs in combination with TR extract, while untreated cells appeared normal (Fig. 6).

Elderbery et al. (2022a) reported that human leukemic (MOLT4) cells treated with CuO-TiO₂-chitosan-escin nanocomposites exhibited higher levels of DAPI and smaller nuclear areas. Furthermore, Chang et al. (2021) observed significant apoptotic cell death in Triple Negative Breast Cancer (TNBC) cells after treatment with rutin-chitosan nanoconjugates, as evidenced by DAPI fluorescence micrographic analysis. Similar results were obtained for a cervical carcinoma cell line, following a treatment with curcumin-loaded ChNPs (Khan et al., 2016). Our findings are consistent with reports noted in literature. Therefore, we suggest that the method of cell death, induced by the tested extract with ChNPs may in turn induce the process of apoptosis, for which the active compounds contained in the extract may be responsible.

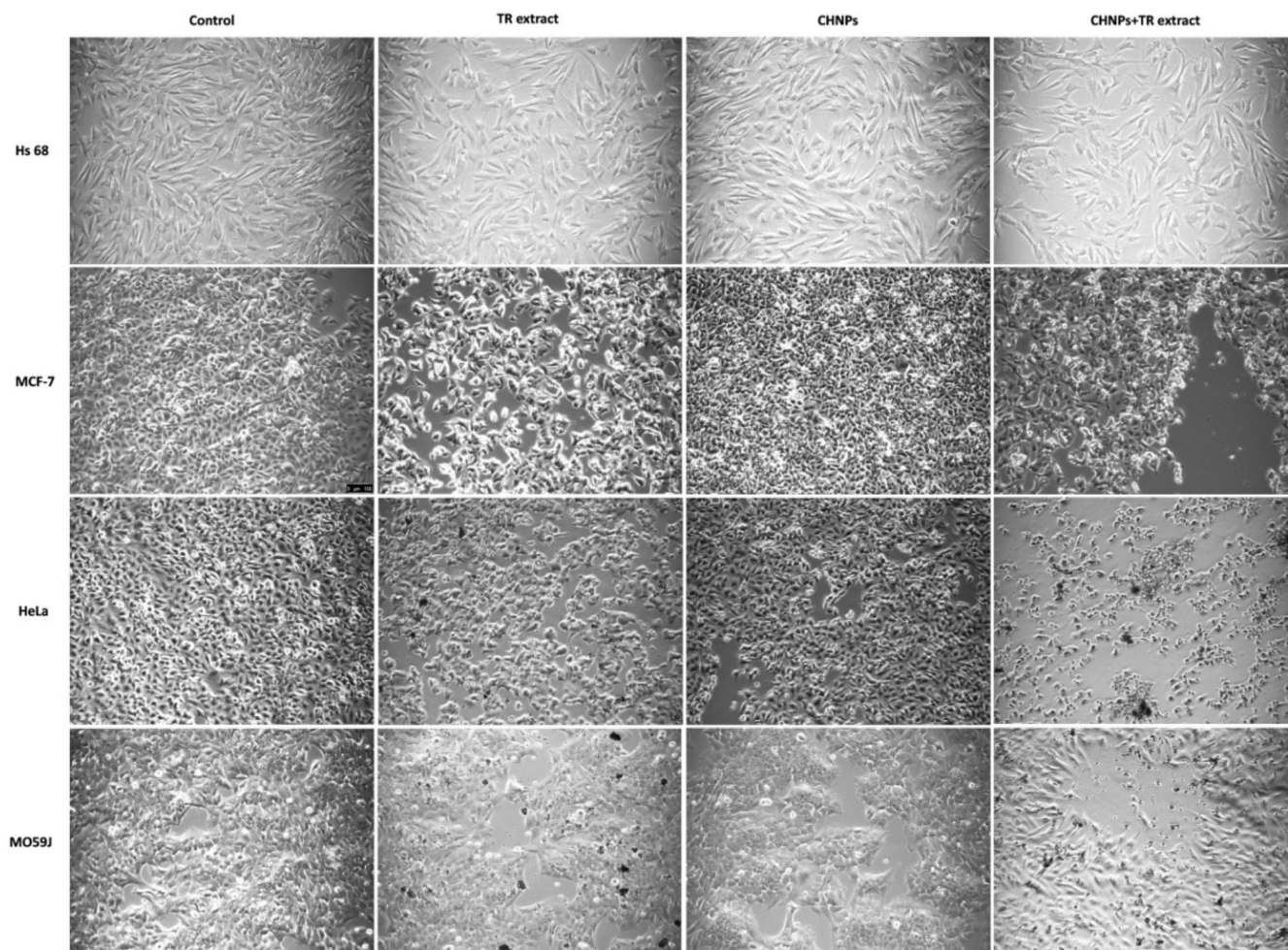


Fig. 5. Morphological observation of Hs68, MCF-7, HeLa and M059J cells (200 ×): Control group; ChNPs, TR extract, and TR + ChNPs after 24 h treatment with IC₅₀. The cell morphology was observed under the light microscope. The most representative fields are shown.

3.5. Genes expression analysis

In this study, the expression of apoptotic genes was checked in breast cancer (MCF-7), glioma (M059J) and cervical cancer (HeLa) cell lines. The cancer cells were treated with ChNPs alone and in combination with *L. nepetifolia* TR and NR root extracts at IC₅₀ concentration. Our results showed a change in the expression of *Bax*, *Bcl-2*, *TP53*, *Casp-3*, *Casp-8* and *Casp-9* apoptotic genes in the tested cell lines with a stronger effect observed in the combination of ChNPs with TR root extract which may indicate enhancement of the biological effect (Fig. 7A, B, C). Our study is consistent with Sahyon et al. (2023) who showed that avocado peel extract with ChNPs showed significantly higher levels of p53 and Bax expression in lung cancer cells, as shown in our study, this effect was stronger for the combination of the extract with ChNPs. Similar results were presented by Haghighi et al. (2020) who showed that encapsulation of *Artemisia scoparia* extract in a chitosan nanogel was more effective and induced apoptosis in a hepatocellular carcinoma cell line (Huh-7) with up-regulation of *Bax* and *miR192* genes along with suppression of *Bcl-2*, *MMP2*, *MMP9* and cyclin D1 than the extract alone. Asghar et al. (2023), on the other hand, showed that *Olive europaea* phenolic extract with ChNPs more strongly increases the Bax/Bcl-2 ratio, a sign of intrinsic apoptosis in liver cancer cells (HepG2). The cited examples show according to our research, that the combination of ChNPs with extracts can enhance the expression of apoptotic genes in various types of cancer and may be a promising candidate for future therapies.

3.6. Caspase 3/7 activity

Caspases are a family of cysteine-aspartate proteases which are important mediators of apoptotic signaling (Julien and Wells, 2017). Caspase 3/7 activity in cells is a direct factor in determining caspase-dependent apoptosis. Caspases 3 and 7 are well-known executive so-called (effector) caspases that play an important role in the execution phase of intrinsic and extrinsic pathways by cleaving many cellular proteins, leading to cell death (Elmore, 2007; Brentnall et al., 2013).

In order to investigate the mechanism of cell death induced by ChNPs as well as NR and TR root extracts from *L. nepetifolia*, we assessed their impact on caspase 3/7 activity. Caspase 3/7 activity is a widely employed technique for detecting apoptosis. Our results showed that the combinations of NR and TR root extracts and ChNPs showed a better effect than the extracts alone by increasing the activation of caspase 3/7 for the tested cell lines (HeLa, MCF-7 and M059J) after 24 h at IC₅₀ concentration, with the best effect observed for the M059J cancer cell line (Fig. 8).

This is the first study of this type showing the effect of NR and TR *L. nepetifolia* root extracts in combination with ChNPs on the activity of caspase 3/7, which is one of the activators of the apoptosis process. Sujima Anbu et al. (2016) demonstrate that extracts of *Gymnema sylvestre* leaves and *Cinnamomum zeylanicum* bark-loaded chitosan nanoparticles induced apoptosis in human cervical cancer (SiHa) cells as evidenced by caspase-3 and -9-activation. Lazer et al. (2022) indicate

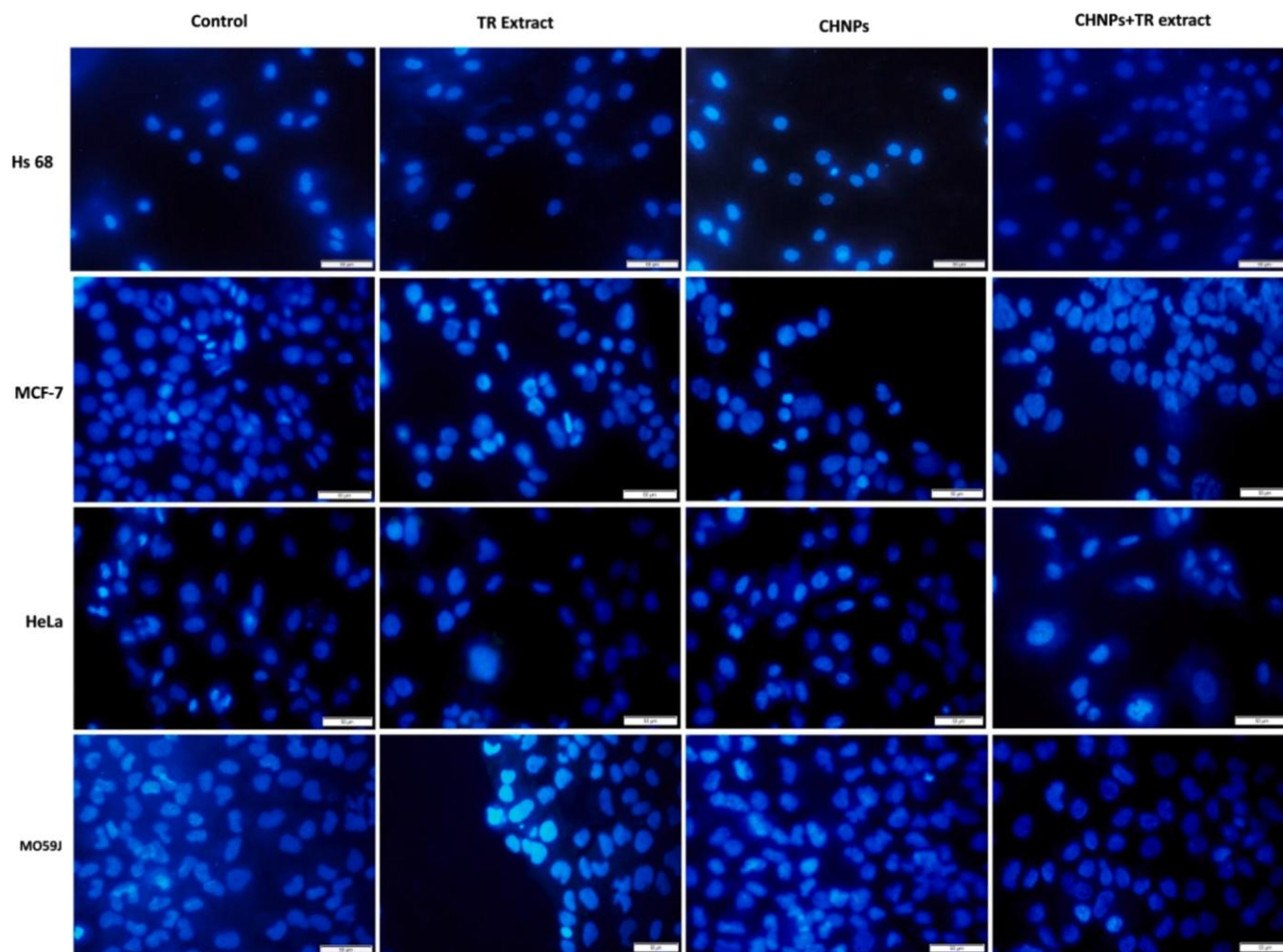


Fig. 6. Nuclear staining using DAPI of Hs68, MCF-7, HeLa and M059J cells (200 ×): Control group; ChNPs, TR extract, and TR + ChNPs after 24 h treatment with IC₅₀. The most representative fields are shown.

that caspase-3/7 assay has shown increased apoptosis in colon cancer (HCT116) cells after treatment with chitosan-folic acid-hesperetin nanoparticles. In addition, Elderderly et al. (2022b) indicate that CuO-TiO₂-chitosan-berbamine nanocomposites significantly elevated caspase-3, 7, 8, and 9 activities in the human chronic myelogenous leukemia (K562) cell line. Observed changes may be a promising indication for further research aimed at exploring potential mechanisms of action of ChNPs combined with phytochemicals.

The preliminary studies obtained in the course of the research show the potential of using nanotechnology and plant biotechnology in the development of new, more effective forms of drug delivery to cells. In addition, the natural origin of nanoparticles and extracts lowers production costs and reduces side effects for patients, which is extremely important in the treatment of many diseases.

4. Conclusions

Based on our preliminary studies, it can be concluded that for the tested NR and TR *Leonotis nepetifolia* root extract in combination with chitosan nanoparticles, the cytotoxic effect was stronger than for each separately using the MTT test, the TR extract with nanoparticles was the strongest, which may be related to the higher content of bioactive metabolites contained in this extract. In turn, morphological changes, apoptosis-related genes expression and caspase 3/7 activity, being the initial activators of apoptosis, were observed. More importantly, our results suggest that chitosan nanoparticles in combination with *Leonotis*

nepetifolia root extracts have the potential for further analyzes that may result in the development of interesting therapies in the fight against various diseases in the future.

CRedit authorship contribution statement

Tomasz Kowalczyk: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing, Visualization. **Monika Owczarek:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Ewelina Synowiec:** Formal analysis, Data curation, Methodology, Resources, Software, Writing – review & editing. **Anna Merez-Sadowska:** Formal analysis, Investigation, Writing – review & editing. **Laurent Picot:** Investigation, Writing – review & editing. **Tomasz Śliwiński:** Project administration, Supervision. **Przemysław Sitarek:** Conceptualization, Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

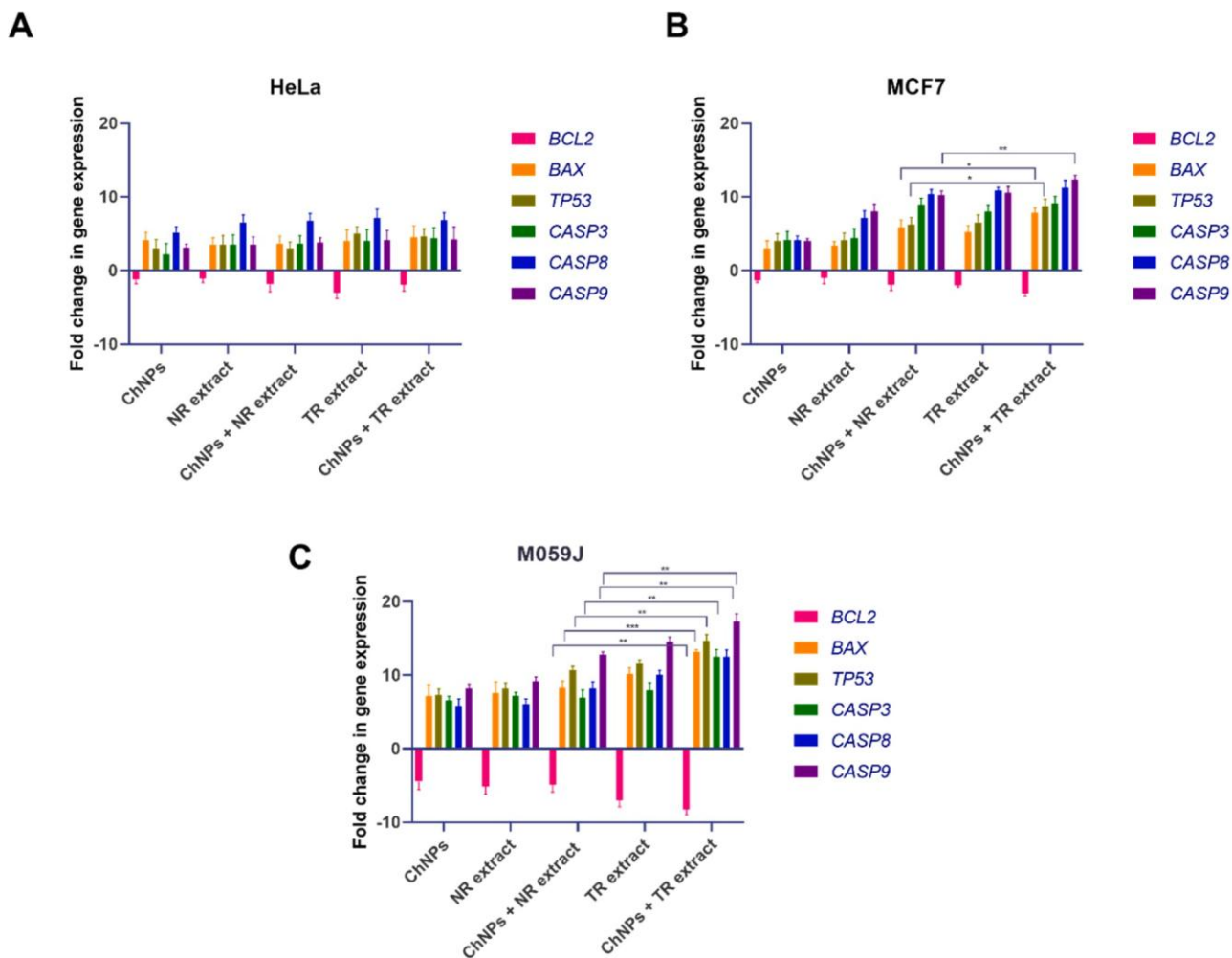


Fig. 7. Effect of NR and TR extracts/ChNPs on mRNA expression levels of *BCL2*, *BAX*, *TP53*, *CASP3*, *CASP8* and *CASP9* genes in HeLa (A), MCF7 (B) and M059J cells (C). Relative expression (Fold change due to treatment) of target mRNA was determined using the $2^{-\Delta\Delta CT}$ methodology. mRNA level of each gene is normalized to 18 S rRNA level. Results are presented as mean \pm SD (n = 3). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control cells - significant differences between ChNPs + NR extract treated and ChNPs + TR extract treated cells.

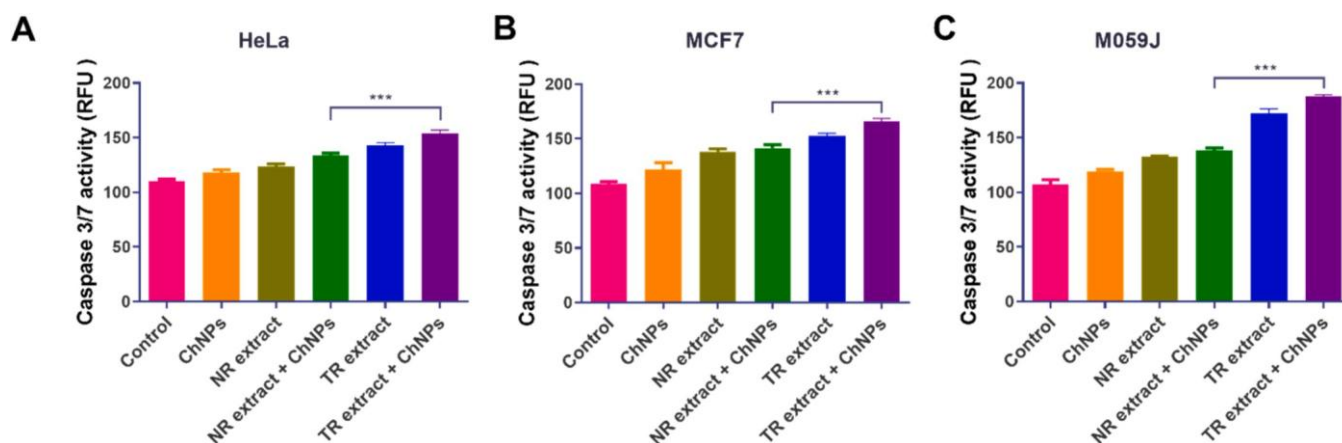


Fig. 8. The activity of caspase 3/7 after 24 h treatment (with IC_{50}) with CHNPs, NR extract, TR extract, NR+ ChNPs and TR + ChNPs on HeLa (A), MCF-7 (B) and M059J (C) cancer cell lines *** p < 0.001 - NR extract + ChNPs vs. TR extract + ChNPs.

Data availability

Data will be made available on request.

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