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Nanoformulated rosemary extract impact on oral cancer: in vitro study

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Abstract

Background: Treating cancer is challenging for both the patient and the physician. This may be explained by the fact that treatment regimens rely on either chemotherapy alone or a combination with radiotherapy. Both modalities have damaging side effects that add to the patient suffer. This inconvenience from the patient part has led scientists to search for discovering new era in cancer management. Plant extracts have proved to be beneficial in many medical fields. Not surprisingly, tumor prevention and therapy is one of these bright fields. *Rosmarinus Officinalis* has attracted many researchers due to its diverse pharmaceutical capabilities. New drug delivery systems must be set to overcome biomedical as well as biophysical barriers. Nanoformulation of therapeutic agents may help bypassing these burdens. The aim of the present study was to investigate the role of rosemary extract (RE) loaded on chitosan nanoparticles as a cancer treatment modality. Synthesis of nanoparticles encapsulating rosemary extract was performed with subsequent characterization by electron microscopy. Assessment of their effect on OSCC cell line (Hep-2) regarding cell cytotoxicity and impact on cell cycle and apoptotic pathway as well as intracellular ROS quantification was performed.

Results: RE nanoparticles were found to be cytotoxic to Hep-2 cells in a dose-dependent manner. They induced apoptotic changes as well as cell cycle arrest at G2/M phase. They enhanced ROS expression in cancer cells. Autophagosomes appeared in treated cells.

Conclusion: Finally, it could be concluded that RE nanoparticles may be a new candidate in the field of cancer management, mainly oral cancer.

Keywords: Rosemary, Cancer, Dental pulp stem cells, Nanotechnology, Nanochemoprevention, Angiogenesis, Nanocarrier

Background

Nowadays, scientists are focusing on developing new anticancer drugs of natural origin. This is because the clinical effectiveness of chemotherapeutic agents is lowered by their damaging side effects (Moore et al. 2016). Cancer cells show DNA instability leading to increased growth rate. This is due to either enhanced cell division or resisted apoptosis or both of them (Pietenpol and Stewart 2002). It is well known that cell replication is controlled through several pathways and mediated by a set of diverse signaling molecules. Dysregulation of these pathways causes cell accumulation which leads to tumor formation (Pietenpol and Stewart 2002). The stage of carcinogenesis determines the regimen of intervention as well as the drug of best choice. For several years, cancer prevention and treatment was mediated through chemical, pharmaceutical agents (Aggarwal et al. 2004). For every newly emerging anticancer agent, studies concerning drug efficacy must be performed on cancer cells, followed by animal models to end by assessment on volunteers to be approved clinically. This gives researchers a background on the effect of this agent on different stages of carcinogenesis, namely initiation, promotion and progression (Ramos 2008).

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Plant-based compounds are promising in the field of cancer prevention and treatment. The Mediterranean region offers the world a gift from the family of Lamiaceae named *Rosmarinus Officinalis* L. (RO). It is a very active son of this family known for its medicinal uses. Its extract contains many polyphenols, while the most important are carnosic acid (CA) and rosmarinic acid (RA). Both of them have anticancer biological activity (Allegra et al. 2020).

Delivery systems have a great role in ensuring effectiveness of drugs. Nanoparticle delivery systems are the newly emerging ones (Park et al. 2001). Polymeric nanoparticles are the best of all known approaches. They are biodegradable, biocompatible as well as their safety. They also have been approved by FDA for tissue engineering and drug delivery (Kong et al. 2010). Chitosan has been used as a carrier for drugs by different routes of administration. It showed less toxicity both in vitro and in vivo. It is mucoadhesive so is the best choice for locally delivered drugs. Chitosan is insoluble in water, but its chemical groups can be modified by alkylation, hydroxylation, carbonylation, etc. When chemically modified, chitosan can achieve many specific goals in medicine especially as a drug carrier. Also, it can be used in vaccine delivery and tissue regeneration (Berger et al. 2004; Agnihotri et al. 2004).

Methods

All experimental protocols were reviewed and approved by the Medical Research Ethical Committee of the National Research Centre (NRC) number (MREC-NRC): 19246. The procedures used in this study adhere to the standards of the Declaration of NRC.

Rosemary nanoparticles formulation

Rosemary nanoparticles composite were prepared by encapsulation of rosemary nanoparticles with biopolymer (chitosan) blended with polyvinyl alcohol (PVA) as carrier. The encapsulation of rosemary nanoparticle with the biopolymer and PVA was carried out via nanoprecipitation method. Briefly, rosemary nanoparticles were blended with biopolymer and PVA at composition ratio 1:5:5 (w/w/w) as follows: the organic solution containing 150 mg of rosemary nanoparticles and 750 mg biopolymer dissolved in 50 ml ethanol then injected in 150 ml aqueous solution containing 750 mg PVA. The obtained mixed solution was homogenized using ultrasonic sonication with 300 W ultrasonic dose for 10 mn.

Nanoparticles characterization

The morphology and particle size of the prepared chitosan nanoparticles were examined using transmission electron microscope (with JEOL Model JSM-T20 TEM).

Zeta potential measurement

The electrophoresis mobility (μe) of the prepared chitosan nanoparticles (0.6 mg/ml) dissolved in acetic acid in different pH values (pH 2:6) was using the Zetasizer from Malvern Instruments (3000-HS model) in 37 °C. The zeta potential (ζ) was calculated from the electrophoresis mobility using the *Smoluchowski's* equation.

$$\zeta = \left(\eta/\varepsilon\right) \times \mu\mathrm{e}$$

where η is the viscosity and ε the permittivity of the medium.

Assessment of anticarcinogenic effect of RE nanoformulations

OSCC cell line propagation and maintenance

Squamous cell carcinoma cell line (Hep-2) was supplied from Cell Culture Department-VACSERA-EGYPT. OSCC cell line was imported from the "American Type Culture Collection (ATCC)" in the form of frozen vials, with the reference number "173"; origin species: Homo sapiens (Human); morphology: epithelial-like cells. Cells were grown in DMEM-medium (4500 mg/ml of glucose, gluta MAX and sodium pyruvate) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 Ig/ml streptomycin, at 37 °C in a 5% CO2 humidified atmosphere. Medium was changed every other day till reaching 70–80% confluence. Then, cells were passaged to increase their numbers. Hep-2 cells were then divided into five groups according to RE concentration (10, 20, 30, 40, 50 µg/ml) and a control group cultured in routine culture medium. Chitosan nanoparticles loaded with RE were added to the experimental plates and kept for 48 h. All subsequent tests were performed simultaneously.

Cell cytotoxicity profile of RE nanoparticles

Measurement of cell viability and proliferation comprises the underlying basis for numerous in vitro assays directed toward the quantitation of a cell population. MTT reagent, supplied ready for use, was obtained from (Biospes, China, Cat n#BAR1005-1). It is a colorimetric assay for assessment of cell metabolic activity. Using the oxidoreductase enzymes under defined conditions can reflect the number of viable cells present, and these enzymes are capable of reducing the tetrazolium dye. The absorbance is directly proportional to the number of living cells in culture. The reduction of tetrazolium salts is now widely accepted as a reliable method for examining cell proliferation (Allegra et al. 2020; Cattaneo et al. 2015).

Steps of the assay

Cells (5×104) were placed in 96-well microplates 24 h before MTT assay to allow microplate adherence. 100 µl of serum-free media was added to cells and 10 µl of MTT solution into each well. The plate was incubated in CO2 incubator at 37 °C for 4 h. After incubation, 100 µl of formazan diluent buffer (Biospes, China, Cat n#BAR1005-1) was added into each well. Plate was wrapped in foil, shacked on an orbital shaker for 15 min and incubated overnight at 37 °C. Color absorbance was read at OD range 450–630 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA). Duplicate readings were taken and averaged for each sample. Amount of absorbance was proportional to cell number.

ROS measurement in OSCC cell line

The Fluorometric Intracellular ROS Kit provides a sensitive, one-step fluorometric assay to detect intracellular ROS (especially superoxide and hydroxyl radicals) in live cells after a 1 h incubation. ROS react with a fluorogenic sensor localized to the cytoplasm, resulting in a fluorometric product proportional to the amount of ROS present.

Note: All reagents were allowed to come to room temperature before starting experiment.

Procedure

All samples and standards were run in duplicate. Cells were plated overnight in growth medium at 10,000–40,000 cells/well/90 ml for a 96-well plate. ROS Detection Reagent was reconstituted with 40 ml of DMSO to generate the 500' ROS Detection Reagent Stock Solution. Cells were mixed well by pipetting. Remaining stock solution was aliquoted and stored at − 20 °C protected from light and moisture and stable for 1 month when stored at − 20 °C. Cells were treated with 10 ml of 10 test compound solution (96-well plate) or 5 ml of 5 test compound solution (384-well plate) in suitable buffer (such as PBS). For control wells (untreated cells), the corresponding amount of buffer was added. To induce ROS, the cell plate was incubated at room temperature or in a 5% CO2, 37 °C incubator for a desired period of time. The Master Reaction Mix was set according to the scheme. 100 ml/well (96-well plate) or 25 ml/well (384-well plate) of Master Reaction Mix was added into the cell plate. Cells were incubated in a 5% CO2, 37 °C incubator for 30 mn to one hour. The fluorescence intensity (lex = 640/lem = 675 nm) was measured.

ELISA measurement of apoptosis-related proteins

Total protein was extracted using RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.). Bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) was used to quantify proteins: BAX, Bcl-2, p53, caspase-3, B-actin.

Cell cycle analysis using FACS (propidium iodide flow cytometry)

Hep-2 cells at the logarithmic growth phase were randomly seeded in 60-mm culture dishes. After reaching 50% confluence, cells were cultured in serum-free medium for 24 h to induce cell quiescence. Subsequently, cells were incubated with various concentrations of RE nanoparticles in complete medium. After 24 h, the cells were harvested by trypsinization followed by centrifugation at 2000 rpm for 5 mn. Next, cold 70% ethanol were added to cells for resuspension. Finally, 1 ml propidium iodide (PI) stain solution (PI, 20 µg/ml and DNase-free RNase A, 100 µg/ml) was added to samples, which would be analyzed on a FACScan (Becton–Dickinson, Franklin Lakes, NJ, USA) within 30 mn. Data were acquired from 10,000 cells and processed using Lysis II software (Becton–Dickinson).

Expression of apoptotic marker Annexin-V using flow cytometric analysis

In normal cells, phosphatidylserine (PS) residues are found in the inner membrane of the cytoplasmic membrane. During apoptosis, the PS residues are translocated in the membrane and are externalized. In general, though not always, this is an early event in apoptosis and is thought to be a signal to neighboring cells that a cell is ready to be phagocytosed. Annexin-V is a specific PS-binding protein that can be used to detect apoptotic cells. Control cells as well as treated cells were collected and pelleted by centrifugation. Cells were washed with cold 1X PBS, and the supernatant was carefully removed. Cells were resuspended in 1X Binding buffer at a concentration of $\sim 1 \times 106$ cells/mL, preparing a sufficient volume to have 100 µL per sample. 5 µl of staining solution was added to tubes and gently swirled to mix. The mixture was incubated for 20 min at room temperature in the dark. 400 µl of 1X Binding buffer was added to each tube, and gently the tube was mixed or flicked. Cells were analyzed immediately (within 1 h) by flow cytometry. To detected cell death, Annexin-V/PI double staining kit (eBiosciencesDx) has been used in flow cytofluorimetric analyses. The Annexin-V corresponding signal provides a very sensitive method for detecting cellular apoptosis, while propidium iodide (PI) is used to detect necrotic or late apoptotic cells, characterized by the loss of the integrity of the plasma and nuclear

membranes. The data generated by flow cytometry are plotted in two-dimensional dot plots in which PI is represented versus Annexin-V-FICT. These plots can be divided in four regions corresponding to: (1) viable cells which are negative to both probes (PI/FITC −/−; Q3); (2) apoptotic cells which are PI negative and Annexin positive (PI/FITC −/+; Q1); (3) late apoptotic cells which are PI and Annexin positive (PI/FITC +/+; Q2); and (4) necrotic cells which are PI positive and Annexin negative (PI/FITC ±; Q4).

Autophagy analysis using transmission electron microscopy

The cells were treated with RE nanoparticles and then collected by trypsinization, fixed with 2.5% phosphate-buffered glutaraldehyde and post-fixed in 1% phosphate-buffered osmium tetroxide. The cells were embedded, sectioned and double stained with uranyl acetate and lead citrate and analyzed using a JEM-1200EX transmission electron microscope (TEM; JEOL, Tokyo, Japan).

Statistical analysis of results

Kruskal–Wallis statistics test was used, followed by multiple correction (using Dunn's test) to compare various measurements (viability, ROS, P53, caspase-3 and Bcl-2) between untreated and cells treated with various concentration of drug. Descriptive statistics were also provided. Linear regression analysis was done to determine if there is a significant trend in the effect of increasing drug dose on each parameter. To calculate the $IC_{50,}$ drug doses and values were log transformed and normalized, respectively, and a sigmoid curve was fit onto the data. The analyses and data visualization were all done using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Results

Rosemary nanoparticles composite characterization

Transmission electron microscope (TEM) informed us on the particle shape as well as their size. TEM micrographs of these nanoparticles are shown in Fig. 1. Electron microscopy analysis confirmed the presence of nanoparticles. RE-loaded chitosan nanoparticles were found to be spherical, distinct and regular with a size of 0.5 µm to 200 nm. Their surface is solid and has a dense structure. Chitosan nanoparticles appeared aggregated in the photomicrographs. The RE particles were well coated by chitosan. The particle size of the prepared nanoparticles ranged from 0.5 µm to 200 nm with an average size of 100 nm.

Zeta potential result

Zeta potential (ζ) for the prepared chitosan nanoparticle with concentration 0.6 mg/ml in different acidic pH values was measured, and data are illustrated in Fig. 2. It is clear that all the zeta potential was found in positive charge values due to the presence of amino group which is protonated

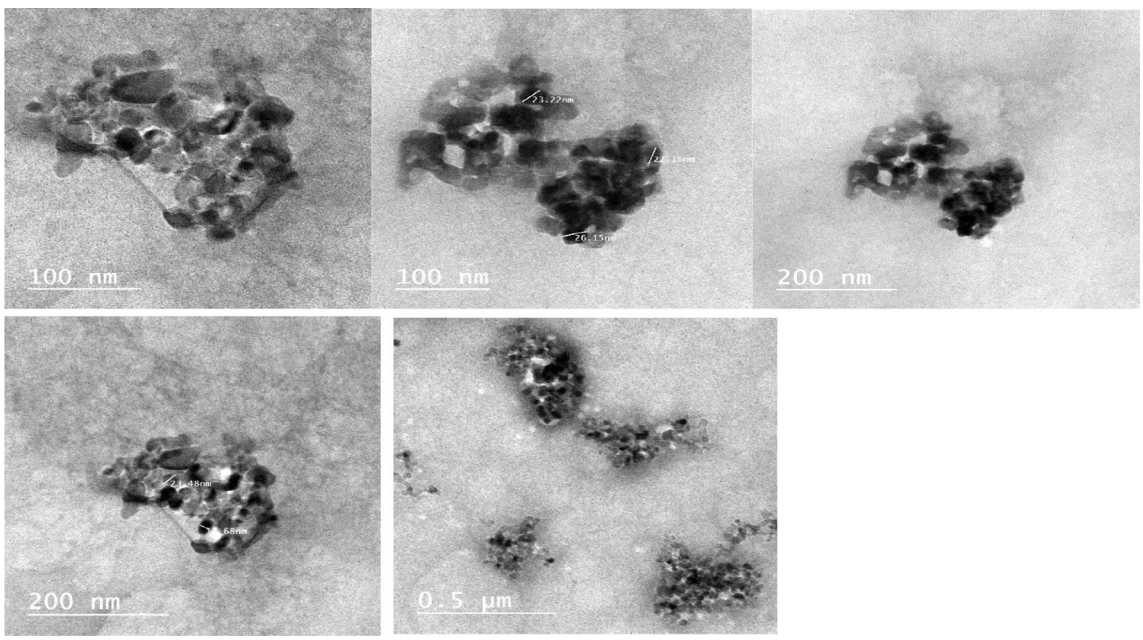

Fig. 1 TEM photomicrographs of chitosan nanoparticles showing particle size ranging from 0.5 µm to 200 nm

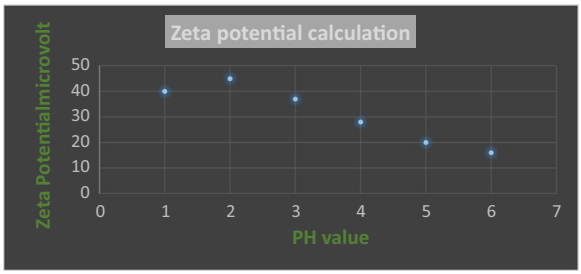

Fig. 2 Zeta potential measurement of RE chitosan-loaded nanoparticles

in acidic medium and gives a net positive charge. The maximum zeta potential value was 45 mV in pH 2.

Cell cytotoxicity profile of RE nanoparticle

RE nanoparticles have a cytotoxic effect on Hep-2 cells as they decreased cell viability in a dose-dependent manner. A highly statistically significant difference was detected using RE concentrations of 40 and 50 µg/ml compared to control cells in routine culture medium ($p=0.0038$, $p<0.0001$). A significant difference was recorded in RE concentration of 50 in comparison with concentration 10 µg/ml ($p=0.0161$) (Figs. 3 and 4).

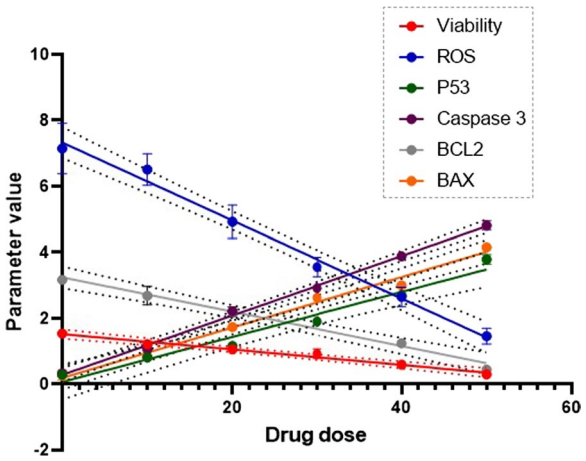

Fig. 4 Summary of potential RE nanoparticles anticancer effect on Hep-2 cells

IC 50 calculated was 19.56 µg/ml.

ROS measurement in OSCC cell line

A highly statistically significant difference was detected using RE concentrations of 40 and 50 µg/ml compared to control cells in routine culture medium ($p=0.0051$, $p=0$ 0.0001)). A significant difference was recorded in

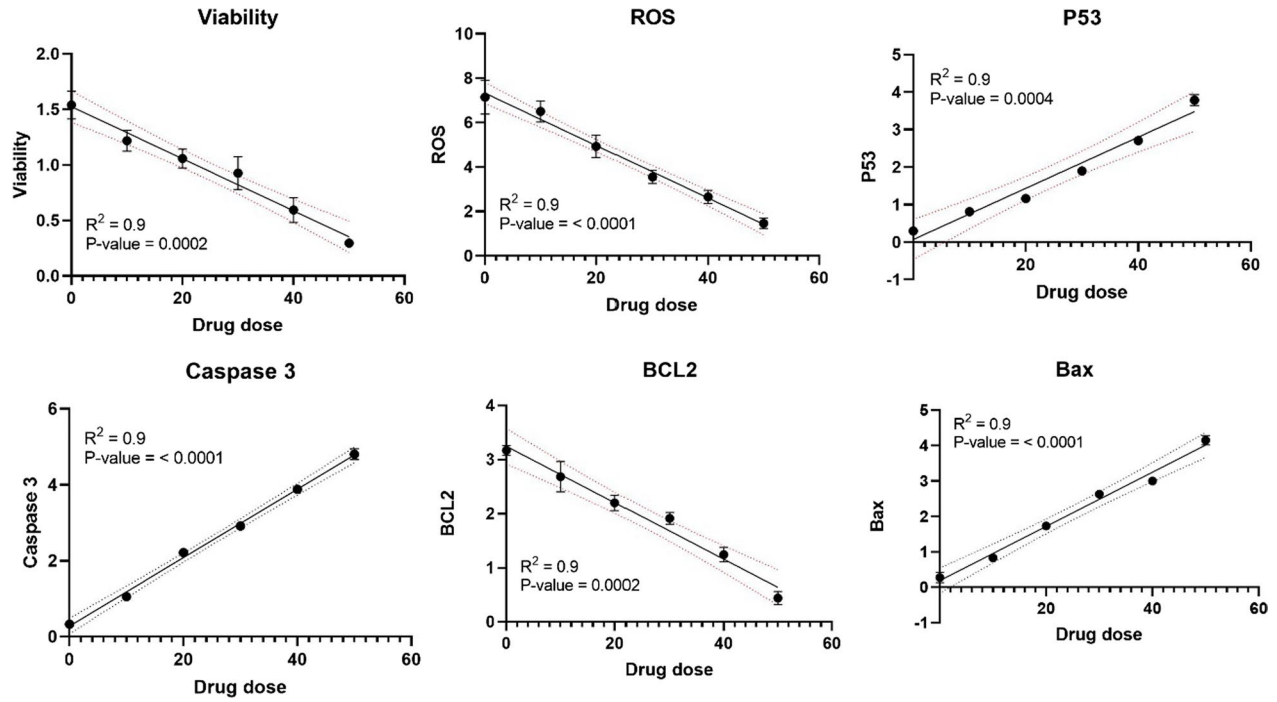

Fig. 3 Linear regression analysis for the effect of RE nanoparticles on each test. In each figure, dots refer to the average (mean) of the replicate cells and the standard error of the mean is shown as error bars. The dashed line refers to the confidence intervals at a confidence level 95%. R^2 is the regression coefficient, and P values show the significant of correlation between both measurements

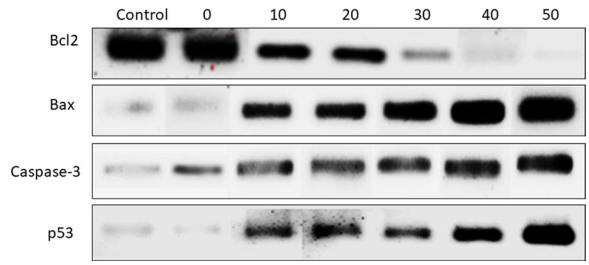

Fig. 5 Western blot analysis of apoptosis-related proteins in Hep-2 cells cultured in different concentrations of RE nanoparticles

RE concentration of 50 in comparison with concentration 10 µg/ml ($p=0.0038$) (Figs. 3 and 4).

ELISA measurement of apoptosis-related proteins (Figs. 3 and 4)

Regarding P53 protein, a statistical significant difference was detected using RE concentration of 50 µg/ml compared to control cells in routine culture medium ($p=0.0131$). For caspase-3 protein, there was a statistical significant difference using RE concentrations of 50 µg/ml compared to control cells in routine culture medium ($p=0.0266$). For Bcl-2, a statistical significant difference was detected using RE concentrations of 50 µg/ml compared to control cells in routine culture medium ($p=0.0166$). For BAX, a statistical significant difference was found using RE concentrations of 50 µg/ml compared to control cells in routine culture medium ($p=0.0266$).

Western blot analysis to investigate the change of apoptosis-associated proteins

To analyze western blot data, we looked for the presence of bands as well as their sizes. Pro-apoptotic proteins as Bax, caspase-3 and p53 are upregulated in Hep-2 cells in a dose-dependent manner, while anti-apoptotic protein Bcl-2 is downregulated also in a dose-dependent manner compared to control cells (Figs. 4 and 5).

Cell cycle analysis

Our cell cycle analysis results indicate alterations of the cell cycle of Hep-2 cells by different concentrations of RE nanoparticles. There is an increase in G0/G1 phase (% DNA content) with a concomitant arrest of cell growth in the G2/M phase (Fig. 6). The FACS results showed that RE inhibited the proliferation of Hep-2 cells during the G0/G1 phase.

Expression of apoptotic marker Annexin-V using flow cytometric analysis

Overall, apoptosis in particular late stage as well as necrosis is enhanced in cells treated with RE nanoparticle (Fig. 7).

Autophagy analysis using transmission electron microscopy

TEM was used to observe the ultra-structural changes induced by RE nanoparticles in Hep-2 cells and to assess the intracellular destruction caused by this compound for 48 h. The morphological changes observed in most cells after treatment with RE nanoparticles IC 50 primarily start with a reduction in cell volume and condensation of the nucleus. Other cells showed extensive DNA damage leading to nuclear fragmentation and appearance of autophagosomes in cell cytoplasm. Rosemary-treated cells revealed morphological changes characteristic of apoptosis. Clear nuclear fragmentation was found in the treated Hep-2 cells (Fig. 8).

Discussion

New bioactive agents may be found in nature. Plant polyphenols have shown a vast pharmacological activities. One of these natural gifts is *Rosmarinus Officinalis*. Recently, it has attracted the attention of scientists due to its potential anticancer effect. For an agent to fight against cancer, it must have the power of either inhibiting cell viability or proliferation or enhancement of apoptosis and death of these malignant cells. In this study, RE nanoparticles were found to be cytotoxic to Hep-2 cells in doses ranging from 10–50 µg/ml. Similarly, Da Rocha et al. (2001) stated that RE concentrations between 0.1 and 100 µg/ml are effective in depressing the proliferation of different cancer cell lines. Cattaneo et al. (2015) assessed the effect of RE from plant leaves on cell proliferation of melanoma cells. They found that after 24 h, the proliferation was inhibited and the impact was accentuated after 48 and 72 h of culture. The whole extract gave better inhibitory effect than each components separately. This may be explained by this cytotoxic effect resulted from combinatorial action of diverse molecules (Cattaneo et al. 2015).

There are many ways to decrease cell viability of cancer cells such as cell proliferation inhibition, enhancement of cellular ROS content, apoptosis and autophagy induction in cells as well as necrotic cytotoxicity. To reveal the exact mechanism by which RE suppressed cell viability, a set of in vitro assays were applied.

Reactive oxygen species (ROS) are generated as a result of the reduction of oxygen during aerobic respiration and by various enzymatic systems within the cell. At physiological levels, ROS contribute to cell signaling and host

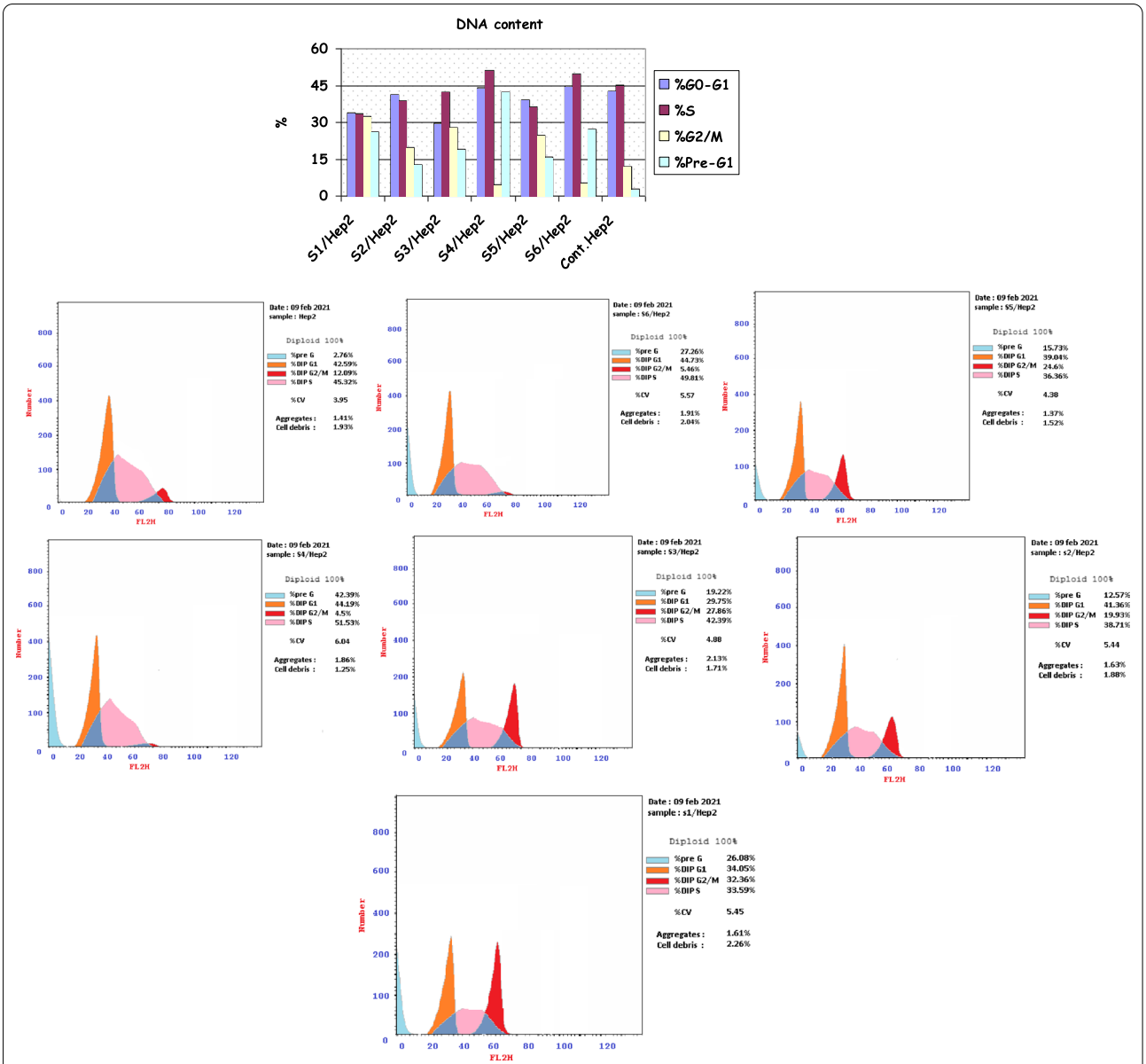

Fig. 6 Cell cycle analysis of RE-treated Hep-2 cells. Cell cycle arrest in G2/M phase was detected

defense. Increased ROS generation, above the detoxification capacity of the biological system, results in oxidative stress and cellular damage. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins and DNA. ROS have been implicated in disease states, such as Alzheimer's disease, Parkinson's disease, cancer and aging (Gutiérrez et al. 2010).

By ROS-level assessment in cancer cells with and without RE treatment, it can be stated that ROS are accumulated in Hep-2 cells in a dose-dependent manner. Unbalanced ROS accumulation in cells guides them toward both apoptosis and necrosis. An increase in the necrotic fraction by flow cytometry proved the presence of necrotic cells in treated cells. Accordingly, Pérez-Sánchez et al. (2019) found that treating colorectal cancer cells with RE in a dose of 20–40 µg/ml resulted in an obvious increase in ROS intracellularly which guided cells toward necrosis and death.

The impact of RE nanoparticles on apoptosis induction in malignant cells was detected using Annexin-V flow cytometry. RE enhanced Annexin-V-positive cells in a dose-dependent manner. We can conclude also that

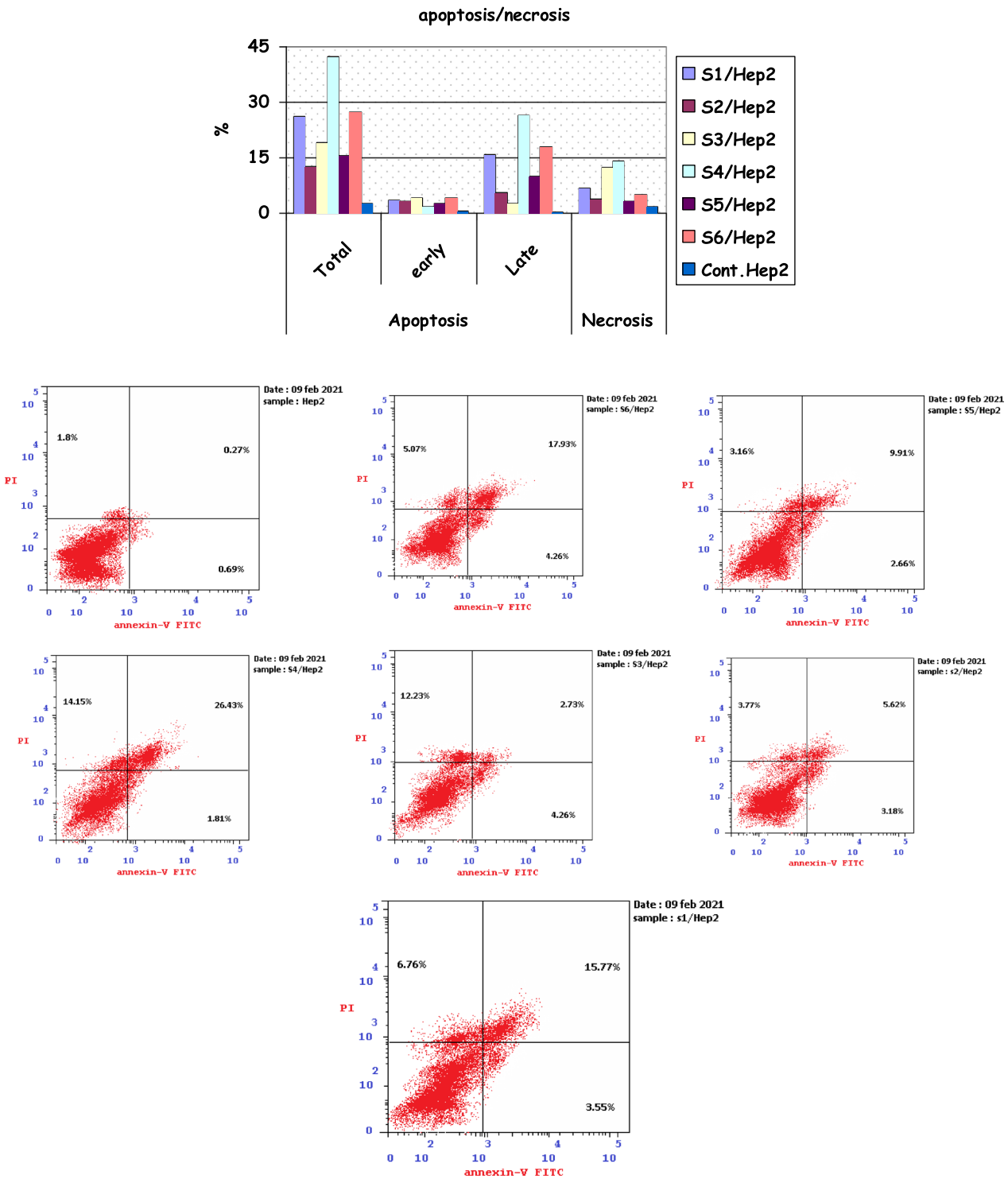

Fig. 7 Apoptosis as well as necrosis was enhanced compared to untreated control cells (first plot)

RE nanoparticles conduct a cell cycle arrest as well as an increase in apoptosis/necrosis cell fraction.

One of the basic processes common between all living cells is the cell cycle. It is divided into two major stages: interphase and M phase (mitosis phase). Based

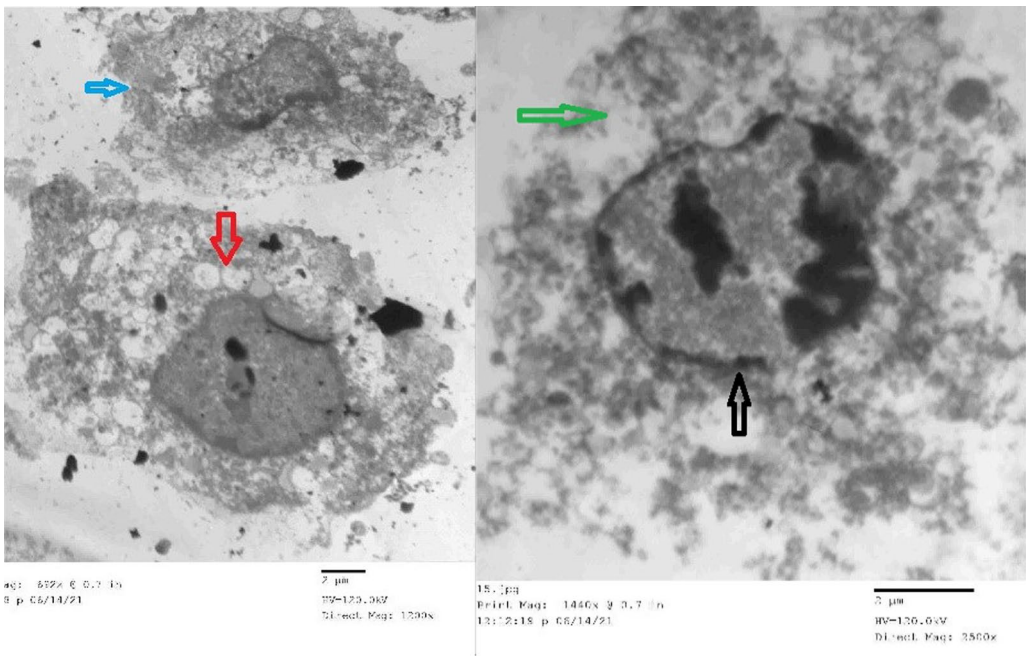

Fig. 8 RE inhibition of cell viability may be mediated by induction of Hep-2 cells to undergo apoptosis and autophagy. TEM images showed autophagic cytoplasmic vacuoles formation in cells treated with RE nanoparticle (red arrow). Loss of cell membrane integrity is noticed in treated cells (blue arrow). Chromatin margination and clumping are marked signs of apoptosis found in Hep-2 cells (black arrow). Loss of integrity of intracellular organelles is found concomitant with cell vacuolation (green arrow)

on synthesis of DNA, we can divide interphase into G1-, S- and G2-phases (Elmore 2007). From the present results, cell cycle analysis indicated that RE nanoparticles arrested Hep-2 cells at G2/M phase. This may suggest that blocking G2/M phase cells may be responsible for RE-induced apoptosis in Hep-2 cells. In a similar way, Pérez-Sánchez et al. (2019) found by cell cycle analysis that 30–40 µg/ml RE caused arrest and accumulation of cancer cells in G2/M phase. Cattaneo et al. (2015) stated that melanoma cells treated with RE extract showed a counterbalance of a cell cycle arrest in the G2/M phase, followed by hyperploid cells appearance. Similarly, Karimi et al. (2017) proved a RE-induced blockade of cell cycle in G2/M phase of GIT malignancy.

Apoptosis in cells is mediated via an intrinsic as well as an extrinsic pathway. Cancer may result from dysregulation of these pathways. Caspases and Bcl-2 proteins are responsible for regulation of apoptosis. Bcl-2 is an anti-apoptotic protein, while Bax is a pro-apoptotic protein. Cell sensitivity to apoptosis is due to balance between these proteins (Elmore 2007). In the present study, the results indicated that the Bax/Bcl-2 ratio increased by RE nanoparticles treatment suggesting that RE suppress Hep-2 cells viability by downregulation of anti-apoptotic proteins and upregulation of pro-apoptotic proteins. Western blot results analysis revealed that caspase-3 protein in Hep-2 cells significantly increased after RE application. In a similar way, Dai and Liu (2021) studied the effects of rosmarinic acid on the proliferation and apoptosis of human liver cancer Huh7 cells. The result showed that rosmarinic acid could effectively inhibit the proliferation of human liver cancer Huh7 cells by increasing the concentration and time in a dose-dependent manner and promote the apoptosis of Huh7 cells by regulating Bcl-2, Bax and cleaved caspase-3.

Autophagocytic vacuoles started to appear in cells treated with RE nanoparticles in a dose-dependent way compared to untreated cells. Signs of enhanced apoptosis as chromatin margination and nuclear membrane fragmentation were noticed.

Chitosan was chosen as a nanodrug delivery in our research as per our aim, and we intended to offer a locally acting formula that may be applicable in managing oral cancerous lesions. Chitosan has a penetration capability as it is able to open tight junctions existing between epithelial cells of the oral cavity lining mucosa. It helps drug transfer both para- and transcellularly (Nagpal et al. 2010). In a similar way, Sailaja et al. (2010) showed that chitosan nanoparticles are more stable, less toxic and easy to prepare. Their submicron size makes them suitable for a wider range of routes of administration in specific, mucosal, noninvasive ones, nasal, ocular

as well as oral mucosal routes. Agnihotri et al. (2004) stated that chitosan as a drug carrier has a good control on releasing water soluble drugs as well as in controlling their bioavailability. Nagpal et al. (2010) proved that when decreasing particle size of chitosan nanoparticles, an increase in the surface-to-volume ratio occurs as well as the specific surface area. This enhances the dissolution of poorly water-soluble drugs so increases their bioavailability. Rampino et al. (2013) stated that chitosan efficacy as a drug carrier is related to its particle size. Smaller sized particles interact better with cell membranes as they are endocytosed in comparison with larger size particles. We blended chitosan with PVA to ameliorate its physical properties regarding mechanical ones, in particular its tensile strength. Berger et al. (2004), in the same way, used a blend of PVA/Chitosan showing better controlled drug delivery due to the interaction between their molecules (Rampino et al. 2013).

Conclusions

Chitosan/RE nanoparticles were successfully synthesized. Results showed a brilliant effect of these nanoparticles in medicine. This novel chitosan/RE nanoparticles may show a new hope in cancer treatment protocols specially local management of oral cancer. In conclusion, we can state that RE compounds show the in vitro capability to inhibit malignant cellular proliferation, cell cycle arrest and enhancement of apoptotic profile of Hep-2 cells. The treatment of cancer cells with RE leads to a strong increase in intracellular ROS that results in cell death. By using this novel therapeutic mix, deleterious side effects of routinely used chemotherapeutic agents may be overcome or even ameliorated allowing a better life quality for cancer patients.

Abbreviations

RE: Rosemary extract; OSCC: Oral squamous cell carcinoma cell line; ROS: Reactive oxygen species; DNA: Deoxyribonucleic acid; CA: Carnosic acid; RA: Rosmarinic acid; NRC: National Research Centre; PVA: Polyvinyl alcohol; Mn: Minute; H: Hour; Ml: Milliliter; Mg: Milligram; TEM: Transmission electron microscope; FCS: Fetal calf serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD: Optical density; PBS: Phosphate-buffered saline; DMSO: Dimethyl sulfoxide; CO2: Carbon dioxide; IC50: Minimum inhibitory concentration; FACS: Fluorescent activated cell sorting; ELISA: Enzyme-linked immunosorbent assay.

Acknowledgements

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Author contributions

ME planned the experiment design and was helped by HE in achieving different steps of the research. All authors have read and approved the manuscript. RA and HE were responsible for material preparation and data collection. ME and RA performed the statistical analysis. ME analyzed the results helped by HE. Both the manuscript draft and the final one were written, revised and approved by all authors.

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

Approval was obtained from the Medical Research Ethics Committee of National Research Centre Number (MREC-NRC): 19246. The procedures used in this study adhere to the standards of the Declaration of NRC.

Consent for publication

Not applicable.

Recommendations

Animal models should be used before preclinical studies on human volunteers to determine the exact dose of RE nanoparticles. Also, the most effective administration route must be stated. These studies will allow measuring plasma level of bioactive ingredients of RE nanoparticles after local application on mucosa. This will help revealing any side effects with prolonged use of local RE nanoparticles.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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