Academic Journal of Cancer Research 11 (1): 01-08, 2018
ISSN 1995-8943

DOI: 10.5829/idosi.ajcr.2018.01.08

Antitumor Activity of Rosmarinic Acid Encapsulated in Chitosan Nanoparticles

[1]Yasmin Y.A. Darwish, [1]Mohamed Abd-Elhakeem,
[2]Ahmed Ibrahim Abd El-Maksod, [3]Hussein Sabit and [1, 4]Mokhtar El-Zawahri

[1]College of Biotechnology, Misr University for Science and Technology, P. O. Box: 77, Giza, Egypt
[2]Genetic Engineering Research Institute, University of Sadat City, Menofiya, Egypt
[3]Department of Genetics, Institute for Research and Medical Consultations,
Imam Abdulrahman Bin Faisal University, P. O. Box: 1982, Dammam, 31441 Saudi Arabia
[4]Research and Development Center, Misr University for Science & Technology, P. O. Box: 77, Giza, Egypt

Abstract: Cancer remains the main health-threatening diseases globally with an estimation of 18.1 million new cancer cases per year. Liver cancer is the fifth most common cancer occurring in males and the ninth most common cancer occurring in females globally, with over 840,000 new cases in the year 2018. Rosmarinic acid (RA) is a naturally occurring phenolic compound with a number of biological activities. Considering it slow bioavailability and low stability, effective and safe delivery methods are essential to enhance RA activity. The aim of the present study was to investigate the potential use of chitosan nanoparticles (CS-NPs) as a drug delivery system to improve the antitumor activity of RA against hepatocellular carcinoma cells (GepG2). Free CS-NPs and RA encapsulated in CS-NPs (RA-CS-NPs) were prepared by ionic gelation using sodium tripolyphosphate (TPP). Particle size distribution analysis and transmission electron microscopy (TEM) confirmed the size ranging from 100 to 1000 nm, while surface charge of nanoparticles was -34 mV. The prepared RA-CS-NPs showed good loading capacity and good release rate as the drug releasing reached 83.8% after 4 hours. The antitumor behavior on hepatocellular carcinoma cell line (HepG2) showed elevated antitumor activity of RA-CS-NPs compared with free RA as revealed by trypan blue test and cytotoxicity assay (MTT). Moreover, RT-PCR-based gene expression analysis showed an up regulation of *P53*,*COX*-2 and *ErbB*2genes in the cells treated with RA-CS-NPs compared to untreated cells.

Key words: Rosmarinic Acid · *Chitosan nanoparticles* · Cancer · Anticancer · Liver Cancer

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death worldwide [1]. It is a cause of death for about half million individual per year [2, 3]. The high morbidity and mortality is related to resistance of HCC to chemotherapeutics as cisplatin, 5-fluorouracil and Adriamycin [4, 5].

The expression of *COX-2* gene is highly correlated with hepatocellular carcinoma as the inhibition of *COX*-2 suppresses growth of hepatoma cell lines [6].

P53 is a tumor suppressor gene that acts as a sequence-specific transcription factor to regulate expression of over one hundred different targets and to modulate various cellular processes like apoptosis, cell cycle arrest and DNA repair [7]. It was implied that elevated *P53* is an early marker of HCC [8].

Rosmarinic acid (RA) is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid commonly found in plants belonging to the *Boraginaceae* and the subfamily Nepetoideae of the Lamiaceae family. RA has important biological activities, e.g. antiviral, antibacterial, anti-inflammatory, anti-cancer [9]. In human hepatoma cells (HepG2), RA causes apoptosis by increasing the expression of apoptosis-related genes, so it might be a promising candidate against hepatocellular carcinoma [10].

Nevertheless, besides the low bioavailability that constrains the transport across biological barriers, RA is unstable and must be protected from degradation in the

Corresponding Author: Hussein Sabit, Department of Genetics, Institute for Research and Medical Consultations, Imam Abdulrahman Bin Faisal University, P.O. Box: 1982, Dammam, 31441 Saudi Arabia. Cell: +966546177974.

physiological environment [11]. Thus, the antitumor efficacy of RA clearly depends on the design of appropriate carriers for their delivery, protection and release [12]. Among the different approaches explored so far, nanocarriers of particular interest, especially those made of natural polymers, assure drug time retention at the absorption site [13]. For this application, chitosan nanoparticles (CS-NPs) has become particularly interesting for the association and delivery of many drugs [14]. CS-NPs introduce an exceptional potential for drug delivery including biocompatibility, stability in contact with physiological fluids and control drug releasing [14].

The main aim of this study was to prepare RA-encapsulated CS-NPs to regulate the drug release rate of RA for bioavailability improvement and meanwhile, enhance the antitumor activity of RA.

MATERIALS AND METHODS

Preparation of Chitosan: Shrimp shells (obtained from local market) were immediately washed several times with water and detergent then allowed drying in open area. 200 g of grounded dry material were soaked in 1L of 1% HCl for 24 hours to remove minerals and then it was treated with 2% NaOH to decompose the albumen into water-soluble amino acids. The deacetylation process was carried out by adding 50% NaOH then was boiled at 100°C for 2 h. on a hot plate. Remaining creamy white material was collected and air-dried [15].

Preparation of RA-CS-NPs: Chitosan flakes were dissolved in 2% (v/v) acetic acid to form a solution with final concentration (2mg/ml). 25 mg RA (Sigma-Aldrich) was dissolved in different volumes CS solution (50, 125, 250 ml); in order to guarantee the best ratio between CS and the RA, as documented previously [16]. Finally, different volumes of sodium tripolyphosphate solution (7, 17, 35 ml respectively) having a concentration of 2 mg/ml was added to the above mixture under mechanical stirring (600 rpm).

The reaction was kept for 1.5 h at room temperature. The obtained nanoparticles were freeze-dried and maintained at -20°C until use, while the supernatant was used to calculate the Association efficiency (AE) and loading capacity (LC). Chitosan nanoparticles (CS-NPs) without RA were prepared by the same method.

Characterization of Nanoparticles: TEM observation was performed on a microscope (JEOL JAM-2100-HR-EM) at National Research Centre (Cairo, Egypt). Zeta potential and particle size distribution was performed by (Nanotrac Wave II, Microtrac, USA) at Nawah Scientific (Cairo, Egypt).

Association Efficiency and Loading Capacity: Association efficiency (AE) and loading capacity (LC) were analyzed by UV-VIS spectrophotometer at 320 nm by measuring the concentration of free RA (unentrapped) in the collected supernatants.

$$AE\% = \frac{\text{Initial amount of RA (mg) - Final amount of RA in supernatant (mg)}}{\text{Initial amount of RA (mg)}}$$

$$LD\% = \frac{\text{Initial amount of RA (mg) - Final amount of RA in supernatant (mg)}}{\text{Amount of CS (mg)}}$$

***In vitro* Release Study:** *In vitro* release assessments from RA-CS-NPs were carried out for 24 h. in phosphate buffered saline (PBS) at pH, 6.6 and 1.2. An aliquot of RA-CS-NPs (100 µg/mL) was placed in dialysis bag (Spectra/Por®, 3, 500 MWCO, Spectrum Medical Industries, CA, USA) and suspended in 15 mL of PBS at 37°C under gentle magnetic stirring (100 rpm). At scheduled times; 2 mL of medium was withdrawn and replaced with an equal volume of fresh medium.

The amount of RA released was estimated by Spectrophotometric analysis. All the experimental procedures were performed in triplicate. The cumulative release percentage (CR%) of RA at each time point was determined using the following equation:

$$CR\% = (Mt/Mi) \times 100$$

where Mi and Mt are the initial amount of drug encapsulated in the NPs and the amount of drug released at the time t. Control experiment using free RA was also carried out under similar conditions

Cell Lines Culture and Maintenance: Hepatocellular carcinoma cell line (HepG2) was purchased from the Holding Company for Biological Product and Vaccines (VACSERA), Giza, Egypt. Cells were cultured in 5% CO_2 and at 37°C. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% AB mix.

Cell Viability Assay: Cell viability was assessed using Trypan blue test. This test was performed to determine the number of viable cells present in cell suspension after treatment of these cells with RA. Cells suspension was mixed with dye and viable cells were detected visually under light microscope. The total number of viable cells was calculated using the following equations:

Table 1: Reverse and forward sequences of P53, COX-2 and ErbB2.

Gene name	Sequence (5-3)
P53 forward	AGAGTCTATAGGCCCACCCC
P53 reverse	GCTCGACGCTAGGATCTGAC
COX2 forward	ACGCGTCACCTTAATATGCG
COX2 reverse	ATCCCTTCACTAAATGCCCTC
ErbB2 forward	GCTTTGTGGTCATCCAGAA
ErbB2 reverse	CTCCAGCCCTAGTGTCAG

$$TC = (X/4) \times 2 \times 10^4$$

where TC was the total number of viable cells in 1 mL; X was the average number of viable cells count from the four squares of the hemocytometer.

MTT Assay: 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay is a simple, rapid and reproducible assay used to determine the activity of cytotoxic drugs by comparing the results before and after treatment. Living cells contains NADPH oxidoreductase, these enzymes are capable of reducing the tetrazolium dye MTT to give purple color. Intensity of color resulted was an indication of the cancer cells viability after treatment of our drugs RA, RA-CS-NP and CS-NP with various concentrations. We plated cells at 1000 to 100000 per well, then the cell were incubated for 6 to 24 hours. Ten µL MTT reagent was added and the cells were incubated for 2 to 4 hours until purple precipitate is visible. One hundred µL of detergent reagent was added and left at room temperature in the dark for 2 hours then we recorded absorbance at 570 nm.

RNA Extraction and cDNA Synthesis: Total RNA was extracted using Total RNA Purification Kit (Jena Bioscience GmbH, Germany). cDNA was synthesized using SensiFast cDNA Synthesis kit of Bioline Reagents Ltd.

Target Genes: In the present study, three genes were targeted; *COX-2*, *P53* and *ErbB2*. The specific primer sequences are presented in Table (1).

RESULTS

Characterization of RA-CS-NPs: Particle size and size distribution are important parameters towards the development of suitable nano-drug for therapeutic purposes. In addition, they can also influence the drug loading, drug release and stability of drug inside nanoparticles [17]. In this study, the obtained RA-CS-NPs were spherical shape and have variety in size that range between 100 nm and 1000 nm as indicated from TEM imaging and Malvern Zetasizer particle analyzer (Figure 1 and 2). The obtained sizes were appropriate for the formation of RA-CS-NPs complex [18].

Zeta potential is an indirect measure of the surface charge. Zeta potential values (high zeta potential values, either positive or negative) are achieved in order to ensure stability and avoid aggregation of the particles [19]. In our study, the formulation was slightly negative and approximately -34mV, which considered a stable form and the negative charge, was related to the charge of RA.

Association Efficiency and Loading Capacity of RA in CS-NPs: This high incorporation capacity in the designed formulation is probably related to the chemical nature of the drug and to its interactions with the NP structure at the pH value of the experimental conditions.

As shown in table (2), the results exhibited a high association efficiency and good loading capacity. The highest AE % (96.08%) was observed by using 500 mg CS-NPs and 25 mg RA (F1), while the highest LC% (7.90%) was achieved by using 250 mg CS-NPs and 25 mg RA (F2). Based on AE% and LC%, F1 was used for further *in vitro* study.

***In vitro* Release Study:** The *in vitro* release study of RA from CS-NPs was performed in PBS buffer solutions at pH 7.4 or pH 2.5 to simulate the different pH conditions of the gastrointestinal system. The pH-dependent cumulative amount of RA released from the RA-CS-NPs is shown in Figure (3). A fast release was observed during the *in vitro* release assays and complete diffusion across the dialysis membrane was found to occur after one hour. Using loaded drug (RA-CS-NPs) the initial burst was at first 0.5 hour about 18%, after 2 hours it was 58.8% at pH 1.2 increasing time and changing pH to be 6.6 the release was 78.5% after 3 hours and the maximum release was after 4 hours about 85.8%.

Trypan Blue Test: Trypan blue is one of the most important techniques to study cell viability. It was employed to assess the cell membrane integrity as an indicator on the cell viability. In the present study, cell viability was assessed using trypan blue and the results were presented in Figure (4).

MTT Assay: The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable

Table 2: The loading formulations of RA-CS-NPs

Formulation	Chitosan (mg)	Initial drug amount (mg)	Final drug amount (mg)	Association efficiency %	Loading capacity %
F1	500	25	0.98	96.08	4.80
F2	250	25	5.24	79.04	7.90
F3	100	25	20.00	20	5.00

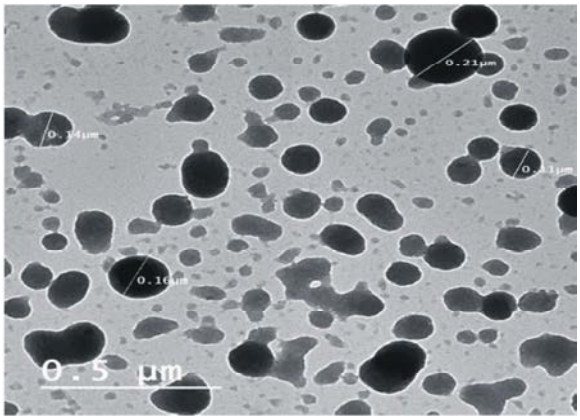

Fig. 1: TEM image of the RA-CS-NPs.

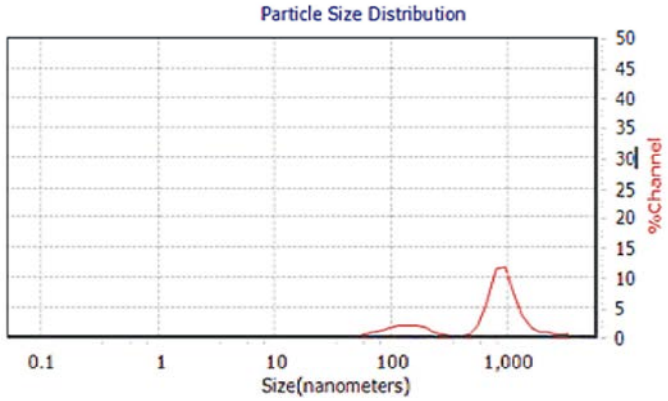

Fig. 2: The particles size has a range between 100-1000 nm

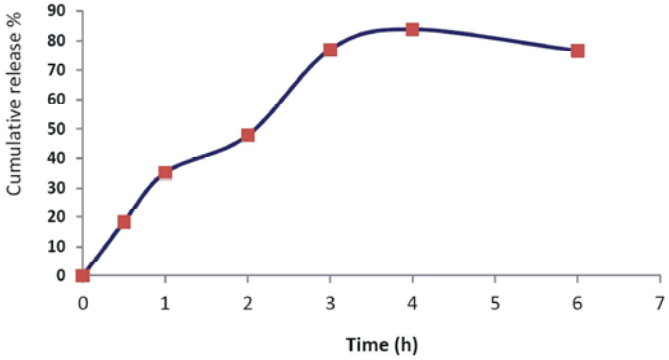

Fig. 3: Illustrate the release of RA from the loaded RA-CS-NPs depending on pH and time

for high throughput screening. The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles [20].

The obtained results (Figure 5) showed that the activity of the mitochondrial reductase enzyme was decreased upon treatment the cells with the specified drugs. Treating the HepG2 cells with different doses of RA killed the cells in a dose dependent manner. Using free CS-NPs responsible for apoptotic effect to the cells in a gradually manner. Loaded RA-CS-NPs has an effect in reducing cell viability but there was some fluctuation.

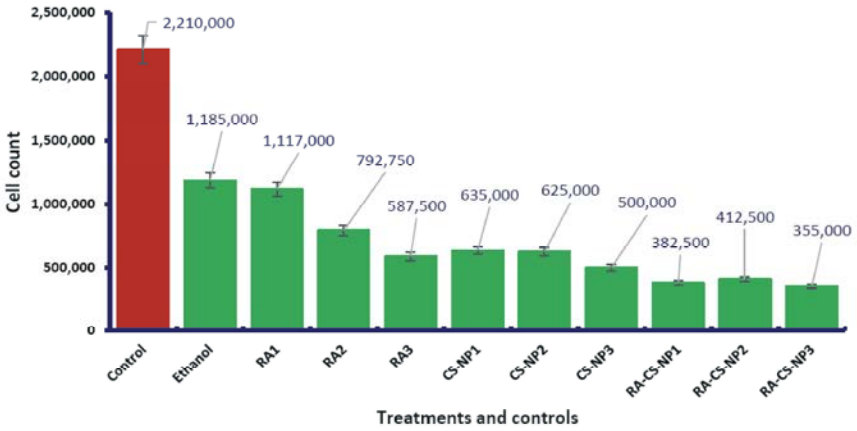

Fig. 4: Effect of RA, CS-NPs and RA-CS-NPs on total cell count of HepG2. RA_1 50 µg, RA_2 (100 µg), RA_3 (150 µg), $CS\text{-}NP_1$ (1mg), $CS\text{-}NP_2$ (2mg), $CS\text{-}NP_3$ (3mg), $RA\text{-}CS\text{-}NP_1$ (50 µg RA+1mg CS-NPs), $RA\text{-}CS\text{-}NP_2$ (100 µg RA+2mg CS-NPs), $RA\text{-}CS\text{-}NP_3$ (150 µg RA+3mg CS-NPs)

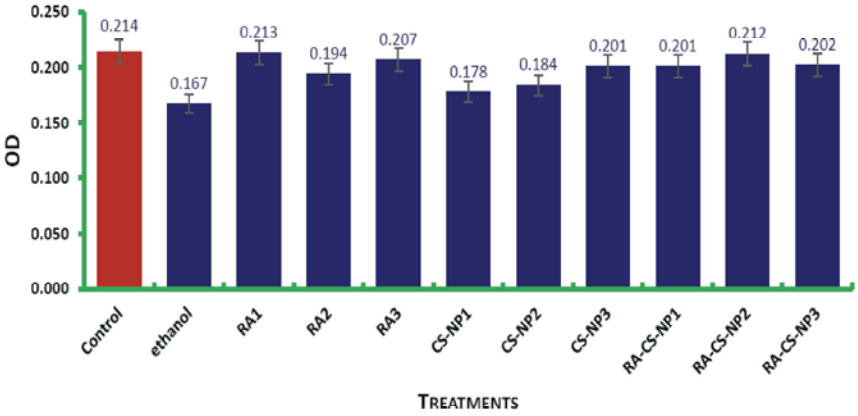

Fig. 5: MTT assay for three doses of RA, CS-NPs and RA-CS-NPs compared with control and ethanol as (-ve control)

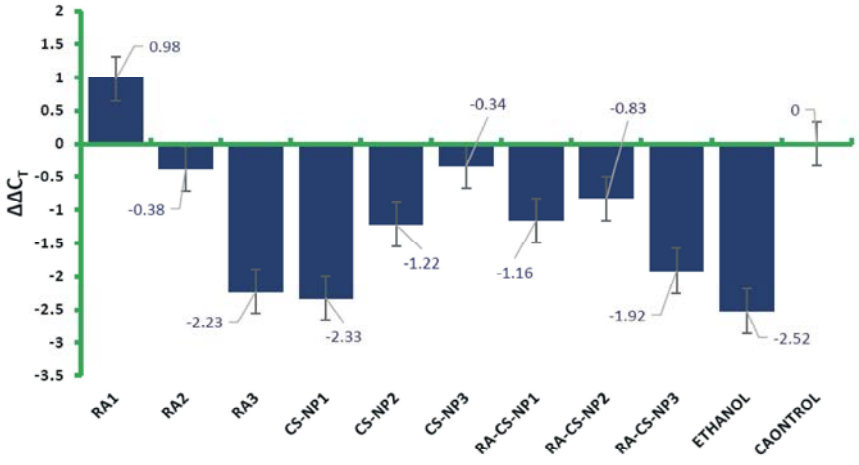

Fig. 6: Illustrate the fold differences (ΔΔCT) for COX-2 between RA, CS-NPs and RA-CS-NPs with different doses. -ve sign means upregulation, and +ve sign means downregulation. RA_1 50 µg, RA_2 (100 µg), RA_3 (150 µg), $CS\text{-}NP_1$ (1mg), $CS\text{-}NP_2$ (2mg), $CS\text{-}NP_3$ (3mg), $RA\text{-}CS\text{-}NP_1$ (50 µg RA+1mg CS-NPs), $RA\text{-}CS\text{-}NP_2$ (100 µg RA+2mg CS-NPs), $RA\text{-}CS\text{-}NP_3$ (150 µg RA+3mg CS-NPs)

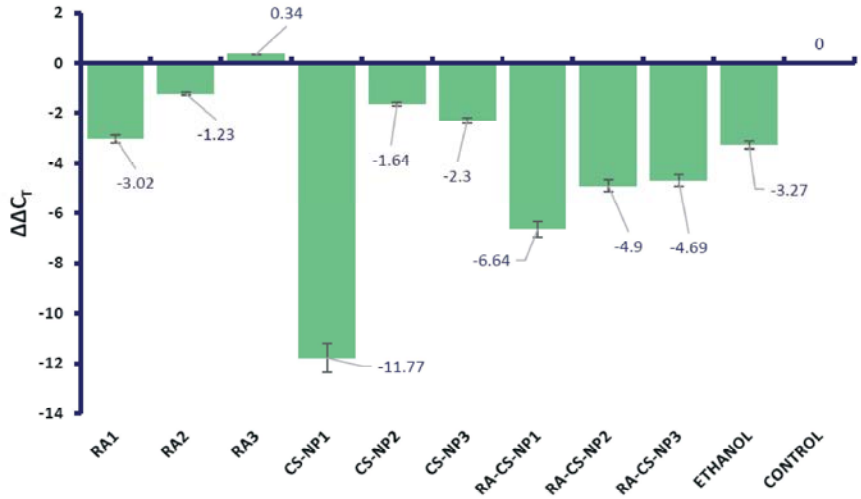

Fig. 7: Illustrate the fold differences (ΔΔCT) for ErbB2 between RA, CS-NPs and RA-CS-NPs with different doses. -ve sign means upregulation and +ve sign means downregulation. RA_1 50 µg, RA_2 (100 µg), RA_3 (150 µg), $CS\text{-}NP_1$ (1mg), $CS\text{-}NP_2$ (2mg), $CS\text{-}NP_3$ (3mg), $RA\text{-}CS\text{-}NP_1$ (50 µg RA+1mg CS-NPs), $RA\text{-}CS\text{-}NP_2$ (100 µg RA+2mg CS-NPs), $RA\text{-}CS\text{-}NP_3$ (150 µg RA+3mg CS-NPs)

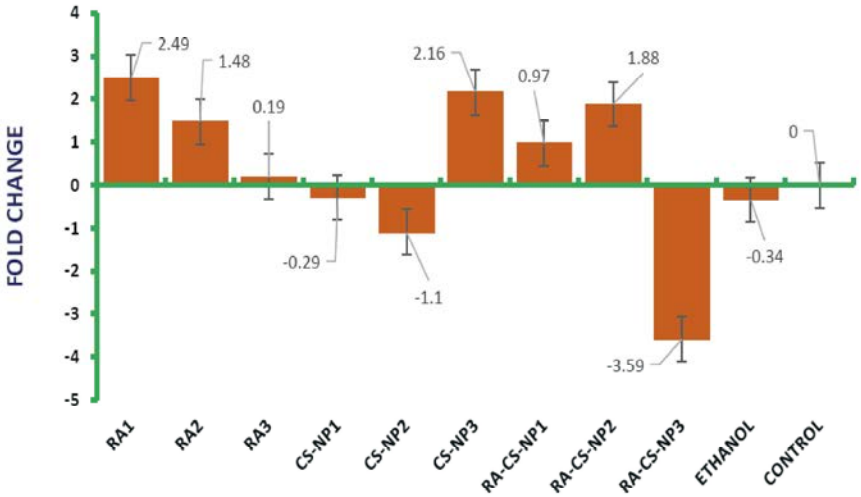

Fig. 8: Illustrate the fold differences (ΔΔCT) for P53 between RA, CS NP and RA-CS NP with different doses. -ve sign means upregulation and +ve sign means downregulation. RA_1 50 µg, RA_2 (100 µg), RA_3 (150 µg), $CS\text{-}NP_1$ (1mg), $CS\text{-}NP_2$ (2mg), CS-NP3 (3mg), $RA\text{-}CS\text{-}NP_1$ (50 µg RA+1mg CS-NPs), $RA\text{-}CS\text{-}NP_2$ (100 µg RA+2mg CS-NPs), $RA\text{-}CS\text{-}NP_3$ (150 µg RA+3mg CS-NPs)

Gene Expression Analysis: *COX-2*, *erbB2* and *P53* genes expression: Gene expression studies were performed to confirm apoptosis induction in HepG2 cells by RA, free CS-NPs and loaded RA-CS-NPs. Gene expression investigated by RT PCR. There was down regulation to COX2 gene using RA with dose of 50 µg. The expression of *ErbB2* was in a dose dependent manner using RA, as the down regulation was (ΔΔCT 0.34) of this anti-apoptotic gene using RA in dose 150 µg. In *P53* expression (the apoptotic gene) the up regulation was increased using CS-NPs of doses 1mg and 2 mg and in loaded RA-CS-NPs in a dose of 150 µg was increased 3.59 folded difference (Figures 6, 7 and 8). There is a fluctuation in cell viability and in *P53* expression using Loaded RA-CS-NPs.

DISCUSSION

The ionic gelation method was chosen to form the NPs because of the ability of CS forming gel, thus by ionic crosslinks with TPP which happens at pH of 5.5. Moreover, at this pH the amino groups of CS are protonated (pKa=6.5). At this pH the ionization of the phenolic group and the carboxylic acid was occurred (pKa =3.57), which gives the chance of attractive electrostatic interactions between the negative charge of RA and positive charge of CS. In which, we can suppose that RA entrapped in CS [21] or may be adsorbed at the surface of the nanoparticles by hydrogen bond or hydrophobic force [22].

Chemotherapies have many adverse effects in cancer treatments, so there are many researches in using the natural compounds in cancer. Both RA and CS-NP have anticancer activity on HepG2 to [10, 23].

The result of this study showed that RA induced apoptotic effect to HepG2 cells in a dose dependent manner. Our results were in agreement with Rahmatullah *et al*. [10] who assessed that RA has antitumor activity on cancer cells as in breast cancer, ovarian cancer and hepatocellular carcinoma.

Using *CS-NPs* inhibited the proliferation of the HepG2 hepatocellular carcinoma by increasing the *Dose and* this in agreement with Loutfy *et al*. [23].

In this study, the inhibition of cell proliferation was markedly observed using the loaded RA-CS-NP, as the cell count decreased compared to RA and CS-NP treatment. This could be explained by alteration in properties of RA after it had been formulated into nanoparticles using chitosan nano form.

We found that The highly expressed gene was P53 gene, which is a tumor suppressor gene, as the $\Delta\Delta C_T$ increase to 3.59 using the loaded RA-CS-NP of a dose 150 µg RA and 3mg of CS-NP.

The fluctuation in cell viability and gene expression using loaded RA-CS-NP may be related to formation of aggregates and precipitates as increasing pH above 6 (pH of the HepG2 7.4), this due to loss of the positive charge density of CS. Solving this problem may be by using thiolated CS, which bends to mucosal cell surfaces with higher avidity [24].

CONCLUSIONS

The core of this work was to underline the potential application of chitosan nanoparticles for nano-drug delivery. This could be a fundamental alternative nanomedicine to enhanced antitumor activity of chemotherapeutic drugs. In the present study, CS-NPs incorporating RA, were successfully prepared and characterized in order to improve their stability, bioavailability, *in vitro* release rate and antitumor activity. The RA-CS-NPs had a higher antitumor activity on hepatocellular carcinoma (HepG2) compared with free RA and free CS-NPs as indicated from total cell count. The decrease in cell count might be indication of the up regulation of *P53*, which is tumor suppressor gene acting as cell guard. On the other hand the *COX-2* gene and *ErbB2* genes which are oncogenes were up regulated, so those genes had no role in the apoptotic activity of loaded RA-CS-NPs. It can be concluded that encapsulation of RA in CS-NPs may be a promising approach for the drug delivery of antitumor drugs, however it needs further study for other chitosan derivatives and different carriers.

ACKNOWLEDGEMENT

The authors thank Dr. Shaimaa E. Abdel-Ghany, Assistant Professor of Genetics, College of Biotechnology, MUST, for her help in the molecular and cell culture laboratories during the conduction of this study.

Conflict of Interest: The authors declare no conflict of interest.

REFERENCES

1. Graf, D., D. Vallböhmer, W.T. Knoefel, P. Kröpil and G. Antoch, 2014. Multimodal treatment of hepatocellular carcinoma. Eur. J. Intern. Med., 25: 430-437.
2. Parkin, D.M., F.I. Bray and S.S. Devesa, 2001. Cancer burden in the year 2000. The global picture. Eur. J. Cancer. 37 Suppl 8: S4-66.
3. Kirk, G.D. and E. Bah, 2006. Montesano R Molecular epidemiology of human liver cancer: insights into etiology, pathogenesis and prevention from The Gambia. West Africa. Carcinogenesis, 27: 2070-2082.
4. Wang, X.Q., W.M. Ongkeko, L. Chen, Z.F. Yang and P. Lu, 2010. Octamer 4(Oct4) mediates chemotherapeutic drug resistance in liver cancer cells through a potential Oct4-AKT-ATP-binding cassette G2 pathway. Hepatology, 52: 528-539.

5. Li , L., X.H. Wei, Y.P. Pan, H.C. Li and H. Yang, 2010. LAPTM4B: a novel cancer associated gene motivates multidrug resistance through efflux and activating PI3K/AKT signaling. Oncogene, 29: 5785-5795.
6. Bae, S.H., E.S. Jung, Y.M. Park, B.S. Kim, B.K. Kim, D.G. Kim and W.S. Ryu, 2001. Expression of Cyclooxygenase-2 (COX-2) in Hepatocellular Carcinoma and Growth Inhibition of Hepatoma Cell Lines by a COX-2 Inhibitor. Clinical Cancer Research, 7: 1410-1418.
7. Bai, L. and W.G. Zhu, 2006. Structure, Function and therapeutic applications. J. Cancer. Mol., 2: 141-153.
8. Liu, J., A. Ahiekpor, L. Li, X. Li, P. Arbuthnot, M. Kew and M. Feitelso, 2009. Increased Expression of c-erbB-2 in Liver is Associated with Hepatitis B x Antigen and Shorter Survival in Patients with Liver Cancer. Int. J. Cancer., 15; 125(8): 1894-1901.
9. Tumur, Z., C. Guerra, P. Yanni, A. Eltejaye, C. Waer, T. Alkam and B.S. Henson, 2015. Rosmarinic Acid Inhibits Cell Growth and Migration in Head and Neck Squamous Cell Carcinoma Cell Lines by Attenuating Epidermal Growth Factor Receptor Signaling. Cancer Science& Therapy, 7: 367-374.
10. Rahmatullah, M., H. Shahadat, R. Shahnaz, A.B.M.A. Bashar, R. Jahan and A. Al-Nahain, 2014. Rosmarinic acid: a review of its anticancer action. World Journal of Pharmacy and Pharmaceutical Sciences, 3(9): 57-70.
11. Hou, Y., J. Wang, W. Jin, H. Zhang and Q. Zhang, 2012. Degradation of Laminaria japonica fucoidan by hydrogen peroxide and antioxidant activities of the degradation products of different molecular weights. Carbohydr. Polym., 87: 153-159.
12. Prabaharan, M. and J.F. Mano, 2005. Chitosan-based particles as controlled drug delivery systems. Drug Deliv., 12: 41-57.
13. Campos, A., Y. Diebold, E. Carvalho, A. Sánchez and M. Alonso, 2004. Chitosan nanoparticles as new ocular drug delivery systems: *In vitro* stability, in vivo fate and cellular toxicity. Pharm. Res., 21: 803-810.
14. Silva, S.B., A. Oliveira, D. Ferreira, B. Sarmento and M. Pintado, 2013. Development and validation method for simultaneous quantification of phenolic compounds in natural extracts and nanosystems. Phytochem Anal., 24: 638-644.
15. Abd-Elhakeem, M.A., M.M. Ramadan and F.S. Basaad, 2015. Removing of heavy metals from water by chitosan nanoparticles. Journal of Advances in Chemistry, 11(7): 3765-3771.
16. Silva, S.B., M. Amorim, P. Fonte, R. Madureira, D. Ferreira, M. Pintado and B. Sarmento, 2015. Natural extracts into chitosan nanocarriers for rosmarinic acid drug delivery. Pharm. Biol., 53(5): 642-652.
17. Mohanraj, V.J. and Y. Chen, 2006. Nanoparticles: A review. Trop J. Pharm. Res., 5: 561-573.
18. Nahar, M., T. Dutta, S. Murugesan, A. Asthana, D. Mishra, V. Rajkumar, M. Tare, S. Saraf and N.K. Jain, 2006. Functional polymeric nanoparticles: an efficient and promising tool for active delivery of bioactives. Crit Rev. Ther Drug Carrier Syst., 23(4): 259-318.
19. Pangi, Z., A. Beletsi and K. Evangelatos, 2003. PEG-ylated nanoparticles for biological and pharmaceutical application. Adv. Drug. Del. Rev., 24: 403-419.
20. Riss, T.L., R.A. Moravec, A.L. Niles, S. Duellman, H.A. Benink, T.J. Worzella and L. Minor, 2004-2013. Cell Viability Assays, editors, Assay Guidance Manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences. The National Center for Advancing Translational Sciences (PubMed. gov).
21. Calvo, P., C. Remuñan-López, J.L. Vila-Jato and M.J. Alonso, 1997. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res., 14: 1431-1436.
22. Gan, Q. and T. Wang, 2007. Chitosan nanoparticle as protein delivery carrier - Systematic examination of fabrication conditions for efficient loading and release. Colloids Surf B Biointerfaces., 59: 24-34.
23. Loutfy, S.A., H.M. Alam El-Din, M.H. Elberry, N.G. Allam, M.T.M. Hasanin and A.M. Abdellah, 2016. Synthesis, characterization and cytotoxic evaluation of chitosan nanoparticles: in vitro liver cancer model, Advances in Natural Sciences. Nanoscience and Nanotechnology, 7: (035008) pp: 9.
24. Salatin, S. and A.Y. Khosroushahi, 2017. Overview on the cellular uptake mechanism of polysaccharide colloidal nanoparticles. J. Cell. Mol. Med., 21(9): 1668-1686.