Functionalization of iron oxide nanoparticles with clove extract to induce apoptosis in MCF-7 breast cancer cells

T. Thenmozhi¹

Received: 22 October 2019 / Accepted: 21 January 2020 / Published online: 1 February 2020
© King Abdulaziz City for Science and Technology 2020

Abstract

It has been reported that iron oxide nanoparticles have various biomedical applications, including cancer diagnosis and treatment. Iron oxide nanoparticles were known to exert cytotoxicity against MCF-7 breast cancer cell lines and in this present study, we have investigated for their apoptosis-inducing potential in the same cell line. The flow cytometry analysis of the MCF-7 breast cancer cell lines treated with functionalized iron oxide nanoparticles showed an increased percentage of cells in terms of viable, early, and late apoptosis. The cell cycle analysis of the MCF-7 cell lines treated with *Syzygium aromaticum* extract coated with polyvinylpyrrolidone (PVP) + iron oxide nanoparticles and PVP + iron oxide nanoparticles showed substantial accumulation of nanoparticles in the sub-G1 phase, confirming induction of apoptosis. The activities of caspase-3, caspase-8, and caspase-9 increased with increasing concentration of the nanoparticles indicating that activities of caspase can be activated by iron nanoparticles. Further, functionalized nanoparticles induced oxidative stress through reactive oxygen species (ROS) formation. Therefore, it is concluded that the functionalized iron nanoparticles induce apoptosis in MCF-7 breast cancer cell lines and further provides an opportunity to explore the iron nanoparticles for apoptosis in cancer treatment.

Keywords *Syzygium aromaticum* · PVP + Fe–O–NPs · Caspase-3 · Oxidative stress · ROS

Introduction

Nanotechnology has a wide range of applications in oncology diagnosis and treatment (Gmeiner and Ghosh 2015). Among the many metal nanoparticles reported, iron oxide nanoparticles are considered as the most prospective candidates for cancer treatment, owing to their super-paramagnetic behavior and surface modification properties (Palanisamy and Wang 2019). In light of their intrinsic physical and chemical properties, iron oxide nanoparticles have become one of the most compelling theranostic candidates (Dadfaret al. 2019). Iron oxide nanoparticles accumulate through the enhanced permeability and retention (EPR) effect, which is a highly heterogenous phenomenon

especially in tumors and has become increasingly recognized over the past couple of years (Golombek et al. 2018). Cellular metabolic activity variability is high in different cancer types, in different patients with the same tumor type, and in the same patient between different cancers and metastasis (Bedard et al. 2013). This variability is considered one of the main causes for the heterogeneous effects of clinical trials in cancer treatments and avoids this nanomedicine formulations with chemotherapy have been tested in patients (Hua et al. 2018).

In general, the anti-cancer chemotherapeutics or immune-modulatory agents are administered orally and/or intravenously in clinical practice, contributing to their systemic distribution, which can lead to severe side effects such as immune deficiency and autoimmunity (Borghardt et al. 2016; Caspi 2008). Further, the doses of intratumoral medications are often too small and monotherapies are often insignificant for active cancer therapy, multimodal therapy is applied to improve treatment effectiveness via synergistic effects (Cheng et al. 2019; Ma et al. 2011). Tumor targeted drug delivery through bio-functionalized nanoparticles could be a potential route for cancer treatment (Sanna et al. 2014). Trastuzumab, for instance, has

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-020-2088-7>) contains supplementary material, which is available to authorized users.

✉ T. Thenmozhi
thyaguthenmozhi@gmail.com

¹ Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh 517 502, India

shown a noteworthy effect in studies with breast cancer cells along with therapeutic agents and drug delivery vehicles (Day et al. 2010; Hathaway et al. 2011). Therefore, the main objective of this paper is to induce apoptosis in MCF-7 cells by iron oxide nanoparticles and to enhance their apoptotic potential by biofunctionalization with *Syzygium aromaticum* flower bud's (clove) extract.

Materials and methods

Chemicals, reagents, and equipment

All the chemicals and reagents used in this work are laboratory grade. Fluconazole, streptomycin, and doxorubicin were obtained from Thermo Fisher Scientifics, India. Breast cancer cell lines (MCF-7) were purchased from NCCS, Pune, India. Dulbecco's modified Eagle's medium (DMEM), MTT reagents, and all other chemicals and reagents were purchased from HiMedia, India. Curcumin (CAS No. 458-37-7; $\geq 80\%$) from HiMedia was used as a standard drug in all the studies of this paper. Equipment used in this study are centrifuge (Remi, India); 12 $\times$ 75 mm polystyrene tubes (BD biosciences); Inverted biologic microscope (Biolink); 37 °C incubator with a humidified atmosphere of 5% CO₂ (Healforce, China) and Flow cytometer (BD FACSCalibur).

Preparation of PVP-coated iron oxide nanoparticles loaded with *S. aromaticum* extract

Extract of the *S. aromaticum* flower bud was done according to the protocol described by Thenmozhi et al. (2019), but briefly, the clove powder was subjected to soxhlet extraction with methanol at 1:4 (w/v) ratio for 18 h. The extract was concentrated, dried, and used for *in vitro* studies. The synthesis, characterization of iron oxide nanoparticles and loading of *S. aromaticum* extract into PVP-coated iron oxide nanoparticles is done by following procedures described elsewhere (Thenmozhi et al. 2019) mixing 8 mL of 1 M ferric chloride and 2 mL of 2 M ferrous chloride solutions followed by dropwise addition of NaOH under stirring until the solution changes into brown color, indicative of iron oxide formation. Later 5 g of PVP was added and sonicated for 30 min, followed by thermal annealing at 600 °C for 30 min. The drug loading was carried out by dispersing 5 mg of PVP-coated iron oxide nanoparticles in 5 mL of *S. aromaticum* extract and mixed in a rotary shaker (200 rpm) at 37 °C for 24 h then the nanoparticles with extract are magnetically separated and dried.

Cell culture

Human breast cancer cell lines (MCF-7) were purchased from the National Centre for Cell Sciences (Pune, India). Frozen cells were thawed and inoculated into 5 mL of DMEM supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). The cells were cultured in T-25 flasks and incubated in 95% humidified incubator with 5% CO₂ at 37 °C. The cell cultures were used when reached approximately 70% confluence for experimental treatments.

Cytotoxicity assay

Cytotoxicity of the biofunctionalized iron nanoparticles was measured by MTT assay as reported elsewhere (Thenmozhi et al. 2019), briefly human breast cancer cell lines (MCF-7) were cultured at of 2000 cells/well density and treated with diluted biofunctionalized iron nanoparticles (50, 150, 250, 350, and 450 $\mu\text{g/mL}$) and incubated for 24 h at 37 °C in a 5% CO₂ incubator. MTT solution was then added to each well and incubated for additional 3 h at 37 °C in the 5% CO₂ incubator. Subsequently, the purple formazan crystals were dissolved using dimethyl sulfoxide (DMSO), the optical density was measured of each well using an ELISA reader at 570 nm. The cytotoxicity and IC₅₀ was recorded.

In vitro flow cytometry assays

To perform in vitro flow cytometry assays, we have divided MCF-7 cell lines as per the drug treatment to respective cell lines as summarized in Table S1. The standard (Curcumin at 25 μM), Test Compound-1 (PVP+Fe-ONPs at 228 $\mu\text{g/mL}$) and Test Compound-2 (*S. aromaticum* + PVP + Fe-ONPs at 209 $\mu\text{g/mL}$) were used in the present study.

Annexin V/propidium iodide (PI) staining

When needed the classical Annexin V/PI staining assay has been used to study early apoptosis induction events using the flow cytometry method described by Panicker et al. (2019).

DNA damage study (terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling [TUNEL] assay)

After the treatment, the cells were suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4) at a concentration of $1\text{--}2 \times 10^6$ cells/ml and placed on ice for 30 min. Subsequently, the cells were centrifuged for 5 min at 300 $\times g$ and the supernatant was discarded. Then cells were washed with 5 ml PBS and the cell pellet was resuspended in the

residual PBS by gentle vortexing. The cell concentration was adjusted to 1.2×10^6 cells/ml in 70% (v/v) ice-cold ethanol. The DNA damage was assessed as per the procedure provided with APO-DIRECT™ Kit.

Cell cycle analysis

Cell cycle distribution studies were performed as per the modified protocol described by McGowan et al. (2011). MCF-7 cells were cultured in a 6-well plate to a density of 3×10^5 cells/2 ml. The test compounds were added to the culture and incubated for another 24 h. Later, the medium was removed and washed with PBS. About 200 μ l of trypsin–EDTA solution was added and incubated at 37 °C for 3–4 min. The cells were added with 2 ml of fresh culture medium into 12 $\times$ 75 mm polystyrene tubes and centrifuged for 5 min at $300 \times g$ at 25 °C. The supernatant was decanted and the cells were washed twice with PBS. Subsequently, the cells were fixed in 1 ml of cold 70% ethanol and washed twice with PBS before DNA staining. To ensure that only DNA is stained, the treated cell pellet was treated with 50 μ l of ribonuclease A to get rid of RNA. The pellet was mixed well and incubated at room temperature for 10 min.

Flow cytometric measurement of reactive oxygen species (ROS)

The production of ROS was detected using DCFH-DA dye essentially according to the procedure mentioned previously by Gao et al. (2004).

BCL2 expression study

The cells were cultured in 6-well plate to a density of 3×10^5 cells/2 ml and incubated in a CO₂ incubator at 37 °C for 24 h. The spent medium was aspirated and the cells were treated with the different concentrations of experimental compounds and incubated the cells for 24 h. At the end of the treatment, the medium was removed from the wells and washed with PBS. About 200 μ l of trypsin–EDTA solution was added and incubated at 37 °C for 3–4 min. The cells were harvested with 2 ml of culture medium and taken into 12 $\times$ 75 mm polystyrene tubes. The tubes were centrifuged for 5 min at $300 \times g$ at 25 °C and supernatant was decanted and the cells were washed twice with PBS. The cells were fixed in 1 ml of prechilled cold 70% ethanol and by adding the solution drop wise to cell pellet while vortexing. This should ensure the fixation of all cells and minimize clumping and the cells were allowed to get fixed for 30 min on ice. Specimens can be left at this stage for several weeks. The cell pellet was made by centrifuging the cells at a higher speed compared to live cells for 5 min and the supernatant was aspirated carefully so that no pellet is lost. The pellet

was washed twice with PBS and 10 μ l of FITC-Bcl2 antibody was added and incubated for 30 min in the dark at 25 °C. After incubation, the cells were washed with 1 $\times$ PBS with 0.1% sodium azide and 0.5 mL of PBS was added, mixed thoroughly, and analyzed by flow cytometry.

BAX expression study

The cells were cultured in a 6-well plate to a density of 3×10^5 cells/2 ml and incubated in a CO₂ incubator overnight at 37 °C for 24 h. The spent medium was aspirated and the cells were treated with the required concentration of experimental compounds in 2 ml of culture medium and incubated for 24 h. At the end of the treatment, the medium was removed from all the wells and washed with PBS. About 200 μ l of trypsin–EDTA solution was added and incubated at 37 °C for 3–4 min. To this 2 ml of culture medium was added and the cells were harvested directly into 12 $\times$ 75 mm polystyrene tubes. The tubes were centrifuged for five min at $300 \times g$ at 25 °C and the supernatant was carefully decanted. Later, the cells were washed twice with PBS and the supernatant was decanted. The cells were fixed in 1 ml of prechilled cold 70% ethanol. Later, the cells were washed twice with PBS and 10 μ l of BAX antibody was added and incubated for 30 min in the dark at 25 °C. After incubation, 10 μ l of Mouse IgG fluorescein antibody was added and again incubated for 30 min in the dark at 25 °C. Later, the cells were washed with 1 $\times$ PBS with 0.1% sodium azide, 0.5 mL of PBS was added, mixed thoroughly, and analyzed by flow cytometry.

Analysis of the mitochondrial membrane potential using the cationic JC-1 dye

The MCF-7 cells were cultured in a 6-well plate to a density of 3×10^5 cells/1 ml and incubated in a CO₂ incubator overnight at 37 °C for 24 h. Later, the cells were treated with the required concentration of experimental compounds in 1 ml of culture medium and incubated for 24 h. At the end of the treatment, the cells were harvested directly into 12 $\times$ 75 mm polystyrene tubes and centrifuged the tubes for five min at $300 \times g$ at 25 °C. The supernatant was decanted and cells were washed with PBS and 0.5 ml of JC-1 working solution was added to each pellet. Later gently resuspended the cells in the JC-1 working solution and incubated for 15 min at 37 °C in a CO₂ incubator. After incubation the cells were washed repeatedly with 1 $\times$ assay buffer and analyzed the cells by flow cytometry.

Caspase-9 expression study

For caspase-9 expression study, we have cultured cells in a 6-well plate to a density of 3×10^5 cells/2 ml and the cells

were incubated in a CO₂ incubator overnight at 37 °C for 24 h. Then spent medium was aspirated and treated with the required concentration of experimental compounds in 2 ml of culture medium and incubated the cells for 24 h. At the end of the treatment, the medium was aspirated from all the wells with PBS. About 200 µl of trypsin–EDTA solution was added and incubated at 37 °C for 3–4 min. After incubation, 2 ml of culture medium was added and harvested the cells directly into 12×75 mm polystyrene tubes. The tubes were centrifuged for five min at 300×g at 25 °C and supernatant was carefully decanted. Later, the cells were washed twice with PBS and decanted. The cells were fixed in 1 ml of pre-chilled cold 70% ethanol by adding the solution drop-wise to cell pellet while vortexing and the cells were allowed to be fixed for at least 30 min on ice. Later, the cells were washed twice with PBS and 5 µL of caspase-9 antibodies were added. The cells were mixed thoroughly and incubated for 30 min in the dark at 25 °C. Later, 5 µL of mouse IgG fluorescein antibody was added and incubated for 30 min in the dark at 25 °C. The cells were washed with 1 × PBS with 0.1% sodium azide. About 0.5 mL of PBS was added, mixed thoroughly and analyzed by flow cytometry.

Caspase-8 expression study

For Caspase-8 expression study, we have cultured MCF-7 cells in a 6-well plate to a density of 3×10^5 cells/2 ml and incubated in a CO₂ incubator overnight at 37 °C for 24 h. Then the supernatant was aspirated and the cells were treated with the required concentration of experimental compounds in 2 ml of culture medium and incubated for 24 h. At the end of the treatment, the medium was removed from all the wells and washed with PBS and then 200 µl of trypsin–EDTA solution was added and allowed for incubation at 37 °C for 4 min. After incubation, 2 ml of culture medium was added and the cells were harvested directly into 12×75 mm polystyrene tubes and centrifuged for five min at 300×g at 25 °C and supernatant was decanted. Later, the cells were washed twice with PBS and the supernatant was decanted. The cells were fixed in 1 ml of prechilled cold 70% ethanol and then washed twice with PBS and 5 µL of caspase-8 antibody was added. The cells were mixed thoroughly and incubated for 30 min in the dark at 25 °C. Later, 5 µL of mouse IgG fluorescein antibody was added and incubated for 30 min in the dark at 25 °C. The cells were washed with 1X PBS along with 0.1% sodium azide, about 0.5 mL of PBS was added, mixed thoroughly, and analyzed by flow cytometry.

Caspase-3 expression study

For caspase-3 expression study cultured the MCF-7 cells in a 6-well plate to a density of 3×10^5 cells/2 ml and incubated in a CO₂ incubator overnight at 37 °C for 24 h. The spent

medium was aspirated and the cells were treated with the required concentration of experimental compounds in 2 ml of culture medium and the cells were incubated for 24 h. At the end of the treatment, the medium was removed from all the wells and washed with PBS. Later, the PBS was removed and 200 µl of trypsin–EDTA solution was added and incubated at 37 °C for 3–4 min. After incubation, 2 ml culture medium was added and the cells were harvested directly into 12×75 mm polystyrene tubes. The tubes were centrifuged for five min at 300×g at 25 °C and the supernatant was carefully decanted. The cells were washed with PBS and then fixed in 1 ml of prechilled cold 70% ethanol and then washed twice with PBS. About 5 µL caspase-3 antibodies was added and then thoroughly mixed and incubated for 30 min in the dark at 25 °C. To this 5 µL of Mouse IgG fluorescein antibody was added and incubated for 30 min in the dark at 25 °C. The cells were washed with 1 × PBS with 0.1% sodium azide, and 0.5 mL of PBS added, mixed thoroughly for analyzed by flow cytometry.

Statistical analysis

The experiments were carried out in triplicates and data presented were the mean $\pm$ standard deviation (SD). The apoptosis data was calculated and analyzed using Graphpad Prism of BD FACSCalibur, CellQuest Pro Software (Version: 6.0) utilizing nonlinear regression analysis. One-way ANOVA followed by Tukey's test was applied to observe the significance of the groups. The difference of $p \leq 0.05$ was considered to be statistically significant.

Results and discussion

Results of preparation of clove extract, synthesis and characterization of iron oxide nanoparticles, and loading of a drug (clove extract) into PVP-coated iron oxide nanoparticles are clearly illustrated in our previously published paper (Thenmozhi et al. 2019).

In vitro flow cytometry assays

Annexin-V-FITC assay or Apoptosis study

Annexin V-PI expression study of the used test compounds against MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0) are shown in Fig. 1. Quadrants showing the expression of given untreated MCF-7 Cells (A) & Standard drug (Curcumin-25 µM) treated MCF-7 cells (B) and Test Compound-1 (PVP + Fe–ONPs) and Test Compound-2 (*S. aromaticum* + PVP + Fe–ONPs) treated MCF-7 cells against the Annexin V-FITC and PI Stain.

UL = % of Necrotic Cells; UR = % Late Apoptotic Cells; LL = % Viable Cells; LR = % of Early apoptotic cells

Fig. 1 Annexin V-PI expression study of tested compounds namely PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs against MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0)

Here, Annexin V-FITC acts as primary stain and PI as a secondary fluorescence stain. Table S2 shows the % of MCF-7 cells that endure apoptosis and necrosis in the untreated, standard and test compounds namely PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs treated cells and overlay of the results plotted in bar graph, Fig. S1. As per Table S2, the percentage of cells gated in the different samples like cell control, cells treated with standard drug curcumin, and cells treated with test samples. The lower left (LL) quadrant population represents the viable cells. 99.94%, 51.40%, 64.67%, and 33.95% of cells were gated in untreated, standard, and test compounds, namely, PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs respectively in the LL quadrant. Upper left (UL) quadrant represents cell debris or necrotic cells. 0.06%, 5.40%, 5.59% and 6.58% of cells were gated in untreated, standard and test compounds, PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs respectively in UL quadrant. The upper right (UR) quadrant represents late apoptotic cells. 0%, 26.88%, 11.58% and 28.46% of cells were in untreated, standard and test compounds, PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs respectively in UR quadrant. The lower right (LR) quadrant represents early apoptotic cells. 0%, 16.32%, 18.16% and 31.01% of cells were gated in untreated, standard, and test compounds, PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs respectively in LR quadrant. The M1

populations are the viable cells that are low in mean Annexin V-FITC fluorescence intensity, while M2 populations are the apoptotic cells that have taken up the Annexin V-FITC and are high in mean Annexin V-FITC fluorescence intensity. Similarly, in a study, the biofunctionalization of Ag nanoparticles with lactonase results in altered cytotoxic properties without harming the host tissue and provides an appropriate template for the design of new antibiofilm drugs to overcome the drug resistance issues (Gupta and Chhibber 2019).

DNA damage study (TUNEL assay)

The results for DNA damage assessment by TUNEL assay are presented in Fig. 2. As shown in Fig. 2, the dUTP-FITC histogram of the gated MCF-7 singlets distinguish cells at the M1 and M2 phases. (Here M1 refers to negative expression/region and M2 refers to the positive expression/region). Moreover, the gating of M1 and M2 phases is approximate and can be refined using software (CellQuest Software, Version 6.0) analysis. The obtained results suggest that in MCF-7 cells, the expression of d-UTP is very low in untreated cells (3.94MFU) compared to curcumin (25 μ M) (96.31MFU). The Test Compound-1 (PVP + Fe-ONPs) and Test Compound-2 (*S. aromaticum* + PVP + Fe-ONPs) are showing the 71.81MFU and 86.12MFU of d-UTP expression (Table S3) indicating the positive influence

Fig. 2 d-UTP expression study of test compounds (PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs) against the MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0)

of tested compounds on DNA damage. As per Table S3, no DNA damage (M1) and DNA damage (M2) expression of FITC-dUTP against the untreated, standard, and test compounds namely PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs treated MCF-7 cells were observed and overlay of the results plotted in the bar graph as in Fig. S2, which indicates the positive influence of the compounds tested on DNA damage.

Cell cycle distribution

The cell cycle distribution assay results are presented in Fig. 3. The obtained PI histogram of the gated cell singlets distinguishes cells at the Sub G0/G1, G0/G1, S, and G2/M cycle phases. Here the gating of cell cycle phases are approximate and can be refined using software (CellQuest

Software, Version 6.0) analysis. As summarized in Table S4, in G0/G1 phase, 73.26%, 47.51%, 55.59%, and 46.24% of cells get arrested in untreated, standard control, and test compounds namely PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs with concentrations 228 µg/mL and 209 µg/mL respectively. In S phase, 5.06%, 11.20%, 4.51%, and 3.69% of cells get arrested in untreated, standard control, and test compounds namely PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs with concentrations 228 µg/mL and 209 µg/mL respectively. On the other hand, in G2/M phase, 19.54%, 31.70%, 31.39%, and 41.93% of cells get arrested in untreated, standard control, and test compounds namely PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs with concentrations 228 µg/mL and 209 µg/mL respectively. About 10,000 singlet cells were selected for the

Fig. 3 Cell cycle analysis of tested compounds (PVP+Fe–ONPs and *S. aromaticum*+PVP+Fe–ONPs) against MCF-7 cells using BD FACS-Calibur

analysis for all samples including cell control. In addition, overlay of the results were plotted in a stacked graph as shown in Fig. S3.

Flow cytometric measurement of ROS

In this study, test compounds PVP+Fe–ONPs and *S. aromaticum*+PVP+Fe–ONPs with 2 concentrations 228 µg/mL, and 209 µg/mL respectively were used to check the ROS on MCF-7 and results are illustrated in Fig. 4. As shown in Fig. 4, the H₂DCFDA histogram of the gated MCF-7 singlets distinguish cells at the M1 and M2 phases. In the obtained results, M1 refers to positive expression/region and M2 refers to the negative expression/region. In addition, gating of M1 and M2 phases is approximate and can be refined using software (CellQuest Software, Version 6.0) analysis. Overlay histogram of the tested compounds are illustrated in Fig. S4. The observations H₂DCFDA expression study by flow cytometry suggesting that in MCF-7 cells, the expression of H₂DCFDA is very low in untreated MCF-7 cells

(10.10 MFU) compared to toxic compound-H₂O₂ (200 µM) is showing 41.45 MFU (Table. S5). The test compound-1 (PVP+Fe–ONPs) with IC 50 concentration (228 µg/mL) and test compound-2 (*S. aromaticum*+PVP+Fe–ONPs) with IC 50 concentration (209 µg/mL) along with the toxic compound H₂O₂ (200 µM) is showing 20.08MFU and 13.30 MFU respectively and the overlay of the results are briefed in Fig. S5. Comparable results were observed when biofunctionalizing selenium nanoparticles with a *Rosa roxburghii* fruit polysaccharide and enhanced protective effects against H₂O₂-induced apoptosis were found (Wang et al. 2019).

BCL2 expression

For BCL2 expression study, test compounds PVP+Fe–ONPs and *S. aromaticum*+PVP+Fe–ONPs with 2 concentrations 228 µg/mL, and 209 µg/mL respectively were used and the results are illustrated in Fig. 5. As shown in Fig. 5, Bcl-2-FITC histogram of the gated MCF-7 singlets distinguishes cells at the M1 and M2 phases. Here M1 refers to positive

Fig. 4 H₂DCFDA expression study of test compounds (PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs) with different Concentrations on MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0)

expression/region and M2 refers to the negative expression/region. Gating of M1 and M2 phases are approximate and can be refined using software (CellQuest Software, Version 6.0) analysis. The obtained data of Bcl2 expression Study by flow cytometry suggests that in MCF-7 cells, the positive expression of Bcl2 is very low in untreated MCF-7 cells (0.28% positive expression region) compared to curcumin treated (25 μ M) which is 99.91% positive Bcl2 expression (Table S6). The test compound, (PVP+Fe-ONPs) with IC 50 concentration (228.46 μ g/mL) has shown the 69.57% positive expression of Bcl-2 and the other tested compound, (*S. aromaticum*+PVP+Fe-ONPs) with the concentration (209.93 μ g/mL) has shown the 76.56% positive expression of Bcl-2 and the overlay of the results were plotted in the bar graph as shown in Fig. S6.

BAX expression

For BAX expression study, test compounds PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs

with 2 concentrations 228 μ g/mL, and 209 μ g/mL respectively were used and the results are illustrated in Fig. 6. As shown in Fig. 6, BAX expression study of tested compounds (PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs) on MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0) is very stimulating. Bcl-2-FITC histograms of the gated MCF-7 singlets distinguish cells at the M1 and M2 phases. Here M1 refers to negative expression/region and M2 refers to the positive expression/region. Gating of M1 and M2 phases is approximate and can be refined using software (CellQuest Software, Version 6.0) analysis. The obtained data of BAX expression study by flow cytometry suggests that the expression is very low in untreated MCF-7 cells (0.19% Positive expression) compared to curcumin-treated (25 μ M) which is 97.67% positive expression (Table S7). The test compound, (PVP+Fe-ONPs) with IC 50 concentration (228.46 μ g/mL) has shown the 70.94% positive expression of Bax and test compound, (*S. aromaticum*+PVP+Fe-ONPs) with the IC50 concentration

Fig. 5 BCL-2 expression study of tested compounds (PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs) on MCF-7 cells using BD FACS Calibur, CellQuest Pro Software (Version: 6.0)

(209.93 $\mu\text{g/mL}$) has shown the 98.65% positive expression of Bax and overlay of the obtained results were plotted in bar graph as shown in Fig. S7.

Mitochondrial-JC1

In this study, test compounds PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs with IC₅₀ concentrations are used to check the mitochondrial membrane potential on MCF-7 cell lines. Mitochondrial membrane potential of the tested compounds against MCF-7 cell lines is presented in Fig. 7 and the quadrants in the figure show that the expression of JC1 stain in the untreated MCF-7 cells, standard drug (Curcumin-25 μM) treated MCF-7 cells, and test compound-1 (PVP+Fe-ONPs) treated MCF-7 cells, and test compound-2 (*S. aromaticum*+PVP+Fe-ONPs) treated MCF-7 cells (D) against the FL1-JC1 and FL2-JC1. Table S8 shows the percentage of cells with mitochondrial membrane depolarization in the untreated, standard, and test compounds namely, PVP+Fe-ONPs and *S.*

aromaticum+PVP+Fe-ONPs with IC₅₀ concentrations and overlay of the results plotted in bar graph as shown in Fig. S8. In Fig. S8, the upper quadrant represents healthy cells stained with JC 1 stain (FL2). In upper quadrant, 99.96%, 39.16%, 57.50%, and 46.10% of cells were found in untreated, standard, and test compounds treated with MCF-7 cells respectively. Similarly, the lower quadrant represents the cells that undergone mitochondrial membrane depolarization by the stain, JC1 (FL1). 0.04%, 60.84%, 42.5%, and 53.9% of cells were found in untreated, standard, and test compounds treated with MCF-7 cells respectively in the lower quadrant.

Caspase-9 expression

In this study, test compounds PVP+Fe-ONPs and *S. aromaticum*+PVP+Fe-ONPs with IC₅₀ concentrations are used to assess caspase-9 expression on MCF-7 cell lines. Figure 8, summarizes the caspase-9 expression of the tested compounds against MCF-7 cell lines. In Fig. 8, the

Fig. 6 BAX expression study of tested compounds (PVP + Fe–ONPs and *S. aromaticum* + PVP + Fe–ONPs) on MCF-7 cells using BD FACS-Calibur, CellQuest Pro Software (Version: 6.0)

caspase-3-FITC histogram of the gated MCF-7 singlets distinguishes the cells at M1 and M2 phases. Here M1 refers to negative expression/region and M2 refers to the positive expression/region). Gating of M1 and M2 phases is approximate and can be refined using software (CellQuest Software, Version 6.0) analysis. The obtained data of caspase-9 expression studies by flow cytometry suggests that in MCF-7 cells, the positive expression of caspase-9 is very low in untreated (0.98%) compared to curcumin (25 μ M) which is 99.57% (Table S9). The compound, (PVP + Fe–ONPs) with IC₅₀ concentration have shown the 97.34% caspase-9 positive expression. Whereas the test compound, *S. aromaticum* + PVP + Fe–ONPs) with IC₅₀ concentration (209 μ g/mL) is showing the 99.22% of caspase-9 positive expression (Table S9) and the overlay of the results plotted in the bar graph as shown in Fig. S9.

Caspase-8 expression

For Caspase-8 expression study, test compounds namely PVP + Fe–ONPs and *S. aromaticum* PVP + Fe–ONPs with

IC₅₀ concentrations are used to assess Caspase-8 expression on MCF-7 cell lines. The used concentrations of the compound to treat the cells are summarized in Table S1. Fig. S10, illustrates the results of caspase-8 expression studies of tested compounds (PVP + Fe–ONPs and *S. aromaticum* + PVP + Fe–ONPs) on MCF-7 cells using BD FACS-Calibur, CellQuest Pro Software (Version: 6.0). Here the caspase-8-FITC histograms of the gated MCF-7 singlets distinguish cells at M1 and M2 phases. M1 refers to negative expression/region and M2 refers to the positive expression/region. Gating of M1 and M2 phases is approximate and refined using software (CellQuest Pro Software, Version 6.0) analysis. The observations of caspase-8 expression studies by flow cytometry suggesting that in MCF-7 cells, the positive expression of caspase-8 is very low in untreated (1.23%) compared to curcumin (25 μ M) which shows 99.03% (Table S10). The tested compound, PVP + Fe–ONPs with IC₅₀ has shown 94.94% and the other compound, *S. aromaticum* + PVP + Fe–ONPs with IC₅₀ has shown 98.91% of caspase-8 positive expression (Table S10) and the overlay of the results plotted in the bar graph as shown in Fig. S11.

Fig. 7 JC1 expression study of test compounds PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs with IC₅₀ concentrations against the MCF-7 cells using BD FACSCalibur, CellQuest Pro Software (Version: 6.0)

Caspase-3 expression

For caspase-3 expression study, test compounds PVP + Fe-ONPs and *S. aromaticum* + PVP + Fe-ONPs with IC₅₀ concentrations are used to assess caspase-8 expression on MCF-7 cell lines. Fig S12 illustrates the caspase-3 expression studies of the tested compounds against MCF-7 cell lines using BD FACSCalibur, CellQuest Pro Software (Version: 6.0). As per Fig. S12, caspase-3-FITC histogram of the gated MCF-7 singlets distinguishes cells at M1 and M2 phases. Here M1 refers to negative expression/region and M2 refers to the positive expression/region. Gating of M1 and M2 phases is approximate and can be refined using CellQuest Software, Version 6.0. As summarized in Table S11, the caspase-3 expression studies by flow cytometry suggests that in MCF-7 cells, the positive expression of caspase-3 is very low in untreated (0.1%) compared to curcumin (25 μ M) which is 97.95 %. The tested compound, PVP + Fe-ONPs with IC₅₀ concentration has shown the 92.46% of caspase-3 positive expression and the other compound, *S. aromaticum* + PVP + Fe-ONPs with IC₅₀ concentration has shown the 94.87% of caspase-3 positive expression and the overlay of the results plotted in the bar

graph as shown in Fig. S13. All the results obtained confirm the enhanced properties of biofunctionalized silver nanoparticles for anticancer applications. Other researchers with biofunctionalized silver nanoparticles have observed similar observations from different extracts of plant parts and these nanoparticles have enhanced biological properties like antioxidant, anticancer, and so on (Srinithya et al. 2016; Chandrasekaran et al. 2016; Yadav et al. 2019).

Conclusion

Breast cancer is the most common malignancy among women worldwide and with an alarming rise in incidence rate recently. To reduce chemotherapy side effects and effectively treat breast cancer the current therapies needed new clinical strategies. Thus, for their apoptosis-inducing ability, iron oxide nanoparticles, previously found to exert cytotoxic exert against MCF-7 breast cancer cell lines, were biofunctionalized with *S. aromaticum* extract and investigated for enhanced apoptosis. The flow cytometric study descriptive dot plots showed an increase in the percentages of cells in early apoptosis and late apoptosis of the treated cells.

Fig. 8 Results of the caspase-9 expression studies of the tested compounds against MCF-7 Cell line

The findings from cell cycle analysis showed a significant increase in accumulation of PVP+ Iron oxide nanoparticles loaded with *S. aromaticum* extract as well as PVP+ Iron oxide nanoparticles by the cells during the sub-G1 state, confirming apoptosis induction. Further, these nanoparticles activated caspase-3, caspase-8, and caspase-9 response and also induced oxidative stress *via* ROS formation.

Acknowledgements The author highly thankful to Dr. John Sushma, Assistant Professor, Department of Biotechnology, SPMVV, Tirupati, India for her kind guidance and for providing instrumentation facilities.

Author contributions T.T. designed the experiments, carried out the experiment and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This work do not require ethical approval.

References

- Anfosso L, Efferth T, Albini A, Pfeffer U (2006) Microarray expression profiles of angiogenesis-related genes predict tumor cell response to artemisinins. *Pharmacogenomics J* 6(4):269
- Bedard PL, Hansen AR, Ratain MJ, Siu LL (2013) Tumour heterogeneity in the clinic. *Nature* 501(7467):355–364
- Borghardt JM, Weber B, Staab A, Kunz C, Formella S, Kloft C (2016) Investigating pulmonary and systemic pharmacokinetics of inhaled olodaterol in healthy volunteers using a population pharmacokinetic approach. *Br J Clin Pharmacol* 81(3):538–552
- Caspi RR (2008) Immunotherapy of autoimmunity and cancer: the penalty for success. *Nat Rev Immunol* 8(12):970–976
- Chandrasekaran R, Gnanasekar S, Seetharaman P, Keppanan R, Arockiaswamy W, Sivaperumal S (2016) Formulation of *Carica papaya* latex-functionalized silver nanoparticles for its improved antibacterial and anticancer applications. *J Mol Liq* 219:232–238
- Cheng CT, Castro G, Liu CH, Lau P (2019) Advanced nanotechnology: an arsenal to enhance immunotherapy in fighting cancer. *Clin Chim Acta* 492:12–19
- Dadfar SM, Roemhild K, Drude NI, von Stillfried S, Knüchel R, Kiessling F, Lammers T (2019) Iron oxide nanoparticles: diagnostic, therapeutic and theranostic applications. *Adv Drug Deliv Rev* 138:302–325

- Day ES, Bickford LR, Slater JH, Riggall NS, Drezek RA, West JL (2010) Antibody-conjugated gold-gold sulfide nanoparticles as multifunctional agents for imaging and therapy of breast cancer. *Int J Nanomed* 5:445–454
- Gao F, Yi J, Yuan JQ, Shi GY, Tang XM (2004) The cell cycle related apoptotic susceptibility to arsenic trioxide is associated with the level of reactive oxygen species. *Cell Res* 14:81–85
- Gmeiner WH, Ghosh S (2015) Nanotechnology for cancer treatment. *Nanotechnol Rev* 3(2):111–122
- GolombekSK MJN, Theek B, Appold L, Drude N, Kiessling F, Lammerers T (2018) Tumor targeting via EPR: strategies to enhance patient responses. *Adv Drug Deliv Rev* 130:17–38
- Gupta K, Chhibber S (2019) Biofunctionalization of silver nanoparticles with lactonase leads to altered antimicrobial and cytotoxic properties. *Front Mol Biosci* 6:63
- Hathaway HJ, Butler KS, Adolphi NL, Lovato DM, Belfon R, Fegan D, Monson TC, Trujillo JE, Tessier TE, Bryant HC, Huber DL, Larson RS, Flynn ER (2011) Detection of breast cancer cells using targeted magnetic nanoparticles and ultra-sensitive magnetic field sensors. *Breast Cancer Res* 13(5):R108
- Hua S, de Matos MBC, Metselaar JM, Storm G (2018) Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: pathways for translational development and commercialization. *Front Pharmacol* 9:790. <https://doi.org/10.3389/fphar.2018.00790>
- Ma J, Chen CS, Blute T, Waxman DJ (2011) Antiangiogenesis enhances intratumoral drug retention. *Can Res* 71(7):2675–2685
- McGowan EM, Alling N, Jackson EA, Yagoub D, Haass NK, Allen JD, Martinello-Wilks R (2011) Evaluation of cell cycle arrest in estrogen responsive MCF-7 breast cancer cells: pitfalls of the MTS assay. *PLoS ONE* 6(6):e20623
- Palanisamy S, Wang YM (2019) Superparamagnetic iron oxide nanoparticulate system: synthesis, targeting, drug delivery and therapy in cancer. *Dalton Trans* 48(26):9490–9515
- Panicker NG, Balhamar SOMS, Akhlaq S, Qureshi MM, Rizvi TS, Al-Harrasi A, Hussain J, Mustafa F (2019) Identification and characterization of the Caspase-mediated apoptotic activity of *Teucrium mascatense* and an isolated compound in human cancer cells. *Molecules* 24(5):E977. <https://doi.org/10.3390/molecules24050977>
- Sanna V, Pala N, Sechi M (2014) Targeted therapy using nanotechnology: focus on cancer. *Int J Nanomed* 9:467–483
- Srinithya B, Kumar VV, Vadivel V, Pemaiah B, Anthony SP, Muthuraman MS (2016) Synthesis of biofunctionalized AgNPs using medicinally important *Sida cordifolia* leaf extract for enhanced antioxidant and anticancer activities. *Mater Lett* 170:101–104
- Thenmozhi T, Rasajna N, Rajesh M, Sushma NJ (2019) Biosynthesis and characterization of iron oxide nanoparticles via *Syzygium aromaticum* extract and determination of its cytotoxicity against human breast cancer cell lines. *J Nanosci Technol* 5:587–592
- Wang L, Li C, Huang Q, Fu X (2019) Biofunctionalization of selenium nanoparticles with a polysaccharide from *Rosa roxburghii* fruit and their protective effect against H₂O₂-induced apoptosis in INS-1 cells. *Food Funct* 10(2):539–553
- Yadav R, Saini H, Kumar D, Pasi S, Agrawal V (2019) Bioengineering of *Piper longum* L. extract mediated silver nanoparticles and their potential biomedical applications. *Mater Sci Eng C* 104:109984