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Anti-aging Effect of Free Curcumin and Niosome Entrapping Curcumin in H_2O_2-induced Aging in Human Fibroblast Cell Lines

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Abstract

Aging is not a disease but it a changes that come with time, aging may make an individual susceptible to disease. Exposure to smoke, UV radiation, chemicals or other exogenous agents over time can damage cells, as can produce DNA replication errors or oxidative stress. The use of antioxidant curcumin can produce intracellular defenses in addition to low expensive and no side effect. In the present study, hydrogen peroxide (H_2O_2) was used to produce oxidative stress in Human Fibroblast cell lines (HFB4) and free curcumin and niosome entrapping curcumin treatments are used for aged HFB4 cell lines. Using Flowcytometry analysis, p 16 INK4a and p53 BP1 genes levels were investigated as aging biomarkers. Result show that H_2O_2 treated cell lines are highly elevate p16 INK4a and p53 BP1 genes. While HFB4 cells treated with free curcumin and niosome entrapping curcumin were significantly protected against aged process.

Keywords: Aging, Curcumin, H_2O_2;,Oxidative stress, HFB4 cell lines.

1. Introduction

Aging is characterized by a progressive loss of physiological function, leading to impaired function and increased vulnerability to death. This deterioration is the primary risk factor for major human pathologies, including cancer [1], diabetes [2], cardiovascular disorders [3], and neurodegenerative diseases [4].

Free radicals, or reactive oxygen species (ROS), form a natural byproduct of energy production in the mitochondria. In high doses free radicals can be damaging to the cell, Increasing ROS production in aged cells produce mitochondrial dysfunction, which causes further increases in ROS production and cellular components damage. Human DNA is modified with epigenetic information that enhances or suppresses the expression of particular genes as required by different tissue types. For example, if a cell should develop into a liver cell, epigenetic modifications will ensure that the parts of the genome specific to liver cells are expressed, while the parts specific to other cell types are ignored. The aging process often involves changes in our epigenetic code, which can lead to changes in gene expression that affect normal cellular function [5]. Senescence is cellular response that induces a growth arrest by activation of p16 INK4a and p53 genes [6, 7]. The sustained induction of this genes produce damage that result in a permanent arrest of the cell cycle [7-10].

Skin is the largest organ of the body and is made up of tissue layers, which protect underlying muscles and organs. The most important role of skin is to protect our body against heat, light, infection, and injury. Skin is the first line of defense to various adverse conditions, such as rashes, burns, injuries, infections, and disorders, such as scleroderma, dermatitis, psoriasis, and cancer [11, 12]. The skin's morphological change that comes with aging is wrinkles, decrease the number of sweat glands and blood vessels, dryness, develop brown spots, heliodermatitis and hyperplasia of elastic fibres [13-17].

Antioxidants is a molecule that inhibits the oxidation of other molecules. Antioxidants are classified into two broad divisions, depending on whether they are soluble in hydrophilic or in lipophilic. In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma [18] while lipid-soluble

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antioxidants protect cell membranes from lipid peroxidation [19]. These compounds may be synthesized in the body or obtained from the lipid diet. The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some present within cells, while others are more equally distributed. Some antioxidants are only found in a few organisms [20]. Many of the phytochemicals present in plants have good health positive effects as being direct antioxidants.

Curcumin (Cur), known as 1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6- hepatadiene-3,5-dione, is a nature yellow colored and low molecular weight polyphenol compound purified from the rhizome of the plant Curcuma longa [21]. There are several studies proved Curcumin health beneficial effects as such phytochemical agent. It has anti-inflammatory, anticancer compound [22, 23] and anti-oxidant effects. However, little is known about molecular mechanisms action of curcumin on normal human cells undergoing aging. Curcumin act as chemopreventive agent against various chemicals and environmental pollutants that cause skin damage due to its nontoxic nature, The antioxidant activity of curcumin arises mainly from scavenging of biological free radicals that are produced during physiological processes which can be useful as skin antiaging [24]. Curcumin has shown to protect skin from wrinkles, leading to healthy younger skin [25]. Various synthetic skin care cosmetics are available to treat premature aging with common adverse reactions such as allergic and irritant contact dermatitis, phototoxic, and photoallergic reactions. Despite of the benefit and the useful pharmacological effects of Cur, the application in clinic has been limited due to its extremely low aqueous solubility, instability, poor oral bioavailability and rapid metabolism [26]. To improve the topical bioavailability of curcumin, curcumin has been loaded into nanocarriers like liposomes, polymer nanoparticles, micelles and dendrimers as drug delivery systems [27, 28]. Nanocarriers increase surface area, higher solubility, improved stability, controlled release of active ingredients, reduced skin irritancy, protection from degradation, increased drug loading, and improved permeation of actives into the skin [28].

Niosome are nanocarrier formed by hydration of non-ionic surfactant dried film result in multilamellar or unilamellar bilayer vesicles which enclose aqueous solution of solutes and lipophilic components are in the bilayer itself [29]. The advantages of the non-ionic surfactant are biodegradable, non-immunogenic and biocompatible [30], it also overcome the problems associated with liposomes like susceptibility to oxidation and high price. In this study hydrogen peroxide is used as an aging inducer for HFB4 cell lines, the doses used is 150 µM [31] to examine the effect of H_2O_2-induced oxidative stress on HFB4 cell lines and also the role of curcumin and niosome entrapping curcumin as antiaging agent through decrease the effect of ROS.

2. Materials and methods

2.2: Materials

Cell line HFB4 (Human Fibroblast cell lines) was provided by the Tissue Culture Department, Vaccines & Sera (VACSERA), Egypt and Fetal bovine serum (FBS) was purchased from Invitrogen Corp., Carlsbad, CA. Penicillin-streptomycin, Trypsin, EDTA, and Hank's buffer were purchased from Gibco, USA. dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Ethanol was purchased from Fisher Scientific UK. Polyoxyethylene-80 (tween 80) (purity ≥ 99%) was supplied from Bio Basic Canada Inc. Phosphate buffer saline (PBS) pH 7.4 at 25 °C (purity ≥ 99%) was purchased from Bio Shop Canada Inc. Curcumin and cholesterol (purity ≥ 99.7 %, M Wt. 386.65) were purchased from Sigma Aldrich, USA. All chemicals were used without any further purification.

2.2: Methods

2.2.1: Preparation of niosomes entrapping Curcumin

Niosomes were prepared by the thin film hydration method. 10 µl Tween 80 and 3 mg cholesterol in the ratio (2:1) were dissolved in 10 ml ethanol in a round bottom flask with 1 ml of curcumin. The ethanol was then evaporated at 45 °C under reduced pressure using a rotary evaporator at 50 rpm producing a dry thin film.

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The hydration of the thin film was then done using PBS (pH 7.4). The formed niosomes were then subjected to ultrasonic sonication (5 min) that led to the formation of small vesicles. Finally, niosomes were precipitated using a high speed cooling centrifuge (VS-18000M, Korea, power 220 V/ 50 Hz) (10000 rpm x 30 min) [32].

2.2.2: Characterization of niosomes

2.2.2.1: Entrapment efficiency

The capacity of niosomes to entrap curcumin was determined by the centrifugation method [33]. Briefly, samples were centrifuged at 12,000 rpm (VS-18000 M, Korea, power 220 V/ 50 HZ) for 30 min, to separate the free curcumin (supernatant) from the encapsulated one (pellet). The clear supernatant was then collected and vortexed to obtain a homogeneous solution, while the pellets obtained after centrifugation were diluted with 10 ml saline buffer (pH 7.4) and sonicated for 5 min for further use. This process was repeated three times for each sample.

The absorbance of curcumin was measured at different concentrations using a UV-visible spectrophotometer (JENWAY 6405, U.K.) at 425 nm (the resonance absorption of curcumin). The calibration curve of curcumin was made by plotting the absorbance against the concentration. The absorbance of the free curcumin in the supernatant was determined spectrophotometrically at 425 nm. The concentration of the free curcumin in the supernatant was calculated from the calibration curve made for curcumin and the encapsulation efficiency for noisome was calculated from the following equation:

$$\text{Entrapment Efficiency } \% = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \times 100$$

2.2.2.2 Study of the morphology using transmission electron microscope (TEM)

Niosome were analyzed using a JEOL JEM.1230, Japan. The TEM microscope was operating at an accelerating voltage of 100 kV. Niosome suspension-entrapping curcumin were negatively stained with a 1% aqueous solution of phosphotungstic acid, left to air-dry about 1 sec then samples were incubated for approximately 10 minutes on perforated carbon-coated grids, and then analyzed.

2.2.3: Cell culture

Human Fibroblast cell lines (HFB4) were provided by tissue from VACSERA, Egypt. HFB4 cells were maintained as monolayer culture in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at 37 °C in a (5% CO2) humid atmosphere. When the cells reached at least 80% confluence, they were washed twice with Phosphate-Buffer Saline (PBS) and were detached with 0.25% trypsin/EDTA in Hank's buffer. An equal volume of medium with FBS for trypsin inactivation was added and the HFB4 cells were collected and counted.

2.2.4: Cell Viability and Aging Biomarker Genes Determination

Cell viability was evaluated using the MTT assay. This method is dependent on the reduction of MTT. The cells were seeded in 96-well plates at a concentration of 5×10^4/well for 24 h, and then pretreated with free curcumin and niosome entrapping curcumin treatments. Two hours later, H2O2 (150 µM) was added to the plate and incubated at 37˚C. After 24 h, the medium was discarded and10 µl MTT was added to each well from the MTT stock solution (5 mg/ml). After 4 h of incubation, the supernatants were removed and the absorbance was measured via ELISA reader (Exert 96, Asys Hitch, Ec Austria) at a wavelength of 570 nm.

The aging biomarker genes levels were measured by flow cytometry technique (Partec PAS, Germany).

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2.2.5: Statistical Analysis

Data are analyzed using the Duncan's multiple range test offered by SPSS software version 17.0. The significant differences between the means of the treated and untreated groups are considered significant at $P < 0.05$.

3. Results

3.1: Entrapment efficiency

In the present study, niosome exhibits 67±3% entrapping efficiency for curcumin.

3.2: Morphology and characterization

TEM examination revealed that the ultrastructure of the prepared niosome entrapping curcumin was non-aggregated spherical shape particles (Fig. 1), with nano-size range. The morphology of each image indicated the homogeneity of the particle size. Niosome samples were negatively stained with a 1% aqueous solution of phosphotungstic acid. Samples were incubated for about 10 minutes on perforated carbon-coated grids, and then examined.

Figure 1a shows niosome hasing spherical-shaped particles with size slightly lower than the niosome entrapping curcumin (fig. 1b), the entrapping drug in nanocarrier is larger than the free niosome [34]. All samples exhibited a more narrow size distribution. The electrostatic charge repulsion between the similar charge (either positive or negative) particles prevents the aggregation and thus ensure a dispersed state of the nanosuspension in samples.

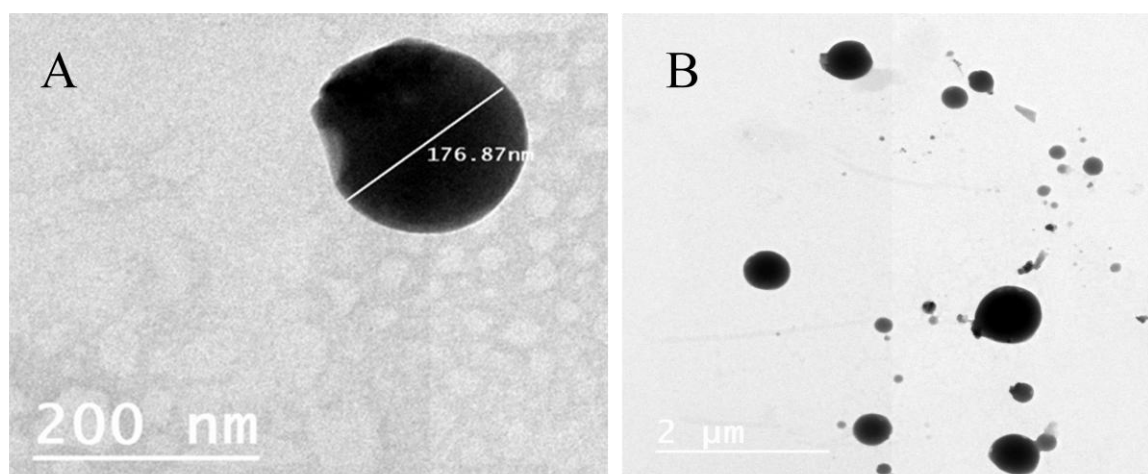

Figure.1. Transmission electron microscope (TEM) images of A) Noisome; B) Niosome entrapping curcumin.

3.3: The effect of free curcumin and niosome entrapping curcumin treatments on HFB4

In figure 2, HFB4 cell lines viability percentage is determined using MTT assay and shows a value ranging between 95-100%. Regarding the apoptotic profile induced in HFB4 cell lines treated using free curcumin and niosome entrapping curcumin. It is noticed that the cell cycle profile of artificially aged human fibroblast cell lines (HFB4) by H_2O_2 showed a significant ($P<0.05$) arrested cells percentage (73.15%) during the G0-G1 phase post 24 hrs. niosome entrapping curcumin treatment followed by H_2O_2 as aging inducing agent show 69.54% , And free curcumin treatment followed by H_2O_2 show 69.52, all significantly difference ($P<0.05$)

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compare to the negative control (non-treated and non-aged) where negative control show 65.71%. In the mean time all cells cases observe a non significant arrest during the S and G2-M phases. Concurrently it is noticed that there is a significant (P< 0.05) percentage (22.17%) of cell arrest during the pre G1 phase of cell cycle in H_2O_2 aged cells as a positive control. also it is noticed that niosome entrapping curcumin treatment and free curcumin treatment show significantly (P<0.05) cell arrest percentage, 10.88% and 7.53% respectively comparing with the negative control (2.76%).

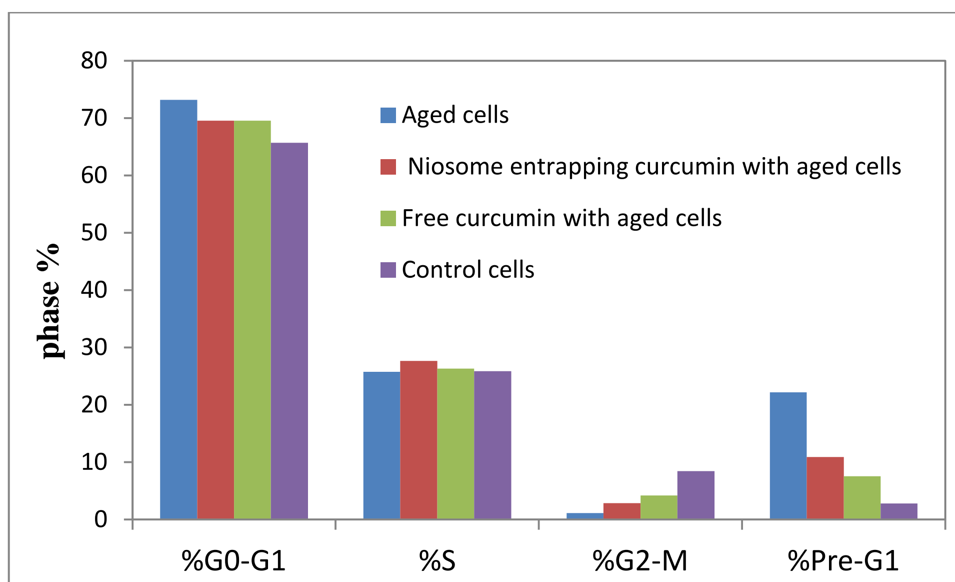


Figure.2. The variation of % arrest for cell cycles G0/G1, S and G2/M and Pre-G1.

In the meantime, The cell cycle arrest is accompanied by apoptotic profile where there is significantly (p<0.05) increase necrotic cells in H_2O_2 treated cells compared with the necrotic cells percentage in free curcumin treatment, niosome entrapping curcumin treatment and negative control respectively. Also there is a significantly (p<0.05) elevated percentage of early apoptotic cells on H_2O_2 treated cells and niosome entrapping curcumin treated cells than in case of early apoptotic cells percentage in free curcumin treated cells and negative control cells. While there was siginificantly (p<0.05) increase percentage of late apoptotic in H_2O_2 treated cells than the rest of treated cells and negative control (fig. 3).

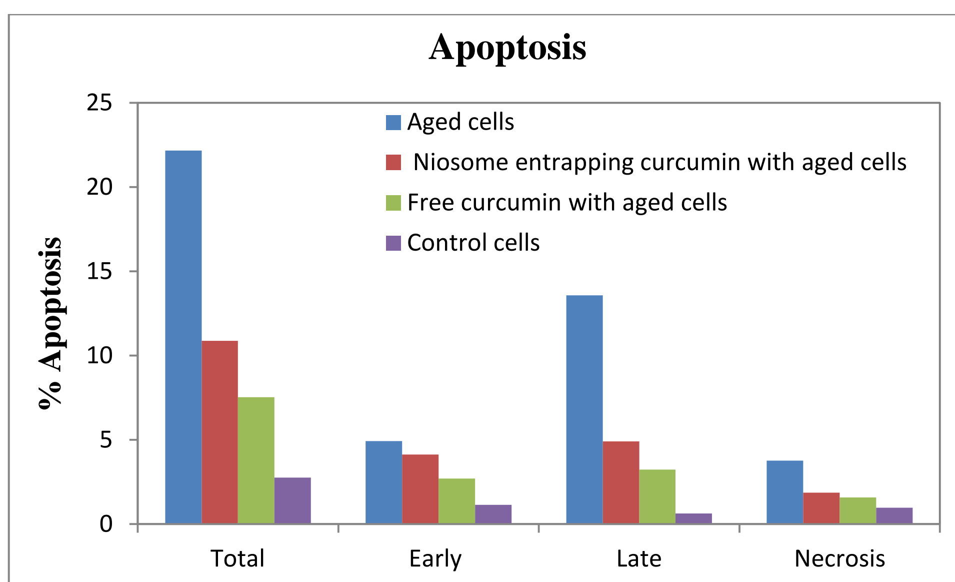
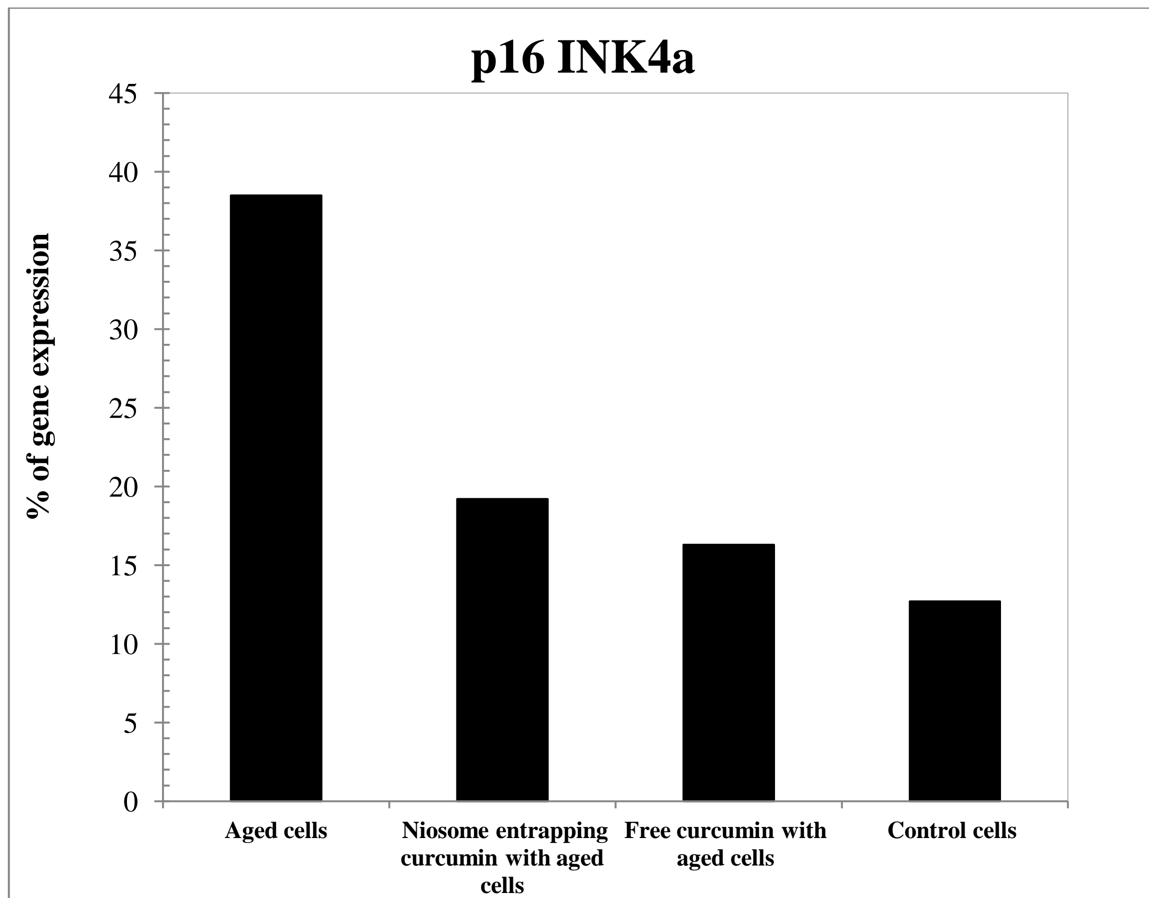


Figure.3. Percentage of apoptosis for aged cells, niosome entrapping curcumin with aged cells, free curcumin with aged cells and control cells.

It is found a highly expression of p16 INK4a and p53 BP1 as aging biomarker in the aged developed cells compared to the non aged cells. Also it was found that there is a positive reactivity of the cells to the treatment schedule using free curcumin and niosome entrapping curcumin were there is a down regulation of target gene expression in a significant way ($p < 0.05$) than the non treated cells. In the mean time it is found that niosome entrapping curcumin treatment showed a lower antiaging potential than in case of free curcumin ($p < 0.05$) (Fig. 4).



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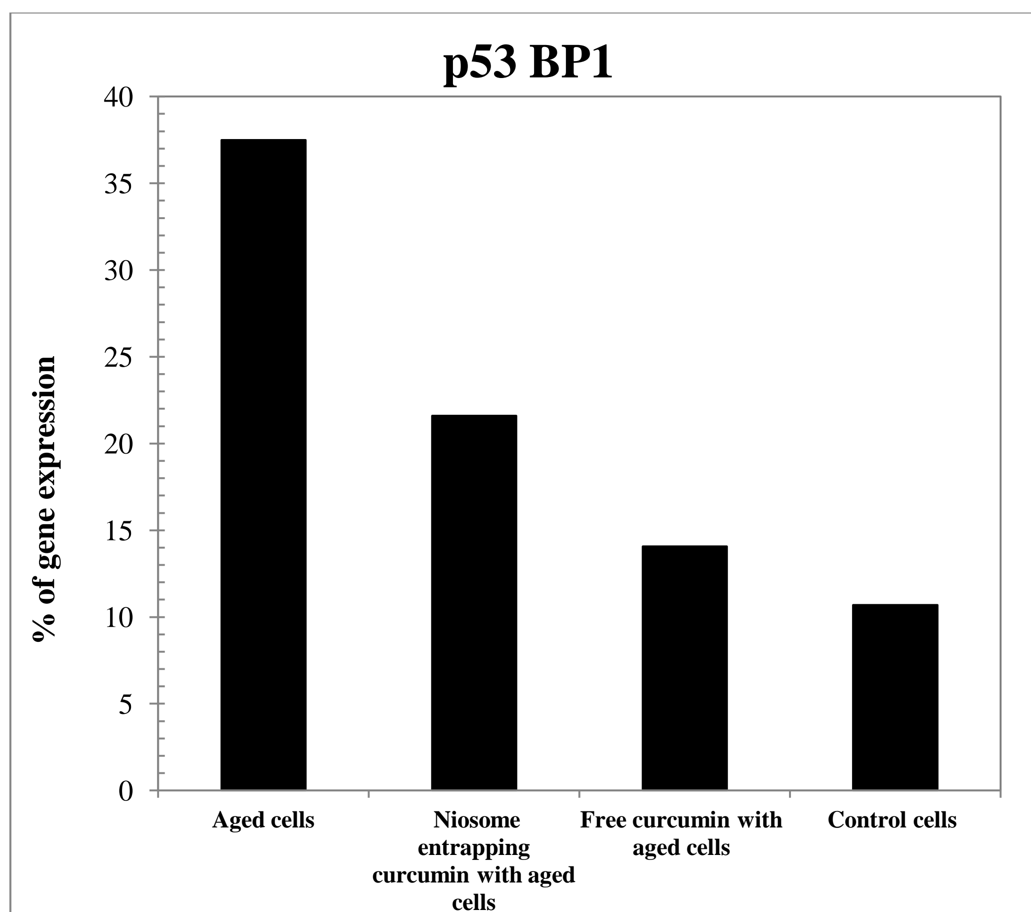

Figure.4. Aging p16 INK4a and p53 BP1 biomarker genes profile induced in s1 aged cells, niosome entrapping curcumin with aged cells, free curcumin with aged cells and control cells.

4. Discussion

The aged cells are characterized by extensive oxidative stress [5]. Thus, removal of excess reactive oxygen species may be effective as anti-aging agent. The new directions on ageing treatments focus on the use of antioxidants to save cell and potential enhancement of the intracellular antioxidant mechanisms. Curcumin with its proven antioxidant properties has been shown to have several therapeutic effects [35] which could act as a free radical scavenger by inhibiting lipid peroxidation and oxidative DNA damage [36]. The strong antioxidant activity of curcumin makes it an interesting agent for overcoming oxidative stress-induced damage. This work also examines the difference between using free curcumin and niosome entrapping curcumin in order to enhance the efficiency of curcumin in aged cells. Our findings indicated that H_2O_2 induced a significant cells aging by increasing the ROS level among the cells. However, pre-treatment of the cells with curcumin can produce anti-aging effects through decreasing intracellular ROS levels, which intern decrease protein oxidation and lipid peroxidation. Oxidation of protein could lead to its aggregation or dimerization and subsequent accumulation of the oxidized proteins in the cytoplasm as inclusions [37]. Moreover, some lipid peroxidation products can alters proteins conformation and function [38]. Therefore, curcumin offered significant anti-aging through antioxidant effect that decrease of ROS levels, inhibition of lipid peroxidation and reduction in protein deformation.It is observed that a significantly ($P<0.05$) arrested cells during the G0-G1 and pre G1 phase in H_2O_2 aged cells. Free curcumin and niosome entrapping curcumin show significantly ($P<0.05$) decrease percentage of cell arrest during both phases. This growth arrest is proceed by the activation of p16 INK4a and p53 tumor suppressor genes. DNA damage by p53 activation [6,7,9,10]. This is a result of damage occurring in telomeres [8,7], that allow for a permanent arrest of the cell cycle. p16 INK4a expression pushes cells to enter irreversible cell-cycle arrest that precludes the growth of

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would-be cancer cells but also contributes to cellular aging. Importantly, loss of p16 INK4a is one of the most frequent events in human tumors. Therefore, regulation of p16 INK4a is essential to maintain a balance between tumor suppression and aging [39]. In the apoptotic profile, the necrotic cells percentage in H_2O_2 treated cells is 3.76% compared with the necrotic cell percentages 1.86% , 1.58% and 0.98% in niosome entrapping curcumin, free curcumin, and negative control respectively. Exposure to H_2O_2 triggers apoptosis through the mitochondrial pathway involving sequential loss of mitochondrial membrane potential, cytochrome c release, and effector caspase-3 activation [31]. These sequences produce cell aging. Flowcytometry analysis show that p16 INK4a and p53 BP1 expression is response to H2O2 treatment and demonstrated that exposure of HFB4 cells to H_2O_2 increase p16 INK4a and p53 BP1 expression. The relatively high levels of p16 INK4a and p53 BP1 expressed in H_2O_2-treated cells suggest that p16 INK4a and p53 BP1 involved in H_2O_2-induced aging. Our present data showed that exposure of HFB4 cells to H_2O_2 activate the aging processes in the form of arrested cells during the G0-G1 and pre G1 phase, high percentage of necrotic cells, high level of early and late apoptotic cells and high expression of p16 INK4a and p53 BP1. However, niosome entrapping curcumin and free curcumin treatments , remarkably decrease the level of these processes leading to anti-aging of HFB4 cell lines exposed to H_2O_2. We observe that the effect of free curcumin treatments is always elevate the effect of niosome entrapping curcumin treatment, this may be due to the long time release effect of the nanocarrier. Based on the present data we hypothesized that curcumin could act as antiaging agent capable of regulating H_2O_2-induced aging. Overall, curcumin could be an excellent choice in synthesis of natural-based drugs for treatment of aging.

4. Conclusion

The present study use H_2O_2 to produce oxidative stress and induce HFB4 cells aging. We showed that H_2O_2 enhance cells phases arrested, apoptosis cells and high expression of some apoptosis genes. The present data concluded that niosome entrapping curcumin and free curcumin treatments protected cells against oxidative stress-induced aging .

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