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Full Length Article

Apoptotic efficiency of aqueous extracts of turmeric, garlic and their active compounds in combination with Tamoxifen in lung and oral cancers: A comparative study

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1. Introduction

Cancers has taken over 'the title of top killer' in 2011, and it is predicted that the number of cancer cases and deaths will be doubled by 2030. Among the known cancers, lung and oral cancer are one of the most prevalent cancers in young adults in India. According to the National Cancer registry program, the incidence of lung and oral cancer affecting the tongue and buccal cavity is very high among males in India mainly due to the chewing of tobacco products (Mishra and Meherotra, 2014). According to the WHO almost 12 of 100,000 people are affected with oral and lung cancers. Even though the chemo and radiotherapy are much preferred method of treatments, which gives immeasurable side effects, hence the search for an alternate is still on, which comes with least sides. Some of the plants derived phytoextracts and dietary substances have anti-cancer potential (Shanmugam et al., 2011; Svejda et al., 2010). Asian countries are known for its rich diversity and its traditional medicines, the herbal plants and their phyto constituents are to known to exert anti-cancer potential etc. India is known for its condiments and there beneficial effects. *Curcumin longa*, *Zingiber officinale* L., and *Allium sativum* are one of the commonly used condiments in Indian cuisines. An Invitro studies done by Liao et al. (2011), reported the anti-cancer properties of *Zingiber officinale* such as enhanced apoptosis (i.e. through over activation of Caspase-3) by inhibiting cell proliferation and cell apoptosis in A549 and SCC-9 cell lines.

The testing of drugs on cancer cell lines derived from different tumors serves as a crucial source for testing the efficacy of various drugs that act against metastasis. Several squamous carcinoma cells are known to express ERα and ERβ on them (Marocchio et al., 2013; Shanle et al., 2013). And treatment of oral carcinoma cells with Curcumin and Quercetin caused apoptotic cell death of squamous carcinoma cells with decrease in adhesion of the cells. The literature suggests that the active compounds Curcumin and Quercetin exert anti-cancer properties by affecting the cell cycle, cell proliferation and viability.

Curcuma longa (turmeric) is been part of Siddha and Ayurveda and it is known to exert anti-inflammatory, anti-microbial, anti-oxidant and anti-cancer properties (Aggarwal et al., 2007). Curcumin is the bioactive constituent present in *C. longa,* and it acts against many molecules like growth factors, transcription factors, kinases, enzymes, cytokines and other receptors (Zhou et al., 2011). Curcumin treatment induces several tumors to be sensitive towards radiation and chemotherapy drugs (i.e. doxorubicin, etoposide, cisplatin, paclitaxel and 5-FU) (Goel and Aggarwal, 2010; Tsubura et al., 2011). Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a flavonoid, which has been studied extensively as it can exert these following activities such as anti-oxidant, anti-inflammatory, and anti-tumor activity (Hertog et al., 1993). Cell proliferation inhibition property of Quercetin was studied in varied cancers such as breast, cervical, prostate, colon etc and it is due induced apoptosis and cell arrest in G2/M arrest or G1 arrest (Choi et al., 2001; Yang et al., 2001; Lee et al., 2006).

We compared the anti-cancer effects of condiments and bioactive compounds of Curcumin and Quercetin with and without Tamoxifen combination. The A549 and SCC-9 cell lines were

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treated with Turmeric, Garlic, Curcumin, Quercetin and Tamoxifen individually and also in combination, simultaneously in order to assess cytotoxic and anti-cancer effects on ER positive lung and oral cancer cell lines along with human embryonic kidney cell line (HEK 293). Here, we show that clinically that the normal HEK cells were not showing any toxicity or cell senescence or apoptosis on treatment with natural extracts, phyto constituents and Tamoxifen (MTT and Immuno cytochemistry assay), The results showed that the combinational therapy were found to be more effective in inducing apoptosis, cell death, inhibition as the cells got arrested in sub-G1 and G1 phases very synergistic effect when compare to individual treatments.

2. Materials

Immortal cell lines such as A549, SCC-9 were obtained from NCCS, Pune and HEK-293 (ATCC CCL1573) obtained from Centre for cellular and molecular biology (CCMB), Hyderabad, Telangana, India. DMEM:Ham'sF12 medium, Hydrocortisone, Hi-Gluta XL-Dulbecco's Modified Eagle's Medium (High Glucose) cell culture medium, recombinant human insulin, L-Glutamine- Penicillin-Streptomycin solution, Dulbecco's Phosphate buffered saline (DPBS), 0.22 μm sterile syringe driven filters, sterile cell scrapers, 0.25% Trypsin-EDTA solution, Bovine serum albumin (BSA) were obtained from Hi-Media India Ltd. Curcumin powder was procured from Calbiochem (Merck-Millipore) Quercetin powder was procured from HIMEDIA. Fetal bovine serum was obtained either from Seralab or Hi-Media. Sterile 15 ml Falcon tubes were purchased from Tarsons India Ltd. The HEPA Class 100-Steri cycle CO_2 cell culture incubator, Sorvall ST-16R centrifuge and micro-pipettes were obtained from Thermo Scientific Corporation, USA. The T-25 cell culture flasks or multi-well cell culture plates were obtained from Axygen Inc., or Corning Inc. cell Image-Based Cytometer (Thermo), applied bio systems Cellular analysis slide, A.B Apoptosis Kit – Annexin V Alexa Fluor 488 & Propidium Iodide and Cell Cycle Kit were purchased from Invitrogen-Life Technologies Inc. Ultrapure, UV-treated water was obtained from Millipore RiOs-DI®3 equipment. EVOS Digital inverted fluorescence microscope was from Motic, Germany. Acetonitrile ULC/MS Grade purchased from Biosolve Chimie SARL (Dieuze, France) and formic acid (Optima LC/MS grade) was purchased from Fisher Scientific (Geel, Beljium, Germany). Methanol (LiChrosolv) was purchased from Merck (Darmstadt, Germany). Deionized water was prepared by passing distilled water through a Milli-Q water purification system (Millipore, Milford, MA, USA). Turmeric extract was prepared using herbaceous perennial plant (*Curcumin longa*), Garlic (*Allium sativam*) extract was prepared by mixing commonly used dietary ingredients in ultrapure water and subjecting the mixture for centrifugation at 4 °C. Curcumin and Quercetin powders were dissolved in 5% DMSO solution in water to form a 10 mM stock solution. All the reagents such as, Turmeric and Garlic extracts, Curcumin and Quercetin suspensions were filtered using 0.22 μm syringe filter before they were added to the cells.

3. Methods

3.1. Cell culture

Human carcinoma cell lines A549 and SCC-9 (Adeno carcinoma of lung, squamous cell carcinoma of tongue) and Human embryonic kidney cell line (HEK-293). The cells were aseptically cultured in cell culture flasks or multiwall cell culture plates in their respective growth medium supplemented with 10% Fetal Bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. A549 cells were cultured in Hi-Gluta XL-Dulbecco's Modified Eagle's Medium, (DMEM-High Glucose). SCC-9 cells were cultured in a 1:1 mixture of DMEM:Ham's F12 medium, supplemented with 2 mM Glutamine, 0.4 μg/ml/ml hydrocortisone and 0.5 mM sodium pyruvate. HEK-293 was cultured in DMEM-based culture media contains DMEM medium (HI-MEDIA INDIA) supplemented with 10% fetal bovine serum (INVITROGEN USA), 50 units/mL penicillin, and 50 μg/mL streptomycin (HI-MEDIA INDIA) and 2 mM L-glutamine. The cells were grown in a cell culture incubator (HEPA Class 100-Steri cycle CO_2 incubator), supplied with 5% CO_2 incubator and maintained at 37 °C.

3.2. Treatment of the cells with natural extracts, Curcumin and Tamoxifen

A-549 and SCC-9 cells were grown in respective media for 48 h, and were serum starved for 24 h. Then the serum free media was replaced with respective media containing 10% FBS. To the cultured cells the following treatments were given Turmeric extracts (1–10 μg/ml), Garlic extract (1–10 μg/ml), Quercetin (1–10 μg/ml), Curcumin (1–10 μg/ml) individually or in combinations. The controls were cultured in control media throughout the experimental duration. The cells were cultured in 6 well plate (Culture media 2 ml/well). Experiments were carried out in triplicates and the experiments were repeated twice. After 24 h or 48 h of treatment, the detached cells were collected along with the medium in a 15 ml polypropylene tubes and the wells were washed using DPBS, later the adhered cells were trypsinized using 0.25% Trypsin-EDTA solution for about 5 min at room temperature and the trypsinization process was neutralized by adding serum and the trypsinized cells were transferred to the 15 ml tube to the same tube which has the detached cells and centrifuged at 2200 rpm, for 5 min at room temperature, in a Sorvall ST-16 R centrifuge. The supernatant was discarded and the cell pellet was re-suspended in 500 μl of DPBS to determine cell death or to perform cell cycle analysis.

3.3. Apoptosis and cell death assay

The cell pellet containing 5×10^5 cells/ml was re-suspended in 100 μl of Annexin V binding buffer and to this suspension 5 μl of Annexin V Alexa Fluor® 488 was added and mixed well. The cell Annexin V Alexa Fluor® 488 mixture was incubated at room temperature in dark for 20 min. The cells were centrifuged at 2200 rpm for 5 min. The pellet was again re-suspended in 100 μl of Annexin binding buffer and 1 μl Propidium iodide (PI) was added to it, mixed well and incubated in dark for 5 min. Then 25 μl of the labeled cell suspension was loaded into the applied biosystem cellular analysis slide and then the slide was inserted into the AB cellular image based cytometer for analysis (Countess). The number and percentage of apoptotic and dead cells were assessed by Annexin V and PI staining, respectively. Unstained cells were used to deduct the auto-fluorescence associated with the cells.

3.4. Cell cycle analysis

The cells treated for 24 or 48 h with different agents were re-suspended in 500 μl of DPBS and centrifuged at 500*g* at 4 °C in a Sorvall ST-8 R centrifuge for 5 min. The cell pellets were immediately put on ice and were re-suspended in 1 ml of ice cold 70% ethanol which was added drop wise while the pellet was gently vortexed to avoid clumping of cells. The cells were fixed in 70% ethanol solution overnight at −20 °C. The following day, fixed cells were centrifuged at 1000*g* at 4 °C and were washed with DPBS and centrifuged again. The corresponding cell pellet was re-suspended in 100 μl Cell cycle solution (consisting of propidium iodide, RNase A and Triton-X-100) and incubated in dark for 30 min after cellular analysis by image based cytometer for cell cycle analysis.

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