Publications:


INTRODUCTION

Cervical cancer is the fourth most common cancer affecting women worldwide with 5, 28,000 new cases every year, after breast, colorectal, and lung cancers (Relese et al., 2013). The cervix is the lower, narrow end of the uterus that connects the uterus to the vagina (birth canal). Cervical cancer usually develops slowly over time. Before cancer appears in the cervix, the cells of the cervix go through a series of changes in which cells that are not normal begin to appear in the cervical tissue. Almost 70% of the global burden of cervical cancer falls in areas with lower levels of development like sub-Saharan and Asian and more than one fifth of all new cases are diagnosed in India (Release et al., 2013). Several studies demonstrated that toxicity and drug resistance are the major drawbacks of anticancer chemotherapeutic therapies. Thus, in view of the severity of the disease and considering the side effects posed by existing synthetic drugs, alternative interventions such as substances from natural herbal sources can be tested either individually or in combinations as anticancer agents. The goal of developing newer agents is to overcome such problems and with no or minimum side effects (Cheah et al., 2009).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is widely used as an antioxidant in the food industry and is shown to exhibit a variety of pharmacological and biological activities, including anti-cancer (Kaur et al., 2009). Recent pre-clinical studies clearly demonstrated the chemopreventive effect of GA on dimethyl hydrazine (DMH) induced colon carcinogenesis in animal models (Giftson et al., 2010). Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3, 5-dione) is a polyphenolic pigment isolated from the rhizomes of Curcuma longa (turmeric), a medicinal plant widely used in the ancient Indian and Chinese medicine. It is reported to possess anti-inflammatory, antioxidant and anticancer properties by inhibition of signaling pathways such as NFkappaB, PI3K/Akt and activator protein-1 (AP-1) (Singh et al., 2006). Doxorubicin (D), an anthracycline antibiotic and antineoplastic agent, was first isolated from Streptomyces peucetius (Arcamone et al., 1969).
It is a potent chemotherapeutic agent that is used in the treatment of solid tumors and malignant hematological diseases (O'Bryan et al., 1977). Thus, we set out to determine if the combination of gallic acid, curcumin and/or doxorubicin is more potent than either agent alone. Our findings suggest that the combination of Gallic acid, curcumin and/or doxorubicin are effective in inhibiting the proliferation, migration and colonies formation of HeLa cells.

MATERIALS AND METHODS

Materials

The human cervical cancer cell line (HeLa), Dulbecco's Modified Eagle's medium (DMEM), Fetal bovine serum (FBS) were purchased from GIBCO Ltd (Life Technologies TM, Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] Curcumin, Doxorubicin and Gallic acid were purchased from Sigma, St. Louis, MO, USA. Curcumin stock was prepared in dimethyl sulfoxide (DMSO) and doxorubicin and gallic acid were prepared in phosphate buffer saline pH 7.4 (PBS; Gibco) and stored at -20°C until use. All other chemicals of analytical grade were purchased from Sigma, USA.

Cell culture and maintenance

HeLa cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. They were cultured in Dulbecco's Modified Eagle's medium with 10% heat-inactivated Fetal-Bovine Serum, 1.2 g/L NaHCO3 and antibiotic solution (10,000 units Penicillin, 10 mg Streptomycin and 25μg amphotericin / ml). Cells were maintained at 37°C in a humidified incubator containing 95% O2 and 5% CO2. The cells were divided in to six groups as mentioned below and cultured in microplates or T-flasks to conduct different experiments.

Group-I: Control (Cntl.) cells

Group-II: Curcumin (C) treated cells

Group-III: Gallic acid (GA) treated cells

Group-IV: Doxorubicin (D) treated cells

Group-V: Curcumin (C) + Gallic acid (GA) treated cells

Group-VI: Curcumin (C) + Gallic acid (GA) + Doxorubicin (D) treated cells

Determination of cell viability by MTT assay

Cell viability and cytotoxic effect of curcumin, gallic acid and/or doxorubicin on HeLa cells and their proliferation was determined by MTT assay (Mosmann et al., 1983). Based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazan product, cell viability can be assessed. Briefly, 5000 cells were seeded in each well of 96-well plate and treated with desired concentrations of curcumin (C) (10, 25, 50 and 100μM), doxorubicin (D) (50, 125, 250 and 500 nM) and gallic acid (GA) (25, 50, 100, 200μM). Cells were treated with different combinations of C + GA and C + GA + D for 24 h and 48 h. Then, 10 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for 3–4 h. The medium was removed and 100 μl of 40 mM acid isopropanol was added to each well and after 20 min of mechanical shaking, the optical density was measured at 570 nm in ELISA platereader.

% Cell Viability = \( \frac{OD \text{ sample}}{OD \text{ control}} \times 100 \)

% Inhibition of proliferation = % untreated cell viability (100) - % drug treated cell viability

In vitro scratch assay for cell migration

Cells (5 × 10⁴) were seeded in 60 mm culture plates for monolayer formation up to 80% confluence then scratch was created by sterile p20 tip and washed with 1X PBS to remove floating cells. Cells were treated for 24 h either with curcumin (41.28 μM) or doxorubicin (232.34 nM) or gallic acid (105.66 μM) and their combination C + GA (40 + 100μM) and C + GA + D (20 μM + 50μM + 200nM).

International Journal of Applied Biology and Pharmaceutical Technology
Available online at www.ijabpt.com
After incubation the drug containing medium was removed, photographic images were taken at 0, 3, 6, 12 and 24 h using inverted phase microscope. Cell migration was expressed as the percentage of the gap relative to the total area of the cell-free region (Liang et al., 2007).

**Colony formation assay**

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into colonies. Only a fraction of seeded cells retains the capacity to produce colonies. Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 5-8 days. Briefly, cells (1000) were seeded in each well of 6 well culture plates, incubated at 37°C with 5% CO₂ for 48 h. Then, media was removed, cells were washed with 1X PBS, then treated with curcumin (41.28 μM) or doxorubicin (232.34 nM) or gallic acid (105.66 μM) and their combinations as indicated before, for 24 h. Then, media containing compounds/drug was removed; cells were maintained with fresh media and incubated for 8 days. Colonies were fixed with glutaraldehyde (6% v/v) stained with crystal violet (0.5% w/v) and counted using a stereomicroscope (Franken et al., 2006).

Calculated plating efficiency (PE) and surviving fraction (SF).

\[
\text{PE} = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100%
\]

\[
\text{SF} = \frac{\text{no. of colonies formed and surviving}}{\text{no. of cells seeded}} \times \text{PE}
\]

**RESULTS**

**Assessment of Cell viability**

Individual combination treatment with curcumin, gallic acid and/or doxorubicin enhances the inhibition of HeLa cell proliferation and cell viability, MTT assay was performed. Individual treatment with curcumin, gallic acid or doxorubicin showed significant inhibition of cell proliferation in a dose dependent manner. Individually, curcumin, gallic acid and doxorubicin showed IC₅₀ values at 41.28 μM, 105.66 μM and 232.34 nM respectively as shown in Fig. 1.

---

**Fig 1A:** Cell viability of curcumin at 24 h

**Fig 1B:** Cell viability of Doxorubicin at 24 h

**Fig 1C:** Cell viability of Gallic acid at 24 h

**Fig 1:** Effect of Curcumin, Gallic acid and Doxorubicin on cell viability of HeLa cells at 24h.
Figure 1D: Effect of Curcumin, Gallic acid and Doxorubicin on cell viability of HeLa cells at 48h.

Figure 2A shows the combination treatment with curcumin and gallic acid where in curcumin (40 μM) and gallic acid (100μM) showed 86.3 % inhibition of cell viability. Interestingly, 91.44 % inhibition of cell viability was noted when curcumin (20μM), gallic acid (50μM) and doxorubicin (200nM) was administered indicating synergistic effect as shown in figure 2B.

Figure 2A: Effect of Curcumin, Gallic acid treatment on cell viability of HeLa cells at 24h.
Figure 2B: Effect of Curcumin, Gallic acid and/or Doxorubicin treatment on cell viability of HeLa cells at 24h.

Combinations cell viability 48 h:

Figure 2C: Effect of Curcumin, Gallic acid treatment on cell viability of HeLa cells at 48h.

Figure 2D: Effect of Curcumin, Gallic acid and/or Doxorubicin treatment on cell viability of HeLa cells at 48h.
Assessment of cell migration

The % of cells that had migrated into the scratch area was calculated after 3, 6, 12 and 24 h and shown in figure 3. Complete gap closure in 24 h was observed in the untreated cells, however, significant change in cell migration was observed when treated with curcumin, gallic acid and doxorubicin in the concentrations mentioned before, either individually or in combinations. Control, curcumin, doxorubicin and gallic acid individually shown inhibition of cell migration by 0.15, 0.75, 0.60 and 0.75 inches respectively. In combination treatment, C + GA shown 0.80 inches inhibition of cell migration while C + GA + D shown 0.85 inches. These results suggest that combination treatment was effective in inhibiting the migration of HeLa cells compared to individual drug treatments (Fig 3).

Figure 3A: Effect of Curcumin, Gallic acid and/or Doxorubicin on cell migration. Curcumin (C) (41.28 μM), Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 μM), C + GA (40 + 100 μM) and C + GA + D (20 μM + 50 μM + 200 nM).

Figure 3B: Effect of curcumin, gallic acid and/or doxorubicin on cell migration. Curcumin (C) (41.28 μM), Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 μM), C + GA (40 + 100 μM) and C + GA + D (20 μM + 50 μM + 200 nM).
Cell migration assay: 24h

![Cell migration assay](image)

Concentrations of compounds/drug

![Concentrations of compounds/drug](image)

**Figure 3C**: Effect of Curcumin, Gallic acid and/or Doxorubicin on cell migration. Curcumin (C) (41.28 μM), Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 μM), C + GA (40 + 100μM) and C + GA + D (20 μM + 50μM + 200nM).

**HeLa cell colonies formation**

The effect of curcumin, gallic acid and doxorubicin on HeLa cells colony formation was assessed. The % of cells that had formed colonies after 8 days was calculated and shown in figure 4. There were more colonies observed in untreated control cells whilst significant changes in colonies formation were observed in cells treated with curcumin, gallic acid and doxorubicin concentrations mentioned before, both individually and in combinations. Curcumin, gallic acid and doxorubicin individually shown 66.67%, 61.11%, 75% colony formation, while C+GA shown 47.22% and C+GA+D shown 30.56% colony formation. These results suggest that combination treatment was more efficacious in inhibiting colony formation of HeLa cells indicating the synergistic effect (Fig 4).

![Clonogenic assay](image)

**Fig 4A**: Effect of Curcumin, Gallic acid and Doxorubicin on colonies formation
Figure 4B: Concentrations of compounds/drugs used. Curcumin (C) (41.28 μM), Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 μM), C + GA (40 + 100 μM) and C + GA + D (20 μM + 50 μM + 200 μM).

Figure 4C: Effect of Curcumin, Gallic acid and Doxorubicin on colonies formation. Curcumin (C) (41.28 μM), Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 μM), C + GA (40 + 100 μM) and C + GA + D (20 μM + 50 μM + 200 nM).

DISCUSSION
Current cancer treatment strategies favour combination therapies which would offer low toxicities to the cancer patients. Natural products have proven to be the most reliable source of new and effective anticancer agents. Chemotherapy, one type of cancer treatment a major role in treating various cancers especially to control advanced stages of malignancies in clinical situations (Kinghorn et al., 2003). Most of these chemotherapeutic agents show severe normal toxicity, and cause adverse side effects. The clinical use of anthracyclines like doxorubicin induces life threatening cardiomyopathy and congestive heart failure, which is a major difficulty for optimum use of doxorubicin (Minotti et al., 2004).
Hence, the present study focussed on combinational therapy of curcumin, gallic acid and doxorubicin in cervical cancer cells. Tumor resistance to apoptotic cell death is an important hallmark of cancer and contributes to increased survival of cells that have acquired oncogenic mutations, eventually leading to uncontrolled cell proliferation, invasion, metastasis, angiogenesis and chemoresistance.

Curcumin inhibits MAPK, p38, c-JNK activation and scavenges reactive oxygen species (ROS) which minimizes the cardiotoxicity of doxorubicin (Misra et al., 2011) and is a potent suppressor of NFκB pathway which promotes cell proliferation and inhibits apoptosis (Yu LL et al., 2011). GA has shown to sensitize a variety of human cancer cell lines for apoptosis induced by different anti-cancer drugs (Hseu et al., 2008).

Cancer development involves many aspects of the cell, treatment with a single agent is rarely effective (Adhami et al., 2007). Two or more cellular processes are usually targeted in therapy. Therefore, combination therapy is now a prominent approach in cancer chemotherapy. There are many advantages of this approach including targeting more than one critical molecular process, delivering lower dose agents with lower toxicity and increasing patient tolerance. Currently there is growing interest in the use of combination chemotherapy allowing the delivery of lower drug dosages each with different modes of activity (Khor et al., 2006).

In the present study, C, GA and exhibited significant suppressive effect on HeLa cells viability in a dose dependent manner (Figure 1). Such an inhibitory activity of cell viability was enhanced significantly when the HeLa cells were exposed to C + GA and C + GA + D combinations (Figure 2). Cell migration was significantly inhibited 60% with C + GA and 65% with C + GA + D combination (Figure 3). Results of the present study revealed that combination exposure of both drugs at same time point exhibited highest inhibition in cell viability, colony formation and cell migration than either C, GA or Dalone. These observations may be of value while carefully considering the combination therapies in a clinical setting and beneficial in cervical cancer therapy. Our results suggest that concurrent treatment of C, GA and D shown synergistic effect in human cervical cancer (HeLa) cells and these drugs may have future clinical utility for treating cervical cancer.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Suresh Yengu, Dept. of Animal Sciences, University of Hyderabad, Hyderabad for providing laboratory facilities to perform the research work.

REFERENCES


IARC., 2013.


