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CURCUMIN IN COMBINATION WITH GALLIC ACID AND DOXORUBICIN EFFECTIVELY INHIBITSPROLIFERATION AND MIGRATION OF HUMAN CERVICAL CANCER CELLS: AN *IN VITRO* STUDY

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ABSTRACT: In view of the severity of cervical cancer and adverse effects of synthetic drugs, natural product-based combination therapy has gained importance. Hence, in the present study we evaluated the *in vitro* anticancer activity of Gallic acid, Curcumin both individually and in combinations with Doxorubicin in HeLa human cervical cancer cells. Cell viability was assessed by MTT assay, cell migration was determined by *in vitro* scratch assay and colony formation was determined by clonogenicassay. Gallic acid, Curcumin and/or Doxorubicin treatment showed inhibition of cell proliferation in a dose dependent manner. However, combination treatment was found to be more effective in inhibiting proliferation, colonies formation and cell migration of HeLa cells demonstrating the synergistic effect.
Key words: HeLa, MTT assay, *In vitro* scratch assay, clonogenic assay.

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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 (Release *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 cervicaltissue.

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 therhizomes of *Curcuma longa* (turmeric), a medicinal plantwidely used in the ancient Indian and Chinese medicine. It is reported to possessantiinflammatory, 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 doxorubicinis 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, migrationand 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 -20ºC until use. All other chemicals of analytical grade were purchased from Sigma, USA.

Cell culture and maintenance

HeLacells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. They were cultured in Dulbecco's Modified Eagle's mediumwith 10% heat-inactivatedFetal-Bovine Serum, 1.2 g/L $NaHCO_3$ and antibiotic solution (10,000 unitsof Penicillin, 10 mg Streptomycin and 25µg amphotericin / ml). Cells were maintained at37ºC in a humidified incubator containing 95% O_2 and 5% CO_2.The cells were divided in to six groups as mentioned below and cultured in microplates or T-flasks to conduct different experiments.

Grioup-1: Control(Cntl.) cells

Group-II: Curcumin (C) treatedcells

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 purpleformazon product , cell viability can be assessed. Briefly, 5000 cells were seeded ineach 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 24h and 48 h. Then, 10 µl of MTT (5 mg/ml in PBS) was added to eachwell 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.

$$\% \text{ Cell Viability} = \frac{\text{OD sample}}{\text{OD control}} \text{X } 100$$

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

In vitro scratch assay for cell migration

Cells (5×10^4) 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).

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 welledculture plates,incubated at 37^0C with 5% CO_2 for 48 h.Then, media was removed,cells were washed with 1X PBS, then treatedwithcurcumin (41.28 µM) or doxorubicin (232.34 nM) or gallic acid (105.66 µM)and their combinationsas 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).

PE = no. of colonies formed/ no. of cells seeded x 100%

SF = no. of colonies formed after treatment/ no. of cells seeded x PE

RESULTS

Assessment of Cell viability

Individual combination treatment with curcumin, gallic acid and/or doxorubicin enhances the inhibition of HeLacellproliferationand cell viability, MTT assay was performed. Individual treatment with curcumin, gallicacid or doxorubicin showed significant inhibition of cellproliferation in a dose dependent manner. Individually, curcumin, gallic acid and doxrubicin showed IC50 values at41.28 µM, 105.66 µM and 232.34 nMrespectively as shown in Fig. 1.

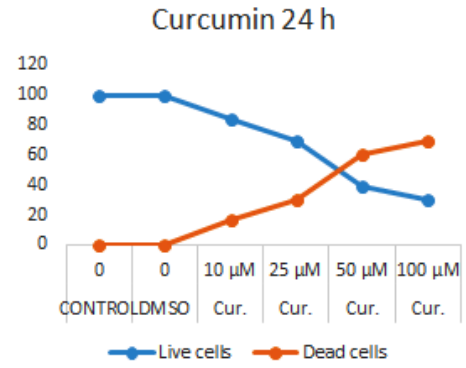

Fig 1A: Cell viability of curcumin at 24 h

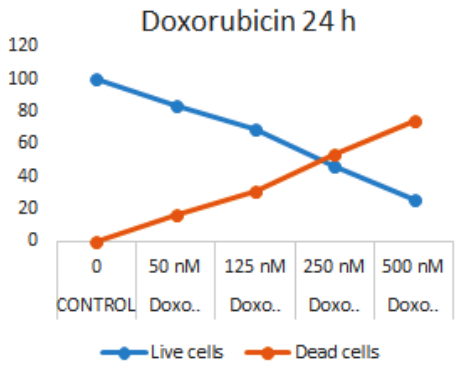

Fig 1B: Cell viability of Doxorubicin at 24 h

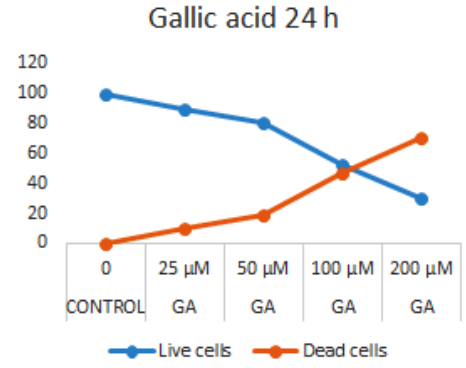

Fig 1C: Cell viability of Gallic acid at 24 h

Fig 1: Effect of Curucmin, Gallic acid and Doxorubicin on cell viability of HeLa cells at 24h.

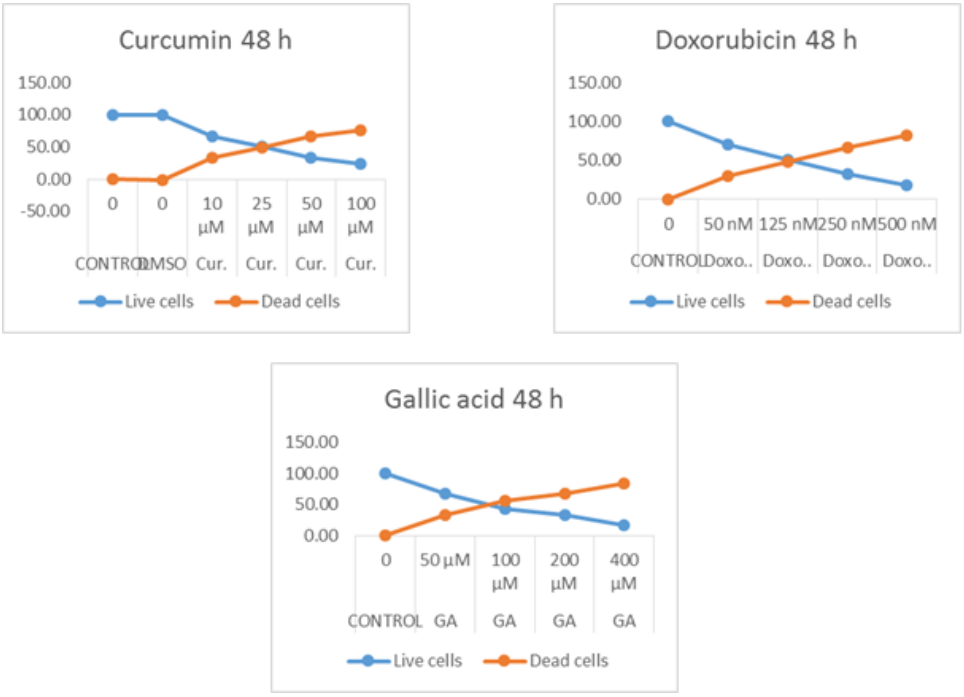

Figure 1D: Effect of Curucmin, Gallic acid and Doxorubicin on cell viability of HeLa cells at 48h.

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 indicatingsynergistic effect as shown in figure 2B.

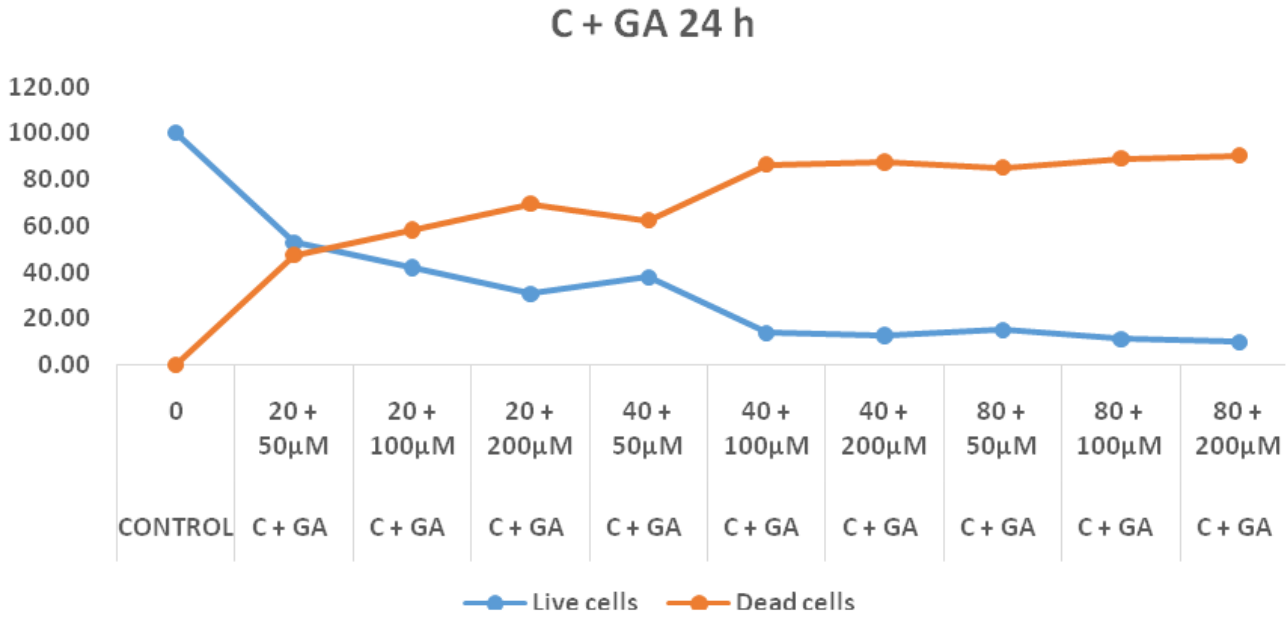

Figure 2A: Effect of Curucmin, Gallic acid treatment on cell viability of HeLa cells at 24h.

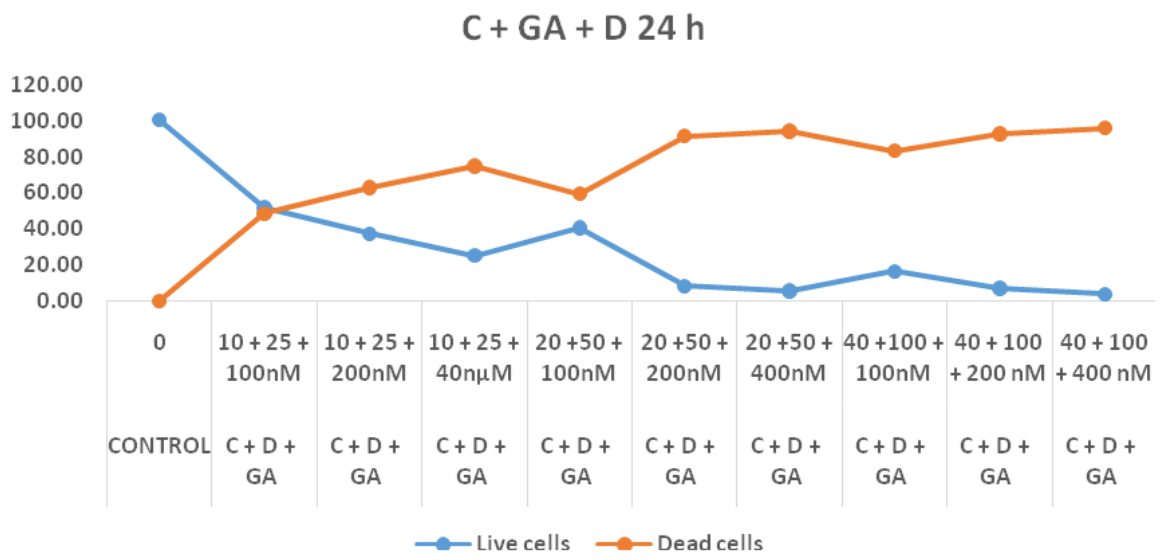

Figure 2B: Effect of Curucmin, Gallic acid and/or Doxorubiin treatment on cell viability of HeLa cells at 24h.

Combinations cell viability 48 h:

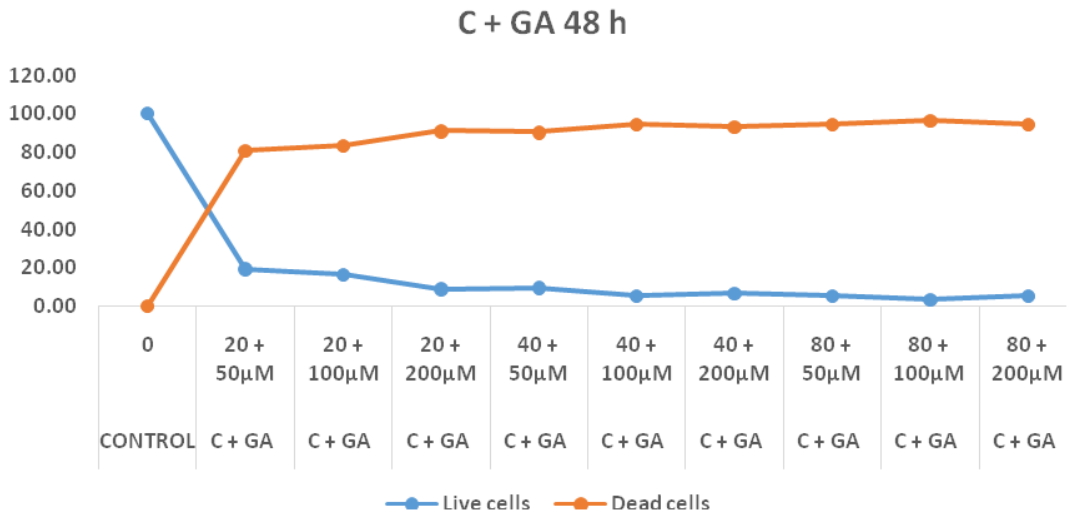

Figure 2C: Effect of Curucmin, Gallic acid treatment on cell viability of HeLa cells at 48h.

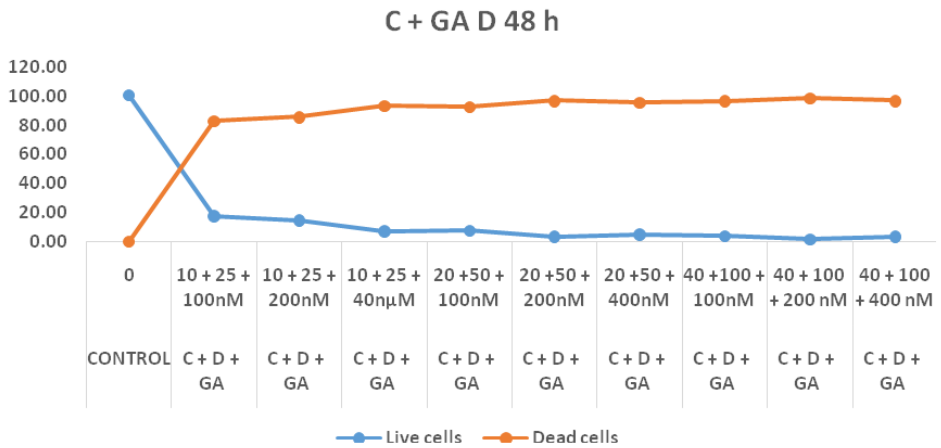

Figure 2D: Effect of Curucmin, Gallic acid and/orDoxorubiin treatment on cell viability of HeLa cells at 48h.

Assessment of cell migration

The % of cells thathad migrated into the scratch area wascalculatedafter 3, 6, 12 and 24 h and shown in figure 3.Complete gap closure in 24 hwas observed in the untreated cells,however, significant changein cell migration was observed when treated with curcumin, gallicacid 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 shown0.85 inches.These results suggest that combinationtreatment was effective in inhibiting themigration of HeLa cells compared toindividual drug treatments (Fig 3).

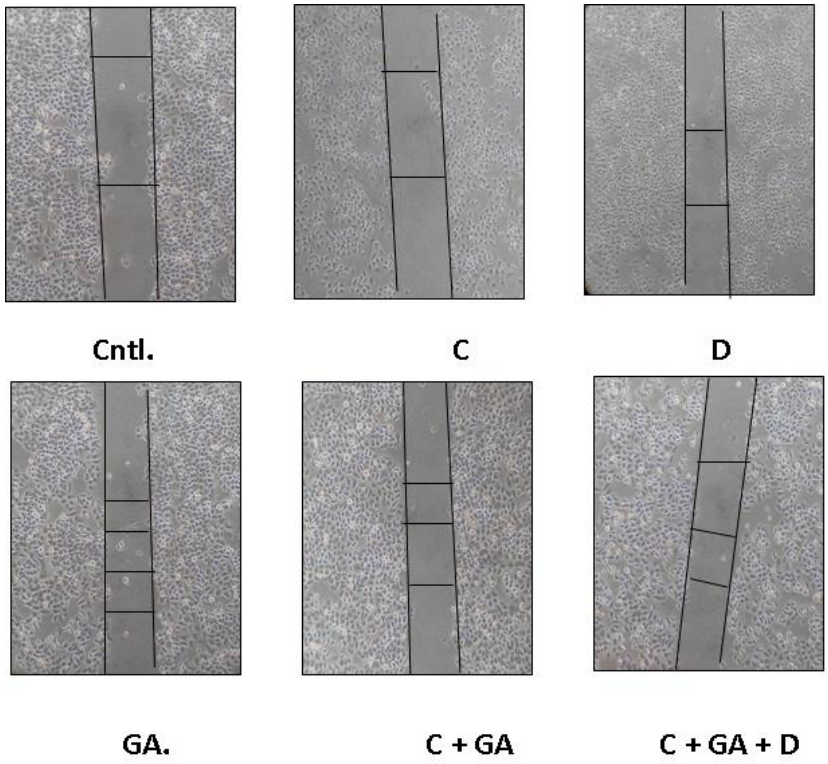

Figure 3A: Effect of Curucmin, Gallic acid and/or Doxorubicin on cell migration.Curcumin (C) (41.28 µM) , Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 µM), C + GA (40 + 100µM) and C + GA + D (20 µM + 50µM + 200nM).

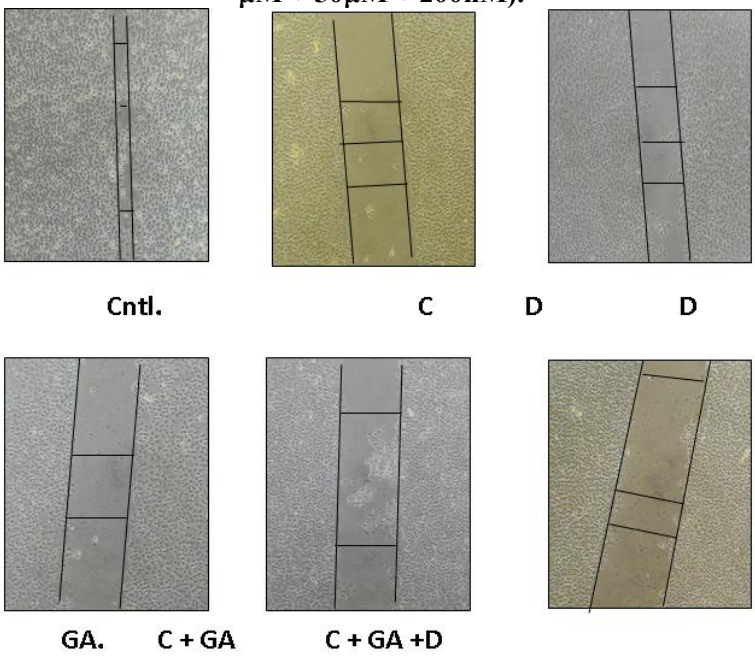

Figure 3B: Effect of curucmin, gallic acid and/or doxorubicin on cell migration.Curcumin (C) (41.28 µM) , Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 µM), C + GA (40 + 100µM) and C + GA + D (20 µM + 50µM + 200nM).

Cell migration assay: 24h

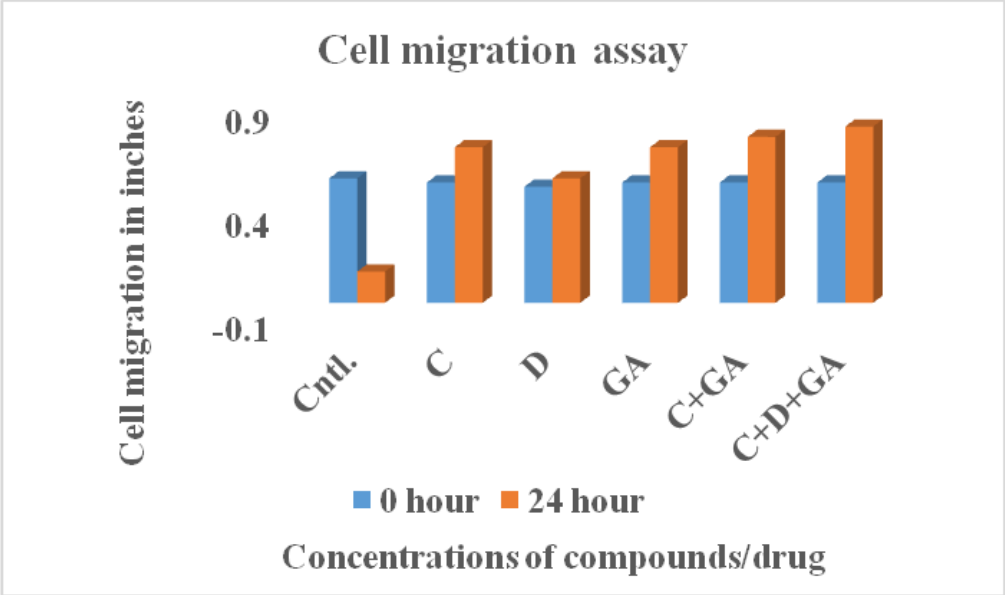

Figure 3C: Effect of Curucmin, Gallic acid and/or Doxorubicin on cell migration.Curcumin (C) (41.28 µM) , Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 µM), C + GA (40 + 100µM) and C + GA + D (20 µM + 50µM + 200nM).

HeLa cell colonies formation

The effect of curcumin, gallic acidand doxorubicin on HeLa cells colony formation was assessed. The % of cells thathad formed colonies after 8 days wascalculated and shown in figure 4. There were more colonies observed in untreated control cells whilesignificant changes in colonies formation were observed in cells treated with curcumin, gallic acid and doxorubicinin 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).

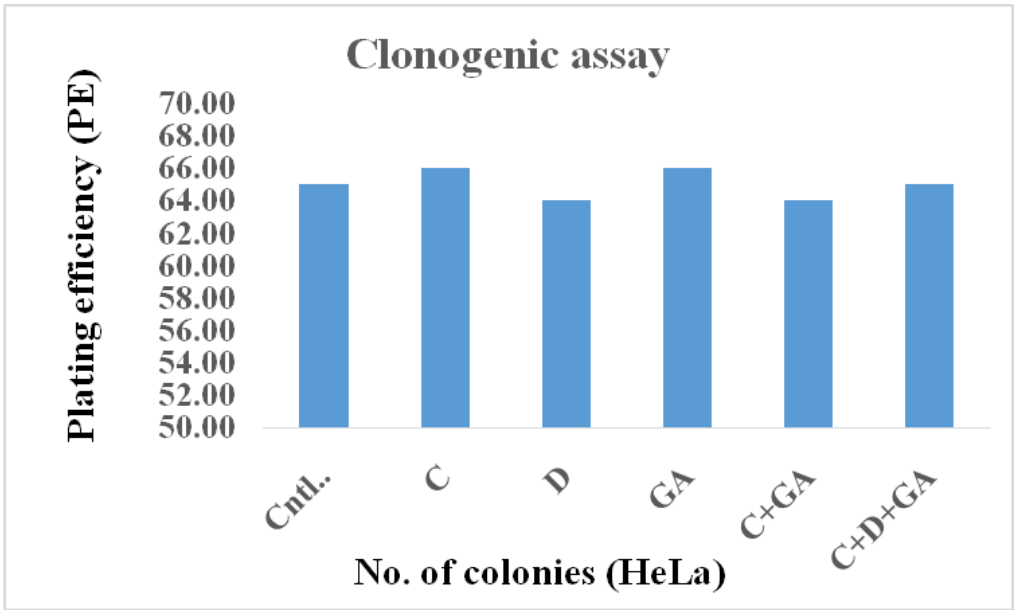

Fig 4A: Effect of Curucmin, Gallic acid and Doxorubicin on colonies formation

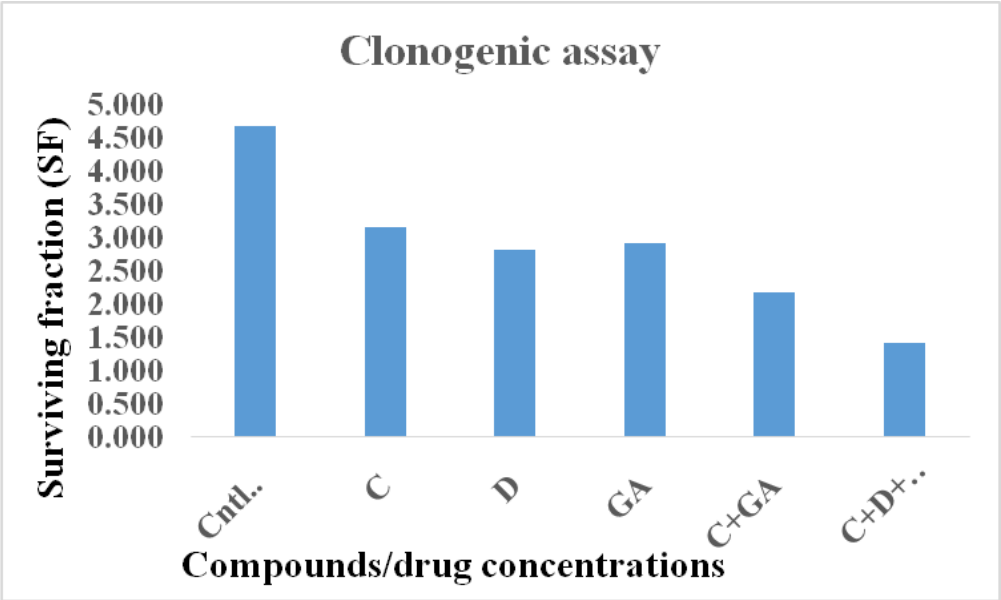

Figure 4B:.Concentrations of compounds/drugs used. Curcumin (C) (41.28 µM) , Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 µM), C + GA (40 + 100µM) and C + GA + D (20 µM + 50µM + 200

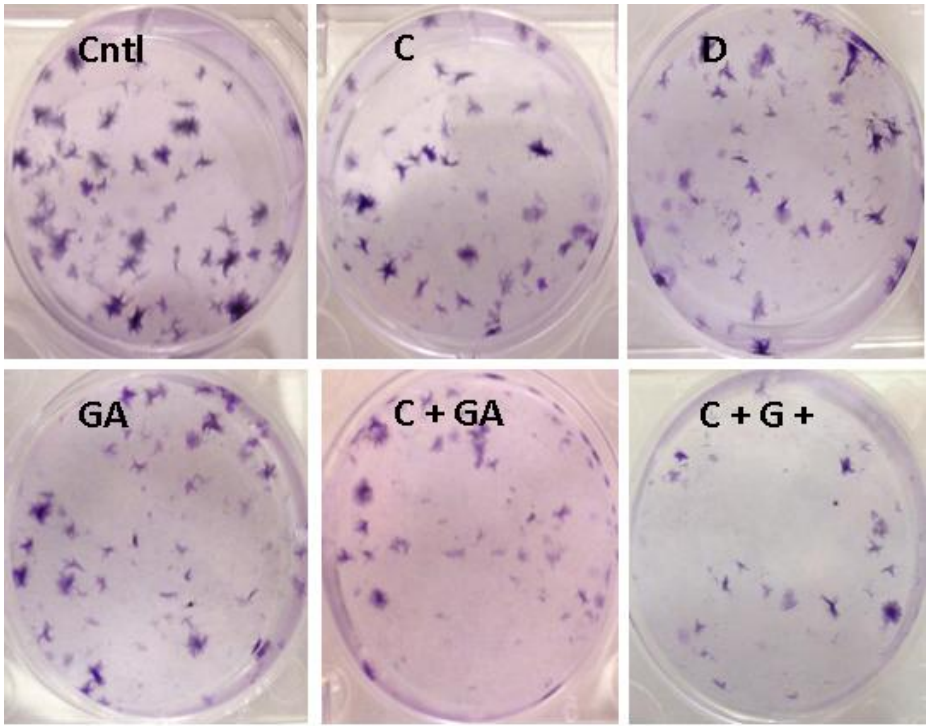

Figure 4C: Effect of Curucmin, Gallic acid and Doxorubicin on colonies formation.Curcumin (C) (41.28 µM) , Doxorubicin (D) (232.34 nM), Gallic acid (GA) (105.66 µM), C + GA (40 + 100µM) and C + GA + D (20 µM + 50µM + 200nM).

DISCUSSION

Current cancer treatment strategies favourcombination 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 ancer 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, thepresent study focussed on combinational therapy ofcurcumin, gallic acid and doxorubicin in cervical cancer cells. Tumor resistance to apoptotic cell death is an important hall mark of cancer and contributes to increased survival ofcells 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 NFkB 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). Suchan inhibitory activity of cell viability was enhanced significantly when the He La 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 drugsat same time point exhibited highest inhibition in cellviability, colony formation and cell migration than either C, GA or Dalone.

These observations may be of value while carefullyconsidering 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.

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