Biochemical and morphological analysis of the anticancer effects of *Cassia auriculata* leaf extract (CALE) *in vitro* against breast and larynx cancer cell lines

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ABSTRACT

The *In vitro* anticancer effect of CALE (*Cassia auriculata* leaf extract) was evaluated in human breast adenocarcinoma MCF-7 and human larynx carcinoma Hep-2 cell lines. CALE was found to inhibit the growth of both cell lines in a dose dependent manner with IC_{50} values of 400μg and 500μg for MCF-7 and Hep-2 cells respectively. The results showed anti cancer effect due to nuclear fragmentation and DNA laddering in agarose gel electrophoresis. Morphological analysis by Transmission electron microscopy showed mitochondrial membrane damage in CALE treated MCF-7 and Hep-2 cells. CALE was found to have cytostatic effect on the growth of both cells measured by the DNA, RNA and total protein contents. Levels of GSH decreased upon CALE treatment indicating the involvement of cellular antioxidants. The activities of LDH and G_R reduced following CALE treatment for both the cell lines. The activities of mitochondrial membrane bound enzymes like Na^+/K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase decreased suggesting mitochondria mediated cytotoxicity of CALE against breast and larynx cancer cells.   © 2010 Trade Science Inc. - INDIA

KEYWORDS

INTRODUCTION

There is a real challenge for designing and synthesizing completely novel drugs for specific cellular targets, very much a chemotherapeutic approach. Therefore existing biological resources can be exploited in search for new molecules, because while there are many compounds already under investigation for their chemo preventive properties. In recent years plant and plant products serve vital sources of blocking and suppressing agents that interfere the carcinogenic process. The plants as such or their established products have been widely reported for their chemo preventive and therapeutic potentials against cancer[6,24]. Epidemiological studies have suggested that dietary habits could influence as many as 30% of cancers. The studies also revel that specific pharmacologically active agents present in diet might reduce the relative risk of cancer develop-

ment. While some compounds of diet have been blamed for inducing cancer, numerous studies suggest that there are also protective agents found particularly in vegetables, fruits, herbs and spices. The exploitation of plant products as chemo preventive agents is very much attractive, just for the reason of least toxicity. A remarkable suge of interest in chemoprevention research has thus led to the identification of many phyto chemicals as effective chemo preventive agents. Ethanomedical plant-use data in many forms has also been highly utilized in the development of formularies and pharmacopoeias, providing a major focus in global health care, as well as contributing substantially to the drug development process.

Cassia auriculata. Linn (Cesalpinaseae) is one of such plant with fast growing, branched, tall, ever green shrub with reddish - brown branches. Dried flower and leaf of the plants are being used for medicinal treatments traditionally. The plant also finds its use in antidiabetic activity[1,15,22], antihyperlipidaemic effect, anti viral, anti spasmodic activities and anti pyreitic activity[15,22]. The leaf extract of *Cassia auriculata* shows emollient effects[10] and anti bacterial activities[25]. The leaf extract of the plant is proved to have effect in treatment of alcoholic liver injury[14]. Therefore our present study was focused on the biochemical and morphological analysis of CALE treated MCF-7 and Hep-2 cell lines.

MATERIALS AND METHODS

Chemicals

Analytical grade reagents were used in the study. Eagle's Minimal essential media (S.MEM) and Fetal bovine serum (FBS) were purchased from GIBCO. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), proteinase K, were purchased from Sigma Chemical Company, St Louis, MO, USA. Molecular weight ladders were from Boringermanhemm-Germany, GIBCO BRL, Geni India Ltd. Nucleo Spin DNA extraction kit was from Macherey-Nagel Gmbg and Co, Germany.

Preparation of *Cassia auriculata* leaf extract (CALE)

Freshly collected leaves of *Cassia auriculata* was

cleaned and shade dried. The dried leaves were coarse powdered in a low speed blendor. 1gm of the powdered leaves was soaked in 100ml of absolute ethanol. The mixture was kept in the rotary shaker for 48 hours. The contents were filtered through muslin cloth and the filter was dried at 55°C. The sediments were re-extracted as mentioned above. The dried extract was scrapped and stored at 4°C in air tight vials.

100mg of the ethanolic extract was dissolved in 10ml of MEM without FCS containing 0.5% dimethyl sulphoxide (DMSO). Working concentrations of CALE ranging from 10micrograms to 1milligram were prepared freshly and filtered through 0.45microns filter before each assay and tested for any fungal or bacterial contaminations. The final concentration of DMSO in the medium was <0.01% which had no detectable effect on cell growth.

Cell lines and cell cultures

MCF-7 (Human breast adenocarcinoma) and Hep-2 (Human larynx cancer) cell lines were obtained from National Center for Cell Sciences, Pune and grown in S.MEM media supplemented with 10% FBS,100 IU/ml penicillin,100µg/ml streptomycin,20µg/ml Kanamycin Acid Sulphate,20µg/ml Amphotericin-B, 3% L-Glutamine and 7.5% Sodium-bi-carbonate solution. The cells were maintained as monolayer in 25cm^2 plastic tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 in air. Exponentially growing cells were used for all the experiments.

Cytotoxicity and anticancer assay

The cytotoxicity of CALE was found using the non tumorogenic breast (HBL-100) and African green monkey epithelial (Vero) cells. The HBL-100 and Vero cells are immortal, but non tumorogenic and do not express most of the specific tumorogenic markers. Both cells at a concentration of 1×10^5 cells per well were seeded in 24 well plates. After overnight growth, the medium was replaced with maintenance medium (SMEM without FBS) containing the various concentrations of the CALE and incubated for 24 h. The plates were microscopically examined for cytotoxicity. MTT assay was used to assess the cell viability based on the reduction of MTT by mitochondrial dehydrogenase enzyme of the viable cells to purple formazon product as described

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by Mosman[20]. Briefly, cells were diluted in the growth medium and seeded in 24 well plate at a concentration of 5×10^4 cells/well. After overnight incubation, the growth medium was replaced with exposure medium (SMEM without FBS) containing desired concentrations of CALE. After 24 h, the cells in each well were washed with 200μl of PBS, and incubated with 100μl of 500μg/ml MTT in PBS at 37°C for 3 h. The so formed MTT-formazon product was dissolved in 200μl of DMSO and estimated by measuring the absorbance at 570nm in an ELISA plate reader. Cell survival was expressed as percentage of viable cells of treated samples to control samples. The test was performed in triplicates and the experiments were repeated at least three times. The same protocol was repeated to check the anti cancer activity of CALE using known tumourogenic MCF-7 and Hep-2cells.

Trypan blue dye exclusion assay

MCF-7 and Hep-2 cells at a concentration of 1×10^5 cells per well were seeded in 24 well plates. After 24 hours, the medium was replaced with maintenance medium (SMEM without FBS) containing various concentrations of CALE and incubated for 24 h. 0.5 ml of TPVG was added to the wells after decanting media. After 2 minutes the TPVG was neutralized by adding 100μl of FCS and then decanted carefully. The plates were incubated at 36°C for five minutes, until the complete monolayer was removed from the surface. Added 1 ml of MEM (without FCS) to the wells and the suspension was aspirated to break the clumps. 0.2ml of the suspension was mixed with 0.2ml of try pan blue and cell count was done as described in cell count assay. Cell count was done for each plate[2,3].

Transmission electron microscopy

Cells were treated with different concentrations (400 and 500μg/ml for MCF-7 and Hep-2 respectively) of CALE for 24 hours and plated. The cells processing for TEM was carried by protocols described by David[7].

Cells were fixed in modified carnoy's fluid buffered in 0.1 M phosphate buffer at pH 7.4. Fixation was for 10 to 18 hours at 4°C, after which the cells were washed in fresh buffer, and post fixed for 2 hours in 1%. Osmium tetroxide in the same buffer at 4°C. After several washes in 0.1M phosphate buffer, the specimens were dehydrated in acetone solutions and embedded in CY 212 oral dite. Ultra thin sections of 60 to 80nm thickness were cut using an ultra-cut E (Reichert Jung) Ultra-microtome and the sections were stained in alcoholic uranyl acetate (10 minutes) and Lead citrate (10 minutes) before examining the grids in a transmission electron microscope (Philips, CM-10) operated at 60-80KV.

DNA fragmentation analysis

MCF-7 cells were treated with test compound for 24 hrs. 10×10^5 cells were collected along with respective controls, centrifuged at 3000rpm for 5 min and supernatant discarded. The cell pellet was washed with ice-cold PBS and re suspended. DNA isolation was carried out using Nucleo Spin DNA extraction kit as per the instruction provided. DNA (2μg) was loaded in 1.6% agarose gel, stained with 0.5μg /ml of ethidium bromide and visualized in a UV illuminator[31].

Assessment of cytostatic effect

Cells were plated in 6 well plates (Corning star, New York) and incubated with 400μg of CALE for MCF-7 and 500μg of CALE for Hep-2 cells for a period of 24 hours. Cytostatic effect of CALE was assessed by measuring the amount of cellular DNA[4], RNA[26] and total protein[17] levels.

Biochemical assays

24 well plates seeded with MCF-7 cells were incubated with their IC_{50} level of CALE and control cells were incubated with MEM for 24 hrs. Following incubation, cells were collected by trypsinization, washed with PBS (pH 7.4) three times and pellet was transferred into extraction solution, which contained 20mM potassium phosphate buffer (pH 7) and a protease inhibitor cocktail. Cells were sonicated using a Biosonic IV sonicator with 15- sec bursts for a total of 4 min on ice and centrifuged at 15000g for 45 min in a refrigerated (Remi, Japan) centrifuge. The supernatant obtained was used in the experiments. Latate dehydrogenase activity was assessed by the method of Nieland et al., 1959.Glutathione reductase was assayed by the method of Dubler and Anderson, 1981. Intracellular levels of GSH were estimated using the method of Patterson, 1955.

Mitochondrial membrane bound enzyme assays

MCF-7 and Hep-2 cells were incubated with 400µg and 500µg of CALE respectively, for 24 hours. The cells were then pelleted and washed once with phosphate buffer and resuspended the same buffer and frozen. The suspension was thawed to break the cells and cooled in ice. The cell extract was made in 2.5mM in $MgCl_2$ and centrifuged at 6000g for 10 minutes to remove the unbroken cells. The membrane fraction was collected by centrifugation at 50,000g for 45 minutes. After washing twice and re-homogenization in the same buffer (ice-cold), the membrane fraction was used for the estimation of Na^+/K^+ ATPase[28], Ca^{2+} ATPase[12] and Mg^{2+}[33].

Statistics

The anti cancer assay data are presented as mean percentages of control ±S.D and linear regression analysis was used to calculate the IC_{50} values for the cytotoxicity assays. All other data were analyzed using analysis of variance (ANNOVA) followed by Tukey-HSD test and the results were considered statistically significant if the p value was <0.05.

RESULTS AND DISCUSSION

Cytotoxicity and anticancer activities of CALE

Any compound that is active against the cancer cells must be checked for its selectivity or non toxicity against normal cells. Therefore we first examined the cytotoxic effects of CALE on the growth of the normal HBL-100 and Vero cells as respective controls. Further anticancer activity was checked in cancerous MCF-7 and Hep-2 cells. CALE showed dose dependent cytotoxic effects on both MCF-7 and Hep-2 cells with IC_{50} values of 400µg (Figure 1a) and 500µg (Figure 1b) respectively, however though not significant the above mentioned concentrations of CALE had its effect on normal cell lines (Figure 1a & b). For all further experiments IC_{50} values were used. The above observations were also confirmed by the Trypan blue dye exclusion assay (Figure 2a & b).

Morphological changes of cells by CALE

Figure 3 shows the transmission electron micro-graphs of MCF-7 and Hep-2 cells treated with 400 and 500µg of CALE respectively for 24 hours. The untreated control MCF-7 cells had smooth surface and reduced microvilli (Figure 3 A). Cell surface microvilli, large nucleoli and scanty cytoplasm were common in cancer cells. Hep-2 control cells had giant mitochondria which are characteristics of the cancer cells (Figure 3 E). Swollen mitochondria and vacuoles were common in cells that were exposed to CALE as evident from figure 3. Lot of vacuoles in cytoplasm, shrunken and broken nuclei and swollen mitochondria were found in cancer cells that undergo apoptosis. The cytoplasm and cell surface were disorganized. The nuclei of CALE treated cells was distinguishable but was found to be badly damaged (Figure 3 C & D). Chromatin was clumped into solid masses, showing irreversible degree of diffuse cytotoxicity, with loss of cell viability. Complete DNA fragmentation was also observed on treatment which suggested apoptotic cell death.

DNA fragmentation assay

In the current study, MCF-7 cells treated with CALE showed characteristic ladder of nucleosomal oligomers at higher concentration with lower molecular weight nucleosomal multimers appearing at lower concentration (Figure 4 A). Hep-2 cells on treatment exhibited apoptotic ladder with small fragments of DNA (Figure 4 B). Chromosomal DNA fragmentation at internucleosomal sites is the earliest one of the nuclear events and the most extensively studied biochemical event in apoptosis. Studies has shown 180-200 bp fragments of DNA to occur, prior to cell death[5,35] which confirms DNA fragmentation in the current study. In many cultured cell line, apoptosis can be readily triggered by DNA-Damaging agents[21] DNA fragmentation is a general component of apoptosis. Thus the most common method to detect apoptosis is the DNA gel electrophoresis, in which DNA fragments are observed. Most of the authors have reported that internucleosomal DNA cleavage occurs during apoptosis in a wide variety of cells and tissues[9,19].

Cytostatic effects of CALE

Figure 5 shows the levels of total protein, DNA and RNA in MCF-7 and Hep-2 cells after treatment of CALE for 24 hours. Figure 5 a shows the significant

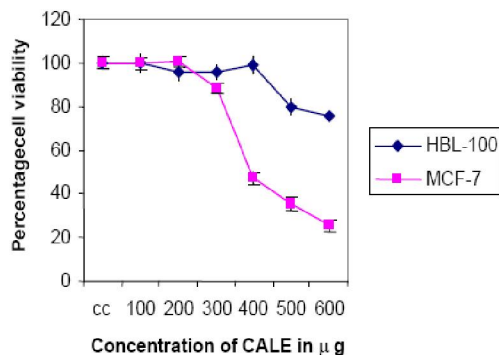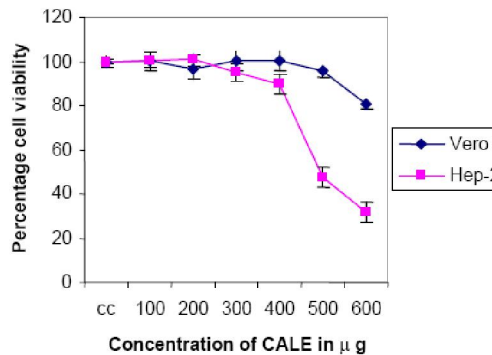*Regular Paper*

Figure 1-a

Figure 1-b

Figure 1: Effects of CALE on MCF-7 and Hep-2 cancerous cells at 24 h incubation time along with HBL-100 and Vero as normal cell lines. Cell survival was measured using MTT assay and expressed as percentage of viable cells of treated samples to untreated control samples. Data are represented by mean ± SD of two independent experiments each performed in tetrads. IC_{50} value was calculated using linear regression analysis.
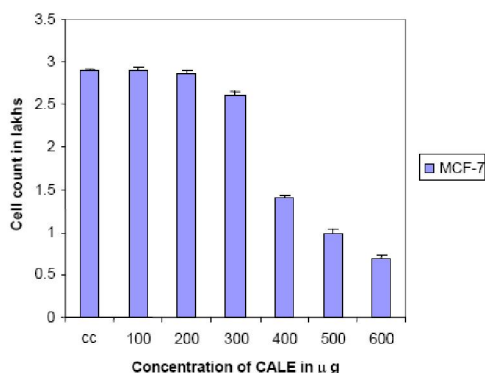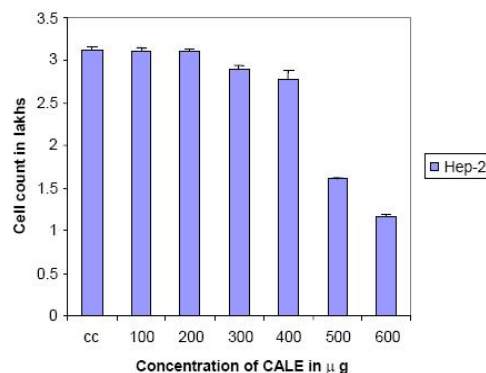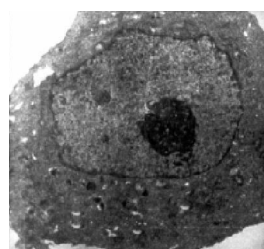
Figure 2-a

Figure 2-b

Figure 2: Cell viability of CALE treated MCF-7 and Hep-2 cells calculated by try pan blue dye exclusion assay. Data represents mean ± SD of two independent experiments each performed in tetrads.
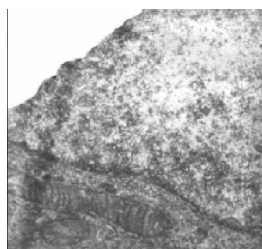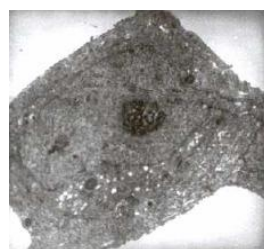

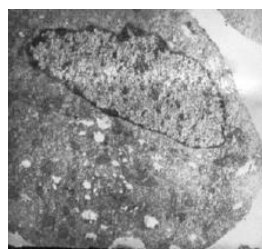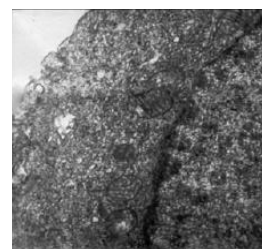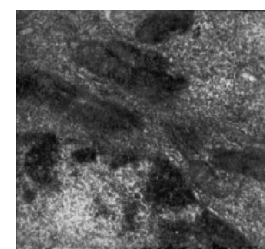| A : MCF-7 Control (X 1950) | B : MCF-7 Control (X 11500) | C : MCF-7 CALE Treated (X 1950) | D : MCF-7 CALE Treated (X 2650) |

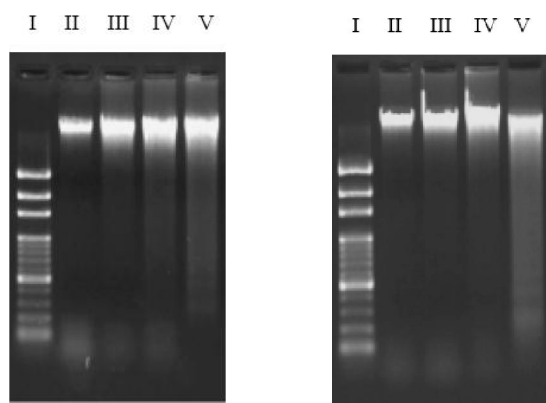| E : Hep-2 Control (X 2650) | F : Hep-2 CALE Treated (X 2650) | G : Hep-2 CALE Treated (X 8400) | H : Hep-2 CALE Treated (X 15500) |
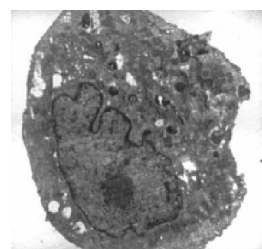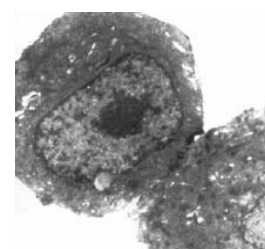
Figure 3: Transmission electron micrographs of CALE treated MCF-7 and Hep-2 cells.

I - Molecular weight ladder
II - Untreated cell control
III - 400µg/ml
IV - 500µg/ml
V - 600µg/ml

I - Molecular weight ladder
II - Untreated cell control
III - 500µg/ml
IV - 600µg/ml
V - 700µg/ml

Figure 4 : Agarose gel electrophoresis of MCF-7 (A) and Hep-2 (B) cells showing DNA ladders reflecting the presence of DNA fragments on treatment with various concentrations of CALE for 24 h.

reduction of total protein levels in MCF-7 and Hep-2 cells when treated with CALE. Figure 5 b shows the levels of DNA for CALE treated cells with respect to their untreated controls. Significant reduction in total DNA content was observed for both the cells. This may be due to the cytostatic effect of CALE. The total RNA levels of CALE treated MCF-7 and Hep-2 cells were found to be reduced at 24 hours (Figure 5c). The decrease in total RNA levels is suggestive of low cellular function which may be due to static or apoptotic stage of cells. The decreased levels of protein as observed in the CALE treated MCF-7 and Hep-2 cells could be related to the decrease in DNA and RNA levels.

Lactate dehydrogenase activity

The inter conversion of pyruvate to lactate is regulated by LDH, which has been found to be useful in recognition of neoplastic disease. Even under aerobic conitions, the tumour cells maintain high glycolytic rate. Thus eventually these tumours produce high levels of lactic acid[29]. In order to counteract the increased amount of lactic acid, the activity of LDH remains high in these tumour cells. It is therefore not surprising that the untreated MCF-7 and Hep-2 cells showed high levels of LDH activities (Figure 6a & b).

LDH activity remains as one of the important marker in assessing cell cytotoxicity[29,32]. The decrease in LDH activity was reported in cancer cells undergoing cyto-

toxicity[30,32,34]. The decreased activity of LDH (Figure 6a & b), could probably be related to the inhibition of glycolysis in the CALE treated MCF-7 and Hep-2 cells. It is established that glucose induces extensive deprivation apoptosis of cancer cells[13]. The reduced glycolytic activity would have been the reason for the decrease of LDH activity observed in the treated cells.

Biochemical analysis of CALE treated MCF7 and Hep-2 cells

Oxidative stress as a consequence of ROS causes damage to the cellular components like nucleic acids and protein. The reduction of DNA, RNA and protein leves by CALE treatment could be due to the production of ROS. GSH is an important antioxidant, which is the substrate for GR. The levels of GSH in both the cells reduced following CALE treatment (Figure7a) suggesting it's utilization either in scavenging free radical or decreased synthesis of GSH resulting from an inhibition of protein synthesis. As mitochondria lack the GSH synthesizing enzymes, free radicals generated promotes mitochondrial uptake of GSH and decreased export of GSH from mitochondria to cytosol. The net efflux of GSH is very slow, when low levels of extra mitochondrial glutathione is consistent with a mechanism that conserves mitochondrial GSH at times of cytosolic GSH depletion causing the swelling up of mitochondria as observed in TEM analysis of CALE treated MCF-7 and Hep-2 cells.

A decrease of GSH can produce peroxides and influx of Ca^{2+} and ultimately cause cell death[16]. Mitochondrial impairment occurred as an early event in the process of apoptosis induced by GSH depletion in neuronal cells[18]. Sato et al.[27] reported that increase in reactive oxygen species or depletion of endogenous antioxidant level promotes cell death. Hydrogen peroxide and reactive oxygen intermediates formed can act as a metabolic signal by oxidizing specific thiol groups and trigger intracellular events leading to apoptosis[11]. Thus the reduction of GSH levels in both cell types could be connected to its free radical scavenging activities. O_2^- radicals could have escaped from the mitochondria by diffusion and could have resulted in the generation of H_2O_2. GPx catalyses the reduction of H_2O_2 utilizing GSH thereby producing GSSG. GSSG reduction to GSH is catalysed by the enzyme glutathione reductase.
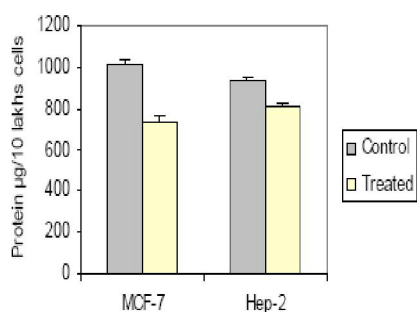
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Figure 5a : Total protein concentration of MCF-7 and Hep-2 cells treated with 400 and 500µg of CALC respectively for 24 h.
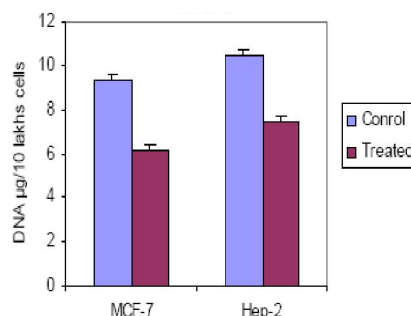

Figure 5b : Total concentration of MCF-7 and Hep-2 cells treated with 400 and 500µg of CALE respectively for 24 h.
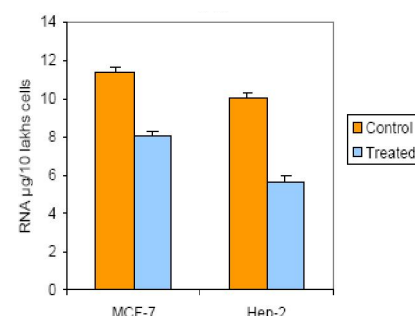

Figure 5c : Total RNA concentration of MCF-7 and Hep-2 cells trated with 400 and 500µg of CALE respectively for 24 h.

Figure 5 : The levels of DNA, RNA and total protein in CALE treated MCF-7 and Hep-2 cells. Data represents mean ± SD of three individual experiments performed in triplicate and results were statistically significant at p < 0.5.
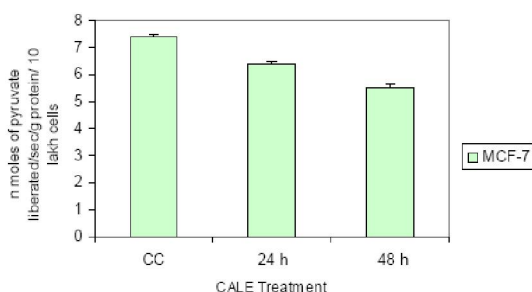

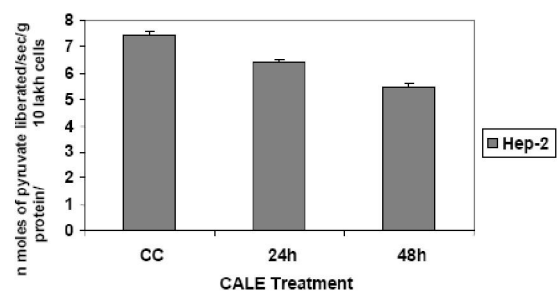**Figure 6a : Levels of LDH in MCF-7 cells treated with 400µg of CALE.**

Figure 6b : Levels of LDH in Hep-2 cells treated with 500µg of CALE.

Figure 6 : The levels of LDH activity in CALE treated MCF-7 (a) and Hep-2 (b) cells. Data represents mean ± SD of three individual experiments performed in triplicate and results were statistically significant at p < 0.5.

Figure 7a : Levels of GSH in MCF-7 and Hep-2 cells treated with 400 and 500µg of CALE respectively for 24 h.

Figure 7b : Levels of G_R in MCF-7 and Hep-2 cells treated with 400 and 500µg of CALE respectively for 24 h.

Figure 7 : The levels of GSH (A) and G_R (b) activities in CALE treated MCF-7 (a) and Hep-2 (b) cells. Data represents mean ± SD of three individual experiments performed in triplicate and results were statistically significant at p < 0.5.

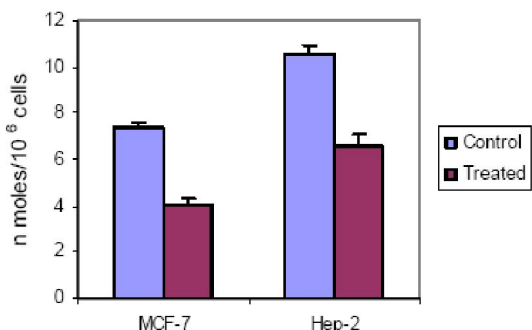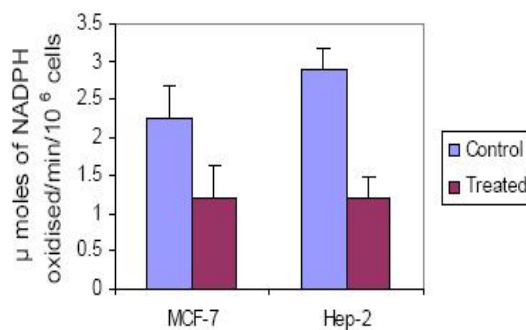This might be the reason for the decrease in activity of glutathione reductase as observed in CALE treated MCF-7 and Hep-2 cells when compared with the control (Figure 7 b). Thus the respiratory stress that was encountered by the cell in response to CALE treatment may be the reason for the reduced activity of glutathione reductase.

Mitochondrial enzyme activities

Significant inhibition of Na^+/K^+ ATPase activities was observed in both cells on CALE treatment (Figure 8 a). There was reduction in activities of Ca^{2+} ATPase in both cells when treated with CALE (Figure8 b). Mg^{2+} ATPase activities also remained inhibited suggesting an alteration in the homeostasis of these ions (Figure 8-c).

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These results are not surprising as they may be due to the mitochondrial membrane damage observed by TEM in CALE treated MCF-7 and Hep-2 cells. The inhibition of Na^+/K^+ ATPase should have resulted from the depletion of ATP resulting in cytosolic acidification. Therefore cytosolic rise in Ca^{2+}, Mg^{2+} and Na^+ with a
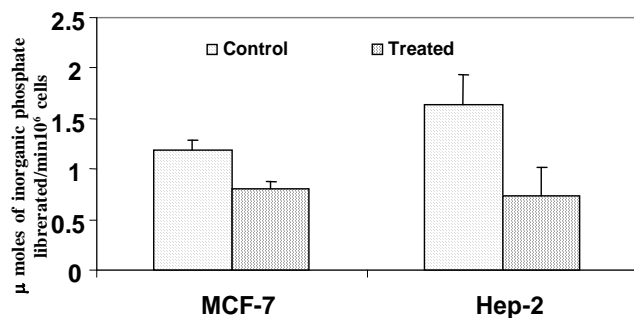

Figure 8a: Levels of Na^+/K^+ ATPase in MCF-7 & Hep-2 cells treated with 400 and 500 μg of CALE respectively for 24 h
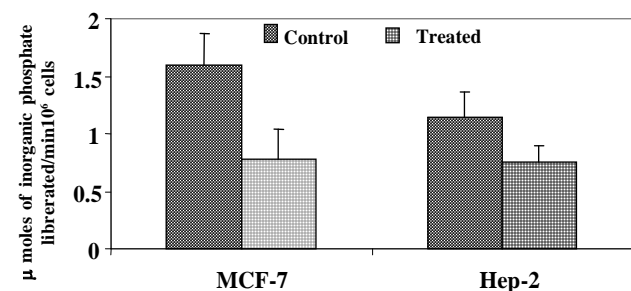

Figure 8b: Levels of Ca^{2+} ATPase in MCF-7 & Hep-2 cells treated with 400 and 500 μg of CALE respectively for 24 h
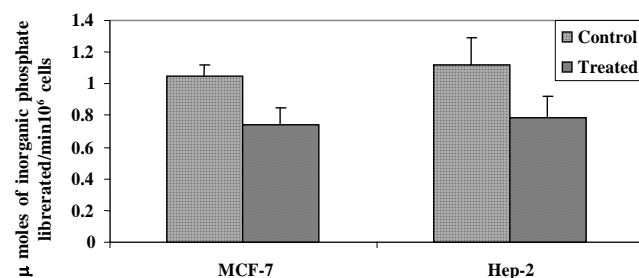

Figure 8c: Levels of Mg^{2+} ATPase in MCF-7 & Hep-2 cells treated with 400 and 500 μg of CALE respectively for 24 h

Figure 8: The levels of mitochondrial membrane bound enzyme activities in CALE treated MCF-7 and Hep-2 cells. Data represents mean ± SD of three individual experiments performed in triplicate and results were statistically significant at $p < 0.5$.

concomitant decrease of K^+ levels could have resulted. Accumulation of GSSG can have deleterious effects on the confirmation activity of thiol containing enzymes. Most of the ATPases have –SH group on the active site. They may react with GSSG causing inactivation of the enzymes. Depletion of GSH would have resulted in raise of the GSSG levels and concominant inhibition of enzyme activities.

Thus in conclusion, CALE brings about morphological and biological changes in breast and larynx cancer cells thus making it offer as a new valuable candidate against these forms of cancers. The molecular mechanisms and pathways of action need to be explored through further research however this study brings in light to an unexplored new candidate that could be focused against cancer.

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