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Chlorogenic acid complex (CGA7), standardized extract from green coffee beans exerts anticancer effects against cultured human colon cancer HCT-116 cells

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Abstract

Coffee is commonly consumed beverage in the world and it has been suggested to have beneficial effect. Chlorogenic acids (CGAs) are main ingredient of coffee beans which has been extensively used in nutraceuticals and medicine. Recently, various therapeutic effects of chlorogenic acids have been investigated. However, there are limited studies to investigate its anticancer properties. In the present study, we have used chlorogenic acid complex (CGA7) a decaffeinated water soluble green coffee bean extract to evaluate its cytotoxic effect on human and mouse cancer cell lines by using different approaches. From our results we found CGA7 treatment induces cell death in a dose and time dependent manner in different cancer cell lines. Further, CGA7 induced apoptosis was characterized by DNA fragmentation, PARP-1 cleavage, caspase-9 activation, and down regulation of Bcl-2, an anti-apoptotic protein and up regulation of pro-apoptotic protein BAX. Overall findings indicated that CGA7 complex a potent anticancer molecule found in green coffee beans could be a safe bioactive ingredient for prevention of cancer.

Keywords: Apoptosis; Chlorogenic acid complex; PARP-1; Green coffee beans; DNA fragmentation

1. Introduction

Cancer is considered as one of the leading causes of death worldwide, which has raised attention of researchers towards the search of effective and novel therapeutic drug for cancer treatment. The current mainstream treatment involves surgery, radiotherapy and chemotherapy, but it has significant negative side effects with limited competence and elevated rate of secondary failures, hence, the researchers have focussed on advancement of effective anticancer drugs with less toxicity [1–3]. Drugs extracted from plants sources is of great importance and many plant-derived compounds like polyphenols, flavanoids and terpenoids are of enormous nutritional and medicinal value and comprehensively studied for their potential as beneficial effects on human health [4–6]. When plant foods are consumed, the bioavailability of plant derived compounds may elicit a variety of important bioactivities [7]. Recently like green tea, extract made from green coffee beans have received much interest from researchers with increasing investigation as a possible health-promoting supplement. Green coffee beans are rich source of chlorogenic acids. Chlorogenic acids (CGAs) are by products of cinnamic acid. Caffeoylquinic acids (CQA) and dicaffeoylquinic acids (diCQA) are the main CGA found in green coffee beans and it is recorded to have strong antibacterial

Abbreviations: LDH, lactate dehydrogenase; PARP1, poly(ADP-ribose) polymerase; AO/EO, acridine orange/ethidium bromide; DNA, deoxyribosenucleic acid.

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and anti-inflammatory activities [8,9]. Published findings from clinical trials have proved the therapeutic effects of green coffee bean extracts containing chlorogenic acids with respect to obesity and diabetes management [10–12]. A array of health benefits have been attributed with the consumption of CGAs such as reduction of the corresponding risk of Alzheimer's disease, diabetes type 2 and cardiovascular disease [13–15]. Chlorogenic acid-rich foods and supplements on the prevention and treatment of metabolic syndrome and associated disorders, including in vivo studies, clinical trials, and mechanisms of action have been reported [16]. Recently, number of pre-clinical and phase I clinical studies have shown that treatment with chlorogenic acid has shown beneficial effects in colon cancer, brain cancer, breast tumours, lung cancer and chronic myelogenous leukaemia [17–19]. However, the molecular mechanisms behind the anticancer response of chlorogenic acid remain yet largely unclear and need to be clarified. In the present study we have made an attempt to screen anticancer activity of CGA7 complex a decaffeinated green coffee bean extract.

2. Material and methods

2.1. Chemical and reagents

All chemicals and reagents were purchased from Himedia, SRL India and Sigma Aldrich, USA. Antibodies were purchased from Santa Cruz Biotechnology, USA.

2.2. Cell culture

Human Cervical (HeLa), Liver (HepG2), breast (MCF7), colon cancer (HCT-15, HCT-116), cell lines and mouse B16-F1, EAC, Normal cell line MEF (STO) were procured from National Centre for Cell Science (Pune, India). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin and 10% fetal bovine serum (FBS; GIBCO Inc., NY, USA). The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and cells were sub-cultured every 3 days.

2.3. Chlorogenic acid complex (CGA7) preparation

Chlorogenic acid complex (CGA7) is composite of seven isomers which consists of three (3-, 4-, and 5-caffeoylquinic acids) and three (3, 4-, 3, 5-, and 4,5-dicaffeoylquinic acids) and one 5-feruloylquinic acids, these seven CGAs accounts for 50% of total CGAs (Supplementary Fig. S1). CGA7; a standardized decaffeinated extract from green coffee beans was procured from Department of Phytochemistry, Vidya Herbs Pvt. Ltd. (Bangalore, Karnataka, India), and dissolved in cell culture media (DMEM) at the appropriate concentrations.

2.4. Determination of cytotoxicity by MTT assay

The cytotoxic effect of CGA7 complex a decaffeinated green coffee bean extract on EAC, B16F1, HeLa, HepG2, HCT-15, HCT-116 and STO was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cells were counted using haemocytometer and 4000 cells/well were seeded in 96 well plates in 100 μL of complete media (containing 10% of FBS). Cells were treated with varying concentrations (0.25, 0.5 1.0 and 2.0 mg/mL) of CGA7 for 24 and 48 h. After completion of incubation, MTT solution (5 mg/mL) was added to each well and incubated for 2 h precipated purple-coloured formazan crystals were dissolved in 100 μL of DMSO by proper mixing. The colour absorbance of each well was read at 570 nm in Multiscan EX reader with a reference serving as blank. Then, IC50 value of CGA7 was calculated.

2.5. Lactate dehydrogenase (LDH) leakage assay

LDH activity was measured in the cell culture medium of each treatment group using an LDH Activity assay kit (Sigma, MAK066), according to the manufacturer's instructions; the supernatant from each sample was used in the LDH assay. Briefly, cells were treated with CGA7 (0.25–1 mg) for 24 and 48 h and the cell culture media of treated and control groups were collected from 96 well plates. The cell culture media were centrifuged and 50 μL of the supernatant transferred to fresh 96 well plates and mixed with equal volumes of reaction mixture containing LDH assay buffer and LDH substrate, incubate the plates at 37 °C for 10 min. Absorbance was read at 450 nm using Multiscan EX reader and the data were represented as milliunits/litre (mU/L).

2.6. DNA fragmentation assay by agarose gel electrophoresis

DNA fragmentation assay was carried out by using agarose gel electrophoresis [20]. Briefly (2×10^6) HCT 116 cells were treated with CGA7 at two different concentrations After completion of incubation cell lysates were treated with 1% sodium dodecyl sulfate and RNase A (10 μg/mL) and keep it for overnight incubation at 56 °C, followed by proteinase K (50 μg/mL) treatment at 56 °C for 8 h. After completion of incubation DNA was extracted and precipitated overnight at −20 °C by adding a half volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol. The DNA obtained was resolved on a 1.5% agarose gel, visualized and photographed under UV Transilluminator after ethidium bromide staining. Control cells were also processed in the similar manner.

2.7. Morphological study under phase contrast microscopy

HCT-1116 cells were treated with different concentrations of CGA7 (0.5 and 1.0 mg/mL) for 48 h. Following incubation, the cells were observed under phase contrast microscope at 20× magnification.

2.8. Fluorescence microscopic analysis of apoptosis

We used acridine orange/ethidium bromide (AO/EB) double staining assay method to detect apoptosis. Briefly 1.5×10^5 HCT-116 cells were seeded in 60 mm dishes and incubated for

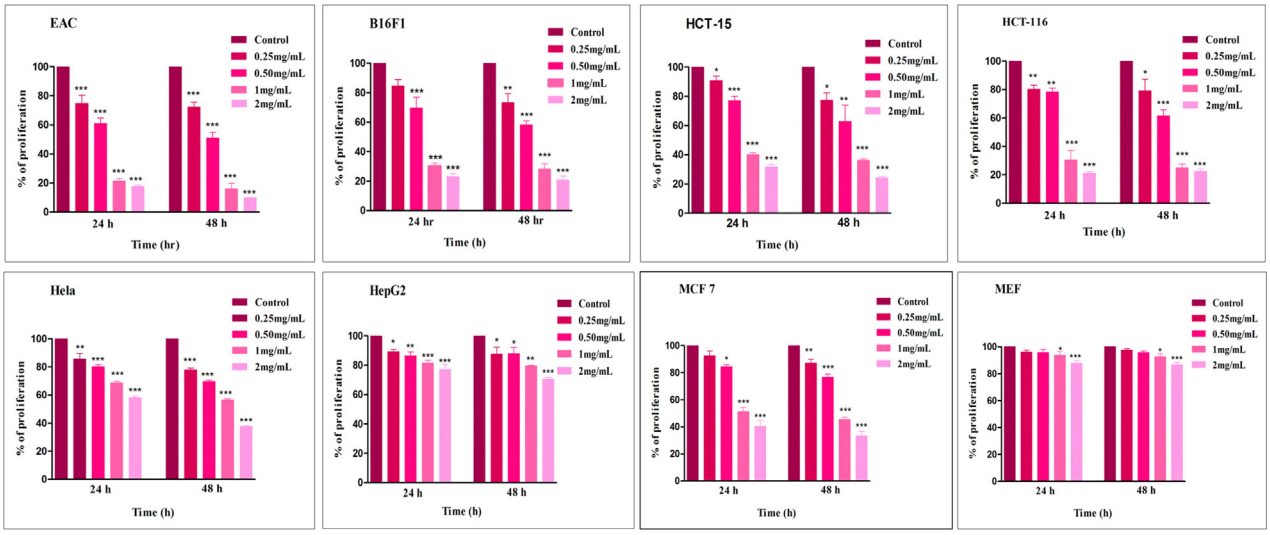

Fig. 1. Evaluation of cytotoxic effect of CGA7 on different cancer cell lines. Effects of CGA7 treatment on cell viability of mouse EAC, melanoma (B16F1) and human colon cancer cell lines (HCT-15 and HCT-116), cervical (HeLa), liver (HepG2), breast cancer (MCF7) and normal cell line MEF (STO). MTT assays were performed in 96-well plates and treated with the indicated concentrations of CGA7 (0.25, 0.5, 1 and 2 mg/mL) for 24 and 48 h. Cytotoxic effects were determined. Data are presented on two independent experiments and error bars represent S.E.M. P value represents $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

24 h followed by replacement of media containing 1 mg/mL of CGA7 complex. After 24 and 48 h incubation the media was removed and washed with phosphate buffer saline, cells were mixed with 400 μL of AO/EB solution and cells were observed under fluorescent microscope.

2.9. Western blotting

For the anticancer study, HCT-116 cells were treated with CGA7 (1 mg/mL) for 12–48 h after incubation, cells were thoroughly washed with PBS and cells were lysed using RIPA buffer. The lysates were centrifuged at 12 000 RPM for 20 min at 4 °C, and the protein concentration was measured by using the Bradford reagent (Bio-Rad). Equal amounts of proteins (100 μg) were loaded in 8%–15% SDS-PAGE. The resolved proteins were transferred to PVDF membrane and blocked with 5% non-fat dry milk in Tris-buffered saline. After blocking, the membranes were incubated with the corresponding primary antibodies separately overnight at 4 °C. The membranes were washed with TBS-T and incubated with alkaline phosphatase-conjugated anti-Rabbit IgG antibody at room temperature in the dark for 3–4 h. The signal was detected by using an ECL detection kit.

2.10. Statistical analysis

All the measurements were made in triplicate and all values were represented as mean ± standard error. Statistical analysis was performed by one-way analysis of variance ANOVA followed by Dunnett's multiple comparisons were used to analyze the significance of differences between control and treated groups. All statistical tests were performed at the $P<0.05$ level of significance.

3. Results

3.1. Cytotoxicity assays

To determine whether CGA7 complex a decaffeinated coffee bean extract was associated with anticancer activities, CGA7 were assessed for their cytotoxicity using MTT assays in human cervical (HeLa), liver (HepG2) and colon (HCT 116 and HCT-15), breast (MCF7) cancer cells and mouse EAC and B16F1 cells along with normal cell line (STO). The MTT assays results indicated (Fig. 1) that CGA7 treated cells exhibited a dose-dependent decline in viability compared to untreated (control) cells, both after 24 and 48 h of treatment. Among the cell lines tested mouse EAC and B16F1 showed maximum sensitivity to CGA7 with an IC50 values of 0.56 and 0.76 mg/mL respectively, followed by human colon cancer cells HCT-116 and HCT-15 with an IC50 values of 0.84 and 0.95 mg/mL, respectively. Whereas MCF7, Hela and HepG2 cells showed limited sensitivity with IC50 values of 1.5, 1.4, and 4.1 mg/mL, respectively at 48 h. While CGA7 induced less cytotoxicity in normal cell line STO (MEF) with an IC50 value of 11.72 mg/mL, compared to untreated (control cells) (Fig. 1 and Table 1). This

Table 1
IC 50 values of CGA7 against various cancer cell lines and MEF (STO) cells.

Cell lines	IC 50 mg/mL	
	24 h	48 h
HeLa	2.4	1.4
HepG2	7.9	4.1
HCT-15	1.2	0.95
HCT-116	1.0	0.84
MCF7	1.5	1.3
EAC	0.74	0.56
B16F1	1.0	0.76
STO	11.9	8.1

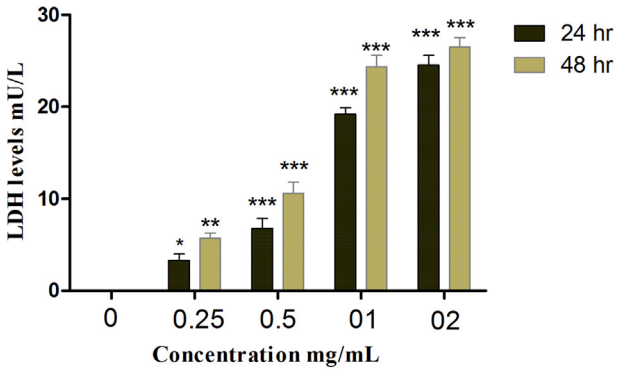

Fig. 2. Lactate dehydrogenase assay. After the exposure of HCT-116 cells with CGA7 at different concentrations (0.25, 0.5, 1 and 2 mg/mL) for 24 and 48 h, the release of LDH was measured at 450 nm. Results are presented as LDH release. Significant levels at $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ compared to vehicle treated controls.

result indicates that CGA7 has potent growth-inhibitory activity against human colon cancer HCT-116 compared to other human cancer cell lines, therefore HCT-116 cell line was used further to evaluate the cytotoxic effect of CGA7.

3.2. LDH levels

LDH assay was carried out to determine cell membrane damaging potential following CGA7 treatment against HCT-116 cells. LDH leakage into the cell culture supernatant following 24 and 48 h treatment with CGA7 was measured (Fig. 2). Treatment with CGA7 showed a concentration dependent increase in the LDH. Overall, our results clearly suggest that CGA7 were capable of inducing cytotoxicity in HCT-116 cancer cell line.

3.3. CGA7 complex induce morphological changes and cell apoptosis in HCT 116 cells

In order to study the morphological alterations, HCT-116 cells were treated with CGA7 (1 mg/mL) at different time intervals i.e. 24 and 48 h. Morphological alterations such as loose arrangement, shrinkage and cell rounding were observed in CGA7 treated HCT 116 cells in comparison to the control cells under a phase contrast microscope (20× magnification) (Fig. 3A). The fluorescent microscopic examination showed that live HCT-116 cells displayed normal green nuclei, while CGA7 treated cells showed yellow or orange condensed or fragmented chromatin aggregated along the periphery of the nuclear membrane and membrane blebbing indicating apoptosis (Fig. 3B).

3.4. CGA7 treatment exhibits DNA fragmentation

DNA fragmentation is one of the hallmark sign of apoptosis. DNA fragmentation assay was performed in HCT 116 cells, after 24 and 48 h of incubation with CGA7 complex. Our experimental results showed formation of distinct ladder in 24 and 48 h of CGA7 treated cells as compared to control cells (Fig. 4). However we could not find DNA fragmentation in control cells upon 48 h incubation with CGA7.

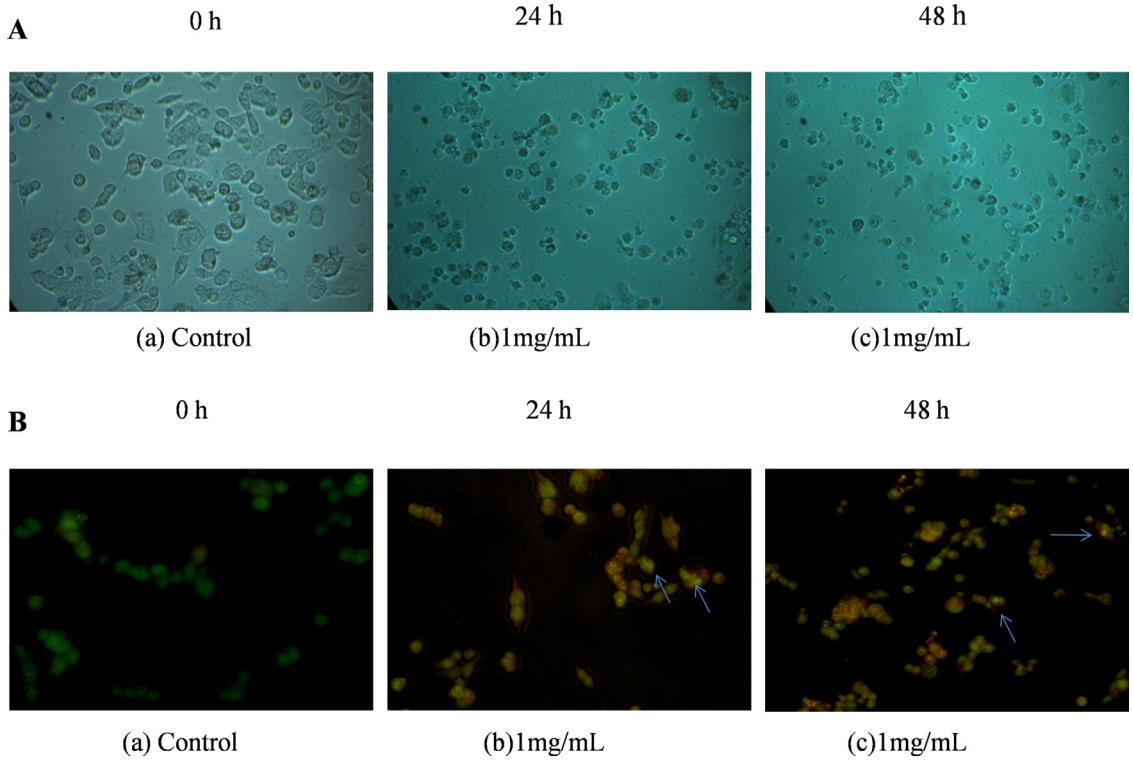

Fig. 3. Morphological changes observed in CGA7 treated HCT-116 cells. A (a)–(c). The morphology changes of HCT-116 cells treated with 1 mg/mL of CGA7 for 24 and 48 h using a phase contrast microscope. B (a)–(c). Morphological observation of HCT cells treated with 1 mg/mL of CGA for 24 and 48 h using a fluorescence microscope (20 × magnification).

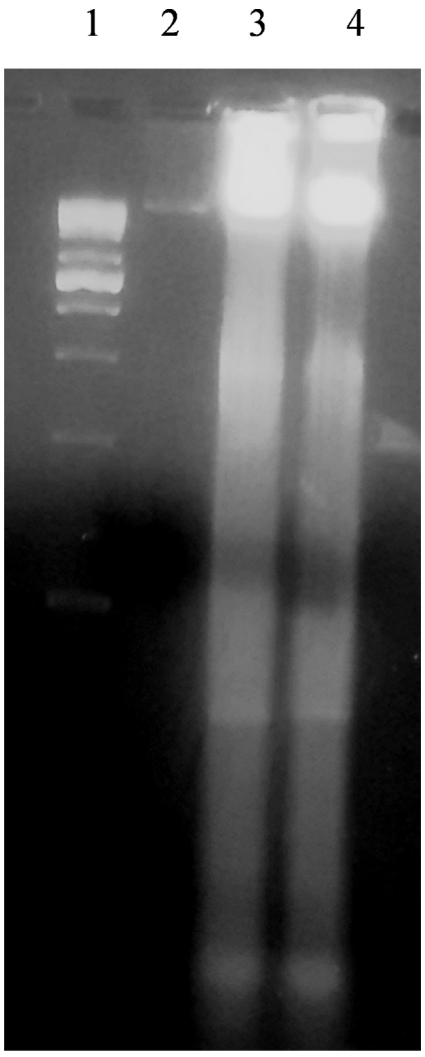

Fig. 4. DNA fragmentation assay. Agarose gel electrophoresis of DNA from HCT 116 cells. Lane 1–3 kb DNA marker (GeNeiTMLowRangeDNARuler); Lane 2 – untreated cells, Lane 3 and 4 cells treated with 1 mg/mL CGA7 for 24 and 48 h.

3.5. CGA7 alters the expression of apoptosis-related proteins in HCT-116 cells

In order to understand the mechanism by which CGA7 induces apoptosis, we examined changes in the expression levels of apoptotic markers following exposure of CGA7. HCT 116 cells were treated with CGA7 (1 mg/mL) for 12–48 h. Whole cell lysates were prepared and then subjected to immunoblotting. Results showed that CGA7 treatment induces very clear PARP1 cleavage where the 116 kDa protein was cleaved to an 89 kDa catalytic fragment and low level of CASPASE 9 activation compared to control after 48 h (Fig. 5). In addition we have also observed a dose-dependent reduction in the expression of anti apoptotic protein Bcl-2 with related increase in the expression level of pro apoptotic protein BAX (Fig. 5). Previously, it has been shown that PARP-1 cleavage triggered by caspases is considered as an important sign of apoptosis. Interestingly, we observed PARP1 cleavage at higher concentrations of CGA7 treatment; this clearly indicates our results suggest

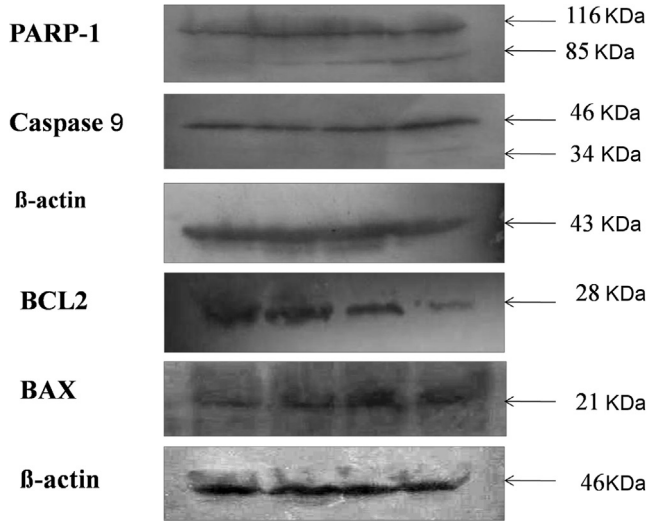

Fig. 5. Effect of CGA7 on expression of different apoptotic markers. Cell extracts were prepared after 12, 24 and 48 h of CGA7 treatment (1 mg/mL) in HCT 116 cells. Control cells grown for 48 h served as control. Cell extract 100 μg was resolved on SDS-PAGE and western blot analysis was performed using antibodies against PARP1, CASPASE9, BCL2, BAX and ß-actin was used as the loading control.

that CGA7 activates the mitochondrial pathway of apoptosis to induce cytotoxicity in cancer cells

4. Discussion

It is widely believed that foods and beverages are rich in polyphenols helps to prevent various kinds of illness associated with oxidative stress, including coronary heart disease and different types of cancer [21,22]. In the recent years, there has been an increasing interest in certain compounds, having massive health effects which include antioxidants and phytochemicals, which are derived from natural resources such as plants and microorganisms. There are many plant derived anticancer drugs. For example the epipodophyllotoxin analogues, the taxanes, the camptothecins and the vinca bisindole alkaloids, are all effective and clinically useful plant-derived anticancer drugs [23,24]. Chlorogenic acid has been reported to have antiproliferative activity. Modern studies showed chlorogenic acid has the ability to induce apoptosis and cellular DNA damage in lung cancer cells without disturbing normal lung fibroblasts. However, the anti-cancer effects of chlorogenic acid have not comprehensively investigated [25].

In this study, we report the anti-cancer efficacy and associated mechanisms of CGA7 complex by various experimental approaches, MTT assay was performed to detect mitochondrial activity in living cells and it is measure of surrogate viability. In the current investigation, cytotoxicity studies in different cancer cell lines showed that CGA7 affected cell viability in a time and dose-dependent manner (Fig. 1), indicating that CGA7 potentially affect the cell survival by disrupting the mitochondrial structure and metabolism [26,27]. LDH assays have been used to determine cell-mediated cytotoxicity and also to identify mediators that induce cytolysis [28]. LDH is a soluble cytoplas-

mic enzyme that is present in most of the cells and is released into blood stream or extracellular space when the plasma membrane is damaged. The leakage of LDH is another marker of cytotoxicity; treatment of HCT-116 cells with CGA7 resulted in significant concentration dependent increase in the LDH levels in cell culture supernatant (Fig. 2). This indicates that the cytotoxicity of CGA7 against HCT-116 cells might be attributed to the cell membrane destructive effects of CGA7.

Morphological alterations such as chromatin condensation, membrane blebbing and or fragmentation, which are considered to be important characteristic feature of apoptosis. In CGA7 treated cells morphology become more round and floated compared to untreated control cells, showing dissimilar cytoskeleton. AO/EO staining demonstrated apoptotic associated changes like membrane blebbing and bright dense granular yellow or orange condensed or fragmented chromatin aggregated along the periphery of the nuclear membrane (Fig. 3B). From our studies we have observed the increase in the number of cells stained orange with or without fragmented DNA at higher doses of CGA7 imply the possibility of late apoptotic and necrotic cell death. Also, gel electrophoresis of extracted DNA revealed characteristic clear ladder formation in case of CGA7 treated cells. However there is no evidence of DNA fragmentation in case of control cells. Both the microscopic and DNA fragmentation assay indicates that CGA7 initiates apoptosis in HCT-116 cells.

Further, to elucidate the anti-cancer mechanism by which CGA7 induce apoptotic cell death, we investigated which proteins are activated during the induction of apoptosis by using western blot experiments. The results showed that the proapoptotic proteins, PARP-1, and caspase-9, were activated by CGA7 treatment in a dose dependent manner. PARP-1 cleavage is reported as a marker for apoptosis and is one of the important targets for caspases [29–31]. In the present study we observed PARP-1 cleavage and Caspase 9 activation (Fig. 5) demonstrates that CGA7 triggers activation of mitochondrial pathway of apoptosis. In addition interestingly CGA7 significantly suppress the expression level of Bcl-2 and increase the expression level of BAX (Fig. 5). Apoptosis involves a multiple network of protein-protein interactions that essentially rely on the equilibrium between the pro-apoptotic and anti-apoptotic proteins which belong to BCL2 family [32]. From our study the observed changes in the levels of Bcl-2 and BAX protein expression indicates activation of intrinsic pathway mechanism of apoptosis following CGA7 exposure. In conclusion, this study demonstrates that CGA7 induced cell death proceeds via apoptosis. Our results suggest the potential of CGA7 from green coffee beans in inhibition of colon cancer; hence CGA7 can act as a good dietary, chemopreventive as well as therapeutic agent for the prevention of cancer. However, more studies are required to test for anticancer activity of CGA7 in advanced tumour models.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fshw.2017.06.001.

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