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Kaempferide, the most active among the four flavonoids isolated and characterized from *Chromolaena odorata*, induces apoptosis in cervical cancer cells while being pharmacologically safe

Lekshmi R. Nath[1]#, Jaggaiah N. Gorantla[2]#, Sophia Margaret Joseph[1], Jayesh Antony[1], Sanu Thankachan, Darsan B Menon, Sankar S[3], Ravi S. Lankalapalli[2]*, Ruby John Anto[1]*

[1]Cancer Research Program, Division of Cancer Research, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India.

[2]Chemical Sciences and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695019, Kerala, India.

[3]Department of Pathology, Government Medical College, Thiruvananthapuram-695011, Kerala, India

# These authors contributed equally to this work.

* Corresponding authors

* Ravi S. Lankalapalli, Chemical Sciences and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695019, Kerala, India.
(Email: ravishankar@niist.res.in), Phone: 91-471-2515352, Fax: 0471-2491585.
* Ruby John Anto, Division of Cancer Research, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India.
(Email: rjanto@rgcb.res.in), Phone: 91-471-2529473, Fax: 0471-2348096.

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Running title: Kaempferide, an apoptotic inducer from the DCM extract of *Chromolaena odorata*

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Abstract

Chromolaena odorata, commonly known as Siam weed, is popular as a traditional medicine. We report the isolation and characterization of four compounds from a cytotoxic fraction F-17, isolated from the DCM extract of *C. odorata* by bioactivity guided fractionation. The organic extracts were screened in five cancer cell lines of various origins for their cytotoxic effect, among which the DCM extract was found to be the most cytotoxic and was purified by silica gel column chromatography to obtain four major compounds. The compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, and HR-MS methods and were found to be acacetin (**1**), dihydrokaempferide (**2**), isosakuranetin (**3**), and kaempferide (**4**). MTT assay was used to study the preliminary cytotoxic evaluation of these four compounds. Among the cancer cell lines that were screened, HeLa was the most sensitive to kaempferide (IC_{50}:16 μM) followed by acacetin (174 μM), dihydrokaempferide (>200 μM) and isosakuranetin (>200 μM). HeLa cells treated with kaempferide (**4**) exhibited morphological characteristics of apoptosis, such as DNA aggregation and chromatin condensation and were non-toxic to rapidly dividing normal human fibroblast cells up to 100 μM. Annexin V staining indicated membrane flip-flop, characteristic of early apoptosis, which was further confirmed by FACS analysis. Induction of apoptosis by this compound was assessed by cleavage of caspases and PARP. FACS analysis showed that the cell death is independent of cell cycle arrest. Acute and chronic toxicity studies conducted *in vivo* proved that the compound is pharmacologically safe. To the best of our knowledge, this is the first study reporting the anticancer potential and pharmacological safety of kaempferide (**4**).

1. Introduction

Cervical cancer, the most dreadful female cancer in developing countries, contributes considerably to cancer-related mortality among women in Africa, Central America and South-Central Asia and has the highest disease frequency in India[1]. The causative agent of cervical cancer is human papilloma virus (HPV) implicated in sexually transmitted diseases and most of the victims of this cancer are from poor socio-economic status who cannot afford costly drugs. *Chromolaena odorata,* formerly known as *Eupatorium odoratum,* is native to North America and has been introduced to tropical Asia, West Africa, and parts of Australia[2]. Although regarded as a serious weed, *C. odorata* has potential medicinal uses. Traditionally, fresh leaf juice or a decoction of *C. odorata* is used to cure amenorrhea, skin diseases, poison bites, wounds, amygdalitis, fever, inflammatory diseases, malaria, jaundice and rheumatism[3,4,5]. In St. Lucia, *C. odorata* is used for early stages of cancer as a decoction in

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which this plant is boiled with four other plants[6]. Also, people in Machang, Kelantan, and Malaysia use this plant to treat wounds, uterus-related problems, and to arrest bleeding[7]. This plant is reported to have anti-inflammatory, antioxidant, wound healing, antimicrobial and haemostatic properties[8,3,9,10]. Previous phytochemical studies on *C. odorata* have led to the isolation of flavonoids, anthraquinones, alkaloids, triterpenoids, and steroids[11-14]. Preliminary studies have been reported on the cytotoxic effects of the ethanolic extract of the whole plant on various cancer types, including cancers of blood, breast, lungs, liver, cervix, and prostate[15,7,16,17]. Recently, chromomoric acid C-I, an activator of Nrf2 has been reported from the methanolic extract of *C. odorata*[18]. Even though some of the compounds isolated from this plant have been shown to have anti-cancer potential, studies pertaining to the anticancer potential of kaempferide (**4**) is not reported. In the present study, we report for the first time the presence of a potent anticancer principle kaempferide (**4**) along with other three less cytotoxic compounds from the DCM extract of the leaves of *C. odorata.* We have also investigated the mode of cell death associated with this compound in inducing cell death in cervical cancer cells. In this study, we report some promising anti-apoptotic activity of kaempferide (**4**) against cervical cancer cells, as evidenced by morphological features such as memberane blebbing, nuclear dye uptake by acridine orange/ethidium bromide and annexin V-PI stain and the activation of caspase cascade, which is a classical marker of apoptotic cell death. We have also conducted *in vivo* studies to ensure the biological safety of the compound.

2. Materials and methods

2.1 Reagents and antibodies

Annexin V apoptosis detection kit and antibodies against PARP (Poly (ADP-ribose) polymerase) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against caspases 3, 7, 8 and 9 were purchased from Cell Signaling Technology (Beverly, MA, USA), silica gel for column chromatography, and silica gel F_{254} for thin layer chromatography were obtained from Sigma Chemicals (St. Louis, MO, USA). Solvents were purchased from Merck (Germany). Curcumin and MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma Chemicals (St. Louis, MO, USA), Immobilon Western blotting reagent was purchased from Millipore Corporation, Billerica, USA.

2.2. Methods

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2.2.1 Extraction from the leaves of *C. odorata*

Fresh plant was collected in August 2009 from local areas in Thiruvanathapuram and was identified by Dr. G. Valsaladevi, Curator, Dept of Botany, University of Kerala, and a voucher specimen has been deposited at the author's laboratory (VOUCHER NO: CRP04). Leaves of the plants were washed thoroughly and shade-dried at room temperature (25–30 °C) for 7 days. The well dried sample was powdered and a defined quantity (100 g) was serially extracted using solvents with increasing polarity viz. hexane, dichloromethane, ethyl acetate and methanol at 37 °C, 120 rpm, 24 h at room temperature. The individual solvent extracts were concentrated separately under vacuum using rotary evaporator; the resulting crude extracts were dissolved in a minimal volume of DMSO and were stored in -20 °C freezer as stock solutions (100 mg/mL or 50 mg/mL).

2.2.2. Isolation of bioactive fraction using bioassay-guided fractionation

The crude DCM extract (3 g) was subjected to silica gel column chromatography (60-120 mesh size, column size 40 cm × 20 cm) using hexane/dichloromethane (100/0, 80/20, 60/40, 50/50, 25/75, 0/100), dichloromethane/chloroform (100/0, 80/20, 60/40, 25/75, 0/100), chloroform/methanol (100/0, 80/20, 60/40, 0/100). A total of 50 fractions were collected, visualized with UV chamber (256 nm and 365 nm) and then pooled the fractions possessing similar TLC profile. Each pooled fraction (F-1 to F-20) was subjected to cytotoxicity and F-17 which was eluted by dichloromethane/chloroform 60/40 ratio was found to be the most cytotoxic fraction. Hence, F-17 was subjected to silica gel column chromatography, eluted with EtOAc/hexane (20/80 to 50/50), to yield a mixture of two compounds. The mixture of compounds in EtOAc/hexane along with a few drops of methanol in a 50 mL rough surfaced glass vial was kept aside for slow evaporation of solvents at room temperature. After complete evaporation of solvents, a mixture of pale yellow and white solids was observed which were carefully separated with the help of sharp steel syringe needle. The pale yellow colored solid acacetin **1** and the white solid dihydrokaempferide **2** (Fig. 2) were isolated as 15 and 10 mg, respectively. Further elution of the column with EtOAc/hexane (50/50) to 100% EtOAc afforded pure isosakuranetin **3** (Fig. 2) as a white solid (60 mg). Some fractions of compound **3** isolated as a mixture (80 mg) with a yellow colored compound. The mixture was then subjected to purification by column chromatography using the mobile phase gradient system hexane/DCM (30/70, 40/60, 20/80) to acetone/DCM (1/99) affording compound **3** (40 mg) as a white solid, and kaempferide **4** (Fig. 2) as yellow crystals (25 mg). All four compounds were found to be reported from *C. odorata* as well as in other plants. See literature[9,12,19-23].

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2.2.3 Cell culture and Cell viability

The cancer cell lines viz. HeLa (cervical), MDA-MB-231 (breast), HCT 116 (colon) and HL60 (leukemia) were procured from the National Centre for Cell Sciences, Pune and are maintained under standard conditions in our laboratory. The lung cancer cell line, H1299 and normal fibroblasts were gifted by Dr. Bharat Aggarwal, MD Anderson Cancer center, Houston, USA.

MTT assay

Briefly, the cells were seeded in 96-well plates (2000 cells/well). After overnight incubation, cells were treated with different concentrations of organic extracts (25-250 µg/ml) and isolated compounds (5-200 µM) along with positive control curcumin (5-30 µM) for 72 h and cytotoxicity was measured. Fresh media containing 25 µL of MTT solution (5 mg/mL in PBS) and 75 µL of complete medium was added to the wells and incubated for 2 h. At the end of incubation, MTT lysis buffer (20% sodium dodecyl sulphate in 50% dimethyl formamide) was added to the wells (0.1 mL/well) and incubated for another 1 h at 37 °C. At the end of incubation, the optical density was measured at 570 nm using a plate reader (Bio-Rad). The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated samples) × 100 [24].The IC_{50} values were extrapolated from polynomial regression analysis of experimental data.

2.2.4. Acridine orange/ethidium bromide staining

Morphological changes characteristic of apoptosis were assessed by fluorescent microscopy using acridine orange/ethidium bromide staining method. Briefly, cells were seeded in 96-well plates and treated with kaempferide **(4)** as in MTT assay for 24 h. After washing once with PBS, the cells were stained with 100 µL of a 1:1 mixture of acridine orange/ethidium bromide 4 µg/mL solutions. The cells were immediately washed with PBS, viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300) and photographs were taken[25].

2.2.5 Detection of apoptosis by Annexin V

As apoptosis causes changes in membrane permeability, there is a transient leakage of phosphatidylserine to the membrane, which is considered to be an early marker of apoptosis. Annexin V preferentially binds to phosphatidylserine as it is a negatively charged phospholipid. Hence, using FITC (Fluorescein isothiocyanate) conjugated Annexin V, apoptotic cells were detected with the help of a fluorescent microscope by manufacturer's protocol (Santa Cruz, CA, USA). Briefly, the cells were seeded in 96-well plates and treated

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with the kaempferide**(4)** as in MTT assay for 16 h. The cells were first washed with PBS and then with 1X assay buffer after which, 0.5–5 µL (0.1–1 µg) of Annexin V FITC per 100 µL assay buffer was added. After incubating for 15 min at room temperature in the dark, the cells were washed with PBS and immediately photographed using a fluorescence microscope[26].

2.2.6. Estimation of apoptosis by FACS

The extent of apoptosis induced by kaempferide was estimated by FACS using Annexin V apoptosis kit (Santa Cruz, CA, USA). Briefly, cells were seeded in 60 mm culture plates, incubated with different concentrations of drugs. After 16 h, cells were trypsinized and pelleted down by low speed centrifugation, washed with PBS and were suspended in 1X assay buffer. To the buffer 5 µL of FITC conjugated Annexin V and 10 µL of propidium iodide were added and incubated for 15 min in dark at room temperature. The cells were then analyzed immediately by flow cytometry to get the % of apoptotic cells (FACS Aria^{TM}, BD Bioscience)[27].

2.2.7 Western blot analysis

For the detection of apoptotic proteins, HeLa cells (10^6 cells/60 mm culture dish) were treated with the kaempferide (**4**) for 24 h. The cells were then washed with PBS and lysed by keeping on ice for 30 min with whole cell lysis buffer containing 20 mM Tris pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.1% Triton, 1 mM DTT (1,4-Dithiothreitol), 0.5 mM PMSF, 4 mM sodium orthovanadate, aprotinin (5 µg/mL) and leupeptin (5 µg/mL). The supernatants were collected by centrifugation at 13,000 rpm for 10 min at 4 °C and boiling in 5X loading dye before separating the proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting them against antibodies of caspase proteins (pro-caspase 3, pro-caspase 7, pro-caspase 8 and pro-caspase 9 antibodies), and poly (ADP-ribose) polymerase (PARP). Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibodies and visualized by enhanced chemiluminescence detection kit (Millipore Corporation, Billerica, MA, USA)[28].

2.2.8 Flow cytometry and cell cycle analysis

Cell cycle analysis helps in distinguishing the distribution of a population of cells to the different stages of the cycle. Briefly, cells were seeded in 60 mm plates and subjected to kaempferide (**4**) treatment for 24h and 48h followed by trypsinization. Curcumin (25µM, 24h) was used as positive control. The cell pellets were fixed in 70% ice-cold ethanol, treated

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with 100 mg/mL RNase A and 50 mg/mL propidium iodide, and subjected to flow cytometry (BD Biosciences)[27].

2.2.9 Toxicological evaluation

Acute toxicity study

Animals

Six to eight-week-old female *Swiss albino* mice (18-22g) were obtained from the RGCB Animal Research Facility and the experiment was done under protocols approved by RGCB Institutional animal ethical committee (IAEC NO:189(a)/RUBY/2012).

The experimental design

Swiss albino mice were randomly divided into 3 groups of 6 animals each and were allowed to acclimatize for a week. Group I was taken as the control, which received only cremophor vehicle, while Group II and III received a single dose of kaempferide(**4**) (50 and 200 mg/Kg body weight respectively) dissolved in cremophor as intraperitonial injection. The mice were observed continuously for 1h, for any gross behavioral changes and death, and then intermittently for the next 6h and 24h. The behavioral parameters monitored were convulsion, hyperactivity, sedation, grooming, food and water intake, etc. The animals were observed frequently for the next 7 days from the day of treatment after which, the animals were euthanized in a CO_2 chamber. The blood was collected for analyzing biochemical parameters of liver function, the abnormal values of which are indicative of toxicity. The liver was fixed in 10% buffered formalin and the thin cryostat sections (LEICA CM 1850UV Cryostat)were stained with haematoxylin and eosin for histopathological evaluation. The weight of the animals as well as that of the individual vital organs were also recorded.[28]

Chronic toxicity:

Swiss albino mice were randomly divided into 2 groups of 6 animals each and were allowed to acclimatize for a week. Group I was taken as the control, which received only cremophor vehicle, while Group II received 75mg/Kg body weight of kaempferide(**4**) dissolved in cremophor as intraperitonial injection on alternate days, thrice in a week, for 3 months . The animals were observed frequently during this period after which, the animals were euthanized in a CO_2 chamber. The blood was collected for analyzing biochemical parameters of liver function, the abnormal values of which are indicative of hepatotoxicity. The liver was fixed in 10% buffered formalin and the thin cryostat sections were stained with haematoxylin and eosin for histopathological evaluation. The weight of the animals as well as that of the individual vital organs were also recorded[28].

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2.2.9. Statistical analysis

For the flow cytometry, data analysis was performed using the BD FACS Diva software version 5.0.2. The statistical analysis was performed using Graph Pad Prism software (Graph Pad software Inc., San Diego, CA, USA). Statistical significance was defined as $p< 0.05$. The error bars represent ± SD , taken from the three independent experiments.

3. Results

3.1. DCM extract of *C. odorata* induces maximum cytotoxicity in cervical cancer cells

The cytotoxic effect of organic extracts of *C. odorata* was evaluated in cancer cells of different origin. The cells were treated with different concentrations of the extracts for 72 h, and cell viability was determined by MTT assay. Among the four extracts screened, DCM extract is active against four cancer cells of different origin except leukemia (Fig. 1A-D). We selected HeLa for further studies since it turned out to be the most sensitive cell line in which the DCM extract induced a dose-dependent cytotoxicity (IC_{50} 37.5 µg/mL) (data not shown) and showed an array of spots in TLC in the solvent system, chloroform/methanol (96/4) (data not shown).

3.2. Kaempferide (**4**) was found to be most cytotoxic among the four compounds isolated from DCM extract of *C. odorata*

The active DCM extract was fractionated as discussed in section 2.2.2. F-17 was found to be cytotoxic against cervical cancer cell line (Fig. 1E-F). Purification of F-17 resulted in four compounds which were characterized (Fig. 2, Table 1). Kaempferide (**4**) (IC_{50} 16 µM) is the most cytotoxic followed by acacetin (**1**) (IC_{50} 178 µM), dihydrokaempferide (**2**) (IC_{50} 277 µM), and isosakuranetin (**3**) (IC_{50} 312 µM) (Fig. 3A). Curcumin was used as positive control for this study. Kaempferide (**4**) brought down the IC_{50} to 16 µM in HeLa cells, while being non-toxic to normal human fibroblasts up to 100 µM (Fig. 3B), while curcumin induced the same effect at 16.67 µM demonstrating that both the compounds have almost the same cytotoxic effect in HeLa cells.

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3.3. Kaempferide (**4**) is non-toxic to normal fibroblasts while inducing morphological changes, membrane flip-flop and nuclear membrane damage, characteristic of apoptosis in HeLa cells.

We compared the morphological effect of kaempferide (**4**) in HeLa cells and normal fibroblasts. While phenotypic changes characteristic of apoptosis were induced in HeLa cells from 10 μM onwards, no significant change was observed in normal fibroblast upto100 μM (Fig. 3C). We also evaluated its cytotoxic effect in other cervical cancer cell lines SiHa (25.86 μM) and Caski (18.75 μM) (Fig. 3B). The percentages of cells in the early and late stage of apoptosis were also evaluated after treating HeLa cells with kaempferide (**4**), which induced a dose dependant increase in the extent of apoptosis (Fig. 3D). The early stage of apoptosis was confirmed by Annexin V staining using fluorescence microscopy. Kaempferide (**4**) treatment produced a gradual increase in the percentage of Annexin V-positive cells in a concentration-dependent manner (Fig. 4A). The extent of apoptosis induced by kaempferide (**4**) was further estimated by FACS analysis of the Annexin V–FITC/PI double stained cells. The apoptotic cell population was increased from 4.8% to 15.1% and 21 % respectively, when treated for 16 h with 10 μM and 15 μM of kaempferide (**4**) (Fig. 4B).

3.4. Kaempferide (**4**) does not induce cell cycle arrest in HeLa cells

To explore whether the growth-inhibitory effect of *C. odorata* on HeLa cells is mediated through cell cycle arrest, we analyzed the distribution of cells in different phases of the cell cycle by measuring intracellular DNA content in each phase. It was very interesting to note that kaempferide (**4**) failed to induce cell cycle arrest even at concentrations above IC_{50} and at different time intervals(24h &48h), while the positive control (curcumin 25 μM) readily induced G2M arrest at 24h. However, there was an increase in the number of cells in sub G0 phase, when treated with kaempferide (**4**) again indicative of enhancement in apoptosis (Fig. 4C).

3.5. Kaempferide (**4**) induces caspase-dependent apoptosis in HeLa cells leading to PARP cleavage

Our next attempt was to investigate the mechanism behind the cytotoxic effect of kaempferide (**4**). First, we checked the role of caspases, the key regulators of apoptotic program[29]. Two distinct pathways of apoptosis have been identified; the mitochondria-initiated apoptosis that occurs through caspase 9 which may further lead to caspase 8 cleavage and the death receptor-mediated pathway in which caspase 8 cleavage occurs

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independent of caspase 9[30,31]. We observed that kaempferide (**4**) induces a dose dependent cleavage of the initiator caspases, caspase 9 and 8 (Fig. 5A-B) which clearly indicates the role of mitochondria in kaempferide-induced apoptotic program in HeLa cells. It also induced a significant and dose dependent increase in the intensity of the cleaved bands of the effector caspases, caspase 7 and 3 (Fig. 5C-D). We then checked the effect of kaempferide (**4**) on the DNA repair enzyme PARP (Poly ADP-Ribose polymerase (116 kDa), the down-stream target of caspases 3 and 7, which cleaved it to fragments of 85 kDa and 25 kDa[32,33]. As observed in Fig. 5E, while 116 kDa PARP remained intact in the untreated cells, 15 μM kaempferide (**4**) cleaved the intact PARP to fragments, which clearly indicates caspase-mediated apoptosis. Hence our results clearly indicate that kaempferide (**4**) isolated from *C. odorata* induces cytotoxicity in HeLa cells through caspase-mediated apoptosis and is independent of cell cycle.

3.6. Kaempferide(4) is non-toxic as assessed by acute and chronic toxicity studies in *Swiss albino* mice, *in vivo*

To rule out the chance of any adverse effect of kaempferide(**4**) *in vivo*, we conducted an acute toxicity study for 7 days and a chronic toxicity study for 3 months in *Swiss albino* mice as described in the "Materials and Methods" and the blood and liver tissues were collected for biochemical and histopathological evaluation. The blood was centrifuged at 1500 rpm for 10 min at 4^0C to separate the serum, which was analyzed for the level of AST, ALT and ALP, elevated levels of which are indicative of hepatotoxicity. Histopathological analysis of the liver tissue was also conducted for toxicological evaluation, using H and E staining. No behavioral changes were noted in the mice at any of the concentrations studied till the end of the experiment. There was no significant change in the weight of the animals (data not shown) as well as that of the vital organs (data not shown) in both the studies indicating that kaempferide is safe, *in vivo*. Results of the serum analysis as well as mouse liver section studied do not reveal any significant change in any of the parameters studied, which was supported by the histopathological data (Fig. 6A&B), confirming that kaempferide is non-toxic and pharmacologically safe.

4. Discussion and conclusion:

Cervical cancer is one of the most common cancers among women in developing countries, including India. Flavonoids have been found to be promising agents toward cervical cancer. They display a wide variety of biological functions including induction of apoptosis, growth

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arrest, inhibition of DNA synthesis and modulation of signal transduction pathways[34]. Our data showed that kaempferide (**4**), a flavonoid isolated from the DCM extract of *C. odorata,* is highly cytotoxic to cervical cancer cells. *C. odorata* has been widely used as a traditional herbal medicine for several inflammatory disorders. Phytochemicals with anticancer and anti-inflammatory potential have become key resources of drugs for the treatment of various malignancies[35]. Several flavonoids, chalcones, flavones and essential oils of diverse biological activities have been isolated from different parts of *C. odorata*[36,12,9,37]. Only very few studies have been reported on the anti-cancer potential of compounds isolated from *C. odorata.* 2'-Hydroxy-4,4',5',6'-tetramethoxy chalcone, isolated from the ethanolic extract of *C. odorata* leaves inhibits growth and clonogenicity in breast cancer cells[15] and flavonoid glycosides isolated from its ethanolic extract induce cytotoxicity in leukaemic cells[16]. The ethylacetate and acetone extracts of the *C. odorata* leaves have been shown to induce autophagic mode of cell death in breast cancer cells[7]. Two cytotoxic flavones have also been reported from the flowers of this plant[9]. Odoratin, a PPARγ agonist and 3-hydroxy-1,2-dimethoxy-6-methylanthraquinone, a compound with weak cytostatic activity towards lung cancer cells have been reported from the DCM extract of the whole plant of *C. odorata*[11].

A study pertaining to induction of melanogenesis in mouse melanoma cells by kaempferide (**4**) has been reported recently [38]. However, to the best of our knowledge, there has been no report on exploiting the anticancer potential of kaempferide **(4)**. On the contrary, a recent study, which reports the isolation of kaempferide (**4**) from *Dillenia Suffruticosa* implies that the compound does not exhibit significant cytotoxicity in breast cancer cells[39]. The present study demonstrates that kaempferide (**4**) induces a dose dependent cytotoxicity in cervical cancer cells, while being nontoxic to normal fibroblasts. The protective effect of *C. odorata* against oxidative damage on cultured skin cells[3] may account for its nontoxic nature to normal cells. The morphological parameters and dose dependent increase in AO/EB staining clearly indicate that the cytotoxic effects of kaempferide (**4**) is mediated by induction of apoptosis[25,26]. The analysis of membrane flip-flop (by Annexin-V staining), the earliest event of apoptosis was carried out at 16h, while all other experiments assessing caspase cascade were done at 24h, since membrane flip fop usually happens before the cleavage of caspases, which further leads to PARP cleavage. Induction of caspase activation and PARP cleavage underscores that caspase cascade has a major role in regulating the cytotoxic activity of kaempferide (**4**). Cell cycle can occur following apoptosis or independent of apoptosis. So we conducted the cell cycle analysis at different concentrations and time points. It was interesting to note that kaempferide**(4)** does not induce cell cycle arrest at any of the parameters studied, though it induces accumulation of

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cells in sub G0 phase, illustrating the induction of apoptosis. A previous study has also reported *in vitro* growth arrest of human colon cancer cells in the G0/G1 phase by kaempferide triglycoside, isolated from *Dianthus caryophyllus*[40].

A major hurdle hampering the efficacy of chemotherapy is the dose limiting toxicity of the drugs, which leads to adverse side effects. Our 7 days acute toxicity study as well as 3 months chronic toxicity study for preliminary identification of toxicity to target organs and to obtain clues to the selection of starting doses for phase 1 human studies rule out any adverse hepatotoxicity owing to kaempferide as evidenced by normal levels of AST, ALT and ALP profiles in serum and normal histopathological staining of the liver tissue, suggesting that the drug is non-toxic and pharmacologically safe.

In conclusion, we demonstrate for the first time that kaempferide (4) isolated from the DCM extract of *C. odorata* induces apoptosis in cervical cancer cells, activating caspase cascade, while being nontoxic as assessed by *in vitro* and *in vivo* studies. Even though kaempferide induces the cleavage of PARP and caspases, which are classical markers of caspase dependent-apoptosis, we cannot confirm that, it is the only mechanism regulating the cytotoxic effect of Kaempferide. Further studies are in progress to elucidate the mechanism of action of this compound. Our finding may be of therapeutic benefit for cervical cancer chemotherapy, if supported by *in vivo* validation, which is currently being pursued in our laboratory. If kaempferide (**4**) isolated from *C.odorata*, a common weed found in almost all continents, can act as an anticancer drug against cervical cancer, which ranks as the most common cancer affecting females in developing countries including India and as the fourth in global scenario, it will be a real boon to the cervical cancer patients, who almost always have a poor socioeconomic status.

Supplementary material

Supplementary material contains ^{1}H and ^{13}C NMR data of flavonoids (**1-4**) (see S2-9).

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Abbreviations: FITC, Fluorescein isothiocyanate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide; DCM, Dichloromethane; EA, Ethyl

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Acetate; *C. odorata*, *Chromolaena odorata*; Hex, Hexane; Met, Methanol; PARP, Poly (ADP-ribose) polymerase; TLC, Thin layer chromatography.

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Figure 1: 17th fraction eluted by 60: 40 (DCM: CHLOROFORM) is responsible for the cytotoxic effect of DCM extract of *C. odorata*

(A-D). Effect of *C.odorata* organic extracts on cancer cells of various origin. A total of 2000 cells in triplicate were exposed to the indicated concentration of four extracts (25-250µg/ml) for 72h and subjected to MTT assay. Relative cell viability was determined as % absorbance over untreated control. Data represent three independent sets of experiments and results are shown as the mean±SD. (E) Cytotoxic effect of different column fractions eluted by column chromatography. The most active DCM extract was subjected to column chromatography using combinations of different solvent systems (Hexane/DCM/Chloroform/Methanol). The most active fraction, F-17 was identified by MTT assay (F). Dose dependent effect of F-17 on HeLa. A total of 2000 cells in triplicate were exposed to the indicated concentrations of F-17 (5-25 µg/ml) for 72h and subjected to MTT assay. The data represents three independent experiments. Relative cell viability was determined as % absorbance over untreated control. Data represent three independent sets of experiments and results are shown as the mean±SD.

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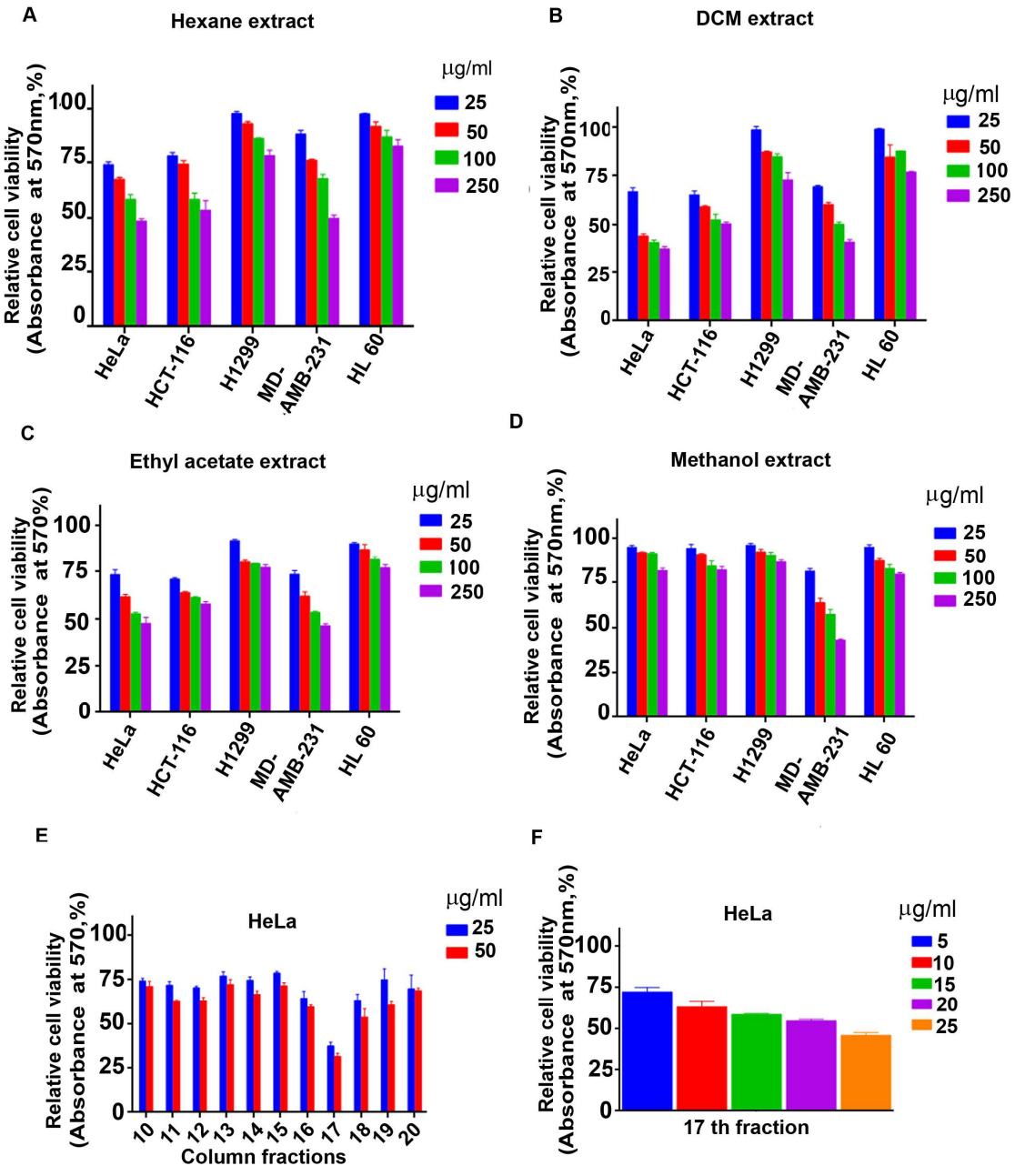

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Figure 2: Structures of Acacetin (1); Dihydrokaempferide (2); Isosakuranetin (3); Kaempferide (4)

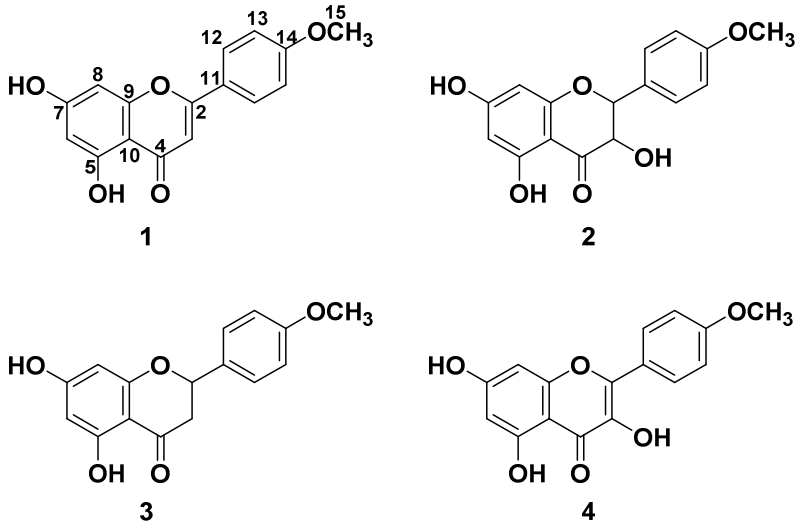

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Table 1. NMR Data for Acacetin (1); DihydroKaempferide (2); Isosakuranetin (3); Kaempferide (4)

	1		**2**		**3**		**4**	
position	δ ^{13}C[a]	δ ^{1}H mult [*J* (Hz)][a]	δ ^{13}C[b]	δ ^{1}H mult [*J* (Hz)][b]	δ ^{13}C[b]	δ ^{1}H mult [*J* (Hz)][b]	δ ^{13}C[a]	δ ^{1}H mult [*J* (Hz)][a]
2	163.7		84.8	5.03 d (12)	78.8	5.34 d (13)	146.7	
3	103.9	6.79 s	73.6	4.54 d (12)	42.6	2.69, 3.08 dd, dd (3, 17)	136.5	
4	182.2		198.4		196.2		176.4	
5	161.9		165.3		164.0		161.1	
6	99.4	6.15 d (2)	97.3	5.93 d (2)	95.7	5.88 s	98.7	6.10 s
7	164.8		168.7		166.9		164.4	
8	94.5	6.45 d (2)	96.3	5.89 d (2)	94.8	5.89 s	94.0	6.40 s
9	157.8		164.5		163.3		156.7	
10	104.1		101.8		101.9		103.5	
11	123.3		130.5		130.8		123.7	
12	128.7	7.98 d (9)	130.3	7.45 d (9)	127.5	7.39 d (9)	129.8	8.07 d (9)
13	115.0	7.05 d (9)	114.7	6.97 d (9)	113.6	6.94 d (9)	114.5	7.05 d (9)
14	162.7		161.6		160.0		160.9	
15	56.0	3.82 s	55.7	3.82 s	54.3	3.79 s	55.8	3.70 s

[a] Measured in DMSO-d_6. [b] Measured in methanol-*d*

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Figure 3: Kaempferide (4) was found to be most cytotoxic among the four compounds, as assessed by cell viability assay and analysis of morphological features and nuclear membrane damage, while being non-toxic to normal fibroblasts

(A) Effect of flavanoids of F-17, isolated from DCM extract of *C. odorata* on HeLa. A total of 2000 cells in triplicate were exposed to the indicated concentration of test sample (2.5-200 μM) for 72 h and subjected to MTT assay. Relative cell viability was determined as % absorbance over untreated control. Data represent three independent sets of experiments and results are shown as the mean±SD. Curcumin as the positive control(5-30 μM) (B) Effect of kaempferide (**4**) on normal fibroblast cell, FS as well as on cervical cancer cells, HeLa, SiHa and caski. A total of 2000 cells in triplicate were exposed to the indicated concentrations of kaempferide for 72 h and subjected to MTT assay. Relative cell viability was determined as % absorbance over untreated control. Data represent three independent sets of experiments and results are shown as the mean±SD (C) Morphological changes induced by kaempferide (**4**) in fibroblast, FS and HeLa by phase contrast microscopy. (D) The early stage and late stage of apoptosis was evaluated by acridine orange/ethidium bromide staining. AO/EB positive cells in various fields were counted, and the average was taken and plotted.

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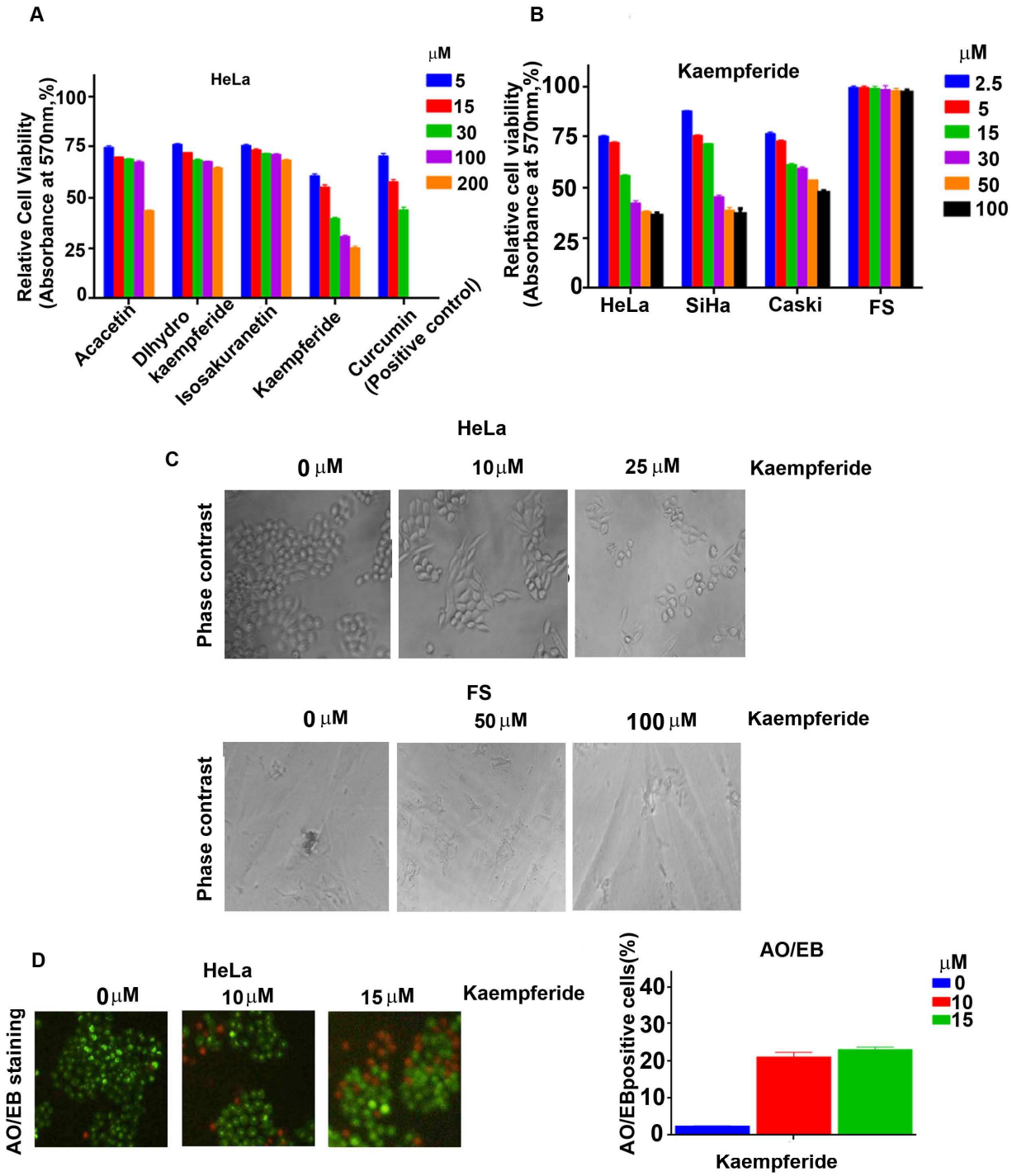

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Figure 4: Kaempferide (4) is inducing membrane flip-flop and accumulation of cells at subG0 phase, characteristic of apoptosis, while being independent of cell cycle.

(A) HeLa cells were treated as indicated with different concentrations of kaempferide for 16 h and stained for Annexin V positivity and membrane flip-flop was captured by fluorescence microscopy. Annexin V positive cells in various fields were counted, and the average was taken and plotted. (B) HeLa cells were treated as indicated with different concentrations of kaempferide for 16 h and stained with Annexin V/PI and estimated apoptosis by FACS.

(C) Cells were harvested after 24h &48 h of kaempferide, fixed in alcohol, stained with propidium iodide, and assayed for DNA content by flow cytometry. Curcumin as the positive control(25 μM) .Representative histograms indicate the percentages of cells in G1, S, G2/M and sub G0 phases of the cell cycle. The percentage of cells with sub-G0 DNA content was taken as a measure of the apoptotic cell population. The data provided is representative of three independent experiments.

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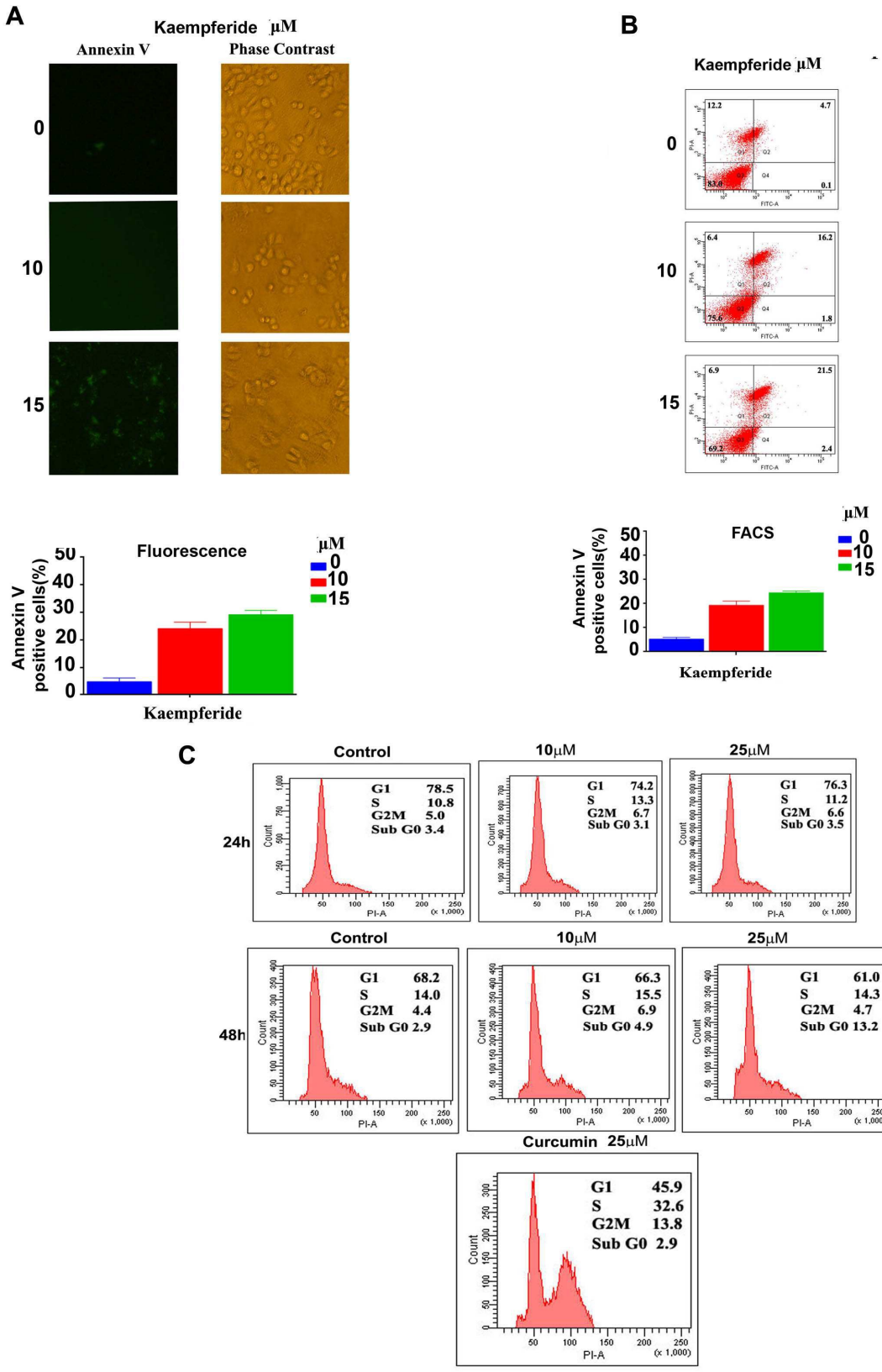

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Figure 5: Kaempferide (4) induces caspase-dependent apoptosis in HeLa cells leading to PARP cleavage: (A-E) Western blots showing caspase activation in HeLa cells. Whole-cell extracts were prepared after treating HeLa cells with indicated concentrations of kaempferide (**4**) for 24 h and subjected to western blotting using antibodies against the caspases 8, 9, 7, 3 and PARP.

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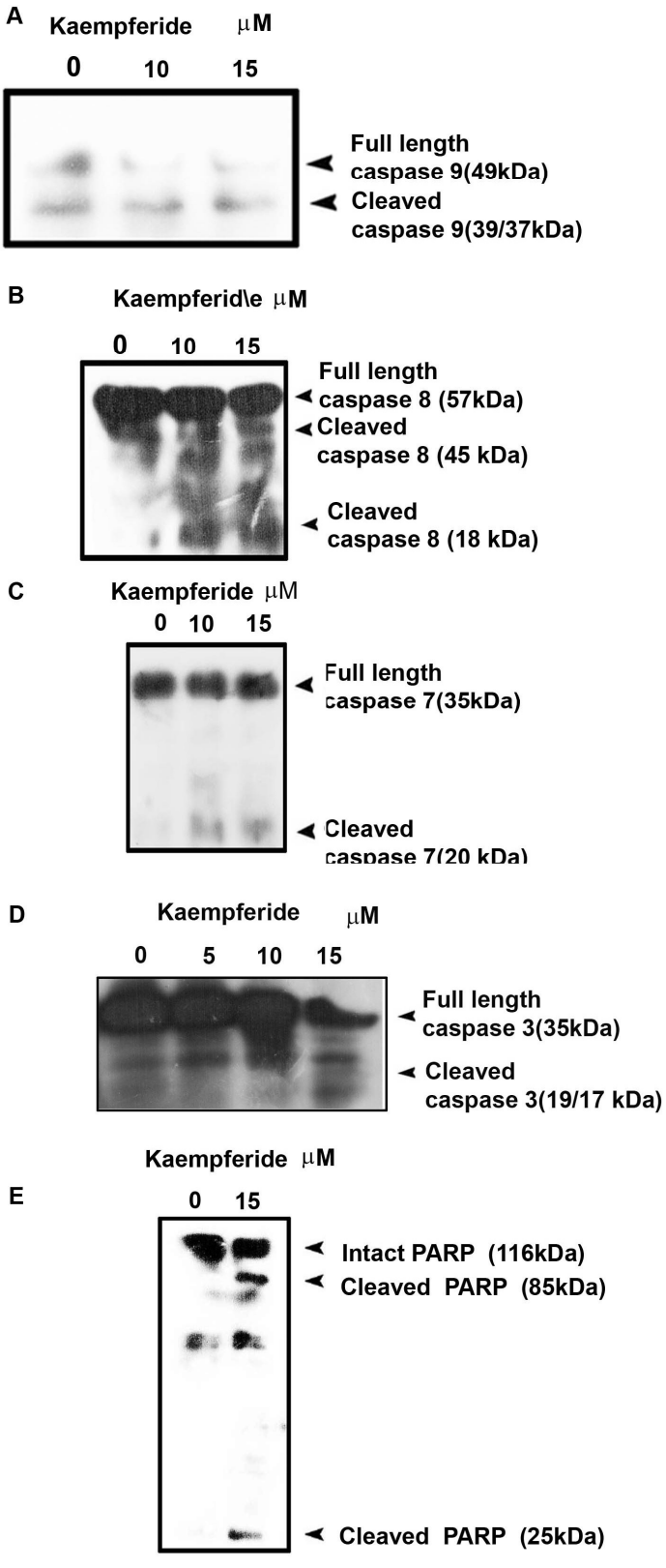

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Figure 6: Kaempferide does not induce pharmacological toxicity as assessed by acute and chronic toxicity studies: **A(a) &B(a)** The activity of toxicological markers such as serum Aspartate Aminotransferase (AST) , Alanine Aminotransferase (ALT) & alkaline phosphatase (ALP)in Kaempferide treated mice for a period of 7 days and 3 months is graphically represented. A(b)&B(b) H&E stained liver tissues of mice treated with or without Kaempferide for a period of 7 days and 3 months.

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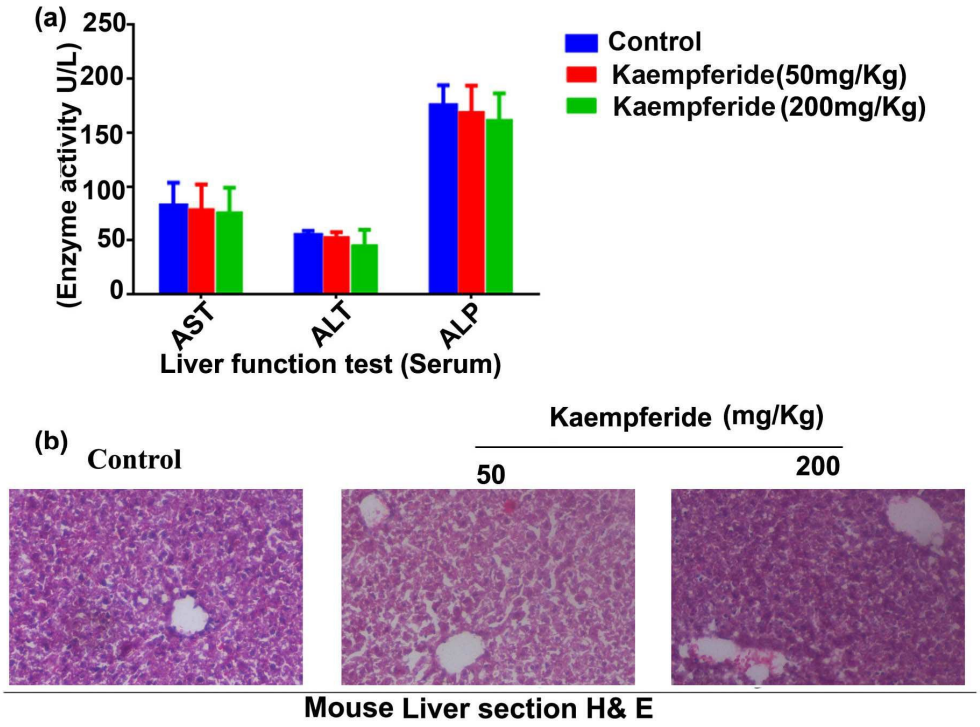
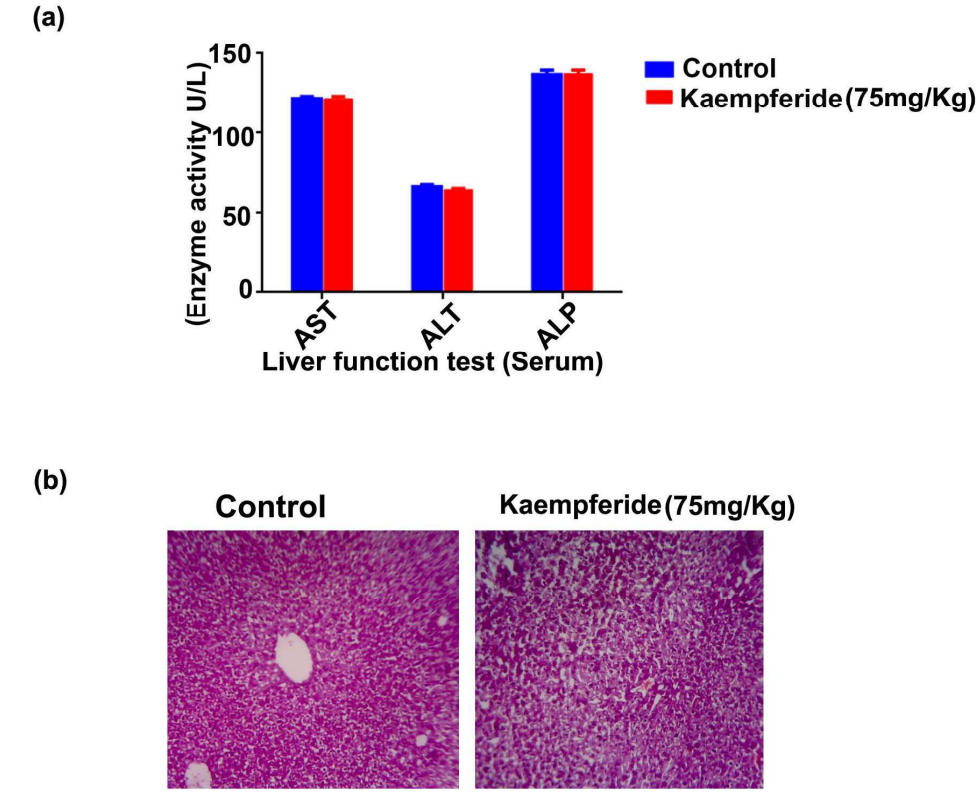
A Acute toxicity study



B Chronic toxicity study



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