

## ORIGINAL ARTICLE

# Anti-proliferative and proteasome inhibitory activity of *Murraya koenigii* leaf extract in human cancer cell lines

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**ABSTRACT**

Proteasome inhibition has been demonstrated to be a promising strategy for cancer therapy. The chymotrypsin-like activity of the 20S catalytic unit of the 26S proteasome is known to be critical for cancer cell survival. Inhibition of the proteasomal activity leads to tumor cell death. *Murraya koenigii* Spreng a medically important herb of Asian origin, is rich in phenolic content, and dietary polyphenols are known to act as proteasome inhibitors. This study was aimed at testing the cytotoxic and proteasomal inhibitory potential of *M. koenigii* leaf extract in four different human cell lines namely; colon, prostate, liver and cervical cancer. The cytotoxicity of *M. koenigii* leaf extract was tested by the MTT assay, inhibition of growth by colony formation assay and inhibition of the chymotrypsin-like (Ch-L) activity of the 26S proteasome using a specific fluorogenic substrate by fluorometry. There was a dose-dependent decrease in cell viability/proliferation with *M. koenigii* leaf extract treatment in all the cell lines tested. In line with the cell viability data there was a dose-dependent decrease in growth as observed by decreased colony formation in the cell lines tested. *M. koenigii* extract decreased the Ch-L activity of the endogenous proteasome in both intact cells and cell extracts in all the four cancer cell lines. Our results suggest that the proteasome is a target for *M. koenigii* leaf extract in various cancer cell lines and that inhibition of the proteasome may be one of the mechanisms responsible for its anticancer potential.

**Keywords:** *Murraya koenigii*, Rutaceae, cancer cell, 26S proteasome, chymotrypsin-like

See commentary of this article, pp 10-11 in this issue

**Introduction**

Cancer is a heterogeneous disease characterized by uncontrolled growth of cells leading to impairment of normal physiological functions. Annually 12.7 million people are diagnosed with cancer and approximately 7.6 million die from the disease.<sup>1</sup> Tumor cells have multiple alterations in their signaling pathways which results in increased growth and proliferation.<sup>2</sup> In recent years, tumor cells have been demonstrated to possess significantly higher proteasome activity than normal cells which is needed for their growth and survival.<sup>3</sup> In tumor cells the proteasome functions to promote cell proliferation and protects against apoptosis.<sup>4</sup> Inhibition of the tumor cell proteasome, specifically the chymotrypsin-like (Ch-L) activity of the 20S proteasome is known to lead to apoptosis and tumor cell death.<sup>5</sup> The eukaryotic proteasome is a highly conserved, large multi-catalytic, multi-subunit complex possessing three distinct protein cleaving activities, associated with three different  $\beta$  subunits respectively: chymotrypsin-like activity ( $\beta 5$  subunit), trypsin-like activity ( $\beta 2$  subunit), and caspase-like activity ( $\beta 1$  subunit).<sup>6</sup> It regulates several cellular processes such as cell-cycle

regulation, apoptosis, signal transduction and cell differentiation.<sup>4</sup> Proteasome inhibitors are now being used to treat solid and hematological malignancies either as mono-therapy or in combination therapy to overcome drug resistance.<sup>7</sup> Natural proteasome inhibitors are now being studied due to toxicity and stability issues with synthetic inhibitors.<sup>8</sup> Anticancer effects of natural products are supported by results.<sup>9,10</sup> from epidemiological, cell culture and animal studies *Murraya koenigii* (commonly called as curry leaf plant), is a small aromatic tree belonging to the family Rutaceae. It is extensively used in Indian culinary practices for seasoning and flavoring dishes.<sup>11</sup> Traditionally, this plant is used as a medicine in eastern Asia for a variety of ailments.<sup>12</sup> The major chemical constituents of the plant are reported to be carbazole alkaloids, coumarins and flavonoids.<sup>13</sup> *M. koenigii* leaf extract (MLE) exhibits hypoglycemic and hypolipidemic effects in experimental animals.<sup>14,15</sup> Carbazole alkaloids and methanolic extracts of *M. koenigii* are reported to possess anti-oxidative<sup>16,17</sup>, anti-diarrheal and anticancer properties.<sup>18,19</sup> Recently we reported that the aqueous-methanolic extract of *Murraya koenigii* inhibits proteasomal activity in breast

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cancer cell lines and that this inhibition was associated with tumor cell apoptosis.<sup>20</sup> Further, the extract inhibited proteasomal activity only in cancer cells but not in normal cells.<sup>20</sup> In recent years there has been a rise in breast, prostate, liver and colon cancers in the developing world including India and this has been attributed to genetic and environmental factors, adaptation of western lifestyle and unhealthy eating habits.<sup>21</sup> Hence, in the present study we examined the effect of the MLE on cell viability and proteasomal activity in a panel of four human carcinoma cell lines namely; colon, prostate, liver and cervical carcinoma cells.

## Materials and Methods

### *Cell lines, Chemicals and Reagents*

Caco2 (human colon carcinoma cell line), HepG2 (human hepatocellular liver cell line), HeLa (human cervical carcinoma cell line) and LNCaP (human prostate carcinoma cell line) were obtained from the National Centre for Cell Sciences, Pune, India. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM Higluta-XL, 100 units/mL penicillin, 100 µg/mL streptomycin and 0.5 ng/mL amphotericin B, 1mM sodium pyruvate and 1 × non-essential amino acid mixture. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethylsulfoxide (DMSO), dithiothreitol (DTT) and crystal-violet, were purchased from Sigma-Aldrich (St Louis, MO,USA). The fluorogenic proteasomal peptide substrate: Suc-LLVY-AMC, and MG-132 (carbobenzoxy-Leu-Leu-leucinal; a specific inhibitor of the 26S proteasome was procured from ENZO Life sciences, USA. All other reagents were procured from Qualigens fine chemicals (Mumbai, India). DMEM, antibiotic antimycotic mix, sodium pyruvate and non-essential amino acid mix were purchased from Himedia (Mumbai, India); fetal bovine serum (FBS) was purchased from (GIBCO, Invitrogen USA).

### *Plant material*

*Murraya koenigii* leaves were collected from the local area from a single tree. Identity of the leaves was confirmed by Dr. B. Pratibha Devi, Professor and Head, Department of Botany, Osmania University, Hyderabad, India. A voucher specimen (voucher no: 068) was deposited in a herbarium at the Department of Botany, Osmania University, Hyderabad, India.

### *Extraction of leaves*

*M. koenigii* leaves were washed and air dried in shade for 3 weeks. After drying, the leaves were ground to a fine powder using an electric mixer grinder. The leaf powder was extracted with 80% methanol in water by keeping on a vortex mixer for 3-4 days. This was followed by centrifugation of the extract at 5000 rpm for 30 min. The supernatant was filtered using a 0.4 µM filter (Millipore). The resultant aqueous-methanolic extract was stored in aliquots at -20°C and was used for all our studies. The leaf extract designated as 'MLE', was used for all the experiments at different doses based on their total phenolic content [equivalent to µg of GAE/mL] measured spectrophotometrically by the Folin-Ciocalteu method.

### *Cell viability/proliferation assay*

The cytotoxic effects of MLE in cancer cell lines were determined by a rapid colorimetric assay, using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells into an insoluble colored formazan product, which can be measured spectrophotometrically at 595 nM after dissolution in DMSO. Briefly, cells were plated in 96-well plates at a density of 50, 000 cells/well. Different concentrations of MLE varying from 1.5–37.5 µg GAE/mL were added and cells incubated for 24h at 37°C. MTT was added to a final concentration of 100µg/well and incubated for further 3h at 37°C. The formazan crystals formed were solubilized in DMSO and incubated for 1h at room temperature and the absorbance was measured at 595 nm in an ELISA plate reader (Biotek, New York, USA). All samples were assayed in triplicate in three independent experiments, and the mean value was calculated. The results are expressed as the mean ± SEM and are expressed as percentage of control which was considered to be 100%.

### *Colony formation assay*

Cells were plated in triplicate at a density of 10<sup>4</sup> cells/well in 12-well plates. The cells were treated with concentrations of MLE ranging from 1-75 µg GAE/mL and incubated at 37°C and 5% CO<sub>2</sub> for one week. After a week, the cells were fixed with 4% paraformaldehyde for 15 min followed by staining with 0.005% crystal violet. The cells were photographed with a digital Nikon D90 camera. Three independent experiments were done with each cell line.

### *Inhibition of proteasome activity in intact tumor cells*

In order to measure the inhibition of the proteasome activity in living tumor cells, cells were plated at a density of  $1 \times 10^4$  in a 24-well plate. Next day, cells were treated with concentrations of MLE ranging from 3-70  $\mu\text{g}$  GAE/mL. After 24h of treatment, the media was aspirated out and 500 $\mu\text{l}$  of  $1 \times$  PBS added, to which was added fluorogenic substrate (20  $\mu\text{M}$  final concentration) specific for the chymotrypsin-like (Ch-L) activity of the 26S proteasome. After 2h incubation at 37°C, 200  $\mu\text{L}$  was transferred into a black plate and the free 7-amino-4-methylcoumarin (AMC) liberated was measured fluorimetrically in a multi-mode reader [Spectra Max M5] at excitation (380 nm) and emission (460 nm). The results are shown as mean  $\pm$  SEM and are expressed as a percentage of the control, which was considered to be 100%. All samples were assayed in triplicate in three independent experiments.

### *Inhibition of proteasome activity in cell extracts*

Cells ( $1 \times 10^7$ ) were harvested, washed twice in  $1 \times$  PBS and re-suspended in 1 mL ATP/DTT lysis buffer [10 mM Tris-HCL (pH 7.8), 5 mM ATP, 0.5 mM DTT, 5 mM  $\text{MgCl}_2$ ]. Cells were incubated on ice for 10 min, followed by sonication for 15 seconds. The lysate was centrifuged at 2000 rpm for 10 min at 4°C. The supernatant enriched in proteasomes was mixed with glycerol (20% final concentration), aliquoted and stored at -80°C, and was stable for at least 1 month. The total protein content of the cell extract was estimated by the bicinchoninic acid (BCA) method using a kit (Bangalore Genei, Bangalore, India). The assay was carried out in a total of 200 $\mu\text{l}$  reaction volume containing cell extract (50  $\mu\text{g}$  protein), 50 mM EDTA, concentrations of the MLE ranging from 15-500 $\mu\text{g}$  GAE/mL were used for Caco2, HepG2 and LNCaP cell lines, whereas the concentration of MLE used for HeLa ranged between 50 $\mu\text{g}$ -2mg GAE/mL. MG-132 was used at concentrations of 2-400 nM for the different cell lines tested. 50  $\mu\text{M}$  of the fluorogenic substrate specific for Ch-L activity of the 26S proteasome was added and incubated for 2h at 37°C. The amount of free 7-amino-4-methylcoumarin (AMC) liberated was measured fluorimetrically. The results are expressed as mean  $\pm$  SEM as a percentage of the control, which was considered to be 100%. All samples were assayed in triplicate in three independent experiments.

### *Statistics*

All experiments were performed in triplicates and repeated at least three times and the data is

represented as mean  $\pm$  SEM. Mean values were compared across concentrations of MLE/MG-132 using non-parametric test of Kruskal-Wallis One-way ANOVA for each cell line using the SPSS statistical software. Differences between vehicle and MLE/MG-132 treatments for a particular cell line were considered significant at  $\alpha$  (probability) level of  $P \leq 0.05$ .

## **Results**

### *M. koenigii leaf extract decreases cell viability of cancer cells*

The Total Phenolic Content (TPC) of the aqueous-methanolic extract of *M. koenigii* leaves was found to be 5 $\mu\text{g}$  of gallic acid equivalents (GAE)/ $\mu\text{L}$  of the MLE. MTT assays were performed with different concentrations of MLE (GAE/ml) in four human tumor cell lines at 24h time point to assess the effect of the extract on cell viability. A time and dose-dependent decrease in cell viability was observed in all the cell lines tested. There was a significant ( $P < 0.05$ ) decrease in cell viability at all doses tested compared to vehicle treated cells in HeLa and Caco2 cell lines. On the other hand in HepG2 and LNCaP cells, decrease in viability was significant ( $P < 0.05$ ) from 15  $\mu\text{g}/\text{ml}$  and 12  $\mu\text{g}/\text{mL}$  respectively. The 50% Inhibitory concentration ( $\text{IC}_{50}$ ) was found to be 8.07  $\mu\text{g}/\text{mL}$  for Caco2 cell line, 4.8  $\mu\text{g}/\text{mL}$  for HeLa cell line, 17.55  $\mu\text{g}/\text{mL}$  for HepG2 cell line and 16.45  $\mu\text{g}/\text{mL}$  for LNCaP cell line respectively (Fig 1A-D).

### *M. koenigii leaf extract alters growth kinetics of cancer cells*

To test the effect of MLE on growth kinetics colony formation assay was used, wherein cells were seeded at a lower density and treated with varying concentrations of the MLE. Colonies were photographed after a week. Similar to MTT assay there was a dose-dependent decrease in the number of colonies in the different cell lines. No colonies were seen at MLE concentrations of 6  $\mu\text{g}/\text{mL}$  for Caco2, 18  $\mu\text{g}/\text{mL}$  for HeLa, 9  $\mu\text{g}/\text{mL}$  for HepG2 and 7.5  $\mu\text{g}/\text{mL}$  for LNCaP respectively (Fig 2A-D).

### *M. koenigii leaf extract inhibits cellular 26S proteasome activity in intact cells*

Whether the MLE inhibits activity of the 26S proteasome in living cancer cells, was assessed in the four cancer cell lines. There was a dose dependent and significant ( $P < 0.05$ ) decrease in the chymotrypsin-like activity of the 26S proteasome in intact LNCaP and HepG2 cells. On the other hand in Caco2 and HeLa cells the decrease in Ch-L activity was significantly ( $P < 0.05$ ) different from 6  $\mu\text{g}/\text{mL}$  compared to vehicle control. The  $\text{IC}_{50}$  values were

12.5  $\mu\text{g}/\text{mL}$  for Caco-2, 7.99 $\mu\text{g}/\text{mL}$  for HeLa cells 43.4 $\mu\text{g}/\text{mL}$  for HepG2 cells and 12.4  $\mu\text{g}/\text{mL}$  for LNCaP cells respectively (Fig 3A-D).

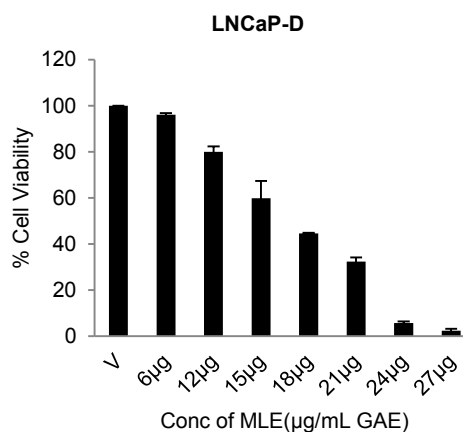
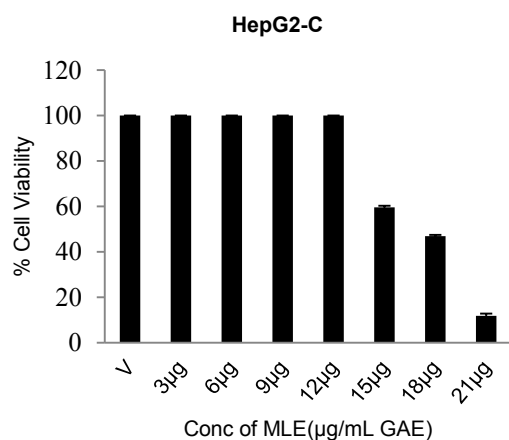
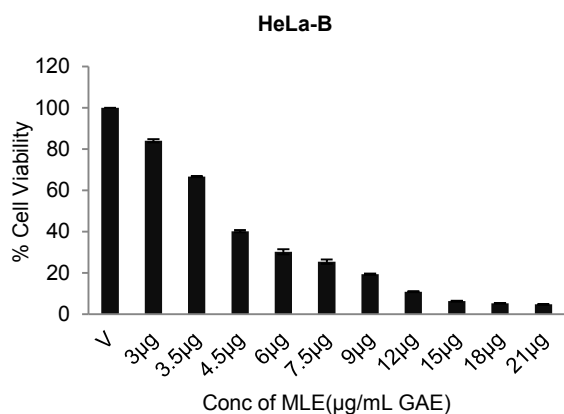
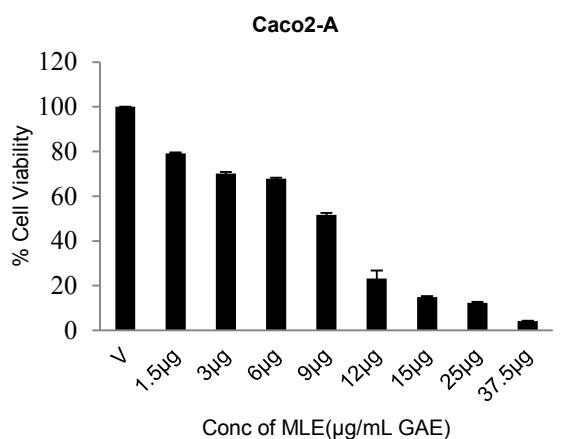


Figure 1. *Murraya* leaf extract decreases cell viability of human cancer cells: Panels A-D shows results of MTT assay after 24h treatment with MLE in Caco2, HeLa, HepG2 and LNCaP cells respectively. The data represents mean $\pm$ SEM of three independent experiments. 'V' in the figure stands for vehicle treated cells.

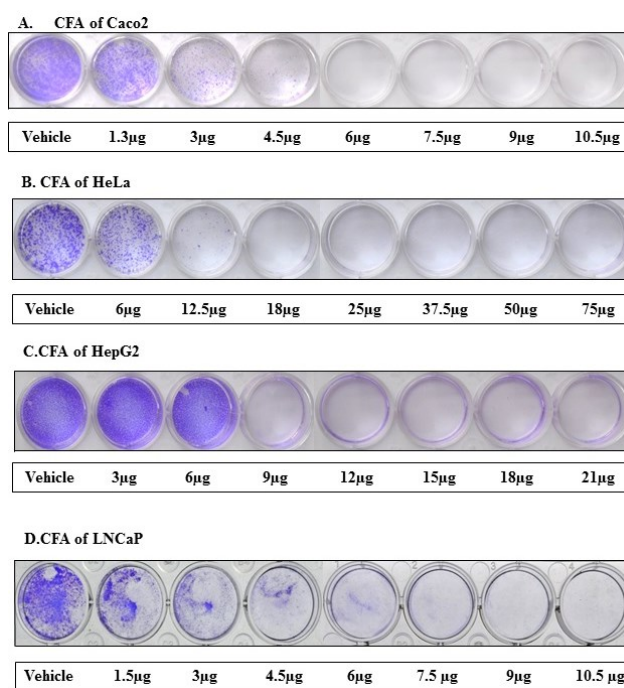


Figure 2. *Murraya* leaf extract alters growth kinetics of human cancer cells: Panel A-D depicts results of colony formation assay in Caco2, HeLa, HepG2 and LNCaP cells respectively. Cells were grown in 6-well plates and treated with various concentrations of the MLE. After a week cells were stained with crystal violet and photographed.

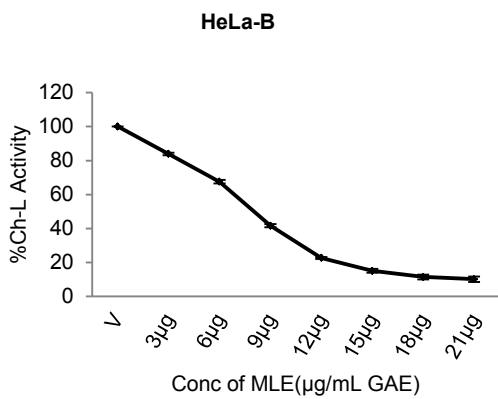
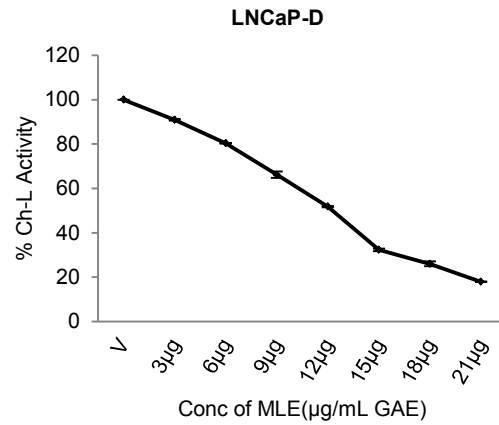
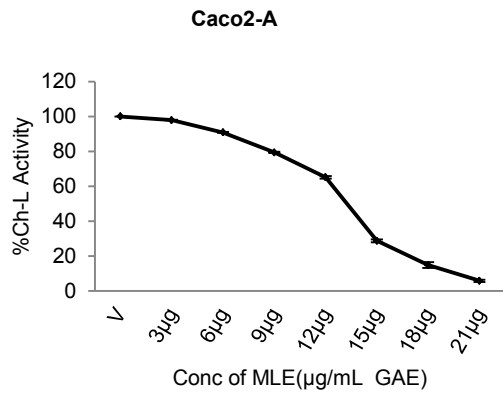
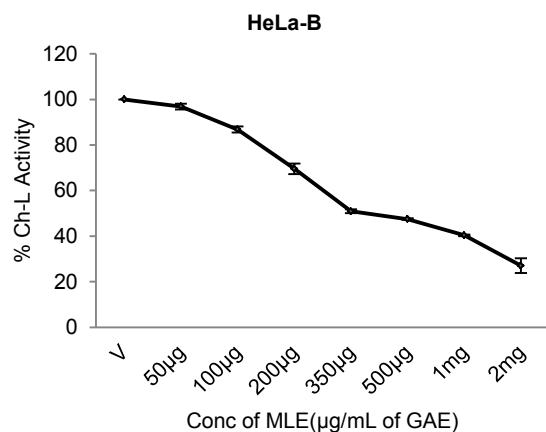
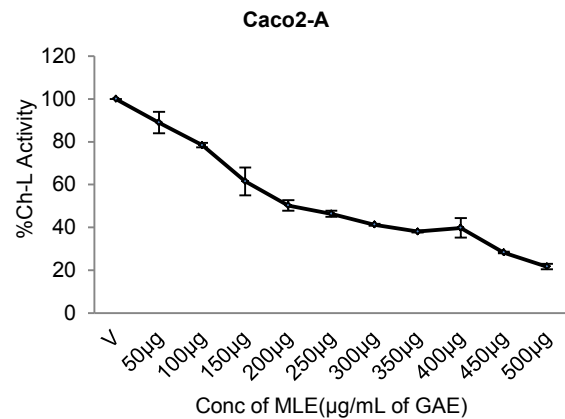
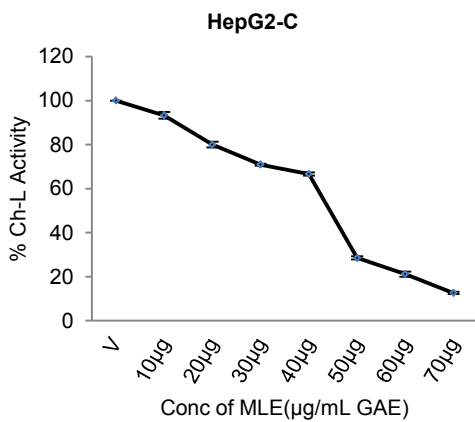


Figure3. Inhibition of the enzymatic activities of the 26S proteasome by the MLE in intact cancer cells: Intact cells were treated for 24h with/without the MLE followed by 2h incubations at 37°C with a fluorogenic substrate for Ch-L activity. The fluorescent intensity of the free AMC's was determined in a multimode reader with excitation at 380 nm and emission at 460 nm. Each activity was expressed as percentage of control (defined as 100%). Panels A-D shows results from Caco2, HeLa, HepG2 and LNCaP cells respectively. The data represents mean +/- SEM of three independent experiments. V in the figure stands for vehicle treated cells.



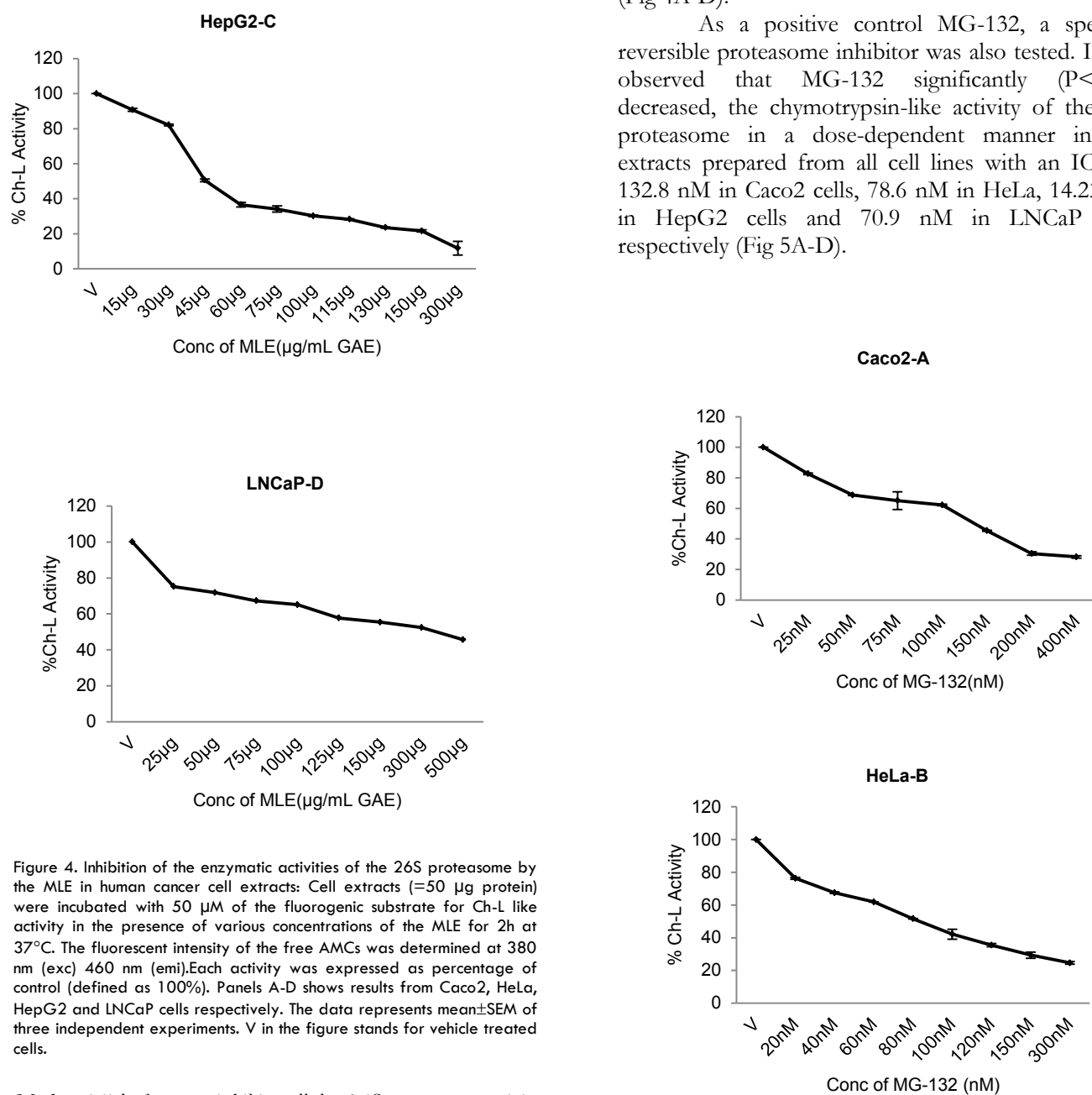


Figure 4. Inhibition of the enzymatic activities of the 26S proteasome by the MLE in human cancer cell extracts: Cell extracts (=50 µg protein) were incubated with 50 µM of the fluorogenic substrate for Ch-L like activity in the presence of various concentrations of the MLE for 2h at 37°C. The fluorescent intensity of the free AMC<sub>s</sub> was determined at 380 nm (exc) 460 nm (emi). Each activity was expressed as percentage of control (defined as 100%). Panels A-D shows results from Caco2, HeLa, HepG2 and LNCaP cells respectively. The data represents mean±SEM of three independent experiments. V in the figure stands for vehicle treated cells.

#### *M. koenigii* leaf extract inhibits cellular 26S proteasome activity in cell extracts

In order to confirm that the MLE does inhibit the 26S proteasome, cell extracts enriched in 26S proteasomes were prepared from all the tumor cell lines. MLE inhibited the Ch-L like activity of 26S proteasome in a dose-dependent manner. There was a significant decrease ( $P < 0.05$ ) in Ch-L like activity in HeLa and Caco2 cells from 100 µg/mL, whereas in HepG2 and LNCaP it was 15 µg/mL and 50 µg/mL respectively. The  $IC_{50}$  values were 216 µg/mL in Caco2, 349.7 µg/mL in HeLa, 51.9 µg/mL in HepG2

cells and 344.7 µg/mL in LNCaP cells respectively (Fig 4A-D).

As a positive control MG-132, a specific, reversible proteasome inhibitor was also tested. It was observed that MG-132 significantly ( $P < 0.05$ ) decreased, the chymotrypsin-like activity of the 26S proteasome in a dose-dependent manner in cell extracts prepared from all cell lines with an  $IC_{50}$  of 132.8 nM in Caco2 cells, 78.6 nM in HeLa, 14.23 nM in HepG2 cells and 70.9 nM in LNCaP cells respectively (Fig 5A-D).

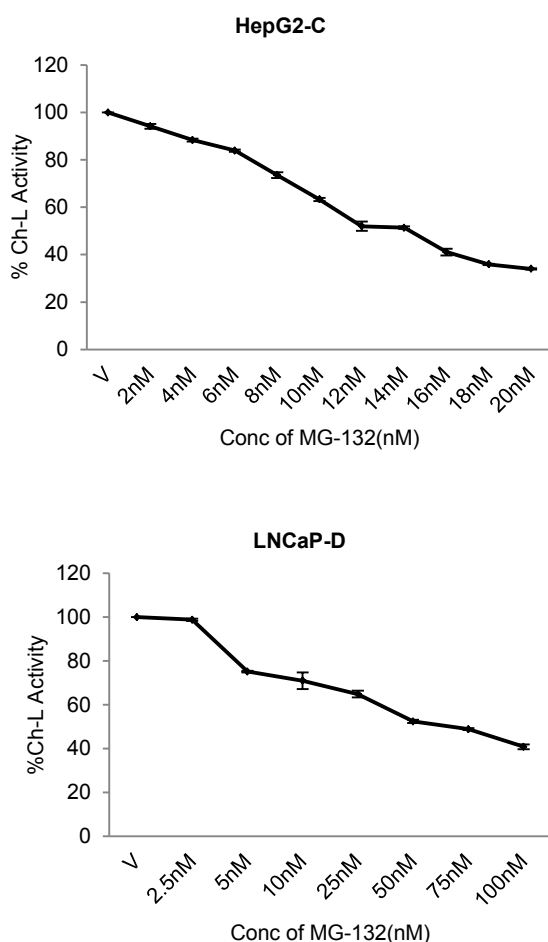


Figure 5. Inhibition of the enzymatic activities of the 26S proteasome by the proteasome specific inhibitor, MG-132 in human cancer cell extracts: Cell extracts (= 50 µg protein) were incubated with 50 µM of the fluorogenic substrate specific for Ch-L like activity in the presence of various concentrations of MG-132 for 2h at 37°C. Each activity was expressed as percentage of control (defined as 100%). Panels A-D shows results from Caco2, HeLa, HepG2 and LNCaP cells respectively. The data represents mean±SEM of three independent experiments. V in the figure stands for vehicle treated cells.

## Discussion

Cancer is a disease where the treatment can be as debilitating as the disease itself. Drug resistance limits the effectiveness of existing treatment options and is a major challenge faced in current cancer research. Use of complementary and alternate forms of medicine for the treatment of cancer is now being explored of which herbal medicine is preferred due to minimal or no side effects.<sup>22</sup> Studies have shown that a diet high in fruits and vegetables is associated with a reduced risk of cancer.<sup>23</sup> A recent approach in cancer therapy is the inhibition of the proteolytic machine – the 26S proteasome.<sup>5</sup> Unlike normal cells, cancer cells are known to have very high proteasome activity, which is needed for their survival and uninhibited

proliferation<sup>3</sup>, and inhibition of the proteasome leads to apoptosis and cell death. Importantly, proteasome inhibitors have been demonstrated to induce cell death only in tumor cells but not in normal cells.<sup>5,7</sup> Bortezomib/Velcade/PS-341 is the first-in-line proteasome inhibitor to be approved by the FDA in 2003 for the treatment of multiple myeloma.<sup>24</sup> Subsequently, bortezomib was tested in a variety of hematological and solid tumors including non-Hodgkin's lymphoma, prostate, breast and non-small cell lung cancer. Nevertheless, synthetic proteasome inhibitors are associated with some toxicity. Dietary polyphenols such as apigenin, quercetin, epigallocatechin gallate (EGCG) have been reported to act as proteasome inhibitors and anticancer agents.<sup>25</sup> Therefore, proteasome inhibitors from natural food sources with minimal or no toxicity can be potential anticancer agents. *M. koenigii* leaves are a rich source of polyphenols/flavonoids.<sup>26</sup> Studies on the anticancer potential of *M. koenigii* leaves are scarce. Mahanine, a carbazole alkaloid isolated from *M. koenigii* leaves is reported to inhibit cell growth and induce apoptosis in prostate and leukemia cancer cells.<sup>19</sup> Recently we reported the cytotoxic and proteasome inhibitory effect of *M. koenigii* leaf extract in breast cancer cells.<sup>20</sup> Interestingly, we observed that *M. koenigii* leaf extract could inhibit the proteasome activity only in cancer cells but not in normal cells.<sup>20</sup> Therefore, we tested the efficacy of *M. koenigii* leaf extract in inhibiting the proteasomal activity of human colon, cervical, liver and prostate carcinoma cell lines. We observed that the MLE dose-dependently decreased the viability in all the cell lines tested, although a differential response was observed in the different cell lines. This was further corroborated by the significant reduction in the number of colonies in MLE treated cells compared to vehicle treated cells. Our cell viability and colony formation results show that MLE altered the growth kinetics of all the tumor cell lines tested. We next tested the potential of the MLE in inhibiting the chymotrypsin-like proteolytic activity of the proteasome in the different tumor cell lines. MLE inhibited the Ch-L activity of the endogenous 26S proteasome in both living cells and cell extracts. The major constituents in *M. koenigii* leaves are known to be alkaloids, flavonoids and coumarins.<sup>13</sup> A few isolated alkaloids have been demonstrated to arrest growth of cancer cells by mechanism(s) other than proteasome inhibition.<sup>16</sup> On the other hand, dietary flavonoids are reported as proteasome inhibitors and anticancer agents.<sup>7</sup> Therefore, it is probable that the proteasome inhibitory activity of MLE is by virtue of its high

flavonoid content. Our results suggest that MLE could induce cell death and inhibit the cellular proteasome in the different cancer cell lines. In summary, our data indicates that *M. koenigii* leaves can inhibit cell proliferation and proteasome activity in four different human cancer cell lines. Proteasome inhibition is probably one mechanism that causes cell death upon treatment with MLE. Therefore, isolation and characterization of active component(s) from *M. koenigii* leaves that possess proteasome inhibitory potential could lead to the development of novel anticancer drugs.

## Conflict of Interest

None

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