

RESEARCH ARTICLE

Aqueous Extract of Anticancer Drug CRUEL Herbomineral Formulation Capsules Exerts Anti-proliferative Effects in Renal Cell Carcinoma Cell Lines

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Abstract

Purpose: Anti-cancer activity evaluation of aqueous extract of CRUEL (herbomineral formulation) capsules on renal cell carcinoma cell lines, and exploration of mechanisms of cell death. **Materials and Methods:** To detect the cytotoxic dose concentration in renal cell carcinoma (RCC) cells, MTT assays were performed and morphological changes after treatment were observed by inverted microscopy. Drug effects against RCC cell lines were assessed with reference to cell cycle distribution (flow cytometry), anti-metastatic potential (wound healing assay) and autophagy (RT-PCR). **Results:** CRUEL showed anti-proliferative effects against RCC tumor cell lines with an IC₅₀ value of $\approx 4\text{mg/mL}$ in vitro., while inducing cell cycle arrest at S-phase of cell cycle and inhibiting wound healing. LC3 was found to be up-regulated after drug treatment in RT-PCR resulting in an autophagy mode of cell death. **Conclusions:** This study provides the experimental validation for antitumor activity of CRUEL.

Keywords: Cruel, uok 146 - achn - renal cell carcinoma - cell cycle - autophagy

Asian Pac J Cancer Prev, 16 (18), 8419-8423

Introduction

Cancer is one of the most deadly diseases for the society and one of the most challenging diseases for the scientists all over the world to tackle with. Drugs and therapies available until now are still not effective enough for most of the cancer types and have side effects. People are, therefore looking towards traditional Ayurveda system of medicine to find out relatively safe and cost effective solution for cancer treatment. Herbometallic and herbomineral therapeutics are being considered as promising alternative medicines for treatment of diseases like cancer. Rasa shastra is the branch of Ayurveda which deals with medicinal properties of herbometallic and herbomineral preparations and the drugs which are used are known as Rasa Aushadhi. These therapeutics are claimed for faster in action and more efficient as compared to only herbal drugs. Herbomineral drugs are prepared by anaerobic cooking which converts the toxic mega particles of metal into safe and efficacious nanoparticles and even smaller picoparticles explaining the usefulness of these drugs as effective medicines for cancer (Sheikh et al., 2012).

Cruel Capsule is an ayurvedic proprietary medicine marketed by Virgo UAP Pharma Ltd. and UNJHA ayurvedic pharmacy India. It is a combination of anti-neoplastic, tissue promoting and anti-inflammatory herbo-minerals. It is claimed to be useful in different type

of benign, malignant and degenerative conditions. Its constituents include extracts of various plants and several bhasmas with claimed anti-cancerous potential along with beneficial anti-oxidant activity. Ingredients are Sarveswar Parpati, Suvarna Bhasma, Rasakarpoor, Swet Mirch, Abhrak Bhasma, Ext Punarnava, Ext. Yastimadhu, Ext. Vasa, Hira Bhasma, Rasasindur, Tamra Bhasma, Lavang, Panna Bhasma, Ext. Saragava, Ext. Rohitak, Ext. Guduchi and Excipients. All the constituents of cruel capsule have reported benefits to enhance the immune response against cancerous cells, some are described here. *Piper nigrum* extract has antimutagenic, antitumor, immunomodulatory, antimetastatic and many more activity shown in different experimental models (Hamss et al., 2003; Sunila et al., 2004; Ahmad et al., 2012). *Boerhaavia diffusa* exhibits antiproliferative and antiestrogenic properties in MCF-7 cells, protective Effects against gamma radiation induced DNA damage in mice and possess hepatoprotective activity in acetaminophen-induced liver damage in rats (Manu et al., 2007; Sreeja et al., 2009; Olaleye et al., 2010). Ethanolic extract of *Boerhaavia diffusa* showed inhibition of S-phase of cell cycle and cell growth in Hela cell line (Sultana et al., 2014). *Glycyrrhiza glabra* inhibits angiogenesis, tumor growth, migration, alleviates tumorigenic effect of endocrine-disrupting chemicals and act as a chemopreventive agent (Fu et al., 2004; Nagaraj et al., 2012; Chu et al., 2014). *Adhatoda vasica* has antimutagenic activity which can be attributed to its

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restoring potential on antioxidant status in CdCl₂ induced renal oxidative stress and genotoxicity in mice (Jahangir et al., 2006). It has protective effect against D-galactosamine induced liver damage in rats as well as radiation-induced hematological alterations in mice (Kumar et al., 2005; Jayakumar et al., 2009). *Syzygium aromaticum* known as clove has chemopreventive potential due to its apoptogenic and anti-proliferative properties (Lee et al., 2001; Banerjee et al., 2006; Jaganathan et al., 2012). *Moringa oleifera* extracts had strong antiproliferative, apoptosis inducing and antioxidant potential and hence it has cancer chemoprevention property (Sreelatha et al., 2009; Verma et al., 2009; Sreelatha et al., 2011; Sharma et al., 2012). *Tecomella undulate* also has antioxidant potential (Kumar et al., 2012), bark extract induce apoptosis in K562 cells (Ravi et al., 2011). *Tinospora cordifolia* has anti-proliferative, differentiation-inducing and anti-migratory/anti-metastatic potential in glioma cells (Mishra et al., 2013). It has antineoplastic activity in HeLa cells and Ehrlich Ascites Carcinoma Bearing Mice (Jagetia et al., 1998; Jagetia et al., 2006). Scientific evidence for the therapeutic potential of Cruel in cancer is limited because formulation complexity does not facilitate investigations. In this study aqueous extract of Cruel was applied on renal cell carcinoma (RCC) cell lines UOK146 and ACHN to understand the anticancerous action of Cruel capsule *in vitro*.

Materials and Methods

Cell culture and drug

UOK146 renal cell carcinoma cell line has (X;1) (p11.2;q21) chromosomal translocation leading to the fusion of PRCC gene at 1q21.2 to the TFE3 gene at Xp11.2 leading to the formation of an oncogenic fusion protein PRCC-TFE3 (Sidhar et al., 1996). ACHN is a cancer cell line derived from a metastatic human renal adenocarcinoma and it bears a deletion of Sav (Tapon et al., 2002) which suggests the compromised Hippo pathway (Zhao et al., 2007). Cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and grown in 5% (v/v) CO₂ in a humidified incubator. Cruel capsule from VIRGO UAP Pharma Pvt. Ltd. was dissolved in DMEM at 50 mg/ml concentration, incubated at room temperature overnight, centrifuged and supernatant was taken for the treatment.

Microscopy:

Changes in morphological characteristics in renal cell carcinoma cells following Cruel treatment were assessed by Phase contrast microscopy (Motic, China). Cells were first cultured in 12 well plate for the microscopic observation. Following Cruel treatment at 4mg/ml concentration (≈IC₅₀ concentration) for 24 hours images were captured by Canon Power Shot A640 camera at 10X objective of microscope.

MTT assay

MTT assay was performed according to Waheed et al., 2013 with slight modification. Briefly, from exponentially

growing 80% confluent T25 flask cells were seeded in 96-well plate in complete DMEM medium. After 24h cells were incubated with varying concentrations (2, 4, 6, 8 and 10 mg/ml) of extract in triplicates for 24h. After that growth media changed with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) containing media and incubated for 4h at 37°C. After that, media was removed and blue coloured formazan was extracted in 600 µl of DMSO at room temperature. The purple color end products were quantified by measuring absorbance (OD 570 nm) with a spectrophotometer (Amersham). Antiproliferative activity was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100$$

Results

Antiproliferative effect

Cruel’s antiproliferative effect on renal cell carcinoma cells was evaluated by MTT cell viability assay. Cells displayed a dose dependent decrease in cell viability following drug treatment for 24 hours. Addition of Cruel at different concentrations i.e. 2, 4, 6, 8 and 10 mg/ml to RCC cells for 24 h inhibited the growth as mentioned and plotted a graph to visualize the percentage inhibition

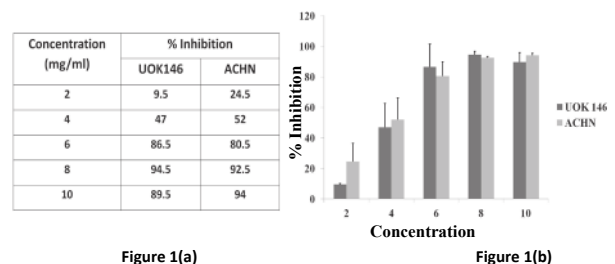


Figure 1. Dose Response Graph: (a) Table showing the dose dependent % cell death of RCC Cells and its (b) Graphical presentation

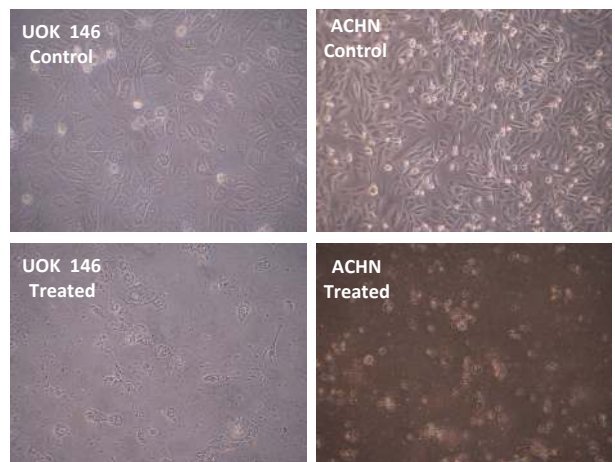


Figure 2 (a) **Figure 2 (b)**

Figure 2. Phase Contrast Microscopy: Images of RCC Cells at same magnification (a) UOK 146 cells: Upper panel is control and lower is 4 mg/ml cruel treated (b) ACHN: Upper panel is control and lower is 4 mg/ml cruel treated

with concentration (Figure 1). IC_{50} was determined by dose response curve plotted between the % inhibition of cell viability and doses concentration, and estimated IC_{50} is nearly 4.8 and 4.0 mg/ml for UOK146 and ACHN respectively.

Morphology

Cruel's effect on RCC cells was evaluated by phase contrast microscopy. After 4 mg/ml of drug treatment cells were incubated for 24 h. Cells became rounded, detached from the flask surface and started floating into the growth media as suspension which is a characteristic feature of cell death (Figure 2).

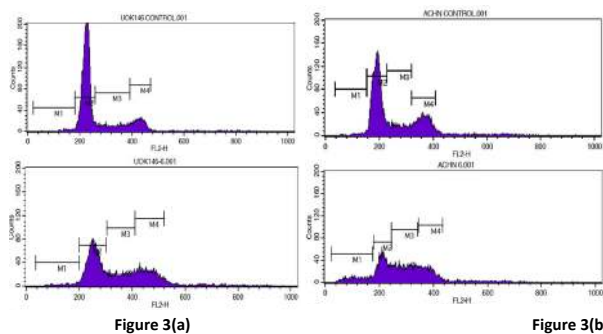


Figure 3. Flow Cytometry Analysis: M1, M2 and M3 and M4 in the histogram denotes the cells in sub-G1, G1, S and G2+M phase respectively (a) UOK 146 cells: Upper panel control and lower is 6 mg/ml cruel treated (b) ACHN: Upper panel Control and lower Panel Treated

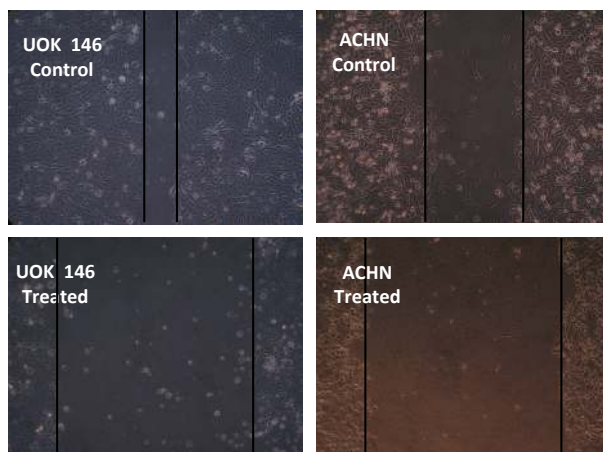


Figure 4. Cell migration assay: (a) UOK 146 cells: Upper panel is control and lower is 4 mg/ml cruel treated (b) ACHN: Upper panel is control and lower is 4 mg/ml cruel treated

Table 1. Primers Sequence Used in the Study

Primer	Sequence (5'-3')	Reference
GAPDH-Forward	AGGGCTGCTTTAACTCTGGT	Ito et al., 2007
GAPDH- Reverse	CCCCACTTGATTTTGGAGGGA	
LC3-Forward	GAGAAGCAGCTTCCTGTCTGG	Jiang ZF et al., 2012
LC3-Reverse	GTGTCCGTTCCACCAACAGGAAG	
BAX-Forward	CCCTTTTGCTTCAGGGTTTC	Liu et al., 2008
BAX-Reverse	TGTTACTGTCCAGTTCGTCC	

Cell cycle analysis

Cruel's effect on cell cycle stages were assessed by flow cytometry after staining with PI, a DNA binding dye. RCC cells were treated with 6 mg/ml Cruel and compared with control cells. Change in cell cycle stages were observed, Cells in S and G2/M population increases after treatment denoting the cell cycle arrest at these stages. Cells in different cell cycle stages and histogram were mentioned in the Table 2 and Figure 3 respectively.

Cell migration

Inhibition in Cell migration was studied by wound healing assay in RCC cells after 4 mg/ml cruel treatment. The extent of scratch wound closure was monitored under phase-contrast microscope and photographed. After drug treatment cell migration into the open space (scratch in the confluent cell layer) is inhibited as compared to control. This denotes that Cruel has property to inhibit the cell migration (Figure 4).

Gene expression analysis

Mode of cell death in RCC cells induced by Cruel treatment was examined at molecular level by gene expression analysis. BAX, a proapoptotic marker and LC3 an autophagy marker gene were used for quantitative RT-

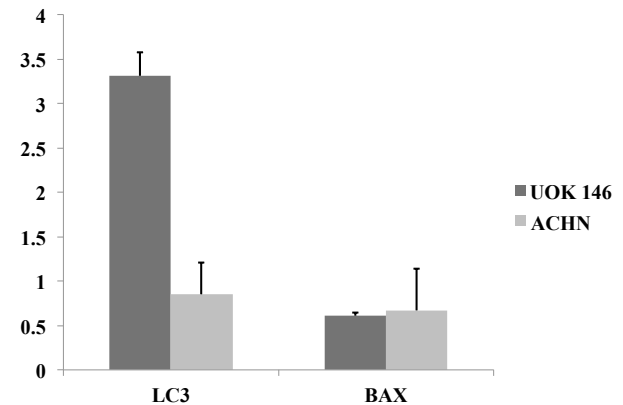


Figure 5. Real Time PCR Analysis: Expression of LC3 and BAX at transcript level after Cruel treatment

Table 2. Percentage of Cell Cycle Stages

Cell cycle stage	UOK146		ACHN	
	Control	6 mg/ml	Control	6 mg/ml
Sub G1 phase(M1)	1.31%	1.94%	1.25%	10.18%
G1 phase(M2)	73.15%	46.29%	58.73%	29.11%
S phase(M3)	13.13%	22.15%	14.36%	34.01%
G2/ M phase(M4)	11.73%	25.33%	22.43%	21.86%

PCR to see the mode of cell death. Quantitative RT-PCR for LC3 showed that cruel treatment increased the level of transcripts up to 3 times as compared to control in UOK 146 and BAX expression is decreased (Figure 5) which indicates autophagy mode of cell death. In ACHN both LC3 and BAX is slightly decreased and hence in this cell line mechanism of cell death was not confirmed. These results primarily indicate that Cruel may induce cell death by autophagy mode.

Discussion

Ayurveda the science of life is a traditional medicine system used by Indian population. Medicinal plants were the primary source for the therapeutics preparation in Ayurveda during the days of Charaka and Sushruta. A lot of plant products have been studied well and described for their medicinal properties. In 8th century AD Indian alchemist Nagrjuna prescribed the use of metals (e.g., mercury, lead, cadmium, iron, and zinc) and minerals (e.g., mica) as medicinal agents. It has been proved that if potentially toxic macro- or micro particles of heavy metals are converted into nano- or pico particles it could be nontoxic (Sheikh et al., 2012). Researchers are looking for some novel phytotherapy for cancer prevention using these minerals in very low, nontoxic and therapeutically significant amount. Minerals are now widely used in Ayurveda alongwith herbal drugs for its medicinal properties leading to the successful invention of many herbomineral drugs. Herbo mineral drugs are now used for many types of cancer for example it has been used as adjuvant therapy for the improvement of quality of life (QoL) in hepatocellular carcinoma (Jayawardhane et al., 2012). Acute promyelocytic leukemia was treated with a proprietary ayurvedic medicine Navajeevan, Kamadudha Rasa and Keharuba Pisti and patient achieved complete disease remission without any side effects (Prakash et al., 2010). Testosterone propionate-induced BPH in male wistar rats was significantly corrected by herbomineral preparation Ural-BPH (Soni et al., 2014). Herbomineral capsule ALG-06 was used for the hypopigmentation especially in vitiligo (Huma et al., 2014). Khamira Marwarid khas is a herbo-mineral preparation with immunostimulatory effect in murine model (Khan et al., 2014). Las01 a novel herbomineral preparation has been found to be effective as a potent anticancer drug in MCF-7 and Hela cancer cell lines (Sheikh et al., 2012).

Cruel a proprietary ayurvedic medicine for cancer treatment is marketed by UNJHA ayurvedic pharmacy and VIRGO UAP pharma in INDIA. This drug is prescribed for the cancer treatment and hence a detailed experimental study for finding its mechanism of action was attempted in this study. We treated RCC cell lines with aqueous solution of Cruel and observed that cells detaching from the surface indicating its cytotoxic effect on cancer cells. Cell cycle analysis following Cruel treatment revealed that cell was arrested at S and G2/M phase of cell cycle implying that Cruel acts at the level of cell cycle regulation. Migratory capacity of cancer cells is the main cause behind cancer metastasis and dissemination to the other body parts which is the most detrimental property of a cancer. Cruel was

tested for its inhibitory action for cancer cell migration and observed that cancer cell migration was significantly inhibited and hence it may be postulated that invasion will also be inhibited qualifying Cruel as a potent anticancer drug with very little to no side effects. Experimental evidences gathered from the present study detailing the mechanism of cell death proves the efficacy of this drug for killing cancer cells. There are mainly three modes of cell death; apoptosis, autophagy and an advanced cell death stage known as necrosis. In apoptosis and autophagy BAX and LC3 are upregulated respectively. In RCC cells Cruel was tested for both autophagy and apoptosis mode of cell death, through gene expression analysis of LC3 and BAX as markers. Down regulation of BAX expression in both the cell lines and upregulation of LC3 expression in UOK146 indicated that autophagy mode of cell death may be the possible mechanism of cell death in RCC cells. Further study, however will be required to see the detailed mechanism of action which will also provide the great opportunity for improvising the drug.

Acknowledgements

We acknowledge W M Linehan, National Cancer Institute, USA and National Centre for Cell Science, Pune, INDIA (NCCS) for providing the UOK 146 and ACHN cell line respectively. DBT- BHU Interdisciplinary School of Life Sciences (ISLS) Banaras Hindu University, Varanasi, INDIA for Flow Cytometry and Real Time PCR facilities. Indian Council of Medical Research (ICMR), Government of India, New Delhi for fellowship support to Shiv Prakash Verma.

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