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Original Research

Anticancer activity of *Cynodon dactylon* and *Oxalis corniculata* on Hep2 cell line

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Abstract: Bioactive chemicals isolated from plants have attracted considerable attention over the years and overwhelmingly increasing laboratory findings are emphasizing on tumor suppressing properties of these natural agents in genetically and chemically induced animal carcinogenesis models. We studied *in vitro* anticancer activity of organic extracts of *Cynodon dactylon* and *Oxalis corniculata* on Hep2 cell line and it was compared with normal human corneal epithelial cells (HCEC) by using MTT assay. Real Time PCR was conducted for p53 and PTEN genes in treated cancer cell line. DNA fragmentation assay was also carried out to note DNA damaging effects of the extracts. The minimally effective concentration of ethanolic extract of *Cynodon dactylon* and methanolic extract of *Oxalis corniculata* that was nontoxic to HCEC but toxic to Hep2 was recorded (IC50) at a concentration of 0.042mg/ml (49.48 % cell death) and 0.048mg/ml (47.93% cell death) respectively, which was comparable to the positive control. Our results indicated dose dependent increase in cell death. P53 and PTEN did not show significant increase in treated cell line. Moreover, DNA damaging effects were also not detected in treated cancer cell line. Anticancer activity of these plants on the cancer cell line showed the presence of anticancer components which should be characterized to be used as anticancer therapy.

Key words: Phytochemicals, Cancer, Apoptosis.

Introduction

Cancer is a multi-step and genomically complex disease and rapidly emerging experimentally verified data is deepening our understanding of wide ranging molecular mechanisms which underpin cancer development, metastasis and resistance against different therapeutic combinations (1,2). Both in-vitro and in-vivo studies have helped in developing a broader landscape of molecular network and it is now known that overexpression of oncogenes, down regulation of tumor suppressor genes, dysregulation of spatio-temporally controlled intracellular signaling cascades and loss of apoptosis. There is an overwhelming list of synthetic and natural agents reported to modulate signaling machinery in cancer cells to induce apoptosis (3). More importantly, bioactive ingredients isolated from plants have been noted to effectively regulate and functionalize extrinsic and intrinsic pathways in drug resistant cancer cells. In line with this approach, secondary metabolites and their derivatives from plants have emerged as a new source to effectively inhibit cancer progression, as evidenced by tumor growth inhibitory activity in xenografted mice. Various phytochemicals have entered in clinical trials and better knowledge of targets will prove to be useful in personalized molecularly targeted cancer therapy (4, 5, 6, 7, 8).

Indole-3-carbinol (I3C), an indole carbinol generated from the metabolism of glucobrassicin produced in cruciferous vegetables effectively prevented NEDD4-1 induced ubiquitination PTEN and proteasomal degradation of PTEN. More importantly, stability of PTEN was significant only in wild type PTEN expressing melanoma cells but not in null or mutant PTEN expressing cells (9). Oroxylin A, a natural active flavonoid has recently been shown to modulate post-translational modification of PTEN by SIRT3. SIRT3, a deacetylase is reportedly involved in deacetylation of PTEN and activation. Functionally active PTEN played contributory role in transcriptional inhibition of MDM2, a protein involved in degradation of wild type p53 (10). Kaempferol (Kae), a natural flavonoid, time dependently increased PTEN expression in treated bladder cancer cells (11).

Therefore it is clear that PTEN is transcriptionally, post-transcriptionally and post-translationally modulated by different phytochemicals.

Cynodon dactylon and *Oxalis corniculata* are medicinally very important used to treat and/or cure different diseases in Ayurvedic system of medicine. *Cynodon dactylon* belongs to the family of Poaceae, extracts of *Cynodon dactylon* shows antiulcer activity (12) cardioprotective and (13) hepatoprotective effect and protein fractions of *Cynodon dactylon* showed antioxidant and immunomodulatory activity in Swiss albino mice (14). *Oxalis corniculata* belongs to the family Oxalidaceae . Juice of plant is used to treat jaundice, stomach infections , also for muscular swellings, pimples and boils (15). *Oxalis corniculata* shows antiseptic, antidiabetic, antihelmintic, anti-inflammatory activites and used to treat urinary tract infections (15). Ethanolic extracts of *Oxalis corniculata* also shows anticancer activity in Ehrlich ascites carcinoma (EAC) induced in Swiss albino mice (16).

The aim of present study is to evaluate the *in-vitro* anticancer activity of *Cynodon dactylon* and *Oxalis corniculata* on hepatic cancer cell line Hep 2 and its comparison with normal cell line.

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Materials and Methods

Media and cell lines

Human corneal epithelial cells (HCEC) were obtained from Vricell, Spain. Hep 2 was obtained from ATCC, (ATCC, USA). The cells were maintained in RPMI supplemented with 10% FCS, 1% GPPS in a humidified atmosphere of 5% CO_2 at 37 °C incubator (SCO6WE Shell Lab Basic CO_2 Water Jacked Incubator, 6 Cu. Ft. USA).

Plant material collection

Whole plants (including leaves, stem and roots) of *Cynodon dactylon* and *Oxalis corniculata* were collected from Margalla Hills, Islamabad.

Preparation of alcoholic extracts

Plants were washed thoroughly with running tap water and final rinse with distilled water, after washing they were air dried and crushed to fine powder. 50 grams of plant material was soaked in 500ml of ethanol and methanol each for 7 days, filtered with Whatmann no. 1 filter paper, residue was again soaked in 500ml solvent and procedure was repeated for 3 times. Filtrate was concentrated to dryness with rotary evaporator at 40 °C, till further use.

Experimental design

Cytotoxicity of ethanolic and methanolic extracts of *Cynodon dactylon* and *Oxalis corniculata* was assessed by MTT assay against Hep 2 cell line and HCEC normal cell line.

Cell viability assay on HCEC (normal) cell line

The cytotoxicity of the plant extracts on HCEC was determined by MTT assay. Cells (1×10^5/ well) were plated in 96 well culture plate (Falcon 3077, Becton Dickinson Labware, New Jersey), with various concentrations of methanolic and ethanolic plant extracts of *Cynodon dactylon* and *Oxalis corniculata* at 37 °C with continuous supply of 5% CO2 in SCO6WE SHEL LAB Basic CO2 Water Jacketed Incubator, 6 (Shel Lab, USA). After 24 hours of incubation 10µl MTT (Sigma, USA) was added. After 4 hours of incubation solubilization solution was added. Viable cells were determined by absorbance at 550nm by ELISA plate reader, positive and negative controls were EGCG extract and cell culture without extract respectively. The effect of the alcoholic extract on the proliferation of HCEC cells were expressed via percent cell viability. The anticancer activity of ethanolic and methanolic extracts of *Cynodon dactylon* and *Oxalis corniculata* were tested on Hep 2 cell line. The cell viability was measured by MTT assay as described above. Percent viability of cells was plotted against the different concentrations of plant extracts. The maximum concentration of the plant extract non-toxic to HCEC but toxic to Hep2 was taken as the effective drug concentration.

Real time PCR

Real time PCR was done for quantitative expression analysis of OGT, p53, Kras, and PTEN. qPCR (quantitative RT-PCR) and data analysis were performed on the ABI 7000 Prism RT-PCR detection system using SYBR green (Thermo Fisher, USA)

Primers were designed using primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For OGT gene sense primer TGGGCAAACATTCTGAAGCG and anti-sense primer TCCAAGCAGACATCAGCCAG, for PTEN sense primer GGACCAGAGACAAAAAGGGAGT and anti-sense primer ACACACAGGTAACGGCTGAG, for p53 sense primer ATG GAG GAG CCG CAG TCA GAT and anti-sense primer GCA GCG CCT CAC AAC CTC CGT C, for Kras sense primer GCCTGCTGAAAATGACTGAATATA and anti-sense primer TTAGCTGTATCGTCAAGGCACTC were used in qPCR.

Caspase-8 and 9 activities

Caspase-8 and Caspase-9 expression were determined after treatment with the plant extracts using Caspase-8 & 9 Calorimetric Activity Assay Kit (Merck, USA).

DNA extraction and DNA ladder assay

The conventional "phenol–chloroform" DNA isolation method was used to isolate DNA from treated and non-treated cancer cells. Cells were dislodged in the culture medium and centrifuged at 5,000g to obtain the pellet containing both intact and apoptotic cells. Re-suspend the pellet in 600µl lysis buffer and vortex to completely re-suspend the pellet. Incubate for 1 hour at 37ºC. Add an equal volume of Phenol/Chloroform and mix well by inverting the tube until the phases are completely mixed. Do not vortex the tube. It can shear the DNA. Spin at maximum speed for 5 minutes at room temperature. Carefully transfer upper aqueous phase to a new tube. Removal of Phenol is done through addition of an equal volume of chloroform to aqueous layer. Tube is inverted and spun at maximum speed for 5 minutes. DNA precipitation was carried out using 2.5 ml of cold absolute ethanol. Tube was incubated at -20ºC for 30 minutes. Supernatant was discarded and DNA pellet was rinsed with 1 ml 70% ethanol. Later after spinning, supernatant was discarded and pellet was air dried. Furthermore, DNA was re-suspended in TE buffer. DNA solution was loaded on 2% agarose gel and visualization of bands was done under UV light.

Results

Overview of the rapidly upgrading list of new medicines approved by the FDA between 1981 and 2010 provided clear evidence of significant contribution of natural agents. It is interesting to note that almost 34% of those medicines were either direct derivatives of natural products or natural products (4,5,6,7,8). Data obtained through preclinical studies is also emphasizing on noteworthy role of phytochemicals in suppressing cancer progression.

Increase in p53 expression was statistically non-significant however, slightly raised p53 was noted in Hep2 cells treated with Methanolic extract of *Oxalis corniculata* and Ethanolic extract of *Cynodon dactylon.* as shown in Figure 1. Increase in PTEN expression was statistically non-significant, however, slightly raised levels of PTEN were detected in Hep2 cells treated with

Table 1. Results of Real Time PCR of *Oxalis corniculata* and *Cynodon dactylon* in terms of CT values, at the concentration of 0.048mg/ml (IC50).

Genes	CT Values			
	***Oxalis corniculata* (Methanol)**	***Cynodon dactylon* (Ethanol)**	**Normal**	**Drug**
Actin	39.11	Undetected	38.60	Undetected
B-Globin	29.48	30.09	30.08	28.59
GAPDH	27.82	31.34	29.25	28.27
KRas	Undetected	Undetected	37.11	38.60
P53	38.94	38.51	35.50	36.43
Pten	36.08	35.27	33.54	35.26
OGT	Undetected	Undetected	36.89	35.54

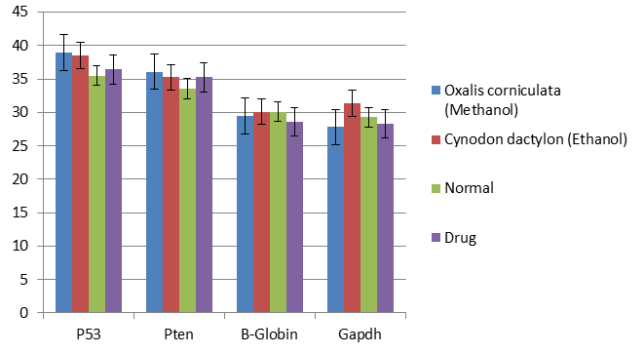

Figure 1. Quantitative expression of tumor suppressor p53 and PTEN genes along with housekeeping genes β-Globin and GAPDH with and without treatment of Hep2 cells by *Oxalis corniculata* and *Cynodon dactylon.*

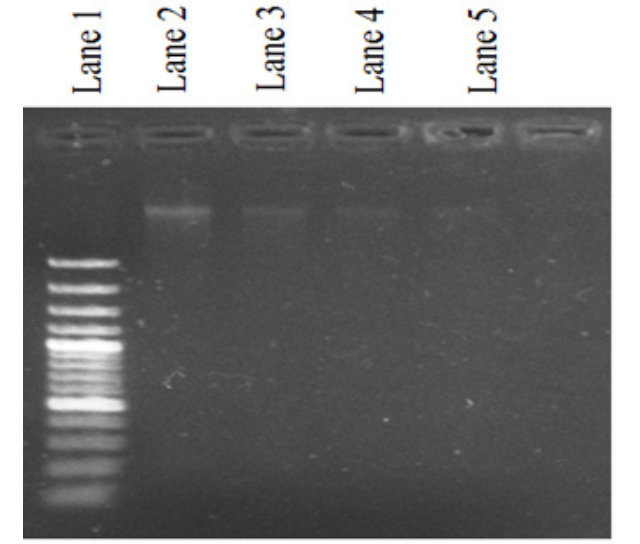

Figure 2. DNA damage in (2) Methanolic extract of Oxalis corniculata, (3) Ethanolic extract of Cynodon dactylon and (4) Non-treated Cancer cells. (5) Drug treated cells. DNA damage was not noted in treated cancer cells.

Methanolic extract of *Oxalis corniculata* and Ethanolic extract of *Cynodon dactylon*. as shown in Figure 1.

The nontoxic dose of the ethanolic extracts of *Oxalis corniculata* and *Cynodon dactylon* on normal human corneal epithelial cells showed that percent viability of the cells was found to be 94% and 97.7% at 0.036 mg/ml and 0.042 mg/ml respectively. Expression analysis of different genes in the cancer cells post-treated with plants extracts is mentioned in table 1 and figure 1. Taxol the anticancer drug was used as a positive control. The minimally effective concentration of ethanolic extract of *Cynodon dactylon* and methanolic extract of *Oxalis corniculata* that was nontoxic to HCEC but toxic to Hep2 was noted (IC50) at 0.042mg/ml (49.48 % cell death) and 0.048mg/ml (47.93% cell death) respectively (table2). Figure 3 shows cell viability.

Ethanolic extract of *Oxalis corniculata* notably enhanced caspase-9 activity at 1mM concentration in Hep 2 cells (figure 4). However *Cynodon dactylon* did not show any significant activity.

Discussion

The promising cytotoxic effect of crude ethanolic extract of Cynodon dactylon was also reported with human colon cancer cell line (17). Ethanolic extracts of Oxalis corniculata also have the cytotoxic effect on Ehrlich acsites carcinoma (EAC) in swiss albino mice (18). Our results indicate gradual increase in cell death with increasing extract concentration. We did not find any DNA damage in cancer cell line after treatment with extracts (Fig. 3).

P53 is a versatile protein reportedly involved in

Table 2. Percent cell death on Hep2 cell line through various concentrations of organic plant extracts.

Concentration mg/ml	% Cell Death	
	***Oxalis Corniculata* (Methanolic Extract)**	***Cynodon dactylon* (Ethanolic Extract)**
0.012	4.6	4.4
0.018	24.5	5.33
0.024	34.1	16.6
0.030	35.5	27.7
0.036	33.4	33.5
0.042	44	49.48
0.048	47.93	56.91

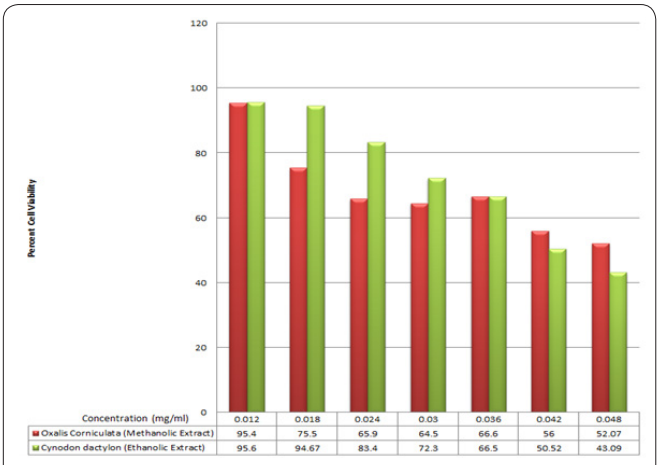

Figure 3. Graphical presentation of cytotoxicity assessment (% Cell Viability) by MTT assay in Hep 2 cells of methanolic and ethanolic extracts of *Oxalis corniculata* and *Cynodon dactylon* respectively.

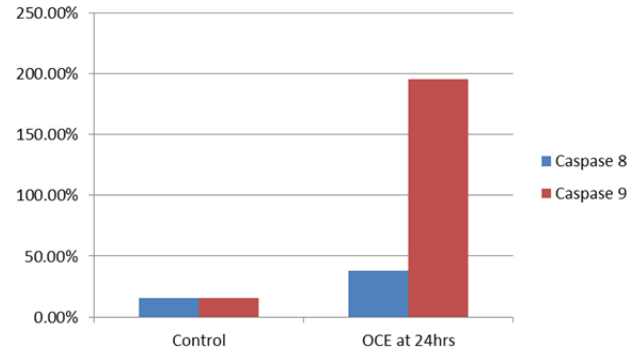

Figure 4. Graphical presentation of Intrinsic and Extrinsic pathway by caspase activity assay in Hep 2 cells of Ethanolic extract of *Oxalis corniculata*.

modulation of wide ranging genes. In this study we did not observe statistically significant increase in p53 expression however, slightly raised p53 was noted in Hep2 cells treated with Methanolic extract of *Oxalis corniculata* and Ethanolic extract of *Cynodon dactylon* (as shown in figure 1). We also did not notice statistically significant increase in PTEN expression, however, slightly raised levels of PTEN were detected in Hep2 cells treated with Methanolic extract of *Oxalis corniculata* and Ethanolic extract of *Cynodon dactylon* (as shown in figure 1).

Keeping in view the potential of ethanolic extract of *Cynodon dactylon* and methanolic extract of *Oxalis corniculata*, detailed analysis of extrinsic and intrinsic pathway will be helpful to evaluate efficacy of extracts and isolated bioactive ingredients. Receptor mediated intracellular signaling to induce apoptosis in Hep 2 cells is still an unexplored area. There are direct pieces of evidence emphasizing on phytochemical mediated upregulation of Death receptors (DR4 and DR5) in Hep 2 cells.

Previously it has been shown that β-phenylethyl isothiocyanate considerably enhanced DR4 and DR5 in HEp-2 Cell Line (19).

Our results show that there is a significant reduction in cell viability in Hep 2 cells in dose dependent manner. Data revealed that ethanolic extract of *Cynodon dactylon* is more effective due to higher percentage of cell death as compared to methanolic extract of *Oxalis corniculata*. Further studies are undergoing in order to know the mechanism of action of these extracts on hep 2 cells. Anticancer activity of these plants on the cancer cell line showed the presence of anticancer components which should be characterized to be used as anticancer therapy. Future studies must converge on mechanistic insights and identification of therapeutic targets to benefit from a boundless source of novel bioactive compounds.

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