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Aqueous Extract of Leaf of *Cyperus Rotundus* Exhibits 'S' Phase Arrest in Oral Cancer Cell Lines

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Abstract:- ***Cyperus rotundus*** **Linn. belongs to the family** ***Cyperaceae,*** **is commonly called 'Nagar motha'. It is a multipotent traditional herbal medicine used worldwide for the treatment of a variety of diseases like diarrhea, skin disease, bowel abnormalities, menstrual irregularities etc. In the present study, the anti proliferative activity of aqueous extract of leaf of** ***Cyperus rotundus on*** **KB oral cancer cell lines were studied by MTT assay, comet assay and cell cycle analysis. The results of MTT showed significant cell death on dose dependent manner. The extent of DNA damage was evident from the number of comet bearing cells in treated sample. Cell cycle analysis by Flow cytometry confirmed, significant increase in S phase attributing to 'S' phase arrest of oral cancer cells. The gene expression studies by RT-PCR, shown significant effects on p53 and p21 regulatory genes. Further studies on bioactive compounds and mechanism can put forth the possibility of** ***Cyperus rotundus*** **extracts a potent anti-cancer drug in therapeutic regimen.**

Keywords:- *Comet Assay, Cell Cycle, Anti Proliferative, Flow Cytometry.*

I. INTRODUCTION

Oral cancer is one among the top ten cancers in the world and top three in India [1]. Around 5% of cancers in men and 2% in women comes under oral cancer. Oral cancer includes any type of malignant neoplasm found on the floor of the mouth, lining of cheek, lips, gingiva, and palate or in the tongue. In India more than 90 - 95% of oral cancer is of squamous cell carcinoma [2]. The major cause of increasing rate of oral cancer in India is due to the habit of tobacco consumption either by smoking or by smokeless consumption along with betal quid [3]. Other causes include viral infections like HPV, poor oral hygiene etc. Treatment strategies employed for cancer now a days is usually multimodality [4]. Even though with these modern approaches, serious side effects are reporting. Therefore, the development and search for novel and effective anticancer agents have become very important issues [5]. Various researches regarding chemo preventive agents revealed that the natural products, especially from plants are the source for over 50% of anti-cancer drugs in clinical trials [6, 7]. *Cyperus rotundus* Linn. coming under the family *Cyperaceae* is a traditional herbal medicine used for the treatment of a variety of diseases [8,9] . Its therapeutic potential and ethno-medical uses have been mentioned even in ancient *Charaka Samhita.* Its tubers are effective in conditions like *Kapha* and *Pitta,* ophthalmia and in inflammations [10, 11]. It is the anti-aging factor in ayurvedic nutraceutical, Chyavanprash in India [12]. *Cyperus rotundus* is effectively used as an analgesic, sedative, anti-spasmodic, anti-malarial and to relieve bowel abnormalities [13, 14, 15]. Traditionally for the treatment of menstrual irregularities and dysmenorrhea, the rhizome of *Cyperus rotundus* was used [16, 17]. Splash and Perfumes were prepared from the aromatic oil synthesized from the rhizome of *Cyperus rotundus* [18]. In this study an attempt is made to investigate the anti-cancer activity of leaf of *Cyperus rotundus* in KB Oral cancer cell lines.

II. MATERIALS AND METHODS

A. Plant Material and Extraction Procedures

The leaves of *Cyperus rotundus* were collected at its flowering stage from Chirakkara village in Kollam District of Kerala, India and were authenticated by Dr. Palanisamy, Scientist D, Botanical Survey of India, Southern Regional Centre, Coimbatore (BSI/SRC/5/23/2016/Tech-279). Fresh leaves were cleaned with running water and dried beneath shade. Then the dried leaves were ground mechanically into fine powder and different solvent extracts were prepared based on polarity. Of these the aqueous extract was selected for anticancer study. The studies were conducted using KB oral cancer cell lines purchased from National Centre for Cell Science (NCCS), Pune and was maintained in at 37ºC in 5% CO2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO2 incubator. The cells were trypsinised (500µl of 0.025% trypsin in PBS/0.5mM EDTA solution (HIMEDIA) for 2 minutes and passaged to T flasks in complete aseptic conditions.

Fig 1:- *Cyperus Rotundus*

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B. Cell Proliferation Assay

The anti-proliferative effect of *Cyperus rotundus* extracts against KB oral cancer cell lines was estimated by MTT assay. Different concentrations of the sample were prepared in serial dilution with (DMSO) dimethyl sulfoxide. The toxicity profiles of the compound were assessed based on the cleavage of the tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide) by mitochondrial dehydrogenases in viable cells. The resulting blue formazan was dissolved in 100ml DMSO and the optical density was measured using a micro plate reader at 595nm [19, 20].

C. Analysis of DNA Damage by Comet Assay

The KB cells were cultured in 6 well plates and treated with IC 50 values of Aqueous extract of leaf of *Cyperus rotundus* (AqLCR) for 24 hours. The cells were trypsinized, washed with fresh media and used for comet assay [21]. Cell suspensions were mixed with 10µl of low melting point agarose (Invitrogen, USA). The slides were incubated at 4^0C, placed in cold lysing solution to allow unwinding of DNA and electrophoresis was conducted. Finally, slides were washed in neutralizing buffer (0.4 mM Tris, pH 7.5) and stained with ethidium bromide (20µg/ml). The slides were photographed using Inverted epi-fluroscent microscope Olympus CKX41 attached with Opitka Pro5 CCD camera. Comets were scored using Tritek comet scoring software and correlated statistically.

D. Analysis of DNA Content and Cell Cycle Distribution by Flow Cytometry

As per standard procedures KB oral cancer cells were cultured with IC 50 values of compounds for 24 hours. The samples were centrifuged and mixed with appropriate volume of PBS (i.e., 1ml of PBS per 1×106 cells). The cells were fixed using 70% alcohol at 20°C overnight. Muse cell cycle reagent was added, incubated at dark for 30minutes and was analyzed using a flow cytometer. Gating was performed with reference to untreated control cells and samples were analyzed.

E. Gene Expression Studies by RT – PCR

Total RNA was isolated using the total RNA isolation kit. Addition of Trizol solution causes the disruption of cells and the release of RNA. The RNA pellet was dried and dissolved in TE buffer. The purity of extracted RNA was determined using fluorimeter Qubit 3.1

One-step RT PCR kit of Thermo scientific, USA was used for the cDNA synthesis and amplification. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis and amplification.

Agarose gel electrophoresis is a method for separating and visualizing DNA fragments. The amplified RNA sample was observed as RNA bands started migrating towards the anode. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen) and the mean density was determined using Image analysis software.

III. RESULTS

A. MTT Assay

MTT assay is based on the reduction of MTT (3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase to purple formazan product. As per the procedure, the aqueous extract of leaf of *Cyperus rotundus* showed more dead cells which indicated the anti-cancer potential against KB oral cancer cells where as less dead cells on treatment with normal cells indicated none cytotoxic reactivity after 24hrs contact. The IC50 value for normal cells is 65.9043µg/ml where as for KB oral cancer cells is 30.573 µg/ml depicts the anti-cancer potential of aqueous extract of leaf of *Cyperus rotundus.*

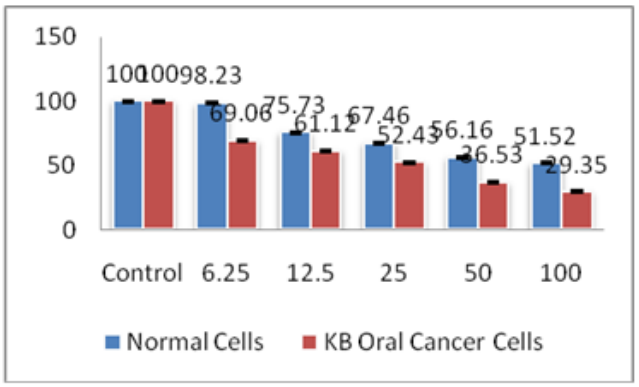

Fig 2:- Percentage viability of Oral cancer cells was significantly decreasing with the increase in the concentration of extract

B. Comet Assay

In the present study, treatment with aqueous extract of leaf of *Cyperus rotundus* showed increased the number of comet bearing cells indicative of DNA damage in KB oral cancer cell lines where as there is no significant effect in the non-cancerous control cell line.

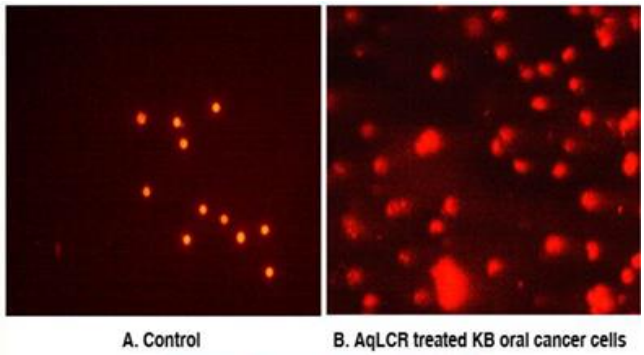

Fig 3:- DNA damage analysis by Comet assay

C. Cell Cycle Analysis by Flow Cytometry

It can be observed that untreated control cells have nearly 72 % cells in Go/G1 phase followed by 20.7 % cells in S phase and 5.5 % in G2/M phase. Treatment with aqueous extract of leaf has shown increase in cells distributed at S phase and concomitant decrease in cells at G0/G1 phase. From the results, it can be observed that leaf extracts exhibited significant inhibition at S phase of cell cycle.

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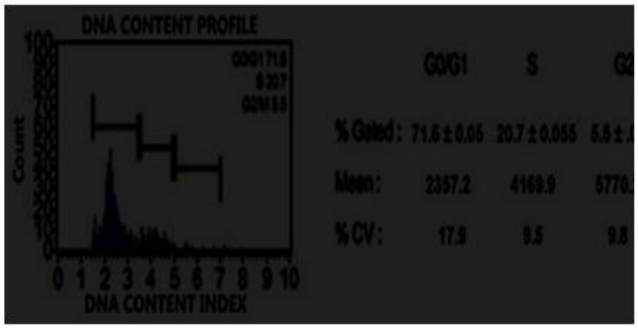

Fig 4:- Flow Cytometric analysis of cell cycle distribution in untreated control cells

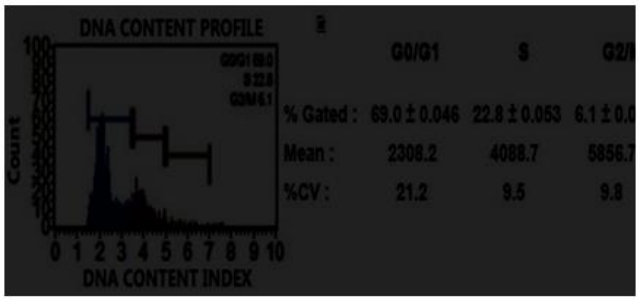

Fig 5:- Flow Cytometric analysis of cell cycle distribution in KB oral cancer cells treated with aqueous extract of leaf of *Cyperus rotundus*

D. Gene expression studies by RT – PCR

The molecular mechanism of cell death induced by aqueous extract of leaf of *Cyperus rotundus* indicated changes in expression of tumor suppressor genes such as p21 and p53. In the present study, mRNA analysis of p53 and p21 gene expression showed up regulation resulting in increased expression of p21 and p53 upon treatment.

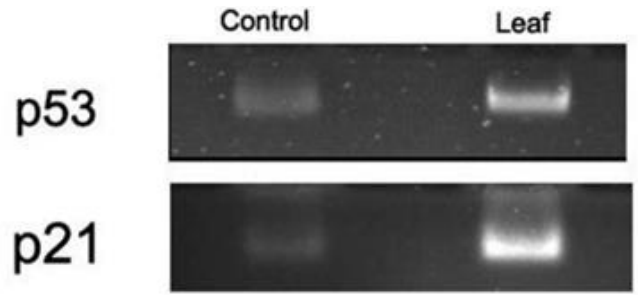

Fig 6:- Expression of tumor suppressor genes p53 and p21

IV. DISCUSSION

Cancer is a complex disease associated with wide range of devastating effects at both molecular and cellular levels. It is characterized by uncontrolled proliferation and spread of abnormal cells. The cancer cells can pervade into other cells and can convert it to cancerous. In normal cells, DNA the genetic material controls all the functions, whenever there is damage to the DNA, it is either repaired or the cell dies. However, in cancer cells, the damaged DNA is not repaired and these cells travel to different parts of the body and begin to grow and form new tumors by replacing normal tissues. If abnormal growth is not arrested it may progress to death of the patient [22].

The cancer that develops in the tissues of the mouth or throat is termed as Oral cancer (OC) which comes under a large group of cancers called head and neck cancers. The prime sites prone to oral cancer are the floor of the mouth and lateral borders of the tongue [23]. Lips, tonsils, gingiva, hard palate, soft palate, salivary glands, oropharynx and nasopharynx are the other sites where oral cancer occurs [24]. As per the annual report of ICMR 2016-2017, in India Oral cancer is the most common cancer amongst men (11.28%), and the fifth most frequently occurring cancer amongst women (4.3%) and the third most frequently occurring cancer in India amongst men and women. The prime risk factors for oral cavity cancer are tobacco and alcohol. Tobacco consumption either by smoking or by chewing, betel quid consumption, alcohol use and HPV infections are the risk factors for oral carcinoma [25, 26]. Current treatment guidelines for Cancer treatment remain stage-dependent which include surgery, with radiotherapy and chemotherapy. These procedures usually produce serious side effects including alopecia, xerostomia etc. [27]. In spite of the latest advances in Medical science and innovations in cancer treatment; it remains as a threat to mankind. So many researches were going on for the development of anticancer drugs from natural sources having fewer side effects [28, 29]. The wide range of therapeutic and pharmacological potential of medicinal plants was revealed in several previous studies [30]. *Cyperus rotundus* Linn. Coming under the Family – *Cyperaceae* commonly known as Nagarmotha is a traditional herbal medicine used for a variety of diseases including dysmenorrhea, bowel abnormalities etc. In the present study the anticancer potential of the leaf of *Cyperus rotundus* was assessed. The anti-proliferative effect of leaf of *Cyperus rotundus* were evident from the results obtained from MTT assay. Number of viable cells was found to be decreasing with increase in the concentration of sample. Morphological changes in extract treated cells were examined and compared with control cells using phase contrast microscope. The extend of DNA damage induced by the leaf extract was better explained from the results of comet assay. Measuring DNA synthesis directly by flow cytometry indicate significant S phase arrest in AqLCR treated cells when compared to control cells. The expression of tumor suppressor genes such as p53 and p21 are found to be more pronounced in AqLCR treated oral cancer cells. All these data reflects the anti cancer potential of leaf of *Cyperus rotundus.*

V. CONCLUSIONS

The results of this study support the efficiency of leaf of *Cyperus rotundus* as an anti-cancer agent for KB Oral cancer cell lines. The anti-proliferative and flow cytometric analysis of *Cyperus rotundus* justifies scientifically, the use of this traditional herbal medicine, paving the way for future studies on bioactive compounds and mechanism can put forth the possibility of formulating effective anti-cancer drug in therapeutic regimen.

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