Int J Pharma Res Health Sci. 2020; 8 (5): 3220-3224
DOI:10.21276/ijprhs.2020.05.01
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CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Triticum aestivum (wheat grass) Exhibited Anticancer Activity on Oral Cancer (KB) Cell Line

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ARTICLE INFO

Received: 14 Aug 2020
Accepted: 12 Oct 2020

ABSTRACT

Objective: Currently oral cancer is the sixth most common cancer in the world with a quarter of world burden being borne by India. Wheat grass, a traditional medicine is used worldwide to treat various ailments, with its anticancer activity being reported on few cell lines like Hep2, Hela and K562.
Aim: Aim of this study was to evaluate the anticancer and cytotoxic activity of WG on oral cancer (KB) cells and mouse embryonic cells (NIH3T3) respectively.
Methods and Material: Standard KB and NIH3T3 cells lines were procured. Ethanol extract of freshly grown WG was prepared in our laboratory and commercially available powder of WG was also procured. Anticancer activity on KB cells and cytotoxic activity on NIH3T3 cells was evaluated by MTT assay with the two forms of WG.
Statistical analysis used: Statistical analysis was performed by using spearman's coefficient correlation to correlate the concentration of extract and cell inhibition.
Results: WG exhibited an inhibition of KB cells in a dose-dependent manner with an IC50 value of 156µg/ml and was non-toxic on NIH3T3 cells.
Conclusion: WG showed anticancer activity on KB cells and therefore can be considered for further studies on animals and eventually on human beings.
Keywords: Cell death, KB cells, NIH3T3 cells, Squamous cell carcinoma, Triticum aestivum.

1. INTRODUCTION

According to the report of the year 2008, oral cancer has been reported to be the sixth most common cancer in the world with approximately 4,00,000 newly diagnosed cases and 2,23,000 deaths every year [1]. The incidence rate of oral cancer in India is 13.5 per 1,00,000 and represents 50% of all cancers [2]. A quarter of world burden of oral cancer is borne by India and its incidence here is higher in lower

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socioeconomic strata of society [3, 4]. Remarkable advancement is noticed year by year in the treatment modalities of oral cancer but the morbidity and mortality associated with it are also increasing. This could be attributed to the side effects and a high cost of current treatment modalities. Therefore the need of the hour is to discover drugs with minimal side effects and low cost. Herbal medication is one such option. The concept of herbal medication in the treatment of cancer is not new because drugs like vinblastin, methotrexate and taxol which are known anticancer drugs are also plant derivatives [5]. Wheat grass (WG) has been traditionally used worldwide to treat various ailments. Anticancer activity of WG on certain cell lines like Hep2 (human epithelioma of the larynx), Hela (cervical cancer) and K562 (chronic myeloid leukaemia) have been reported [6, 7, 8]. However, the data on oral cancer and WG is very sparse. Therefore the aim of this study was to evaluate anticancer activity of WG on oral cancer cells (KB-cells) and cytotoxic activity on mouse embryonic fibroblast cells (NIH 3T3 cells). Objectives of the study were to evaluate the cytotoxicity of ethanol extract of WG and commercially available powder of WG on KB cells and NIH3T3 cells, to quantify the inhibition of KB cells and NIH3T3 cells by both forms of WG, to analyse the dose dependent cytotoxic activity of both forms of WG on KB cells and NIH3T3 cells.

Young grass of common wheat (Triticum aestivum, belonging to the family: Gramineae) plant is known as wheat grass, which can be consumed in the form of juice after grinding with water [9]. Charles F Schnabel was a food scientist who introduced WG to the western world in the 1930s. He conducted an experiment in which he used various mixtures of grain to feed chickens and observed that chickens which were fed on mixtures containing a high proportion of WG exhibited better growth, were healthier and produced 150% more eggs [10]. Dr. Ann Wigmore, from Boston USA, developed and popularized the use of wheat grass juice (WGJ) for therapeutic purposes, as part of herb therapeutic nutritional approach [11]. The therapeutic qualities of WG have been attributed to its nutritional content, including flavonoids, coumarins, tannins, steroids, carbohydrates, saponins and phenols [12].

Invitro studies and clinical trials conducted have shown that WG is beneficial as an anticancer agent. These benefits might be due to antioxidant activity of WG preventing oxidative damage to DNA and lipid peroxidation, stimulation of gap junction communication, an effect on cell transformation and differentiation, inhibition of cell proliferation and oncogene expression, consequences on immune function and suppression of endogenous formation of mutagens [13, 14].

2. METHOD

The source of data: Oral cancer cells (KB-cells) (figure 1) and Mouse embryonic fibroblast cells (NIH3T3-cells) were procured from National Centre for Cell Sciences (NCCS) Pune, India.

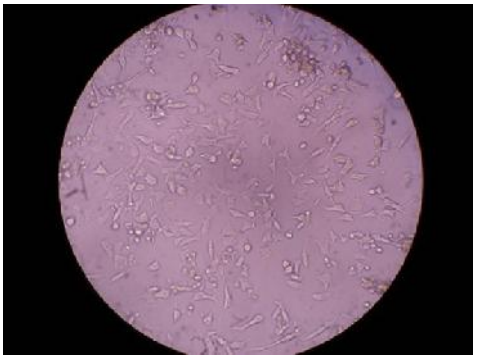

Fig 1: Oral cancer cells (KB-cells)

Methodology:

Young WG was obtained on the eighth day after sowing. An authentication certificate was obtained from Regional Medical Research Centre, Belagavi, Karnataka, India. Ethical clearance was obtained from ethical committee of the institute. Fresh ethanol extract of WG was prepared by maceration technique. The commercially available powder form of WG was procured. These two forms of WG were dissolved in dimethylsulphoxide (DMSO) (10mg/ml) and 10 serial dilutions were prepared. 100µl of each dilution was added to the two cell lines, KB and NIH 3T3 to determine anticancer and cytotoxic effect respectively. The dose-dependent anticancer and cytotoxic effect was analyzed by MTT assay. Quantification of the inhibition of cell proliferation was done by manual counting of cells under an inverted microscope (Labomed, USA) by Neubauer counting chamber.

200ml of ethanol and 1% chloroform water were added to 50gms of freshly grown and chopped WG in a conical flask and kept for maceration with continuous 6 hours shaking for 7 days. The extract obtained was transferred to ceramic bowls, which were placed in a water bath to allow for evaporation of ethanol. WG extract obtained was then stored at 4°C for further use.

MTT assay procedure:

KB and NIH3T3 cells, approximately $2x10^4$ cells were maintained in a 96 well microtiter plate containing Dulbecco's Minimum Essential Medium (DMEM) from HIMEDIA© Laboratories Pvt Ltd, Mumbai, India supplemented with 10% foetal bovine serum (FBS, HIMEDIA©, Mumbai, India) containing 5% mixture of gentamicin (10µg), penicillin (100units/ml) and streptomycin (100µg/ml) GIBCO© by life technologies, NY, USA in presence of 5% CO_2 at 37°C for 48-72 hrs. Desired concentrations of WG was added to the respective wells containing 100µl of medium with cells and incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2. Then, the stock solution of MTT (HIMEDIA©, Mumbai, India) was added to each well and incubated for further 4 hours. After 4 hours incubation, the supernatant was carefully aspirated. DMSO (100µl) was added to the wells so as to dissolve the

precipitated crystals of formazan blue and optical density (OD) was computed using ELISA reader. Percentage of cell viability and percentage of cell death were calculated by applying the following formulae [7]:

Percentage of cell viability = Mean OD of wells receiving each plant extract dilution/Mean OD of control wells x 100.

Percentage of cell death = 1-(OD of sample/OD of control) x100

Principle of MTT assay:

Dehydrogenase enzymes which are present in metabolically active cells will reduce the yellow tetrazolium MTT (3(4,5 dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) to generate reducing equivalents such as NADH and NADPH. The end result is intracellular purple formazan production which can be solubilized by DMSO and quantified by spectrophotometer. MTT assay measures cell proliferation rate because formazan crystals are formed in live cells. Also, conversely we can measure the reduction in cell viability when metabolic events have led to apoptosis or necrosis [7].

Data interpretation:

Data interpretation is done by comparing the absorbance values of control wells (untreated wells) and that of treated wells. Lower absorbance values than untreated wells indicate a reduction in cell proliferation rate whereas, high absorbance values indicate that there was an increase in cell proliferation.

3. RESULTS

In this study, the anticancer and cytotoxic activity of WG on KB and NIH3T3 cells were evaluated by MTT assay. The outcome of this study showed that there was an increase in cell inhibition of KB cells with increasing concentration of WG (ethanol extract and commercially available powder) with an IC_{50} value of 156µg/ml for both forms of WG. The IC_{50} value of WG on NIH3T3 cells at the tested concentrations was not observed, which suggests the nontoxic nature of WG on NIH3T3. The line diagram (Figure 2) shows an inverse relation between cell viability percentage of KB cells and NIH3T3 cells and the concentrations of WG. Statistical analysis (SPSS Inc. Released 2001. SPSS for Windows, version 11.0 Chicago) was performed by using Spearman's correlation coefficient to correlate the concentration of the WG (%) and the cell inhibition (wavelength-nm) and was observed that p value was <0.05 in all four cases (Table 1). The percentage cell viability and percentage cell inhibition calculated by applying the formulae are tabulated in Tables 2 and 3 for KB and NIH 3T3 respectively.

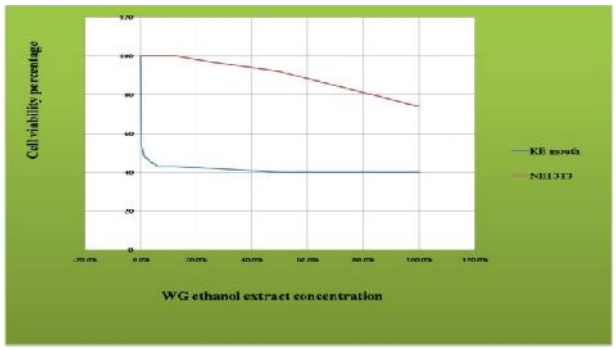

Fig 2: The line diagram shows an inverse relation between cell viability percentage of KB cells and NIH3T3 cells and the concentrations of WG.

Table 1: Spearman's correlation coefficient to correlate the concentration of the extract (percentages) and the cell inhibition (Wavelength- nm)

Correlation between	Wheat Grass	Spearman's correlation coefficient	p-value*	Significance
KB	**Ethanol extract**	-0.992	<0.001	Significant
KB	**Powder**	-0.983	<0.001	Significant
NIH3T3	**Ethanol extract**	-0.772	<0.001	Significant
NIH3T3	**Powder**	-0.494	0.019	Significant

***P<0.05**

Table 2: Anticancer activity of ethanol extract and commercially available powder of WG on KB cell line

Sr. No.	Concentrations(mg/ml)	Ethanol Extract			Commercial Powder		
		Absorbance at 492nm wavelength	% cell viability	% cell Inhibition	Absorbance at 492nm wavelength	% cell viability	% cell Inhibition
1	10	0.27	40%	60%	0.22	38%	62%
2	5	0.27	40%	60%	0.22	38%	62%
3	2.5	0.28	42%	58%	0.225	39%	61%
4	1.25	0.29	43%	57%	0.235	41%	59%
5	0.625	0.29	43%	57%	0.255	44%	56%
6	0.3125	0.31	46%	54%	0.275	47%	53%
7	0.156	0.32	48%	52%	0.29	50%	50%
8	0.078	0.35	52%	48%	0.445	77%	23%
9	0.039	0.36	53%	48%	0.575	99%	1%
10	0.0195	0.43	64%	36%	0.815	100	0
11	control	0.67	100%	0	0	100%	0

Table 3: Cytotoxicity of ethanol extract and commercially available powder of WG on NIH3T3 cell line

Sr. No.	Concentrations(mg/ml)	Ethanol Extract			Commercial Powder		
		Absorbance at 492nm wavelength	% cell viability	% cell Inhibition	Absorbance at 492nm wavelength	% cell viability	% cell Inhibition
1	10	0.225	74%	26%	0.355	115%	0%
2	5	0.28	92%	8%	0.39	125%	0%
3	2.5	0.295	97%	3%	0.395	127%	0%
4	1.25	0.305	100%	0%	0.41	132%	0%
5	0.625	0.320	104%	0%	0.415	133%	0%
6	0.3125	0.325	106%	0%	0.46	148%	0%
7	0.156	0.335	110%	0%	0.46	148%	0%
8	0.078	0.375	123%	0%	0.47	151%	0%

9	0.039	0.425	139%	0%	0.53	170%	0%
10	0.0195	0.44	144%	0%	0.60	193%	0%
11	control	0.305	100%	0%	0.31	100%	0%

4. DISCUSSION

KB cells were derived from human mouth epidermal carcinoma, exhibit epithelial morphology and produce keratin. NIH3T3 cells are mouse embryonic fibroblast cells isolated from a cell line isolated and initiated in 1962 at New York university school of medicine Department of Pathology. The utility of cell lines developed from tumours or from normal tissues for the purpose of investigation of the action of new drugs in a simplified and controlled environment in-vitro helps in future applications.

In our study the IC_{50} value of both forms of WG on KB cells was observed to be 156µg/ml which signifies that WG extract caused 50% inhibition of KB cells at this concentration and is in accordance with the study by Patel J B *et al,* 2013 who conducted an invitro study to evaluate anticancer activity of WG on Hela cell line [7]. Another study conducted by Patel J B *et al*, 2013 to evaluate anticancer activity of WG on Hep2 cell line showed an IC_{50} value of 625µg/ml which is much higher than that of our study [6]. Ponugoti *et al.* 2014 conducted a study in which it was observed that the IC_{50} value of WG on MCF (breast cancer) cell line was 400µg/ml which is also higher than that of ours [15]. The reason for this difference in IC_{50} value could be due to the wheat grass extraction technique employed due to which the active ingredient released could vary. Aydos *et al.* 2011 conducted a study to evaluate antiproliferative effect of WG on K562 (chronic myeloid leukaemia) cell line and was observed that WG extract inhibited the growth of K562 cells in a time-dependent manner where as in our study we observed a dose-dependent inhibition of KB cells [8]. Arora *et al.* 2013 conducted a study on normal kidney cell line NRK-52E cells to evaluate the cytotoxic effect of methanol extract of WG and observed an IC_{50} value of 356.09+0.26µg/ml [16]. Arora *et al*. compared the results with 5-fluorouracil (5-FU) and observed that treatment with WG led to minimum cell damage in comparison to 5-FU. In our study, when the cytotoxic activity of WG on NIH3T3 cells was tested, the maximum cell inhibition observed was 26% at 100% concentration of WG. 50% cell inhibition was not observed at any of the tested concentrations (0.0195mg/ml to 10mg/ml) which suggest the non- toxic nature of WG on normal mouse embryonic fibroblasts which is desirable in cancer treatment so that there is minimal damage to normal tissues. It is a well known fact that currently used anticancer drugs induces severe cellular toxicity which leads to normal tissue damage and related side effects. New drugs with minimal side effects would be welcome. Various metabolic processes occurring in the living organisms continuously generate reduced oxygen species (ROS) and free radicals which are required for cellular activities at normal concentrations but at higher concentrations they may be toxic leading to oxidative stress. They may be deleterious to major cellular components and have been associated with various human diseases like different forms of cancers. The injurious effects of free radicals and ROS can be nullified by antioxidants. The free radicals generated in a normal healthy state will be counterpoised by the endogenous antioxidants but, for efficient restoration in the diseased state, supplementary antioxidants from the diet and other sources like the medicinal plants are necessary. Seeds exhibit voluminous changes after germination. Germinated wheat for a period of 6-10 days is known as 'wheat grass' which contains vanillic acid, ferulic acid, vitamin C and E and β-carotene, the concentrations of which increase as the germination period increases and is maximum on the seventh day of growth. The study conducted by Kulkarni *et al.* 2006 showed that WG had higher oxygen radical absorbance capacity (ORAC) values which are used as a standard tool for comparing the antioxidant capacities of food products. Their study also showed a strong and significant correlation between the flavonoid content and the antioxidant activity of WG [17]. WG contains the enzyme superoxide dismutase (SOD) which transforms the hazardous free radical reactive oxygen species (ROS) into hydrogen peroxides and oxygen molecule [17, 18, 19] Several *in-vitro* studies have shown that WG contains chlorophyll which inhibits the metabolic activation of carcinogens and oxidative DNA damage [20, 21, 22] Certain clinical reports have been reported as by Verma *et al.* 2013 who observed that WG in combination with other herbs showed beneficial effect on oral cancer patients [23]. Though, Verma's is a clinical study and in combination with other herbs it does suggest that WG has great promise for further studies as an anticancer agent. Another pilot study (clinical) conducted by Sela *et al.* 2007 suggested that WG when supplemented with chemotherapy may prevent myelotoxicity in breast cancer patients thereby suggesting that WG can be used as an adjuvant to chemotherapy [24]. Further, studies at the molecular level action of WG on KB cells can be conducted to determine the active ingredient which may be useful as chemotherapeutic agent.

5. CONCLUSION

In conclusion our study showed that WG showed dose-dependent anticancer activity on KB cells and was non toxic to NIH 3T3 cells. This is an *in-vitro* study, the data of which can be useful for further studies on animals and then eventually on human beings.

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Conflict of Interest: None
Source of Funding: Nil