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ORIGINAL RESEARCH

Inhibitory Effect on Cell Growth and Cytotoxicity of Kouchner Plant (*Alpinia galanga* L) Extract on Squamous Cell Carcinoma Cell Line *in vitro*: A Case–control Study

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ABSTRACT

Introduction: Malignancy is the main cause of death in developed countries and the second leading cause of death in developing countries. The aim of the present study was to carry out an investigation of inhibitory and cytotoxic effect of Kouchner (*Alpinia galanga* L) extract on cell growth with a potential of selective effect on malignant cells of squamous cell carcinoma (SCC) compared with normal cells.

Materials and methods: In this *in vitro* study, in order to assess the cytotoxicity of the extract, a photometry method using the microculture tetrazolium test (MTT) was used. All stages were repeated three times on normal and malignant cell lines. A two-way analysis of variance (ANOVA) was used to investigate the effect of extract concentration on the percentage of viable cells among different cell types. To evaluate the effect of the extract concentration on the percentage of viable cells, one-way and two-way ANOVA were used for each cell line ($\alpha = 0.05$).

Results: The results showed that the percentage of viable cells is significantly correlated with different concentrations of Kouchner (*A. galanga* L) extract ($p > 0.001$) and is significantly correlated with the type of cell in the extract concentrations above 20% ($p = 0.023$). However, there is no significant relationship on the extract concentrations below 20% ($p = 0.123$).

Conclusion: The results of this study showed that cytotoxicity of the Kouchner extract at concentrations above 20% on malignant cell line (SCC) has been higher than normal cell lines, and, in concentrations below 20%, they are similar. According to this study, the Kouchner extract may be used as a natural substance with targeted antimalignancy properties and with less side effects against SCC.

Keywords: Carcinoma, Kouchner (*Alpinia galanga* L) extract, Squamous cell carcinoma.

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INTRODUCTION

Malignancy is one of the main causes of death in many countries, especially in developed countries. One of the malignancies is head and neck squamous cell carcinoma (HN SCC).[1]

In cancers of the oral cavity, surgery is an important part of the treatment. The timing and extent of the surgery have variation in the proposed regimens of oral cancers. Surgery of cancers are associated with severe complications, such as disorders in eating, talking, face form, and so forth.[2]

The side effects of conventional medical regimens include peripheral neuropathy,[3] ventricular fibrillation,[4] gastrointestinal system toxicity,[5] and gastrointestinal symptoms, including diarrhea, nausea, vomiting and constipation,[6] neurological dysfunction,[6] alopecia,[7] mucositis,[8] ocular complications,[9] etc.

Now, compounds derived from plants are discussed as an important source of chemotherapy drugs. Among these drugs, we have vinblastine, vincristine, camptothecin, and Taxol.[10]

Zlotogorski-Hurvitz et al[11] showed that curcumin and green tea have antimalignancy properties of HN SCC. Unlike chemical anticancer drugs, which target both normal and malignant cells, these natural substances affect only malignant cells. One of the studies led to the identification of anticancer compounds of various plants, such as yarrow plant, *Camptotheca*, Red drug, etc.[10]

Kouchner is a type of plant that is often used in East Asian countries. The rhizomes of this plant are largely used as a flavoring for foods. These rhizomes have been investigated for their different biological activities. These activities include antibacterial, antiviral, anticancer, and anti-*Trypanosoma* activities. Whelan and Ryan[12] indicate the presence of high amounts of 1,1-diphenyl-2-picryl hydrazyl in the Kouchner plant. This material has antimalignant effects, which include absorbing free radicals present in the malignant cells and increasing the activity of enzymes superoxide dismutase, catalase, and glutathione peroxidase. According to a study by Lee and Houghton,[13] their results showed a cytotoxic effect of Kouchner on lung cancer and breast cancer cell lines. The

effect is such that, based on the study of Kiuchi et al,[14] the inhibitory effects of the compounds of this extract on the inhibition of HT-1080 cells (fibrosarcoma cell line) are higher than 5-fluorouracil drug.

Considering the aforementioned studies and including failure of routine oral SCC treatments and failure to improve the prognosis of this disease during the past four decades, the aim of this study is to investigate inhibitory and cytotoxicity effect of the Kouchner extract on cell growth of malignant cell lines compared with normal cells.

MATERIALS AND METHODS

This study was an *in vitro* experimental study. All stages were repeated three times on normal and malignant cell lines.

Preparation of Plant Extracts

To prepare the herbal extract, the Kouchner plant was first dried at laboratory temperature for 3 days. Then, the dried plant (300 gm) was ground to powder in a mortar. The powder was then combined with n-hexane ($CH_3[CH_2]4CH_3$) (Sigma, Germany). The plant extract was obtained by a distillation rotary device (Vargha Tajhiz, Islamic Republic of Iran) in vacuum. The operation lasted for approximately 24 hours. After extraction, the solvent-removing operation was performed using a Rota Vapor (R300, BUCHI, Switzerland). During this operation, the solvent was evaporated and the extract was concentrated. The remaining extract from the previous step was dissolved in chloroform ($CHCl_3$) (Sigma) to prepare chloroform fraction.

Cell Culture

The cell lines KB (keratin-forming based on isoenzyme pattern oral SCC) and L929 (mouse fibroblast cells) were bought from the Cell Bank of Pasteur Institute of Iran and were cultured in Dulbecco's Modified Eagle's medium (Merck, Germany) containing 10% fetal bovine serum and L-glutamine. Then, the cells were incubated in 5% CO_2 (HF212UV, Heal Force, China) and the culture was subcultured every 3 days.

Evaluation of Cytotoxicity using MTT

To study the cytotoxic effect of the plant extracts, the photometry method was used using MTT (Sigma). This method is based on the enzyme activity of mitochondrial succinate dehydrogenase of viable cells that converts the yellow solution of MTT to purple formazan crystals. In order to perform this cell test, 180 mL of cell suspension was poured per well, in 96-well plates (Gene Fanavaran, Islamic Republic of Iran). Then, 20 µL of different concentrations of the extract was added to the wells and the final volume per well reached 200 µL. A well containing medium and 5% dimethyl sulfoxide (DMSO; Sigma) was considered as a negative control without extract, and a well containing medium alone was considered as blank. Plates were incubated for 48 hours in 5% CO_2 and the temperature was set at 37°C. Then, 20 µL of MTT was added to each well and it was incubated for 2 hours at this time. After this, 100 µL of DMSO was added to dissolve the formazan grains and, finally, the absorption of each sample was measured at a wavelength of 560 nm by enzyme linked immunosorbent assay reader (Biotech, USA). Cell viability in the control wells was considered 100% and cell viability in the wells tested was calculated by the following formula. The concentration of the herbal extract that halves the cell viability was defined as IC50.

$$\text{Cell viability percentage} = \frac{\text{Absoption of treated cells} - \text{Blank absorption}}{\text{Negative control absorption} - \text{Blank absorption}} \times 100$$

Statistical Analysis

The results were entered into the software Statistical Package for the Social Sciences, version 12. To investigate the effect of the concentration of Kouchner extract and type of cells (normal cells—malignant cells) on the viable cell, two-way ANOVA was used. In order to investigate the effects of plant extracts on the percentage of viable cells remaining in each cell line separately, one-way ANOVA and two-way ANOVA were performed at a significance level of $\alpha = 0.05$.

FINDINGS

The results showed that the percentage of viable cells is significantly correlated with different concentrations of the extract ($p < 0.001$) and is significantly correlated with cell type in concentrations above 20% ($p = 0.043$). However, it is not significant in concentrations below 20% ($p = 0.332$) (Tables 1 and 2).

In Graph 1, the percentage of viable cells after exposure to different concentrations of the Kouchner extract, in normal and malignant cell lines, has been displayed.

The statistical difference between the percentage of viable cells after exposure to different concentrations of extract in each cell line has been given separately in Tables 3 and 4 for the Kouchner plant, as well as green tea.

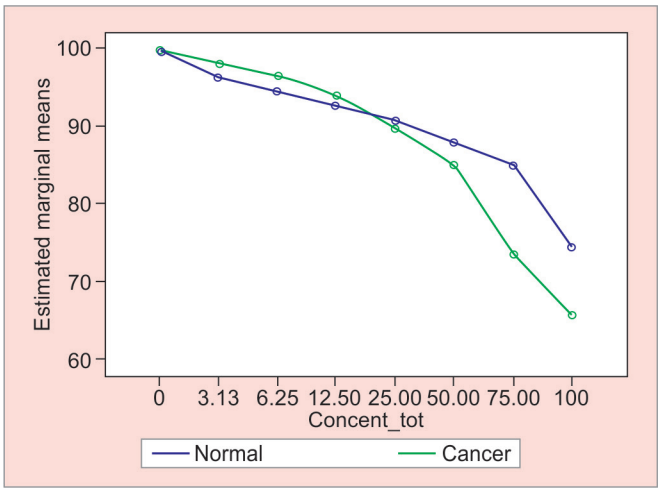
For example, according to the results obtained in a normal cell line of viable cells, a Kouchner extract concentration of 100% has no significant relationship with concentrations of 50% ($p = 0.148$) and 25% ($p = 0.066$), but has a significant relationship with the extract concentrations of 12.5% ($p = 0.038$), 6.25% ($p = 0.022$), and 3.13% ($p = 0.014$).

Table 1: Mean and standard deviation of normal viable cells remaining after exposure to different concentrations of Kouchner plant

Extract concentration (%)	*Mean percent of viable cells*	*Standard deviation*
100	74/5	0/7
75	85/22	5/18
50	88/08	6/72
25	90/83	5/58
12/5	92/73	5/43
6/25	94/73	3/71
3/125	96/44	2/42
0	100	0

Table 2: Mean and standard deviation of malignant viable cells remaining after exposure to different concentrations of Kouchner plant

Extract concentration (%)	*Mean percent of viable cells*	*Standard deviation*
100	71/05	0/36
75	81/24	6/05
50	89/26	4/81
25	94/16	6/43
12/5	96/36	1/26
6/25	97/9	1/16
3/125	98/95	0/35
0	100	0



Graph 1: Percentage of viable cells after exposure to different concentrations of Kouchner extract in normal and malignant cell line

DISCUSSION

The null hypothesis of this study regarding the Kouchner plant was as follows. First, the Kouchner extract does not have effects of growth and cytotoxicity inhibition on any of the cell lines tested, including malignant cells of SCC and normal cell. Second, if they have this property, its effect on the SCC line is similar to normal cells. During this study, the first and second parts of the null hypothesis regarding the Kouchner plant were rejected.

As the results showed, the Kouchner extract inhibits the growth and causes cytotoxicity in both malignant SCC and normal lines. In addition, between the concentrations of 20 and 100%, cytotoxicity of this extract and the tumoral line is higher than normal cell line (mouse fibroblasts). Also, in both malignant and normal lines, the effect of cytotoxicity and inhibition of cell growth has a linear relationship with the extract concentration. Previous studies have shown the antitumor effects of Kouchner and its compounds on various malignancies, including breast carcinoma,[15] adenocarcinoma of the lung,[16] leukemia,[17] and SCC.[18]

Nam et al's[19] study showed that two combinations of coumaryl alcohol and stokis caucul acid are compounds of rhizome extract with cytotoxicity and tumor inhibitory effects against malignancies, including HL60 (human promyelocytic leukemia cells), HT1080 (fibrosarcoma cell line), and HCT116 (human colorectal carcinoma cell line).

Jaiswal et al[20] demonstrated that Galangin, which is one of the polyphenolic compounds in Kouchner, causes

Table 3: Summary (Tukey HSD) of statistical difference between the percentage of viable cells after exposure to different concentrations of Kouchner extract in normal cell line

Concentration of Kouchner extract (%)	*Subgroups based on statistical differences*			
	1	*2*	*3*	*4*
100	65.72			
75	73.50	73.50		
50		85.14	85.14	
25			89.95	89.95
12.5			94.18	94.18
6.25			96.7	96.7
3.125			98.30	98.30
Control				100

The numbers in each column are similar in terms of the percentage of viable cells after exposure to different concentrations of Kouchner extract; HSD: Honest significant difference

Table 4: Summary (Tukey HSD) of statistical difference between the percentage of viable cells after exposure to different concentrations of Kouchner extracts in malignant cell lines

Concentration of Kouchner extract (%)	*Subgroups based on statistical differences*	
	1	*2*
100	74.5	
75	85.22	75
50	88.08	88.08
25	90.93	90.83
12.5		92.73
6.25		94.73
3.125		96.44
Control		100

The numbers in each column are similar in terms of the percentage of viable cells after exposure to different concentrations of Kouchner extract; HSD: Honest significant difference

the destruction of 19 proteins involved in tumorigenesis, leading to the induction of apoptosis in malignant cells.

Murakami et al's[21] study showed that Stokes acetate Acetoxychavicol acetate (ACA) is one of the compounds of the Kouchner rhizome extract. It is an antitumor compound that inhibits carcinogenicity.

The results of the present study, according to Suja and Chinnaswamy,[22] Samarghandian et al,[15] Lee and Houghton,[13] and An et al,[23] indicated that the Kouchner rhizome extract inhibited the growth of tumor cell line compared with the normal cell line. However, in this study, the extract at concentrations of 20% or more led to cytotoxicity of cancer cells more than normal cells, while at concentrations lower than 20%, cytotoxicity of the extract on normal cells was equal to malignant cells. However, in the articles mentioned, at all concentrations the lethal effect and the growth inhibitory of the extract on malignant cell line tested has been more than the normal cell line.

Also, the study of Hadjzadeh et al[24] investigated the cytotoxicity of the Kouchner rhizome extract on gastric cancer cells. It was shown that this extract, at all concentrations, resulted in cytotoxicity and the inhibiting of the growth of both cell lines of malignant and normal alike. This study also demonstrated that concentrations below 20% of the Kouchner extract have a similar cytotoxicity on both malignant and normal line. However, unlike Hadjzadeh et al's study,[24] at concentrations above 20% of the extract the cytotoxicity effect on the malignant cell line was more than the normal cell line.

This difference in the results of this study with previous papers may be due to differences in the composition of the Kouchner extract used in this study, and also differences in the malignant cell line tested. Therefore, the growing season, geographic location, time, and duration of maintenance can affect the composition of the Kouchner extract.[25]

CONCLUSION

The results of this study showed that the cytotoxicity of the Kouchner extract at concentrations above 20% on malignant cell line SCC is higher than a normal cell line and is similar in both categories at concentrations below 20%. According to this study, the Kouchner extract may be used against SCC as a natural substance with targeted antimalignant properties and fewer side effects.

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