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## Effect of Transferulic acid on oral squamous carcinoma cells

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### Abstract

This study was aimed to investigate the anticancer effect of Transferulic acid (Tfr), a most abundant phenolic compound present in wheat, rice, oats, fruits like oranges, apples and its underlying mechanism. Oral squamous carcinoma cell line SCC-4 was treated with Tfr with (20-100µg/ml) concentrations for 24h and 48h. Cell proliferation, cells damage, nuclear fragmentation and chromatin disintegration was examined by MTT assay and DAPI fluorescence staining. Cell death percentage was measured by flow cytometric analysis after PI staining. Pro-apoptotic Bax and anti-apoptotic gene Mcl-1 expression was assessed using Real Time PCR. Tfr (20-100µg/ml) caused cytotoxicity in SCC-4 cells by enhancing apoptosis in a dose-dependent manner. Treatment of oral cancer cells with Tfr (60µg/ml) for 24h, 48 enhanced apoptosis as observed by morphological and cell death assessment in PI staining. Tfr (60µg/ml) significantly induced apoptosis through intrinsic pathway. Transferulic acid is a potent anticancer agent, which may be used as a chemotherapeutic agent.

**Keywords:** Oral cancer, Trans-ferulic acid, apoptosis

### Introduction

Oral cancer is still a major concern of global people health because of late diagnosis, high fatality and low cure rate. Cancer arises in oral cavity area are 90% of cancers of squamous carcinoma cells. Oral carcinogenesis is a complicated process that starts when epithelial cells get damage with various genetic changes. There are many risk factors known to cause oral cancer mainly includes tobacco and intake of alcohol and tobaccos [1]. Either because of genetically or exposure of carcinogens, cancer takes time to initiate, to promote and to progress. Currently available cancer treatment options have restricted success rate with lot of side effects. Since ancient time plants are being used to treat numerous diseases. Multiple studies have shown encouraging results in this emerging field of pharmaceutically important natural anticancer compounds research. Mechanism based research on plants derived natural compounds as anticancer agents provide an important alternative approach that can be effectively use to prevent, cure and minimise side effects of cancer therapies [2, 3]. here in this study we have examined one such natural phenolic compound i.e. Transferulic acid, a phenolic phytochemical, most abundant phenolic acid found in spinach, wheat bran, sugar beet and corn kernels. It has found to a potent antioxidant compound that is used as a preservative in food industry [4, 5]. It has been studied in lung cancer, cervical cancer and colon cancer but not yet explored in oral cancer [6]. Therefore we have studied the effect of Transferulic acid on oral squamous carcinoma cell line SCC-4

### Material and methods

#### Chemical Reagents and cell line

Transferulic acid, DMSO, propidium iodide (PI), ribonuclease-A purchased from Himedia, MTT assay kit, DAPI were obtained from Genetix. Revert Aid first strand Cdna synthesis Kit (K1622) from Genetix. Real Time PCR kit was purchased from Qiagen. RNA Sure® Mini Kit (NP-84105), Ethanol(PG-391-500ml), DMEM Media(SH30261.01), Penicilin Streptomycin Solution(PG-259-100ML), Trypan Blue solution, 04% in PBS(CC4021.010L), Fetal Bovine Serum, South American origin (CCS-500-SA-U), Phosphate Buffer Saline (CC3029) were obtained from Genetix.

#### MTT assay

SCC-4 cells were plated in 96 well plate at density of 1 million/well and grown for 24 hrs. Transferulic acid added at concentration (20, 40, 60, 80, 100, 120) µg/ml while control cells set were DMSO treated cells and cells without any chemical. MTT assay experiment was done after 24 h and 48 h of experiment. 20 µl MTT (5mg/ml in PBS) was added and incubated at

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37°C for 3h then it was removed from all wells. formazan crystals were dissolved with 200 µl of DMSO and incubated at 37°C for 10 min. Further we have recorded absorbance at 492 nm using ELISA microplate reader. The viability percentage was calculated and value that decrease half absorbance (50%) compared to untreated cells was considered as IC 50 that is 60µg/ml.

#### DAPI Analysis

DAPI (4',6-diamidino-2-phenylindole) emits blue coloured fluorescence as it binds to AT regions of DNA. SCC-4 cells were treated with 60µg/ml Transferulic acid for 24 and 48 hrs to determine morphological changes in oral squamous carcinoma cells. The cells were stained with DAPI (300 nanomole solution) incubated for 10 min after that washed with PBS. Finally, Images were taken and examined for cells

damage, nuclear disintegration and chromatin condensation.

#### Real Time Pcr

We have checked the expression levels of genes related to apoptosis pathway i.e. Pro-apoptotic gene- Bax, anti-apoptotic gene-Mcl-1 and beta-actin as housekeeping gene for normalization using BIORED CEFX96 qRT-PCR system (BioRed, USA). We have used following protocol: 10 µL reactions with 0.2 µM each primer, 10 µL SYBR Select Master Mix (2 x) and 1.5 µL template cDNA. Cycling conditions were: initial denaturation 1x 94°C for 3 min; amplifications were done for 40 cycles 93° C for 45 s; 58° C for 45 s; and 72° C 1 min and 1x 72° C 10 min. Relative fold-change of mRNA expression levels were examined by the comparative method that is  $2^{-\Delta CT}$  method and shown as mean  $\pm$  SD. All reactions were carried out in triplicates.

BAD-	
FP: TTCTGACGGCAACTTCAACT	RP: CAATGTCCAGCCCATGAT
Mcl-1-	
FP: TGAAATCGTTGTCTCGAGTGA	RP: TCACAA TCGCCCCAGTTT
Beta-actin -	
FP: TGAGAGGGAAATCGTGCGTG	RP: TGCTTGCTGATCCACATCTGC

#### FACS-PI analysis

Flow cytometry method is a fast and reliable model to quantify viable cells in a cell suspension. Propidium iodide is a membrane impermeant dye that is normally excluded from viable cells. In this experiment we have assessed Cell viability using propidium iodide (PI).  $2 \times 10^5$  cells were treated with PBS (as control) and *trans*-FA concentration 60µg/ml for 48 h. cells were detached using cell scraper and washed with PBS then fixed with 75 % ethanol overnight. After washing cell pellets were stained with 1µg/mL PI and 1µg/mL RNase A in PBS buffer for 30 min at 37 °C in the dark. The samples

were analysed using FACSLSR 2 flow cytometer (Becton–Dickinson Jamia India) and the results were assessed using Flowjo 6.3.3 software.

#### Results

##### MTT assay

To determine whether Transferulic acid has any effect on proliferating cells, SCC-4 cells were treated for with various concentrations i.e. (20, 40, 60, 80,100) µg/ml and analysed for cytotoxicity. We have found that 60 µg/ml significantly reduced proliferation while increasing apoptosis at 48 hr.

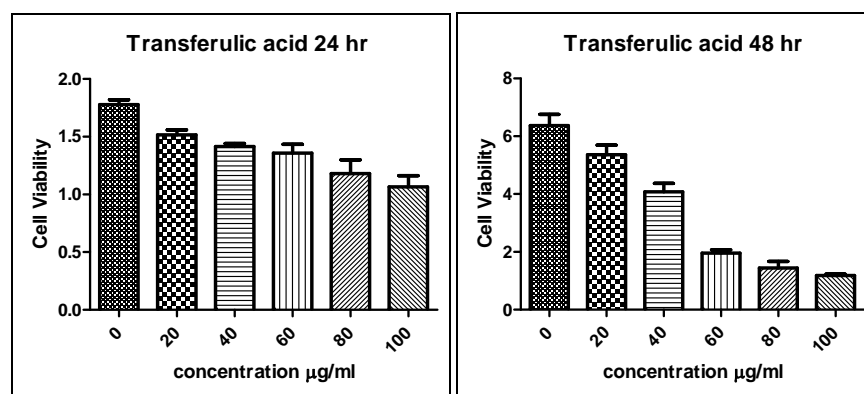
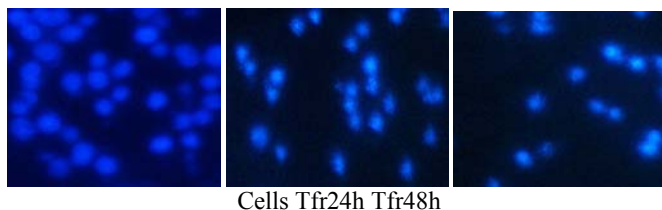


Fig 1: Cytotoxic effect of Transferulic acid on SCC-4 cells as analysed by MTT assay.

#### Assessment of apoptosis using DAPI staining by fluorescence microscopy

In this experiment, DAPI staining is used to assess the effect of Transferulic acid i.e. 60µg/ml on oral carcinoma cells SCC-4 for 24h and 48h with untreated cells as control. It has been observed that apoptotic cells nuclei and apoptotic bodies

appears bright blue fluorescent bodies while control cells nuclei appears uniformly stained as normal blue in colour with round margins. In Transferulic treated cells, nuclear appears damaged and brightly stained with condensed chromatin at 24h and 48h. Cells death was significantly higher at 48h Transferulic acid (Tfr) treated SCC-4 cells.

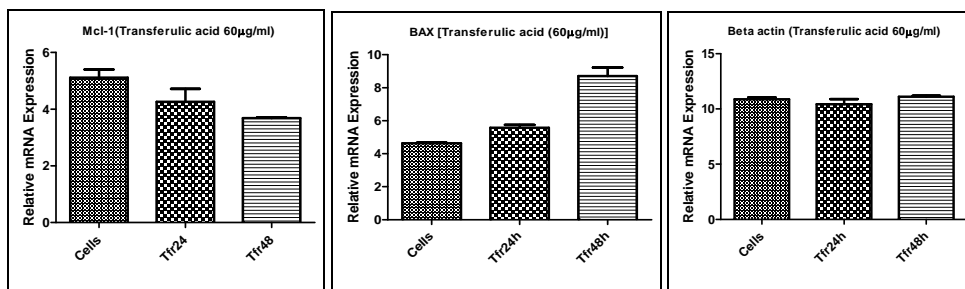


**Fig 2:** Morphological analysis: Transferulic acid (60µg/ml) inducing apoptosis in SCC-4 cells i.e. disintegrated nucleus, damaged cells and chromatin condensation as shown in figure.

**Quantitative expression of pro-apoptosis and anti-apoptosis gene**

BAX and mcl-1 gene play an important role in programmed cell death maintenance. Various studies explained that Mcl-1, an anti-apoptotic gene is overexpressed in oral cancer cells. It

is also found to be responsible for chemotherapy resistance whereas Bax, a pro-apoptotic gene expression is found to be decreased or insufficient in cancer cells compared to normal healthy cells.



**Fig 3:** Transferulic acid downregulates Mcl-1 and upregulates BAX gene expression in oral carcinoma cells. Beta-actin gene expression used to normalise results in Transferulic acid treated SCC-4 cells.

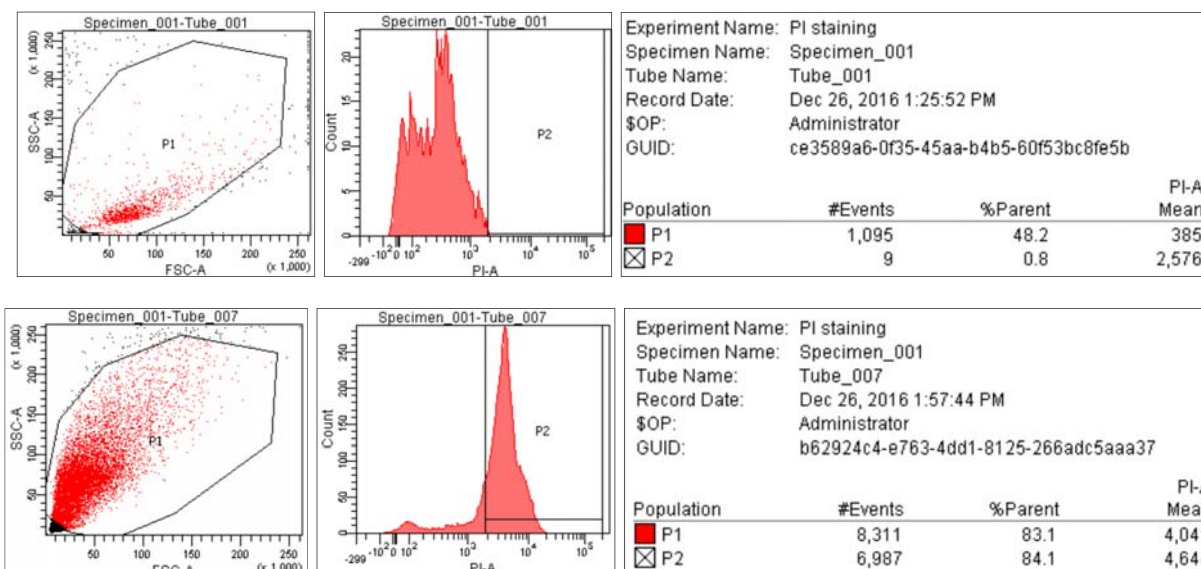
Our results found that the overexpression of MCL-1 was decreased by Transferulic acid treatment along with the increased expression of Bax in SCC-4 cells, suggesting that Tfr reduces the anti-apoptotic action of Mcl-1 and released more pro-apoptotic gene products like Bax, which is involved in apoptosis process. Thus Transferulic acid activates the proteolytic cascade to trigger apoptosis of oral cancer cells SCC-4.

**Effect of Transferulic acid on cell death**

Propidium iodide (PI) is a fluorescent dye that intercalates into double-stranded nucleic acid. It is excluded from viable

cells, but can penetrate cell membranes of dead or dying cells. Therefore, it is widely used for evaluation of cell death and apoptosis or for determination of DNA content in cell cycle analysis. The fluorescence emission maximum for DNA-bound PI is about 615–620 nm.

The induction of cell death was examined by PI staining by FACS analysis in the oral cancer cell line SCC-4 after Transferulic treatment. Our Results revealed that Transferulic acid effectively induced cell death in oral carcinoma cells at 48h. The cell death induced in SCC-4 was 84.1% at 48h compared to control cells 0.8% at 48h.



**Fig 4:** Cell death assessment by PI staining; It was found that in the presence of the Transferulic acid treatment, the proportion of cells being stained by PI increased distinguishably in SCC-4 cells as compared with the control cells at 48 h.

## Discussion

Transferulic acid, a neutraceutical compound with broad range of phytochemical function has been assessed as anti-oxidative, anti-inflammatory, and antidiabetic. Its antiproliferative significance has been studied in cervical cancer, colon cancer and lung cancer but not yet in oral cancer [6-10]. This is the first study to investigate the effects of Transferulic acid on oral squamous carcinoma cells.

We examined this potent anti-oxidant natural compound for cytotoxicity analysis using MTT assay, apoptosis assessment by morphological observation and PI staining using flow cytometry and our results revealed that Transferulic acid (60µg/ml) enhances the programmed cell death in SCC-4 cells effectively at 48hr.

We next confirmed its apoptosis inducer property by quantitative analysis of anti-apoptotic gene Mcl-1 and pro-apoptotic gene Bax. We have shown that Transferulic acid potentially down regulated cancer promoting gene Mcl-1 while up regulated apoptosis inducing gene Bax. Transferulic acid, a polyphenol natural compound shows a promising therapeutic strategy because of to their ability for reducing toxicity.

Thus we conclude that Transferulic acid, exerting apoptosis inducing properties via mitochondrial intrinsic pathway, is a potent anticancer polyphenol compound.

## Conclusion

In conclusion, the idea was to explore effect of Transferulic acid on mitochondrial apoptosis pathway in oral squamous carcinoma cells. We wanted to explore this phenolic phytochemical for its targets leading to apoptosis induction. We have found that Transferulic acid inducing apoptosis via down-regulating (Mcl-1) anti-apoptotic gene expression and up-regulating pro-apoptotic BAX gene expression. Morphological and flow cytometric assessment results of apoptosis induction by Transferulic acid confirmed the same. Our results suggested that Transferulic acid significantly induced apoptosis in human oral cancer cell line.

This natural antioxidant has been explored for its anticancerous, anti-migratory and various neutraceutical properties that make it a potent candidate to be useful as single agent for cancer prevention, treatment, chemoprevention, or in synergistic therapies. In future, more studies are required to reveal mechanism behind anticancer property of Transferulic acid in various types of cancer in vitro and in vivo including oral cancer.

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