Cytotoxic effect of matcha green tea and green coffee on oral squamous cell carcinoma cell line. A comparative study

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Abstract:

Objectives: This study was performed to compare the cytotoxic effect of matcha green tea (MGT) and green coffee (GC) on tongue squamous cell carcinoma (TSSC) cell line through the expression of p53 and Bcl-2 apoptotic genes. **Materials and methods:** TSSC cell line was treated with increasing doses of MGT and GC. IC50 values for MGT and GC extracts were determined. Untreated cells were used as a control. Annexin V staining was used to study apoptosis of cancer cells after treatment with MGT and GC while PCR was used to investigate the expression of p53 and Bcl-2. **Results:** MTT assay showed that MGT and GC are toxic to SCC-15. MGT showed a higher cytotoxic effect than GC. The IC50 for MGT at 24 and 48 h were 33 and 15 µg/ml, respectively. The IC50 value for GC at 24 and 48 h were 73 and 40 µg/ml, respectively. The percentage of apoptotic cells was increased significantly in cancer cells treated by MGT and GC compared to control as detected by annexin V staining assay. Our results demonstrated that MGT and GC treatment could significantly increase p53 protein expression; and suppress Bcl-2 compared to the control. MGT showed higher p53 and decreased Bcl-2 expression compared to GC. **Conclusion:** Our findings indicate that MGT and GC targeted oral cancer cells. They reduce cell viability and induce apoptosis. We also observed for the first time that MGT is superior to GC in suppression of TSSC cells which implies great potential for use in the field of carcinogenesis.

Keywords: Tongue squamous cell carcinoma, MTT assay, Apoptosis, PCR.

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1. Introduction

Oral cancer is a main malignant tumor of the head and neck region (1) . Oral squamous cell carcinoma (OSSC) is mostly detected late, with a low survival rate and poor prognosis (2). Its treatment is still challenging, even with the advancements in immunotherapy and targeted therapy (3). However, the unfavorable side effects drove researchers to develop novel therapeutic strategies with reduced side effects and enhanced chemo-preventive qualities and anti-carcinogenic properties (1).

Several bioactive compounds found in natural products proved to be effective in reducing cancer cell viability without affecting the non-cancer cells. This has raised the interest in cancer research to benefit from their effects against cancer (4).

Coffee and green tea (GT) are common worldwide beverages and have been investigated for their anticancer properties (5). Polyphenols present in coffee and GT exhibit antioxidant, chemo-preventive and chemotherapeutic effects by preventing the development and progression of malignant cells. Epigallocatechin-3-gallate (EGCG) represents the most bioactive phenolic component of GT(4).

Matcha is a powdered form of Japanese green tea, thus the leaf is utilized entirely, unlike other forms of loose-leaf tea, in which the extraction of the soaking leaves is utilized (6). Matcha green tea (MGT) is remarkably rich in antioxidants and many bioactive compounds with anti-cancer properties (7) However, there is not enough research that has investigated the effects of these compounds in whole matcha (6).

Coffee is a beverage that contains a variety of phytochemical compounds including polyphenols as Chlorogenic Acid (CGA), cafestol and caffeine (8). The presence of numerous antioxidant substances in coffee has been suggested to suppress the proliferation of malignancy. Green Coffee (GC) represents an unroasted coffee type. It consists of a variety of phytoconstituents with varying physical and chemical properties (1).

The molecular pathways that are implicated in the physiopathology of cancer are quite limited. However, tumor-suppressor proteins, including p53, are a promising area of oncology research. P53 is implicated in a multitude of anticancer mechanisms, such as the activation of apoptosis and cell cycle arrest. Furthermore, p53 is regarded as a chemotherapeutic target of polyphenols (9). On the other hand, the anti-apoptotic protein

Bcl-2 has been linked to the chemoresistance of malignancies. and so, inhibits apoptosis and promotes cancer cell survival (3). There have been very few studies on the influence of green coffee on OSCC and as far as we know, the anti-carcinogenic effect of matcha green tea against OSSC is not adequately investigated.

Additionally, no prior study has compared the cytotoxic effect of MGT and GC on OSSC cell line. Thus, the present work was performed to compare the cytotoxic effect of MGT and GC on tongue squamous cell carcinoma cell line through the expression of Bcl-2 and p53 apoptotic genes.

We selected tongue squamous cell carcinoma (TSCC) cell line for analysis in this work as it is the most aggressive form of oral cancer which requires new therapeutic approaches with limited side effects (10).

2. Materials and methods

This work was performed at the cell culture department, VACSERA, Egypt. It was approved by the Medical Ethical Committees, Ahram Canadian University.

2.1. Matcha green tea and green coffee preparation

One hundred grams of commercial brand of powdered raw green tea (Matcha) and powdered green coffee were obtained from a local market in Egypt and soaked separately with ethanol. The mixture was allowed to steep for 15 to 30 minutes to obtain the bioactive ingredients from the MGT and GC. After steeping, the mixture was filtered to eliminate any solid elements. Different concentrations of the MGT and GC extracts were prepared by diluting them in appropriate solvent (phosphate buffered saline).

2.2 Cell Culture

We used in this study human commonly utilized TSSC cell line (SCC-15). They were received from the American Type Culture Collection (ATCC®CRL-1628, USA).

All other chemicals employed in this study were received from (Gibco, Grand Island, NY, USA) including MTT.

Cells were cultured in a monolayer in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, NY) containing 1% penicillin, 10% fetal bovine serum (FBS) and streptomycin (PS) (Gibco, Grand Island, NY, USA) with 5% CO2 atmosphere and 37 °C. The medium was

refilled every two days. All the following experiments were conducted in triplicate.

2.3 Cell Viability Assay

MTT assay (3-(4, 5- dimethylthiazol-2-yl)-2, 5-dipheny ltetrazolium bromide) was used to assess the cell viability as directed by the manufacturer.

96-well plates were used for seeding of cancer cells at a density of 1×10^6 cells/well. Cells were subjected to a series of MGT and GC concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µg/ml). Incubation was allowed for 24 and 48 h. Untreated cells served as a control. 50 µl of MTT (2 mg/ml) was added after the incubation. At 600 nm wavelength, the optical density was detected after 3 h. The half-maximal inhibitory concentration (IC50) for MGT and GC was determined based on MTT results. The IC50 value is the extract concentration which induces a 50% decrease in cell viability.

2.4 Annexin V-FITC staining assay for apoptosis

Annexin V staining (KeyGen Biotech, Nanjing, China) was used to study cancer cells apoptosis after incubation with MGT and GC. It was done according to the protocol of the manufacturer. The malignant cells were treated with IC50 values of MGT and GC for different time intervals (24, and 48 h). The control group was left without any treatment. The treated cells were subsequently stained with propidium iodide (PI, 10 µg/ml) and annexin V-FITC. Flow cytometry was used to detect the apoptotic cells percentage by FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5 Real-Time Polymerase Chain Reaction (RT-PCR) for expression of p53 and Bcl-2

1×10^5 cancer cells/well were cultured in a six well culture plate. Grown cells were treated with IC50 values of MGT and GC for 24 and 48 hours. Cells were trypsinized, harvested and centrifuged. RNA extraction was done using Thermo Fisher Scientific Inc. Germany (Gene JET, Kit, #K0732) following the manufacturer's instructions. Real-time PCR was used to detect genes expression.

1000 ng of the total RNA from each sample was used for cDNA synthesis by reverse transcription. Changes in the expression of each target gene were normalized relative to the mean critical thresh- old (CT) values of the β-actin RNA housekeeping gene.

Primers sequences used were as follows:

Bcl-2 F 5’-ATGTGTGTGGAGACCGTCAA-3’,

Bcl-2 R 5'-GCCGTACAGTTCCACAAAGG-3'

p53 F 5’-ATGTTTTGCCAACTGGCCAAG-3’

p53 R 5’-TGAGCAGCGCTCATGGTG-3’

β-actin F 5’-GCACCACACCTTCTACAATG-3’

β-actin R 5’-TGCTTGCTGATCCACATCTG-3’

2.6 Statistical analysis

The statistical analysis was carried out using SPSS 16 ® (Statistical Package for Scientific Studies), windows excel and Graph pad prism. Data of all groups were presented as mean and standard deviation. Exploration of the given data was performed using Shapiro-Wilk test and Kolmogorov-Smirnov test for normality which demonstrated that all data is obtained from a normal distribution (parametric data) similar to the typical Bell curve. Consequently, a comparison was conducted between various groups using One Way ANOVA test and the Tukey's Post-Hoc test was employed for multiple comparisons.

3. Results

3.1. Effect of MGT and GC on cell viability

The MTT cell viability assay showed that MGT and GC are toxic to SCC-15 in a time- and dose- dependent manner. MGT showed a higher cytotoxic effect than that of GC. The IC50 for MGT at 24 and 48 h were 33 and 15 µg/ml, respectively. While the IC50 value for GC at 24 and 48 h were 73 and 40 µg/ml, respectively (Figure 1).

the MTT assay of MGT and GC showed the highest toxicity effect on SCC-15 after 48 h of treatment compared with 24 h of incubation. We used IC50 values of MGT and GC for the following experiments.

3.2. Assessment of Apoptosis Using Annexin-V FITC/PI Dual Staining Flow Cytometry Assay

Since treatment with MGT and GC led to reduced viability of cancer cells, we examined Induction of apoptosis by annexin V staining assay. Our results showed that the percentage of apoptotic cells was increased significantly in cancer cells treated by IC50 dose of MGT and GC compared to the control (Table 1), (Figure 2). In addition, the cytotoxicity of the extract increases with the increase in the time of incubation.

Comparison between apoptotic cell percentage in SCC-15 cell line in different groups showed that there was a significant difference between them as P<0.001, followed by multiple comparisons which showed that control group demonstrated significantly the lowest apoptotic cell percentage, followed by GC after 24 hours, then MGT after 24 hours, then, GC after 48 hours, while MGT after 48 hours demonstrated significantly the highest apoptotic cell percentage (Figure 3).

3.3. PCR results

To elucidate the molecular mechanism by which MGT and GC trigger cancer cells apoptosis, PCR was used to investigate the expression of apoptotic regulatory proteins p53 and Bcl-2 in cancer cell line treated with IC50 of GC and MGT for 24 h and 48 h compares to the control untreated cell line. Our results demonstrated increased p53 expression and decreased Bcl-2 expression after treatment with MGT and GC compared to the untreated cells. The difference was statistically significant.

The effect of MGT and GC on the expression of p53 and Bcl-2 was time-dependent as treating cells for 48 hours decreased their Bcl-2 expression and increased their p53 when compared with the cells treated for 24 hours.

When comparing MGT and GC, p53 was higher in cells treated with MGT than that treated with GC while Bcl-2 decreased in in cells treated with MGT than that treated with GC. The difference was statistically significant (Table 2).

Comparison between p53 and Bcl-2 expression in SCC-15 cell line in different groups showed that there was a significant difference between them as P<0.001, followed by multiple comparisons which showed that: In p53, control group demonstrated significantly the lowest expression, followed by GC after 24 hours, then MGT after 24 hours, and GC after 48 hours, while MGT after 48 hours demonstrated significantly the highest expression. On the other hand, in Bcl-2, control group demonstrated significantly the

highest expression, followed by GC after 24 hours, then MGT after 24 hours and GC after 48 hours, while MGT after 48 hours demonstrated significantly the lowest expression (Figure 4).

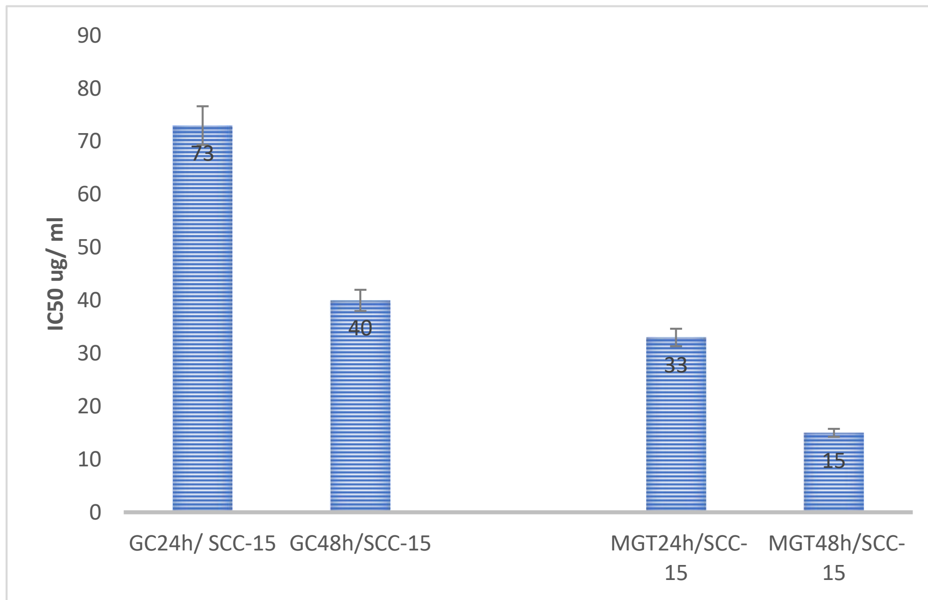

Figure (1): IC50 values for MGT and GC, after 24 h and 48 h of incubation.

Table (1): Average percentage of apoptotic cell in SCC-15 cells incubated with MGT and GC compared to control

Control		MGT				GC				P value
		24 h		48 h		24 h		48 h		
M	SD	M	SD	M	SD	M	SD	M	SD	
2.93 [a]	0.07	18.59 [b]	0.11	33.0 [c]	0.02	7.86 [d]	0.07	11.03 [e]	0.03	<0.0001*

**Significant difference (P<0.05).*
Significant difference for means with different superscript letters (P<0.05).
Insignificant difference for means with same superscript letters (P>0.05).

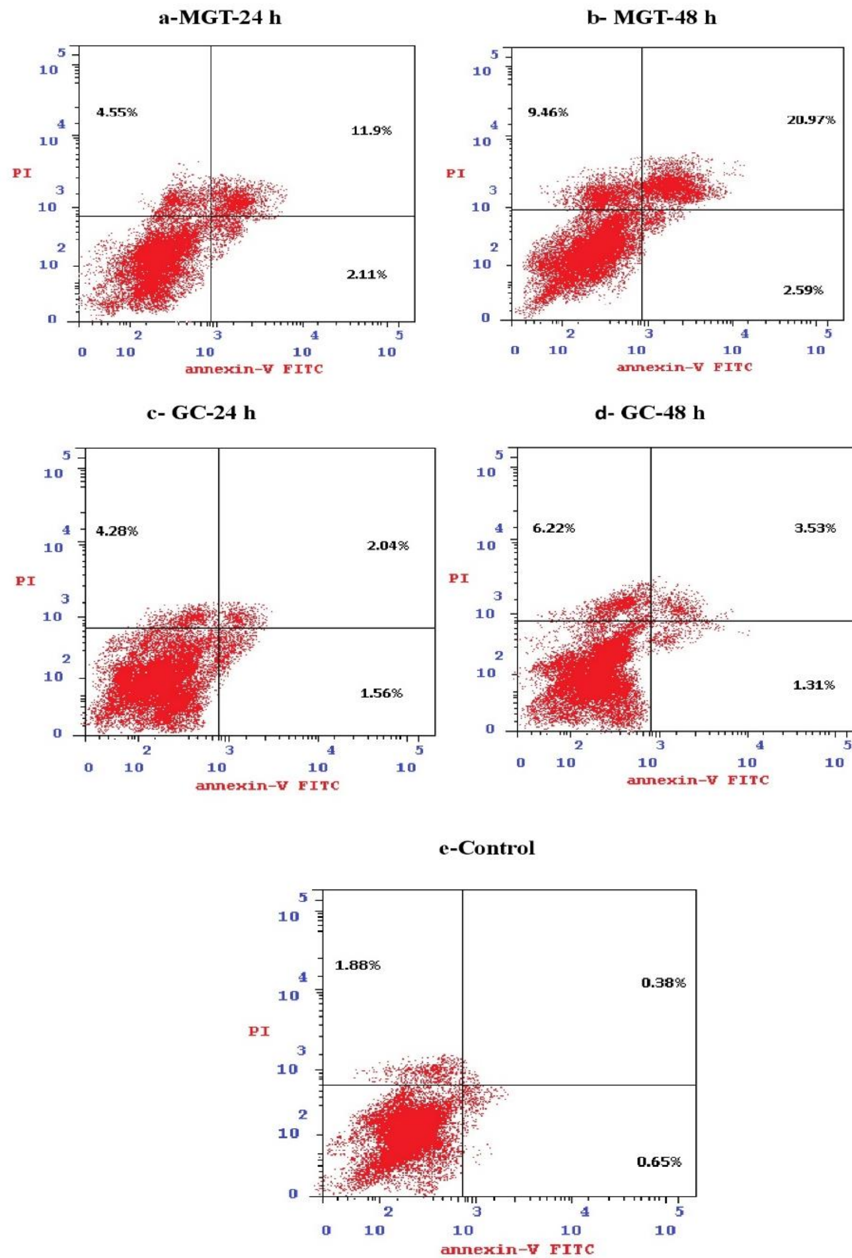

Figure (2): Annexin V assay of SCC-15 cells incubated with MGT and GC for 24 h and 48 h compared to control. Early apoptotic cells are shown in the lower right quadrant and late apoptotic cells in the upper right quadrant. Necrotic cells are represented in the upper left part and viable cells in the lower left part.

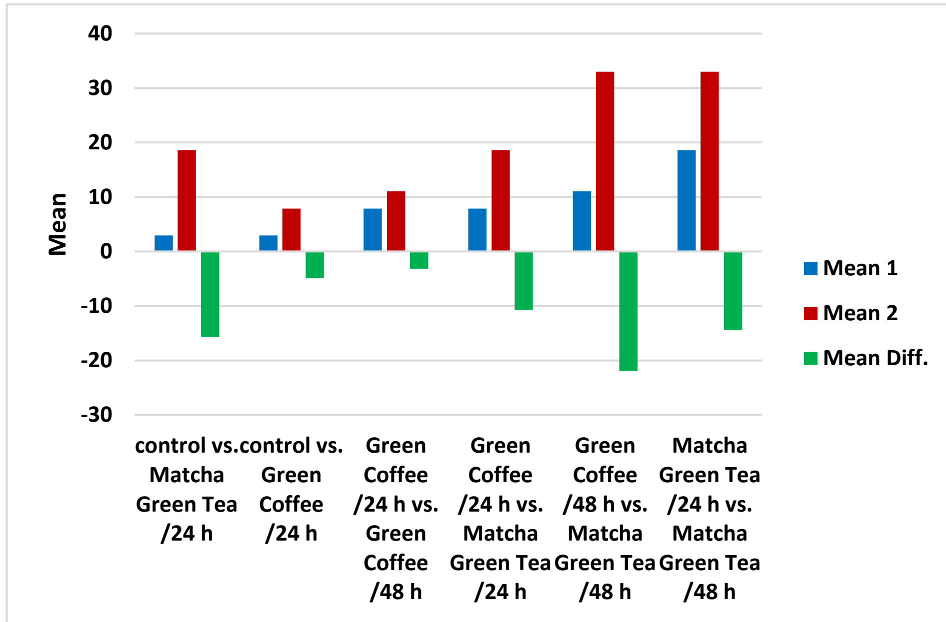

Figure (3): Bar chart showing multiple comparisons between different groups regarding percentage of apoptotic cell in SCC-15.

Table (2): Comparison of p53 and Bcl-2 expression in SCC-15 cells after treatment with IC50 concentration of MGT and GC relative to time and compared to control

	Control		MGT				GC				P value
			24 h		48 h		24 h		48 h		
	M	SD	M	SD	M	SD	M	SD	M	SD	
p53	0.280 a	0.010	2.047 b	0.083	5.147 c	0.148	0.850 d	0.053	2.135 b	0.156	<0.0001*
Bcl-2	1.233 a	0.107	0.511 b	0.011	0.204 c	0.007	0.796 d	0.007	0.515 b	0.006	<0.0001*

**Significant difference (P<0.05).*
Significant difference for means with different superscript letters (P<0.05).
Insignificant difference for means with same superscript letters (P>0.05).

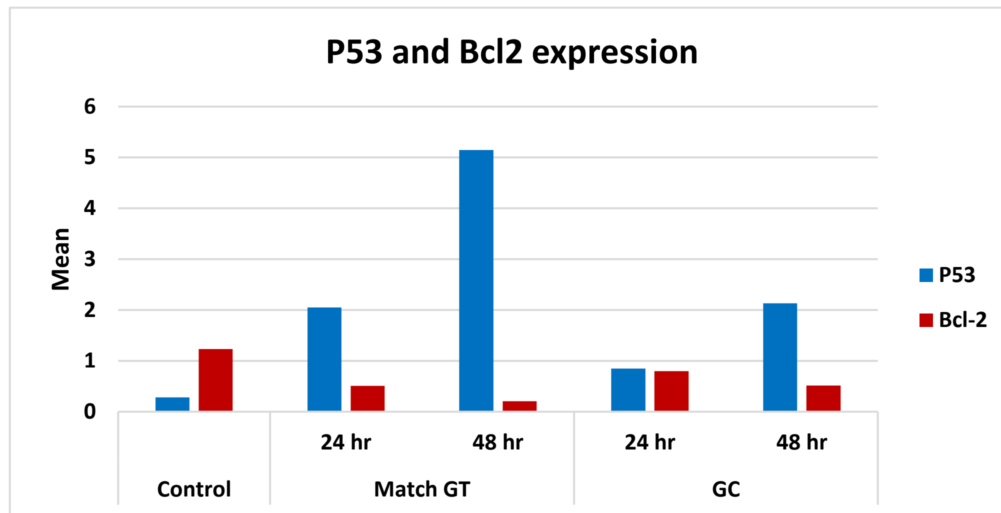

Figure (4): Bar chart showing comparison of p53 and Bcl-2 expression in SCC-15 after treatment with IC50 concentration MGT and GC relative to time and compared to control.

4. Discussion

Carcinogenesis is a multistage and complicated process, and its regulatory mechanisms are not completely comprehended (11).

The antitumor properties of GT polyphenols, particularly their primary compound, EGCG has previously reported in various studies (4). However, rather than using isolated catechins, it is crucial to understand their effects in dietary products to understand its bioavailability and antioxidant potential. Though it is important to conduct studies that fill this gap (12) .

Numerous studies have compared matcha, the powdered form of green tea, to the classic loose-leaf type of green tea. The results revealed that matcha may act differently owing to its greater polyphenol and antioxidant levels (13, 14).

A previous study on coffee discovered that phenolic chemicals, particularly CGA are more efficient in triggering cell apoptosis than caffeine. GC has a higher total phenolic content than other types of coffee, which promotes a greater cytotoxic impact on TSSC cell line. (1).

So far, there are limited publications that discuss the potential cytotoxic effect of GC on OSSC. The antioxidant effect of GC necessitates additional exploration, particularly in oral cancer, and to the best of our knowledge, there is no previous work about the effect of MGT on OSCC cell lines.

Additionally, no prior study has compared the cytotoxic effect of MGT and GC on OSSC cell line. Thus, the current study was performed to compare the cytotoxic effect of matcha green tea and green coffee on tongue squamous cell carcinoma cell line through expression of p53 and Bcl-2 apoptotic genes.

First, the MTT test was carried out to assess the impact of MGT and GC extracts on the viability of SCC-15 cell line. In this work, we found that MGT and GC reduce proliferation of TSCC cells in a time- and dose-dependent manner. The IC50 for MGT at 24 and 48 h were 33 and 15 µg/ml, respectively. While the IC50 value for GC at 24 and 48 h were 73 and 40 µg/ml, respectively.

Our findings revealed that MGT and GC extracts can suppress TSCC cell proliferation in vitro at concentrations that are consistent with other investigations (15, 16).

The results of Rashad et al. (1) revealed that the viability of the tongue squamous cell carcinoma cell line was significantly reduced in a dose-dependent manner after GC extract treatment. This could be related to the elevated level of phenolic content in GC extract. In this regard, Rao et al., (17) reported that GC had a strong cytotoxic impact on ovarian and cervical cancer cell lines.

On the other hand, MGT inhibits cancer cell growth in cell lines of diverse origins (18,16,19). In addition, previous studies revealed an inhibitory effect of EGCG on OSCC cell proliferation in a time- and dose-dependent way, especially after treatment for 48 hours (5,16, 20). All these previous data supported our findings regarding the effect of MGT and GC on the viability of SCC-15 cell line.

In the current work, MGT showed a higher cytotoxic effect compared to GC and it was necessary to evaluate if SCC-15 cell death was provoked by apoptosis. So, we examined the apoptotic potential of MGT and GC utilizing the Annexin V-FITC/PI test.

In our study, we detected a significantly elevated percentage of apoptotic cells in cancer cells treated by IC50 dose of MGT and GC in comparison to untreated control cells. Moreover, the percentage of apoptotic cells increased with increasing the time of incubation of MGT and GC from 24 h to 48 h.

Our findings can be justified by Rashad et al., study (1) in which TSSC cell line treated with GC showed the largest proportion of apoptotic cells than other coffee type. In addition, the antiproliferative impact of

coffee extracts was detected in prostate cancer cell lines (21).

Regarding MGT, a previous study on retinoblastoma cancer cell line concluded that , MGT can trigger cell death via apoptosis (13). In addition, other studies have found that green tea polyphenols induce apoptosis in prostate and breast cancer cell lines (4, 22).

Nevertheless, our results confirmed that MGT and GC inhibit TSSC cell proliferation by triggering apoptosis where MGT demonstrated significantly a higher apoptotic cell percentage than GC.

Apoptosis is a highly controlled process that involves several anti- and pro-apoptotic proteins. The chemoresistance of malignancies has been linked to the overexpression of anti-apoptotic proteins whereas elevated levels of pro-apoptotic proteins induce apoptosis and render tumor cells more susceptible to a variety of anticancer therapies. In this setting, the balance between pro- and anti-apoptotic proteins affects whether cancer cells undergo apoptosis (4).

However, to investigate the potential pathways by which MGT and GC induce apoptosis, we investigated their impact on the expression of two apoptosis-regulating proteins p53, Bcl-2.

The Bcl-2 gene is responsible for the suppression of apoptosis and the enhancement of cancer cell survival (23). On the other hand, p53 gene is a tumor suppressor gene that promotes apoptosis by inducing a permanent cell cycle arrest where there is an inverse relationship between p53 and Bcl-2. Moreover, cell proliferation relies on the balance between cell survival regulators such as Bcl-2, and cell death regulators such as p53 (24).

The current study revealed a significant increase in p53 expression and a significant reduction in Bcl-2 expression in cancer cells treated by MGT and GC compared to the untreated control group.

Moreover, our results revealed that p53 protein expression is increased; and Bcl-2 expression is decreased when increasing the time of incubation of MGT and GC from 24 h to 48 h. The difference was statistically significant.

When comparing MGT and GC treated malignant cells, we found that p53 was higher in cells treated with MGT than that treated with GC while Bcl-2 decreased in in cells treated with MGT than that treated with GC. The difference was statistically significant. These findings could reflect a higher apoptotic potential of MGT compared to GC.

Our results regarding p53 and Bcl-2 expression in cancer cells treated with MGT and GC could be justified by the results of previous studies done on GT and GC. In previous studies, coffee was found to promote apoptosis that is increased with increased p53 expression (1, 25).

In addition Santos et al., (4) showed that GT obtained from the food matrix could reduce the viability and migration of breast cancer cells by modulating p53 levels. Moreover, it was found that EGCG promotes apoptosis in HNSCC cell lines. This could be related to an upregulation of the proapoptotic proteins and downregulation of the antiapoptotic proteins including Bcl-2 proteins (26).

Our data, along with the published results collectively indicate that MGT and GC exhibit an essential anticancer effect. However, additional research is necessary to fully understand the pharmacologic impacts of MGT and GC and to validate the potential relevance of MGT and GC as adjuvant in cancer treatment. A further in-depth investigation should be undertaken to determine the best treatment dose of MGT and GC.

Our study demonstrated for the first time that MGT exerted a more apoptotic effect on oral cancer cells than GC rendering it a potential chemo-preventive and chemotherapeutic agent for the treatment of oral cancer.

5- Conclusions

Our findings indicate that MGT and GC targeted oral cancer cells, though reducing cell viability and induce apoptosis. We also observed for the first time that MGT is superior to GC in suppression of TSSC cells which implies great potential for use in the field of carcinogenesis.

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Conflict of interest

There is no conflict of interest.

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