EGYPTIAN
DENTAL JOURNAL
FOR RESEARCHERS

WWW.EDA-EGYPT.ORG edjr

VOL. 4, 525:532, APRIL, 2020

PRINT I.S.S.N 2682-4752 • ONLINE I.S.S.N 2682-4760

EFFECT OF APIGENIN AND APIGENIN LOADED NANOPARTICLES ON SQUAMOUS CELL CARCINOMA CELL LINE (AN-IN VITRO STUDY)

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ABSTRACT

Background: Apigenin has effective cytotoxic anticancer properties and was used for treatment of variety of malignancies.

The aim of the study is to investigate the effect of Apigenin (Apig) and apigenin loaded nanoparticles (Apig NPs) on squamous cell carcinoma cell line (HEP-2).

Material & method: Treatment of cell line (HEp-2) by different concentrations of Apig and Apig NPs was done to assess the viability of the treated cells to determine the percentages of viable cells that was monitored using MTT assay.

Results: The mean viability percentage of Apig treated cells (in relation to control cells) decreases from 103.69% to 14.41% as Apig concentration increased from 1.22 to 2500 mM with an IC_{50} of 51.46 mM and Apig NPs decreased the cells viability from 98.11% to 12.25% as Apig NPs concentration increased from .24 mM to 500 mM with an IC50 of .89**.** Morphometric analysis showed a decrease in nuclear area factor (NAF) which is an indicator of apoptosis as concentrations increased. The results were statistically significant.

Conclusion: Apig and Apig NPs have a significant anticancer effect on Hep-2 cell line.

KEY WORDS: Apigenin, Apigenin loaded Nano particles, oral squamous cell carcinoma cell line (HEp-2), MTT assay.

Abbreviations: Apigenin (Apig), Loaded Nano particles (NPs), nuclear area factor (NAF), Squamous cell carcinoma (SCC)

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INTRODUCTION

Head and neck cancer is one of the life threatening problems and is accredited to high percentage of deaths worldwide. Squamous cell carcinoma (SCC) of head and neck encompasses the majority of head and neck cancers; it represent the sixth most common malignancy The etiology of SCC is multifactorial including genetic, social, behavioral, environmental risk factors and Human Papilloma Virus infection (Solomon, B. *et al*., 2018) & (Seiwert, T. Y. *et al*., 2015)

Advanced stage HNSCC is associated with poor prognosis, and the median overall survival is 1-year. This poor outcome evokes the need for novel treatment options. For many years, chemotherapy has been the backbone of treatment for patients with unresectable HNSCC. However, different adverse effects of the chemotherapeutic agents create limits in the treatment of HNSCC cancer. (Tang, D. et al., 2017)

Recent studies have shown that molecules as flavones can be used as safer anticancer drugs as they have low toxicity and non-mutagenic properties in the human body. (Winquist, E. et al., 2017) .

Apig, a naturally plant flavone copiously presents in fruits as oranges, grapefruit and vegetables as parsley, tea and chamomile has gained particular interest in recent years as a cancer chemo-preventive agent owing to its potent antioxidant, anti-inflammatory activities, anti-mutagenic and low intrinsic toxicity(Sharma, H. et al., 2014)(Bao, Y. Y. et al., 2013).

Apig inhibited cancer cell proliferation by triggering cell apoptosis, inducing autophagy and modulating the cell cycle. Apig also decreases cancer cell motility and inhibits cancer cell migration and invasion. Recently, Apig was reported to show anticancer activities by stimulating an immune response (Bao, Y. Y. et al., 2013;Shankar, E. et al., 2017).

The Nano-emulsifying systems are a promising nanoscale approaches that offer an effective delivery systems for bioactive components. Premeditated for hydrophobic drugs due to the increase of the effective particle surface area, and hence improve bioavailability (Karim, R. et al., 2017).

MATERIAL AND METHOD

A. Cell line: Originating from Homosapiens (Human), confirmed as human by immunofluorosence and cytotoxic-antibody test (dye exclusion), HNSCC Cell line HEP-2 obtained from cell Culture Department VACSERA- EGYPT. HEp-2 cells imported from the "American type Culture Collection (ATCC). Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS) 2 mM glutamine and sodium bicarbonate (Invitrogen, USA).

B. Fetal bovine serum (FBS): FBS was purchased from GIBO COBRAL limited, Scotland as a sterile serum in 500 ml bottle (stored at -20C till used).

C. Phosphate buffer saline (PBS): It was obtained from Sigma Aldrich USA. The pH adjusted by using HCI or NaOH, PBS was dissolved and sterilized by autoclaving for 20 minutes at 4°C.

D. Trypsin solution: 0.25% in PBS, pH 7.5 obtained from cell culture Media Department VACSERA- EGYPT.

E. Absolute ethanol :(Sigma Aldrich- USA)

F. 70% ethanol in distilled water (BDH- England)

Drug: Apig was purchased from Med Chem Express and was dissolved in pure DMSO using orbital shaker (vortex).The Nano particles were prepared using caprylic capric triglycerides and solutol HS and phospholipids using the phase inversion technique producing Nano-emulsions in the size range from 50-60 nm.

Cell culture and drug treatment:

HEp-2 cells were grown in the recommended medium with 10% FBS and incubated in 5% CO2 at 37°C for 24 hours with 10% FBS. The cells were treated with different concentrations of Apig and Apig NPs and maintenance medium containing DMSO as a negative control was considered cells were incubated at 37c for 24 hours.

MTT assays was performed to evaluate the effect of different concentrations of Apig and Apig NPs on HE-p2 cells. Microscopic examination of hematoxylin and eosin stained slides: Using a digital video camera of light microscope in Oral Pathology Department, Minia University at the power of 1000X then analysis of the images by image analysis software (Image J, 1.27z, NIH, USA). The surface area and nuclear circularity were measured. Nuclear area factor (NAF) was calculated using the formula: NAF= Circularity × Object area.

Statistical analysis:

The mean values of nuclear area factor (NAF) of different concentrations of the drug and their combinations to the control results were assessed statistically using the statistical package for social science (SPSS 16.0) software for windows. The statistical tests performed included analysis of Variance (ANOVA) for comparison of means of different concentrations of any definite drug and comparison of means of different drugs. The results were considered significant when P value <0,05. Student t-test for mean comparisons was performed.

RESULTS

1- Cytotoxicity assay:

MTT assay results showed marked reduction in cell viability, as Apig concentrations increased from 1.22 to 2500 mM with an IC_{50} of 51.46 mM as Apig NPS increased from .24 to 500 mM with an IC_{50} .89 mM as shown in table (1,2).

2- Microscopic examination and Cytologic evaluation:

Control cells: showed regular cellular outline with hyperchromatic nuclei. Only few cells showed the morphological criteria of apoptosis fig1A.

Drug treated cells: Cells treated by Apig and Apig NPs revealed nuclear morphological alterations correlated with the morphological parameters of apoptosis in its various stages. Including peripheral condensation of chromatin against the nuclear membrane, irregularities in the nuclear and cellular membrane, nuclear contraction, nuclear fragmentation and apoptotic bodies. Moreover, few cells showed nuclear changes that coincide with the morphological changes of necrosis characteristically coarse staining with relative preservation of nuclear morphology, nuclear and cellular swelling as well as rupture of cell membrane as shown in fig 1B&C

TABLE (1): The mean viability percentage of HEp-2 cells treated with different concentrations of Apig for 24 h.

Apig conc mM	2500	1250	625	312.5	156.25	87.25	39.06	19.53	9.67	4.88	2.44	1.22
Viability%	14.41	16.31	17.12	19.91	26.94	37.75	76.49	85.14	92.79	101.44	103.51	103.69

TABLE (2): The mean viability percentage of HEp-2 cells treated with different concentrations of Apig NPs for 24h

Apig NPs conc mM	500	250	125	62.5	31.25	15.6	7.8	3.9	1.95	.97	.49	.24
Viability%	12.25	12.88	13.60	14.86	14.95	16.67	19.37	19.82	31.44	41.71	86.58	98.11

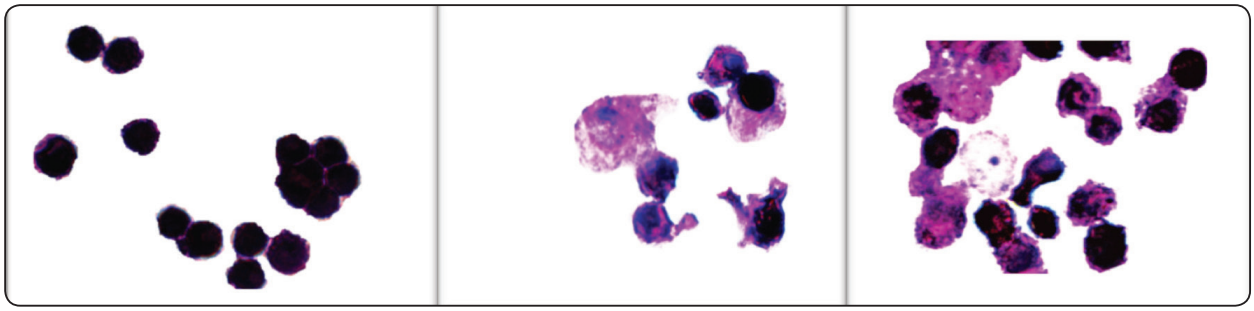

Fig. (1) **(A)** photomicrograph of control Hep-2 cells showing Pleomorphism, hyperchromatism, **(B)** a photomicrograph of Hep-2 cell post treatment with IC50 concentration of Apig revealing secondary necrotic cell, nuclear fragmentation, apoptotic body and nuclear shrinkage. **(C)** photomicrograph of Hep-2 cell post treatment with IC50 concentration of Apig NPs showing necrotic cell, nuclear fragmentation and cell debris (H&E x 1000 oil).

3- Morphometric analysis:

Nuclear Area Factor (NAF): The mean value of NAF of HEp-2 cells treated with Apig and Apig NPs revealed the largest value of NAF is in the control cells that accompanied with a significant decrease IC_{50} in Apig also a significant decrease in case of Apig NPs as shown in table 3.

TABLE (3): Comparing the effect of treating HEp-2 cells with the IC50 concentration of Apig and Apig NPs on the mean values of NAF

NAF	Group 1 (control)	Group 2(Apig)	Group 3 (Apig NPs)
Mean	0.411	0.198	0.119
SD	0.285	0.217	0.126
Range	0.20-1.917	0.000019-1.066000	0.000006-0.69

4- Flow Cytometric Analysis

Cell cycle analysis: The IC_{50} concentration of Apig and Apig NPs produced Pre-G1 apoptosis and cell growth arrest with higher percentage of the Apig NPs. The percentages of cells in Pre-G1 and G2/M phases increased with Apig loaded nanoparticles and Apig as shown in fig (2)

Effect of Apig and Apig NPs on cell apoptosis:

Data recorded revealed that total apoptosis of cells treated with IC50% concentration of Apig was significantly elevated with p value ($P<0.05$). Apig NPs induced apoptosis was significantly elevated more than Apig ($P < 0.05$).The early and late apoptosis % was significantly elevated in both Apig and Apig NPs compared with the apoptotic profile of control cells with higher elevation in Apig NPs. Finally the necrotic cells % was high significantly in the Apig NPs treated cells than in the control free form and in Apig ($P<0.05$). Table 4

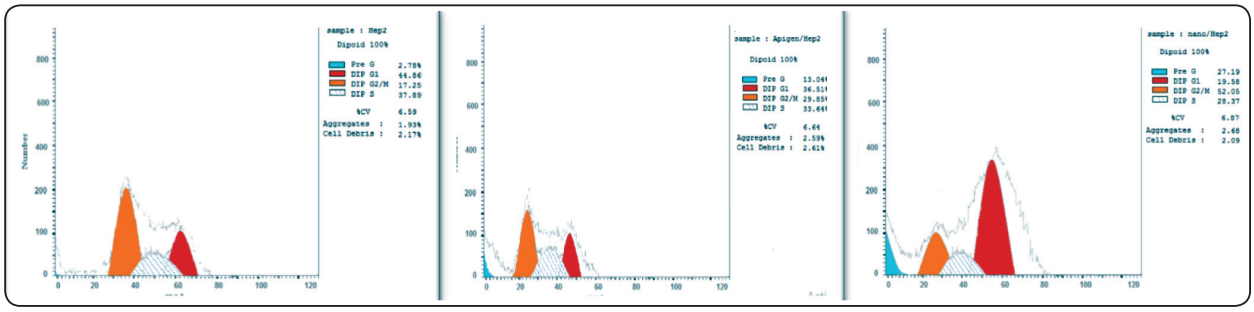

Fig. (2) The effect of Apig and Apig loaded Nano particles on cell cycle distribution

TABLE (4): Effect of Apig and Apig loaded Nano particles on cell apoptosis

	conc.ug/m1	Apoptosis			Necrosis
		Total	Early	Late	
1	Apigen	13.04	4.21	6.49	2.34
2	nano	27.19	7.29	15.79	4.11
3	cont.Hep2	2.78	1.63	0.42	0.73

4- ANNEXIN-V staining and Propidium iodide kit for detection of apoptosis

The highest fraction of control cells are living cells (in lower right quadrant) with only few necrotic and apoptotic cells. With Apig and Apig loaded Nano particles the number of living cells decreased with increase in apoptotic and necrotic cells (apoptotic cells in the upper and lower left quadrant and necrotic cells in upper right quadrant). As shown in Figure 3

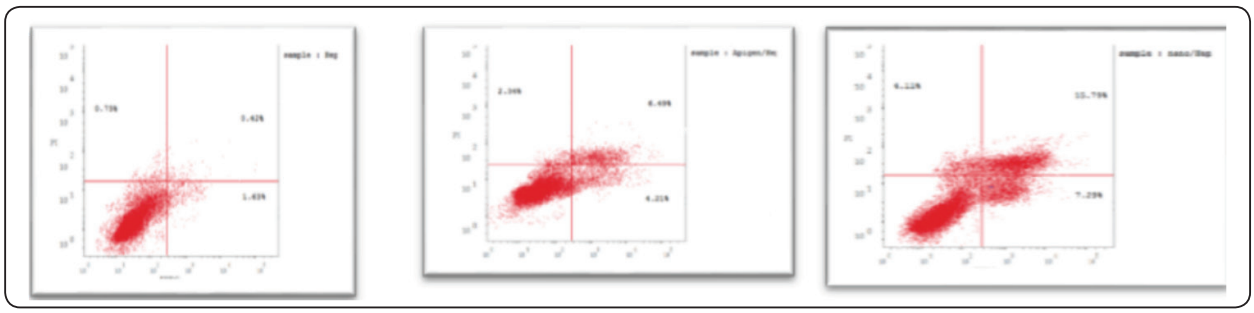

Fig. (3) Annexin V and Propidium iodide showing effect of Apig and Apig loaded Nano particles on cell apoptosis

DISCUSSION

Head and neck squamous cell carcinoma is one of the lethal malignancies of head and neck and represent a worldwide problem. Among its major risk factors are tobacco smoking, alcohol consumption and HPV infection. Despite the exerted efforts toward improving the outcome of the different modalities of therapy, the overall survival rate has not improved yet. Therefore, Apig may have particular relevance for development as a chemotherapeutic agent for cancer treatment.

In the present study, we investigated the effects of Apig and Apig NPs on the viability, morphology, cell cycle distribution, and apoptosis of HE-p2 cells. In the present study marked reduction in cell vitality in a dose dependent manner was found, as the mean viability percentage of Apig and Apig loaded NPs treated cells (compared to control cells) decreased as Apig concentrations increased. Our results of MTT assay were found to be in line with the results of Maggioni, et, al, on OSCC which demonstrated that Apig inhibits cancer cell growth in vitro; in SCC-25 cell line and were consistent with data reported with Shi, et al, that Apig reduced the viability of the human bladder cancer T-24 cells in a dose dependent manner (Maggioni, D. et al., 2013; Shi, M. D. et al., 2015).

While in Apig NPs, the data obtained showed that a minimal concentration of .97% reduced cell viability to less than 50% with respect to untreated controls. This was in line with results of Wu, W. et al who investigated and compared the anticancer activity of Apig nanoparticles and Apig by MTT assay in liver cells HE-pG2, and found that Apig nanoparticles inhibited the cell proliferation more effectively than raw Apig and in addition, Apig nanoparticles had inhibited growth of HepG2 cells at higher rate and by lower IC50 than that of raw Apig (Wu, W. et al., 2017).

The results of current study of the photomicrographic examination was in line with Kilani-Jaziri, S. et al, who found that Apig caused cell shrinkage, cell disintegration, and reduction in cell number in K562 and RT112 cells after 24 hours of incubation with RT112 and K562 cells when observed cell morphology using an inverted microscope at 200× magnification.(Kilani-Jaziri, S. et al., 2012)

Also, Das, S. et al., confirmed that Apig -induced condensed or fragmented nuclei in A375 and A549 cells when the Apig treated cells imaged using fluorescence microscope. Also Chen, X. et al, found that Apig caused nuclear condensation, membrane shrinkage and blebbing of the cells of the colon cancer cells HT-29 when imaged by electron microscopy (Das, S. *et al*., 2012),(Chen, X. *et al*., 2019).

In the present study, the statistical analysis of the NAF values showed significant decrease in the value of NAF of HEP-2 cells treated with Apig NPs and Apig in relation to control cells. Which confirm that Apig and Apig NPs initiated apoptotic events in the HEp-2 cells. This was in agreement with the study of Areeba Anwar on colorectal cancer cell after treatment with lawsonaringenin (LSG) another member of flavonoids who found a significant decreased in NAF. (Zhu, H. et al., 2016)

In the present study, the cell cycle analysis of HEp-2 cells treated with the IC50 dose of Apig and Apig NPs induced increased in the number of cells in Pre G1 phase from 2.78 % control cells to 27.19 % for Apig NPs and 13.04 % for Apig. This increase was associated with a statistically significant decrease in number of cells in G0-G1 phase from 44.86% control cells to 19.58% Apig NPs and 36.51% Apig. Corresponding to decrease in the number of cells in the S phase from 37.89% control cells to 28.37 % for Apig NPs and 33.64% for Apig. Which indicate cell cycle arrest at G0/G1 checkpoint.

Moreover, an increase in the percentage of cells in the G2/M phase cells from 17.25 % control cells to 52.05% by Apig NPs and to 29.85% by Apig. Suggesting cell cycle arrest at G2/M checkpoint in addition to cell cycle arrest at G1 checkpoint. With marked increase in the percentage of cells in case of treatment by Apig NPs.

These results were in agreement with Maggioni, D et al, on a tongue oral cancer-derived cell line (SCC-25) and on a keratinocyte cell line (HaCaT) who addressed that Apig modulated the cell cycle in SCC-25 cells by cell cycle arrest at both G0/G1 and G2/M checkpoints. And with, Zhao et al reported, that Apig treatment caused cell cycle arrest at both G0/G1 and G2/M checkpoints in an oral SCC cell line SCC-25, also Masuelli, L, et al, reported that Apig-induced decrease in cells in both G1 and G2/M phases after Apig exposure in SCC in immortalized keratinocytes and in primary cultures of OSCC.(Maggioni, D. et al., 2013) (Masuelli, L. *et al*., 2011; Zhao, G. *et al*., 2017)

In addition, in human colon carcinoma HCT-116 cells Wang, B. et al reported Apig triggered G0/G1 phase cell cycle arrest and stimulated cell cycle factors. Also, a study demonstrated by Kilani et al., showed that treatment of RT112 cells with 50 μM of Apig for 24 hours caused a significant increase in the pre G1 fraction of cells, decrease in the S-phase fraction, and accumulation of cells in the G2 /M phase.(Kilani-Jaziri, S. et al., 2012; Wang, B. et al., 2017)

While in human epidermoid carcinoma (A431) cells Indra Rajendran, et al found that cells treated with ap-AuNPs showed gradual decrease in G0/G1 phase and a corresponding increases in cell population in S-phase and Sub-G1 fraction reporting cell cycle arrest in S-phase and Sub-G1 arrest. (Indra Rajendran, A Harini Dhandapani, A Rajaram Anantanarayananb *et al*., 2015)

Results in the present study of the tested IC50 concentration of Apig NPs and of Apig on HEp-2 cells both induced both early and late apoptosis phenomena, as well as necrosis with small percentage. That was represented by the elevation

in the cells total apoptotic profile to 27.19% in Apig NPs while in cells treated with IC50 concentration of Apig was elevated to 13.04% compared to control cells 2.78%.

The significant elevation of the late apoptosis in Apig NPs to 15.79 % and to 6.49 % in Apig compared with the apoptotic profile of control cells that were detected to only have 0.42 % indicating that Apig NPs have more apoptotic potential than Apig.

In addition, the early apoptosis was elevated to in 7.29 % Apig NPs and 4.21 % Apig compared with the apoptotic profile of control cells 1.63 %with higher elevation in Apig NPs. Finally, the necrotic cells percentage was significant in the Apig NPs treated cells than in the control free form and in Apig.

This was in agreement with Wang, B et, al, who confirmed that Apig induced increase in the apoptotic cells (early plus late apoptotic cells) in esophageal carcinoma cells, also Zhu et al studied the effect of Apig with concentration 100 on human esophageal cancer cells which confirmed that the cytotoxic effects of Apig were due to both, necrosis, and apoptosis.(Zhu, H. et al., 2016; Wang, B. et al., 2017)

Also, Apig induced apoptosis in human cholangiocarcinoma cells in HuCCA-1 cells via apoptosis, and confirmed with DNA fragmentation by Subhasitanont et, al. In melanoma Das,S,et al, assessed the anticancer potentials of Apig using the melanoma cell line A375 and a lung carcinoma cell line A549 and demonstrated that Apig triggered both early and late apoptotic cells significantly in a concentration-dependent manner whereas the increase in percentage of necrotic cells was negligible in both cell types.(Das, S. et al., 2012; Subhasitanont, P. et al., 2017)

In human epidermoid carcinoma (A431) cells **Indra Rajendran,** et al. results showed that ap-AuNPs induced early apoptosis on A431 cells when investigated via flow cytometry. Also **Nooshin Samadian** compared the effects of Apig-loaded nanogel and raw Apig on the Human Chronic Myeloid Leukemia K562 cells and concluded that apoptosis of K562 cells was induced by Apig and the Nano drug, no toxic effect was observed in normal blood cells and Apig-loaded nanogel exhibited superior apoptotic activity compared to that of Apig. (Indra Rajendran, A Harini Dhandapani, A Rajaram Anantanarayananb *et al*., 2015; Nooshin Samadian, Mehrdad Hashemi, 2017)

CONCLUSION

-Apig and Apig NPs have antiproliferative and chemo preventive role due to its growth inhibition properties, the apoptotic inducing capabilities and its ability to induce cell cycle arrest in different phases. The Nano formulation enhanced and doubled the anticancer effects of Apig at low concentration indicating a direction for future anticancer research.

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