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NUCLEAR AREA FACTOR FOR ESTIMATION OF APOPTOSIS IN GRAVIOLA-TREATED SQUAMOUS CELL CARCINOMA CELL LINE. (EX-VIVO STUDY).

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Abstract

Aim and Objectives: This work is conducted to estimate the apoptotic effect of Graviola on tongue squamous cell carcinoma cell line by calculating the nuclear area factor (NAF) of the Graviola-treated cells.
Material and Methods: Tongue squamous cell carcinoma cell line (HNO-97) was used in the current work. The drug tested in the current work was Graviola (Annona Muricata). The cytotoxicity of Graviola on cultured HNO-97 cells was evaluated by using the MTT cytotoxicity assay. Software for image analysis was used to estimate the NAF of Graviola-treated cells at every concentration. Obtained data were then statistically analyzed.
Results: The obtained data showed a decrease in the NAF mean values of HNO-97 cells which treated with various concentrations of Graviola at various durations as compared to untreated cells.
Conclusion: The current data indicated that estimation of NAF is a sensitive technique for prediction of early apoptosis of Graviola-treated squamous cell carcinoma cell line.
Key Words: Graviola, Nuclear Area Factor, MTT assay.

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INTRODUCTION

Cancer begins and progresses as a multistep process in which malignant cells require some combined properties which include the ability to proliferate out of the body control, self-adequacy in growth signals and resistance to agents which promote apoptosis and suppress proliferation **(Luo et al., 2009)**.

Head and neck cancer (HNC) is considered the sixth most prevalent malignancy throughout the world, with about 550,000 newly diagnosed cases yearly (squamous cell carcinoma (SCC) of lip and oral cavity account for about 90% of them), accounting for about 3.2% of all incident cancers **(Siegel et al., 2020)**.

World Health Organization defined mouth cancer or oral cancer as the malignancy that affects the oral cavity and the lips **(Paraguassu et al., 2019)**. Oral cancer representing about 2–4% of all cases with cancer throughout the world **(Singh and Tripathi, 2018)**.

The conventional and most efficient treatment approaches of cancer are surgery, chemotherapy and radiotherapy, but these approaches are very aggressive and have multiple adverse effects **(Villa and Akintoye, 2018)**. For the time being, using natural products (NPs) in treatment of malignancy gained a marked interest because they have obvious less adverse effects when compared to conventional approaches **(Greenwell and Rahman, 2015)**.

Graviola (Annona muricata) belongs to the family of Annonaceae, which has several names, such as guanabana, soursop, custard apple, and several other names. It is at most found in the tropical and subtropical areas **(Qazi et al., 2018)**. Graviola has multiple curative uses as it has anti-arthritic antimalarial, antiparasitic, anticonvulsant, hepatoprotective, antidiabetic and anticancer effects **(Ioannis et al., 2015).**

To investigate the cytotoxic effect of anticancer agents, various techniques are available involving cytotoxicity assay, DNA fragmentation assay to evaluate the changes of apoptosis, microscopic evaluation of cellular apoptotic and necrotic morphological alterations, Annexin V and Propedium Iodide double fluorescent staining to detect both necrosis and apoptosis and immunohistochemical staining using markers for apoptosis. Each method is not efficient enough by itself to evaluate the efficiency of the anticancer drugs because of lack of accurate assessment of optimal concentration of the drug that enhance maximum effect of the drug with least adverse effects **(Ali et al., 2019).**

This work is conducted to investigate the role of calculating NAF in assessment of the anticancer effect of Graviola on HNO-97 cells

MATERIAL AND METHODS

I. Material

A. Cell line: Human tongue carcinoma cell line (HNO-97), provided from the Department of Cell Culture – Nawah Scientific Research Center, Cairo, Egypt was used in the current work. HNO-97 cells were provided from the ‘’American Type Culture Collection (ATCC)’’ as frozen vials.

B. Reagents: The drug tested in the current work was Graviola (VACSERA- EGYPT) which have a molecular formula of C26H46O7 and molecular weight of 470.6 g/mol. DMEM

(Invitrogen/Life Technologies) was the used growth medium, which was used at a pH of 7.2, stored at 4ºC in the dark and supplied with 10% fetal bovine serum (Hyclone), 10 ug/ml of insulin (Sigma Aldrich- USA) and 1% antibiotic (streptomycin-penicillin).

C. MTT assay kit: In vitro toxicology assay kit (Sigma Aldrich- USA), MTT (3, 4, 5-dimethylthiazol-2, 5-diphenyltetrazolium bromide) 1 gm/vial in MTT dissolving solution 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, 125 ml was used for viability testing.

II. Methods:

Cell culture: HNO-97 cells in culture medium were incubated for 24 and 48 hours at 37°C and a monolayer of HNO-97 cells were obtained.

Viability assay (MTT assay) and IC50 determination: HNO-97 cells, in growth medium, were placed for 24 hours in a 96-well plate before the MTT assay. Every test involved a blank which contains complete medium without cells. HNO-97 cells were treated with serial concentrations (8, 16, 31.25, 62.5, 125, 250, 500, 1000, 2000 µgm) of Graviola. Non-treated control wells were methanol treated. Plates were incubated for 24 and 48 hours at 37ºC. Reconstituted MTT was added in an amount equal to 0.5 mg/ml of PBS. Measuring of absorbance of each well plate was done using ELX-800, Bioteck- USA Elisa reader at a wavelength of 450 nm. The percentage of viable cells was calculated. The data obtained from the MTT assay indicated that IC50 of Graviola at 24 hours post treatment was 68.18 µgm (calculated by using Master Plex 2010 software).

Grouping of the Cells: Letter C was used as a code for the control group. The used concentrations of Graviola are IC50 of 24 hours (68.18 µgm) and double IC50 (136.36 µgm). For the durations of application, the codes were T1 for 12 hours, T2 for 24 hours and T3 for 48 hours.

Evaluation of H&E stained HNO-97 cells: All groups of NHO-97 cells, listed before, was distributed on the glass slide, dried and fixed with alcohol to prepare for microscopic evaluation of H&E stained HNO-97 cells.

Photomicrography and cytological evaluation: Microscopic slides of C groups and the treated cells with different concentrations of Graviola, at the three post-treatment durations T1, T2 and T3, were photomicrographed at the precision measurements unit, Oral Pathology Department, Ain Shams University. The used magnification was 1000x oil. The picking of the field was depending on the existence of the largest number of apoptotic cells.

Nuclear Area Factor (NAF): The nuclear circularity and nuclear surface area of HNO-97 cells were then measured automatically using image analysis software (ImageJ, 1.37v, NIH, USA). NAF was calculated using the following equation: (NAF = nuclear circularity x nuclear surface area) **(DeCoster M., 2007)**.

Statistical analysis:

The NAF mean values of Graviola-treated cells with various concentrations, compared to the control values at various post-treatment durations (T1, T2 and T3), were statistically estimated with the Statistical Package for Social Science (SPSS) software. The statistical tests used involved the one way ANOVA to compare between NAF mean values of control and Graviola treated cells at any specific duration. When P value ≤ 0.05, the results were considered significant.

Post Hoc Tukey test was done to make the comparison between various concentrations and various durations.

RESULTS

Morphometric Results

The NAF mean values of HNO-97 control cells and Graviola-treated cells with various concentrations at various durations are demonstrated in table (1). The obtained data indicated a decrease in the NAF mean values of HNO-97 treated cells with various concentrations of Graviola at various durations as compared to untreated cells (Figures 1 and 2).

T1, T2 and T3 Post Treatment: One way ANOVA test indicated a statistically high significant variance between the NAF mean values of control and various groups of Graviola treated HNO-97 cells (C, IC50 and double IC50) at the three durations (T1, T2 and T3) (P value < 0.001) (Table 2).

Post Hoc Tukey test indicated a statistically high significant variance in the NAF mean values between control group and both groups treated by IC50 and double IC50 of Graviola at the three durations (P1 value < 0.001). However, it indicated a statistically non-significant difference between both groups treated by IC50 and double IC50 of Graviola (table 2).

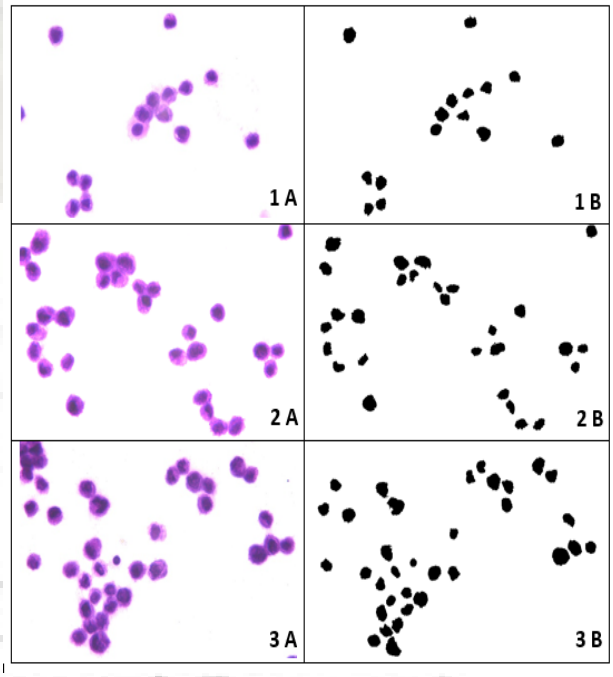

Fig. (1): Cytological changes of the control untreated HNO-97 cells (C) group after T1, T2 and T3 (1A, 2A and 3A sequentially) with its corresponding estimated NAF (1B, 2B and 3B sequentially).

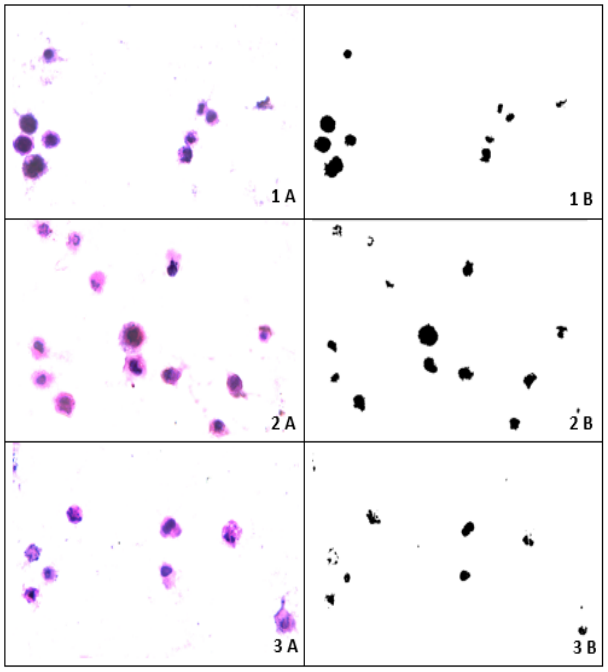

Fig (2): Cytological changes of the treated HNO-97 cells with IC50 of Graviola after T1, T2 and T3 (1A, 2A and 3A sequentially) with its corresponding estimated NAF (1B, 2B and 3B sequentially).

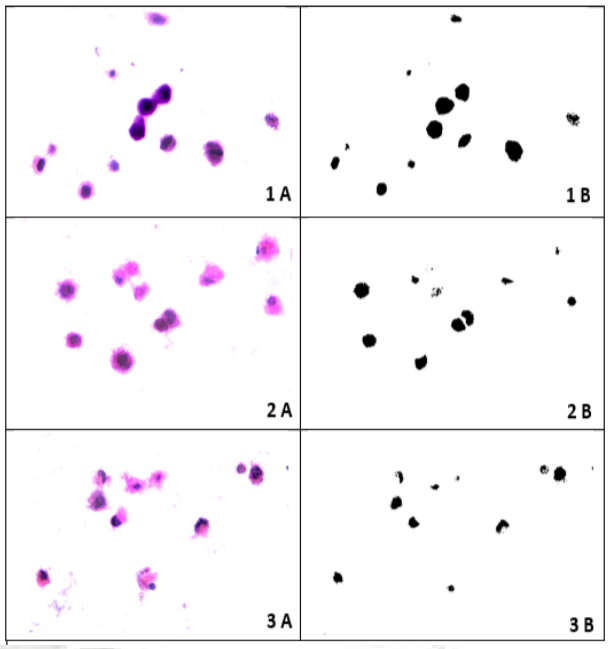

Fig. (3): Cytological changes of the treated HNO-97 cells with double IC50 of Graviola after T1, T2 and T3 (1A, 2A and 3A sequentially) with its corresponding estimated NAF (1B, 2B and 3B sequentially).

TABLE (1): The NAF mean values of control and Graviola-treated HNO-97 cells with various concentrations of Graviola at various durations.

	Control (C)	IC50	Double IC50
NAF mean T1 (12 hrs)	19049.67	8090.571	7510.71
NAF mean T2 (24 hrs)	11782.56	6422.076	5417.39
NAF mean T3 (48 hrs)	9147.796	3919.276	3502.97

Table (2): one-way ANOVA test and Post Hoc Tukey test for the NAF mean values of control and Graviola-treated HNO-97 cells with various concentrations of Graviola at the three various durations.

	Control (C)	IC50	Double IC50	P value
NAF mean T1 (12 hrs)	19049.67±5836.78	8090.571±3448.84	7510.71±3497.96	**< 0.001**
Post Hoc		**P1=< 0.001**	**P1=< 0.001** **P2=< 0.99**	
NAF mean T2 (24 hrs)	11782.56±1469.42	6422.076±1844.93	5417.39±1106.64	**< 0.001**
Poat Hoc		**P1=< 0.001**	**P1=< 0,001** **P2=< 0.51**	
NAF mean T3 (48 hrs)	9147.796±1765.25	3919.276±1110.84	3502.97±1106.06	**<0.001**
Post Hoc		**P1=< 0.001**	**P1=< 0.001** **P2=< 0.96**	

*Data represented as mean ± standard deviation P: Probability, *: Significance ≤ 0.05, P1: Significance vs C, P2: Significance vs IC50*

DISCUSSION

There are multiple and variable techniques used for assessment of the occurrence of either apoptosis or necrosis or both, to evaluate the effect of any anticancer agent against both cancer and normal cells. The effectiveness of anticancer agents is straightway related to its ability to induce the optimal apoptotic effect on cancer cells which is a programmed cell death, and in parallel the minimal ability to induce necrosis that can occur in both cancer and normal cells which is considered as a side effect

The MTT assay is one of the most commonly used techniques to evaluate drug sensitivity in various cell lines. The reduction in the number of the cells indicates inhibition of cell viability and growth and thereafter the drug sensibility is often defined as the drug concentration which is required to reach 50% inhibition of the viability in comparison with the viability of the untreated cells (50% inhibitory concentration, IC50). However, MTT assay gives no information about if the decrease in the cellular viability is a result of either apoptosis or necrosis, which is an important information to assess the effectiveness of any anticancer agent **(Meerloo et al., 2011 and Liu W and Dalgleish A, 2009)**.

The current study used a unique technique based on morphometric analysis for calculating the NAF by measuring the nuclear circularity and nuclear surface area using software for image analysis, which can provide a combination between the morphological changes of the nucleus and quantitative data of both necrosis and apoptosis **(Kashyap et al., 2017)**. NAF is calculated by multiplication of other two substantial apoptotic morphological parameters which are nuclear surface area and nuclear circularity **(DeCoster, 2007)**.

The data obtained from nuclear morphometric analysis indicated that after the three durations (T1, T2 and T3) of treatment there was a decrease in the nuclear circularity and nuclear surface area mean values of Graviola-treated cells with IC50 and double IC50 when compared to control cells. The values which approached 1 indicated more circular nucleus, while values lower than 1 indicated more irregular nuclei.

Statistical results of NAF revealed a statistically high significant difference in the NAF mean values between control group and both groups treated by IC50 and double IC50 of Graviola at the three durations. These results indicate that calculation of the NAF is a direct predictor of apoptosis, especially nuclear changes of early apoptosis as nuclear irregularity and shrinkage.

CONCLUSIONS

Depending on the obtained data from the current work, we could conclude that:

Calculation of NAF is an accurate indicator of early apoptosis in Graviola-treated carcinoma cell line.

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