Original Article

The Possible Protective Effect of Graviola Extract on Salivary Gland Against Cytotoxicity During Cisplatin Treatment (Histological, Biochemical and Immuno-histochemical Study)

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ABSTRACT

Objectives: Chemotherapeutic agents affect both normal and cancer cells. Salivary glands are easily damaged. Protecting normal salivary tissues against the harmful side effects of cisplatin therapy is of great need. This study was designed to evaluate the cytoprotective effect of Graviola extract against cisplatin cytotoxicity on parotid and submandibular salivary glands.

Methods: 30 adult male albino rats weighing 200-250 g were divided randomly into 3 equal groups: control group receiving only saline, cisplatin group receiving 6 mg/kg of cisplatin intraperitoneal and Graviola group receiving 6 mg/kg of cisplatin intraperitoneal followed by 100 mg/kg of Graviola extract daily orally for 14 days. At the end of the experiment parotid and submandibular glands were processed for H&E, VEGF and Ki-67 stains. Glutathione levels were detected from gland homogenates.

Results: Histological evaluation of cisplatin group with H&E showed total loss of architecture and vacuolization in the parotid gland and serous part of the submandibular gland while the mucous part showed separation between the acinar cells and the intra-lobular ducts. With immunohistochemical stains, decreased number of proliferative cells was observed with Ki-67 stain and with VEGF stain the glandular cells showed the least levels of positive staining. Also, glutathione levels recorded the least values among the study groups. In the Graviola group, partial improvement of glandular architecture and decreased vacuolization in both glands, increased proliferative cells with Ki-67 and increase of VEGF expression and the highest value of glutathione levels were observed.

Conclusion: Graviola extract partially ameliorates the cisplatin deleterious effect on the parotid and submandibular salivary glands at the histological and biochemical levels.

Received: 04 March 2021, **Accepted:** 24 April 2021

Key Words: Cisplatin, cytoprotection, graviola extract, parotid gland, submandibular gland.

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ISSN: 1110-0559, Vol. 45, No.2

INTRODUCTION

Mammalian salivary glands are exocrine glands that produce saliva through a system of ducts. Humans have three paired major salivary glands (parotid, submandibular, and sublingual) as well as hundreds of minor salivary glands[1].

Cisplatin (cis-diammine dichloro platinum II, CP) is a potent and highly effective chemotherapeutic against solid cancers. It has reported high incidence of cellular toxicities in different organs.[2]

The anticancer effects of CP depend on triggering mechanisms of cellular apoptosis. This happens either through the formation of intra-strand DNA crosslinks, by coordinative bonds between the platinum atom and DNA[3,4] or through the induction of reactive oxygen species (ROS) that trigger cell death.[5]

Other mechanisms of CP cytotoxic effect on tumor cells include mitochondrial damage, decreased ATPase activity and altered cellular transport mechanisms.[5,6] Despite the CP potent chemotherapeutic effects, its cytotoxicity is not selective for cancer cells. Cytotoxicity also reported against normal tissues.[7]

Graviola (Annona muricata), a member of the Annonaceae family, is a plant in Central and South America and tropical countries.[8,9] Traditionally the dark green leaves of Graviola are used as a treatment for asthma, cough, fever, headache, hypertension and toothache.[10,11] In animal models, Graviola leaves showed scavenging activity either through 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, ferric reducing antioxidant power, or hydroxyl-scavenging activity techniques.[9,10,12] addition, the leaves demonstrated suppression of pro-inflammatory cytokines providing protection against acute and chronic inflammations.[13]

Cancer patients receiving conventional therapy suffer from severe side effects on the quality of their life with no more than extended 6 months of life expectancy.[14] Therefore, new drugs need to be developed that include bioactive natural molecules such as Graviola that do not have similar toxic side-effects and have selective impact on cancer cells only.[15,16]

 DOI:10.21608/ejh.2021.65531.1439

The current study was designed to investigate the possible protective role Graviola to counteract the toxic side effects of CP on normal cells of salivary glands and the possible improvement of the biochemical markers.

MATERIAL AND METHODS

Animals

30 adult male albino rats weighing from 200-250 g were used in this study. The animals were housed in individual cages and received standard food for rodents and tap water. All experimental procedures were carried out in accordance with the laboratory guide lines of Ethics committee of Faculty of Dentistry, Mansoura University, Egypt. The 30 rats were divided into 3 equal groups (10 rat each) as follows:

I. **Group I:** animals receiving only saline (control group);

II. **Group II:** animals receiving 6 mg/kg body wt. of cisplatin intraperitoneal (i.p.) single dose injection (Cisplatin group);[17]

III. **Group III:** animals receiving 6 mg/kg body wt. of cisplatin once by i.p. injection, then after 3 days receiving 100 mg / kg body wt. of Graviola[18] (RAINFOREST PHARMACY®) for 14 days orally[19] Graviola group).

Histological evaluation

The rats were euthanized with overdose halothane after 14 days of Graviola treatment. Parotid and submandibular salivary glands were rapidly excised and then fixed in 10% neutral buffered formalin for 24 hours. The excised fixed glands were then embedded in paraffin and sectioned at 5 microns. Following that they prepared for Hematoxylin and Eosin (H&E) stain.

Immunohistochemistry evaluation

The sectioned glands were immune-stained using avidin-biotin technique. The Sections were stained with rabbit anti-rat vascular endothelial growth factor (VEGF) and Ki-67 (Sigma- Aldrich, St Louis, USA).

Five non overlapped different fields from each slide e were digitized using Olympus® digital camera installed on Olympus® microscope. The images were analyzed on an Intel® Core I3® based computer using Video Test Morphology® software with a specific built-in routine for immune-stain quantification. The targeted positive immuno-stained areas were automaticlly selected and seperated from the original image depending on the positive stain hue range, the selected area was thresholder and defined as region of interest and then a 3D histogram was constructed, and integrated density of the area was calculated.

Biochemical analysis

Parotid and submandibular samples were homogenized in 5-10 ml cold buffer (50 mM potassium phosphate- pH 7.5. 1 mM EDTA). Homogenates were centrifuged at 10000 g for 15 minutes at 4°C and the supernatant was kept at -80°C until it was used for analysis of glutathione levels. The supernatant was analyzed using colorimetric kit (Sigma- Aldrich, St Louis, USA). The values were expressed as nmol/g tissue.

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS) version 20.0. Descriptive statistics were calculated in the form of Mean ±Standard deviation (SD). In the statistical comparison between the different groups, the significance of difference was tested using one-way ANOVA followed by post hoc LSD test. For all tests, statistical significance accepted for probability *P values* < 0.05.

RESULTS

Histological evaluation

Examination of the control group sections of the parotid glands showed normal architecture of serous acini with pyramidal cells and basal spherical nucleus surrounding a narrow lumen (Figure 1A). The examination of submandibular glands reveled normal mucoserous architecture with serous acini like that of parotid and mucous acini with oval basally located nuclei bordering wider lumen. Intra-lobular ducts of both glands were lined with columnar cells with centrally located large rounded nucleus (Figure 2A).

Examination of parotid and serous parts of the submandibular gland sections of the CP group revealed total loss of normal acinar structure with signs of degeneration and intracytoplasmic vacuolization. The nuclei were irregularly arranged with different sizes and shapes, some appeared darkly stained, congested blood vessels also were observed (Figure 1B). In the mucous part of the submandibular sections the separation between acini and intra- lobular ducts were observed and some acini showed loss of architecture. Intra- lobular ducts of both glands showed signs of degeneration and loss of normal structure. Stagnation of secretory material was observed in the submandibular sections (Figure 2B).

The Graviola group sections of both glands revealed incomplete improvement of the acinar structure with signs of mitotic activity. Although in serous acini of both parotid and submandibular sections the acini restored their normal structure, some cytoplasmic vacuoles were still observed. Intra-lobular ducts restored their normal architecture as well (Figures 1C, 2C).

Immunohistochemistry evaluation

I. Ki- 67 examination

In the control group, the brown nuclei of the positively stained proliferative cells as well as negative cells were observed in acinar and ductal cells of both parotid and submandibular gland sections. In the CP

group, the proliferative cells decreased in number when compared to the control group. The acinar cells showed less proliferation than the intra-lobular duct cells. In the Graviola group, the proliferation of the cells was improved in comparison to CP group, and the intra-lobular duct cells showed more positive cells when compared to the acinar cells. Although the number of positive proliferative cells increased, it wasn't equivalent to that of normal sections (Figures 1 A1, B1, C1) (Figures 2 A1, B1, C1) (Table 1).

II. VEGF examination

In the control group, cytoplasmic positive staining was observed more in the intra-ductal cells than in the acinar cells in both the parotid and submandibular glands. In the CP group, the positive reaction was decreased and was limited to the intra-ductal cells. In the Graviola group, acinar cells restored some positivity but didn't reach that of the control group. The intra-ductal cells showed more positive reaction when compared to that of the CP group (Figures 1 A2, B2, C2) (Figure 2 A2, B2, C2) (Table 1).

Biochemical evaluation

Glutathione levels

Graviola group recorded higher levels of glutathione in parotid glands (1.8±0.3) and submandibular gland (2.02±0.5). In comparison, the CP group showed a reading of 1.3±0.3 in the parotid and 1.4±0.2 in the submandibular samples. The control group recorded 2.5±0.5 for the parotid gland and 2.1±0.8 for the submandibular glands (Table 1).

Statistical analysis

One-way ANOVA showed a significant statistical difference for all glands in all groups for VEGF and Ki-67 expressions and for glutathione levels. Post hoc LSD test showed a significant difference between all groups in both glands except for the glutathione levels. In the control and Graviola groups there was no significant difference in glutathione levels for both gland types. The CP group showed a significant difference for glutathione levels in both gland types (Tables 2,3).

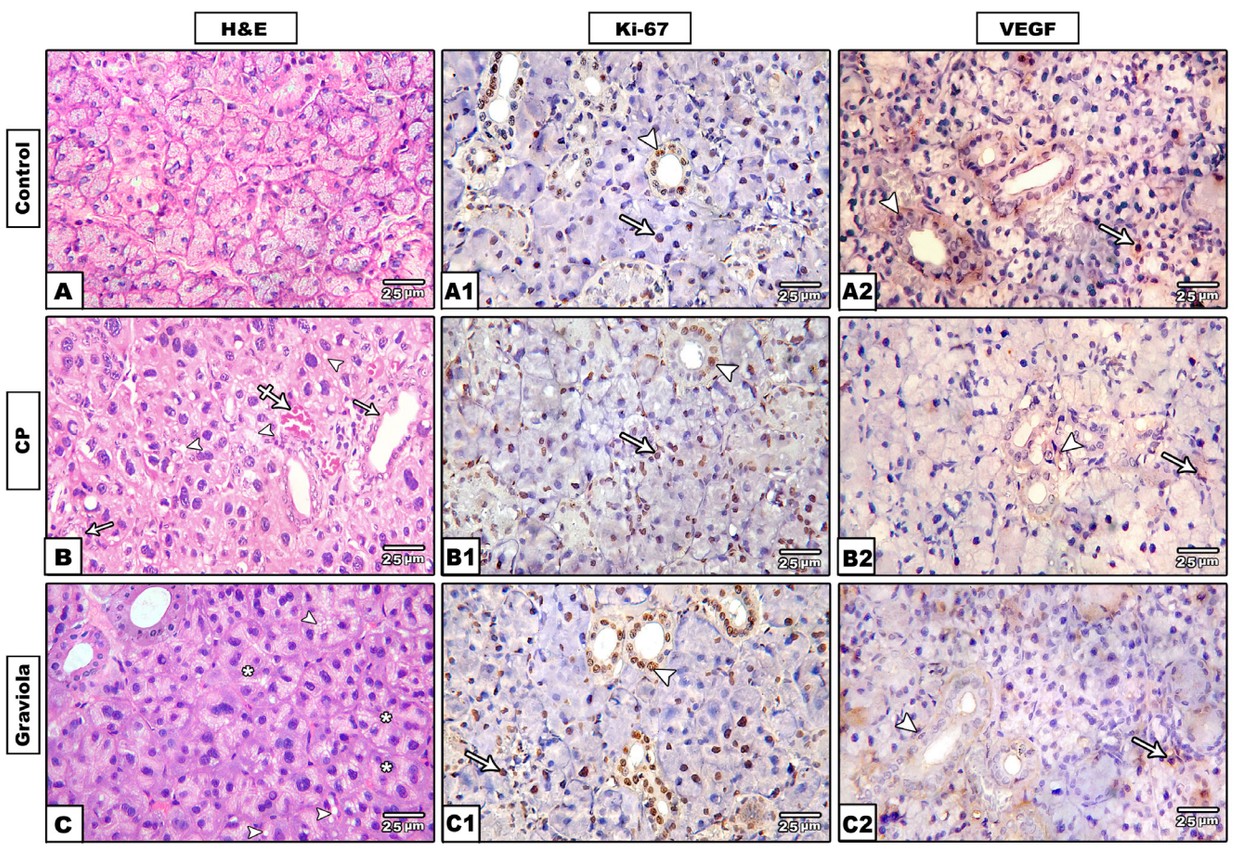

Fig. 1: photomicrographs showing a section of the parotid gland (A) of the control group showing normal architecture of serous pyramidal cells with basal spherical nucleus surrounding a narrow lumen. (B) CP group showed loss of normal acinar structure with signs of degeneration, intracytoplasmic vacuolization (arrow heads), nuclei were irregularly arranged with different sizes and shapes (looped arrow) and some darkly stained nuclei, notice degenerated thin epithelial lining of intra-lobular ducts (arrows) and congested blood vessels (crossed arrow). (C) Graviola group showed apparent improvement of the acinar structure (star) with signs of mitotic activity and some cytoplasmic vacuoles (arrow heads) (H&E stain X 400). Ki-67 positive nuclear reaction (A1) control group, positive reaction observed in both acinar (arrow) and ductal cells (arrow head) (B1) CP group, acinar cells (arrow) showed less positive reaction than ductal cells (arrow head), (C1) Graviola group, increased number of positive cells acinar (arrow) and ductal cells (arrow head) (Ki-67 X400). VEGF cytoplasmic positive reaction, (A2) control group, ductal cells (arrow) showed more positive reaction than acinar cells (arrow head), (B2) CP group, positive reaction was minimal in acinar cells (arrow) and ductal cells(arrow head), (C2) Graviola group, reaction increased in acinar (arrow) and ductal cells (arrow head), (VEGF X400).

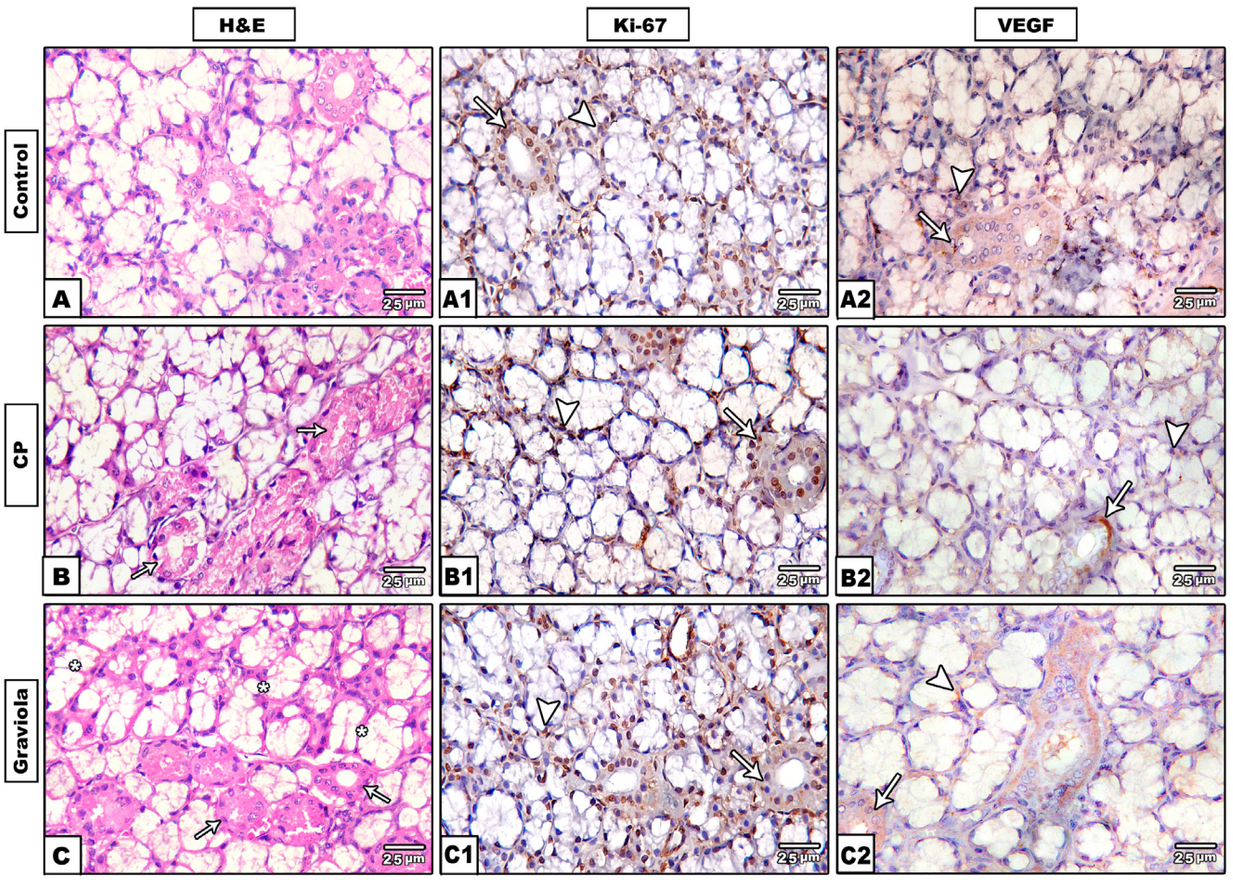

Fig. 2: photomicrographs showing Submandibular gland, (A) Control group with normal mucous acini and oval basally located nuclei bordering wide lumen. (B) CP group showed signs of degeneration in Intra-lobular ducts showed and loss of normal structure (arrow). (C) Graviola group showed apparent improvement of the acinar structure (star) and intra-lobular ducts restored their normal architecture (arrow) as compared to CP group (H&E stain X 400). Ki-67 positive nuclear reaction (A1) control group, positive reaction observed in both acinar (arrow) and ductal cells (arrow head) (B1) CP group, acinar cells (arrow) showed less positive reaction than ductal cells (arrow head), (C1) Graviola group, increased number of positive cells acinar (arrow) and ductal cells (arrow head) (Ki-67 X400). VEGF cytoplasmic positive reaction, (A2) control group, ductal cells (arrow) showed more positive reaction than acinar cells (arrow head), (B2) CP group, positive reaction was minimal in acinar cells (arrow) and ductal cells(arrow head), (C2) Graviola group, reaction increased in acinar (arrow) and ductal cells (arrow head), (VEGF X400).

Table 1: Mean ±Standard deviation (SD) of ki-67, VEGF expression and Glutathione levels in parotid gland groups

		Ki-67 expression		VEGF expression		Glutathione	
		Mean	SD	Mean	SD	Mean	SD
Control Group	Parotid	35.6	3.6	5.3	0.5	2.5	0.5
	Submand.	95.2	3.9	3.8	0.7	2.1	0.8
CP Group	Parotid	24.2	2.6	1.4	1.3	1.3	0.3
	Submand.	56.8	2.6	1.3	0.4	1.4	0.2
Graviola Group	Parotid	30.8	2.4	2.2	0.4	1.8	0.3
	Submand.	77.8	3.6	2.7	0.4	2.02	0.5

Table 2: One-way ANOVA and Multiple comparisons of LSD post-hoc for ki-67, VEGF expression and Glutathione levels in parotid gland groups its significance

One-way ANOVA		LSD post hoc		
Ki-67 expression	0.01	Control group	CP group	0.04
			Graviola group	0.01
		CP group	Control group	0.04
			Graviola group	0.01
		Graviola group	Control group	0.01
			CP group	0.01
VEGF expression	0.01	Control group	CP group	0.01
			Graviola group	0.01
		CP group	Control group	0.01
			Graviola group	0.04
		Graviola group	Control group	0.01
			CP group	0.04
Glutathione levels	0.01	Control group	CP group	0.02
			Graviola group	0.01
		CP group	Control group	0.02
			Graviola group	0.01
		Graviola group	Control group	0.01
			CP group	0.01

* The mean difference is significant at .05 level

Table 3: One- way ANOVA and Multiple comparisons of LSD post-hoc for ki-67, VEGF expression and Glutathione levels in submandibular gland groups and its significance

One-way ANOVA		LSD post hoc		
Ki-67 expression	0.01	Control group	CP group	0.01
			Graviola group	0.03
		CP group	Control group	0.01
			Graviola group	0.01
		Graviola group	Control group	0.03
			CP group	0.01
VEGF expression	0.01	Control group	CP group	0.04
			Graviola group	0.01
		CP group	Control group	0.04
			Graviola group	0.01
		Graviola group	Control group	0.01
			CP group	0.01
Glutathione levels	0.01	Control group	CP group	0.01
			Graviola group	0.5
		CP group	Control group	0.01
			Graviola group	0.03
		Graviola group	Control group	0.5
			CP group	0.03

* The mean difference is significant at .05 level

DISCUSSION

Cytotoxicity of chemotherapy affects normal cells as well as cancer cells. The effect varies in severity depending on the type, amount and duration of the chemotherapeutic agent used.[20]

Cisplatin induced toxicity affects both human and rodent in the same manner, this study aimed to investigate the toxic effect of cisplatin as chemotherapeutic agent, dose of 6 mg\kg used in this study was reported to induce nephrotoxicity in a non-life threating condition and with least rate of death.[21]

Kitashima[22] reported that cisplatin causes abnormal architecture of the submandibular acinar cells and numerous intracellular vacuoles and abnormal nuclei. These findings are in accordance with our current histological results of CP group. Another study reported that cisplatin caused considerable damage to the salivary gland tissues in *vitro*[23] These changes, known as cytotoxic effect of cisplatin, resulted from induced lipid peroxidation by ROS.[24]

Intracytoplasmic vacuolization observed in the present study could be explained by damage of intracellular structures and membrane fusion adjacent secretory granules.[25]

Graviola is a strong antioxidant that protects the cells against hydrogen peroxide DNA damage.[9,26] This can explain the improvement in acinar architecture, normal nuclei, intra-lobular ducts architecture and the decrease in the number of cytoplasmic vacuoles in Graviola group of the present study.

Chemotherapeutic drugs such as cisplatin arrest cell cycle through transcription inhibition.[27] The reduction of Ki-67 expression following neoadjuvant chemotherapy was reported and was considered a sign of better prognosis in breast cancer.[28] Hey *et al* reported that cisplatin prevents glandular cell regeneration through stabilizing DNA strands.[29] In the current study, immuno-histochemical evaluation revealed marked reduction of Ki-67 expression in the CP group in both parotid and submandibular glands.

Ki- 67 expression levels were elevated again in rats receiving Graviola after cisplatin in the current study, Nawwar *et al*. reported that A. muricata leaves extract increased the viability of non-cancerous cells,[30] Also Dai *et al* reported that Graviola selectively inhibited the growth of MDA-MB-468 cells without any effect on non-tumorigenic cells.[31] The results from studying the same family of a plant is an indicator that Graviola has a protective effect on non-cancerous cells.

The immuno-histochemical evaluation of VEGF in CP group showed marked decrease on VEGF in the acinar cells of both glands. these data were in agreement with Shen *et al*'s who demonstrated that low doses of cisplatin could inhibit the growth of blood vessel endothelial cells in *vitro* and showed anti-angiogenic ability in *vivo* through the downregulation of VEGF.[32] The good regulation of oxidative stresses mediated by antioxidant activity of Graviola can explain the increased levels of VEGF expression in the current study. The Long-term instability and high concentrations of ROS would eventually lead to inhibition of angiogenesis.[33]

Cisplatin was found to decrease glutathione, catalase, super oxide dismutase and glutathione peroxidase levels in mice kidneys.[34] These data are in accordance with the current findings of decreased levels of glutathione in both parotid and submandibular glands in CP group.

In a wound healing rat model, Annona muricata extracts accelerated wound healing by elevating levels of antioxidant such as catalase, glutathione peroxidase and superoxide dismutase as well as the reduction of MDA levels.[35] These findings can explain the current results of elevated levels of glutathione within the glandular tissue in the Graviola group. The administration of glutathione protected rat kidney against the cytotoxic effect of cisplatin without affecting its anticancer activity.[36]

Cisplatin is a widely used effective chemotherapeutic agent against solid tumors known for its cytotoxic effect on normal cells. In the current study, the antioxidant properties of Graviola extract is a promising approach for attenuating the degenerative effect of Cisplatin on normal salivary gland cells.

ETHICS STATEMENT

This work was approved by Ethics committee of Faculty of Dentistry, Mansoura University, Egypt.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير الوقائي المحتمل لمستخلص الجرافيولا على الغدة اللعابية لذكور الفئران البيضاء ضد السمية الخلوية الناتجة عن العلاج بالسيسبلاتين (دراسة هستولوجية و بيوكيميائية وهستوكيميائية مناعية)

أميرة أحمد رزق معوض، منة الله محمد الهنداوى

قسم بيولوجيا الفم ـ كلية طب الأسنان ـ جامعة المنصورة ـ مصر

الأهداف: تؤثر عوامل العلاج الكيميائي على الغدد اللعابية بسهولة، كما أن حماية الأنسجة الطبيعية من آثارها الجانبية الضارة أمر بالغ الأهمية وقد صممت هذه الدراسة لتقييم التأثير الوقائي لمستخلص الجرافيولا ضد السمية الخلوية للسيسبلاتين.

المواد والطرق المستخدمة: تم تقسيم ثلاثون من ذكور الفئران البيضاء البالغ وزنها ٢٠٠-٢٥٠ جم إلى ثلاث مجموعات: المجموعة الضابطة تتلقى فقط محلول ملحي، مجموعة سيسبلاتين تتلقى ٦ مجم / كجم من السيسبلاتين داخل الصفاق مره واحدة وتتلقى مجموعة الجرافيولا ٦ مجم / كجم من السيسبلاتين داخل الصفاق مره واحدة تليها ١٠٠ مجم / كجم من مستخلص الجرافيولا يوميًا لمدة ١٤ يومًا. تمت معالجة الغدد النكفية وتحت الفك السفلي من أجل بقع H&E وVEGF وKi-٦٧. وكذلك تم الكشف عن مستويات الجلوتاثيون من متجانسات الغدة.

النتائج: أظهر التقييم النسيجي والكيمياء الهيستولوجية المناعية لمجموعة السيسبلاتين فقدان البنية والتخلخل مع H&E، وانخفاض الخلايا التكاثرية مع صبغة Ki-٦٧ ومع صبغة VEGF أظهرت الخلايا الغدية أقل مستويات التلوين الإيجابي. أيضا، سجلت مستويات الجلوتاثيون أقل القيم بين المجموعات. في مجموعة الجرافيولا، لوحظ تحسن في بنية الغدد، وزيادة الانتشار مع Ki-٦٧، وزيادة ظهور VEGF وأعلى قيمة لمستويات الجلوتاثيون.

الاستنتاج: مستخلص الجرافيولا له تأثير وقائي ضد السيسبلاتين مما يؤدي إلى تحسن في الغدد اللعابية على المستوى الخلوي.