ASDJ
AINSHAMS DENTAL JOURNAL

Print ISSN 1110-7642
Online ISSN 2735-5039

AIN SHAMS DENTAL JOURNAL
Official Publication of Ain Shams Dental School
June2025 • Vol. 38

Effect of Virgin Coconut Oil and Propolis Against Methotrexate Toxic Effect on Submandibular Salivary Glands in Albino Rats

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Aim: This study aimed to evaluate the protective effect of virgin coconut oil (VCO) and propolis against toxicity induced by methotrexate (MTX) on submandibular salivary glands.
Materials and Methods: Fifty rats were randomly and equally divided into five groups (n=10), Group 1 (Control): Rats received saline by oral gavage for 17 days. Group 2 (MTX): Rats were injected intraperitoneally (Ip) with MTX (20 mg/kg) on day 14 only. Group 3 (Propolis and MTX): Rats were administered propolis by oral gavage at a dose of 100 mg/kg/day for 17 days and injected (Ip) with MTX (20 mg/kg) on day 14. Group 4 (VCO and MTX): Rats were administered VCO by oral gavage at a dose of 5 ml/kg body weight for 17 days, with MTX (20 mg/kg) injected (Ip) on day 14. Group 5 (VCO, Propolis, and MTX): Rats received propolis (100 mg/kg/day) and VCO (5 ml/kg) for 17 days, with MTX (20 mg/kg) injected (Ip) on day 14.
Results: The study demonstrated that propolis and VCO effectively mitigate MTX-induced cytotoxicity in submandibular glands at both histological and ultrastructural levels. Propolis preserved gland structure and reduced caspase-3 immunoexpression, while VCO improved gland architecture and blood vessel integrity, significantly reducing caspase-3 levels. The combination of both agents provided substantial protective effects, resembling the control group.
Conclusion: This study highlighted the protective effects of VCO and propolis against MTX-induced cytotoxicity in submandibular glands, suggesting their potential as adjunctive therapies in chemotherapy, potentially reducing salivary gland dysfunction and improving patient quality of life.

Keywords: Methotrexate, submandibular salivary glands damage, propolis, virgin coconut oil, apoptosis.

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Introduction

Cancer is a worldwide health problem resulting in one in six deaths worldwide. In 2020, there were approximately 19.3 million new cancer cases and around 10 million cancer deaths worldwide. For many decades, there were only limited alternatives of cancer treatment that include radiation therapy, surgery, and chemotherapy either as single treatments or combined. Lately, novel approaches, like drugs, biological molecules, and immune-mediated therapies, are used for cancer treatment.[1]

Methotrexate (MTX) is a folic acid analogue, the fundamental difference between them is that folic acid has a hydroxyl group at the 4th position of the pyridine ring while MTX has an amine group at the same position. Because of this similarity in structure, cells internalize MTX through same transport systems as folates, thus lead to inhibiting dihydrofolate reductase (DHFR), an important enzyme in the folic acid cycle and plays a key role to regulate homeostasis, which leads to reduce viability of cells and eventually lead to cell death.[2]

As folate receptors are overexpressed on the cell membrane of many types of cancer cells, MTX has been confirmed to be an efficient targeting agent and had a powerful anti-cancerous effect. MTX has been used for the treatment of breast cancer, sarcoma, acute leukemia, osteogenic, pulmonary carcinoma and intratracheal chemotherapy.[3]

Therapy with MTX leads to increase apoptosis depending on the dose and duration of treatment. This effect is thought to be as a result of generation of reactive oxygen species (ROS) as even using a low dose of MTX can cause severe side effects. Commonly known adverse effects are gastrointestinal signs such as vomiting, nausea, mucosal ulcers, and loss of appetite while using MTX in high doses can make patients suffer from mucosal ulceration.[4, 5] Meanwhile, toxic changes by MTX in the submandibular glands of male Wistar albino rats were in the form of significant degenerative changes in the gland tissue both on the histopathological and ultrastructural levels. [6]

Antioxidants are the molecules included in the counterbalance of free radicals and ROS. They may prevent cellular macromolecules oxidation (any biomolecules in the body) at minimal concentrations. Antioxidants have a lot of inhibitory effects on different disease conditions by decreasing these oxidative stress. Oxidative stress appears as result of imbalance between the production and the elimination of ROS which have a crucial role in different disorders' pathogenesis and pathophysiological processes like diabetes, cardiovascular diseases, and cancer.[7]

Excess ROS production has been linked with carcinogenesis, which leads to damage to nucleic acids, triggering mutations, and leads to DNA strands breaks and eventually leads to abnormal DNA linkages which happen during cancer formation. Antioxidants could be endogenous antioxidants such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and Glutathione (GSH) to protect the organism from oxidative stress-mediated diseases or exogenous antioxidants from natural dietary agents which have drawn a great deal of attention due to their important role in halting cancers by lowering oxidative stress.[8, 9]

An example of natural exogenous antioxidants is propolis. Propolis extract has a complex mixture of natural substances like phenolic acids, phenolic acids esters, flavonoids, amino acids and caffeic acid. Propolis has been used around the world in traditional medicine due to its immune stimulation, anti-inflammatory, anti-tumor, DNA protection and elimination of free radical effects. Propolis' medical properties have been proven to be mostly due to its

flavonoids. its mechanism of action is to inhibit the free radical's formation and release of these reactions to other body parts.[10,11,12]

Another example of natural exogenous antioxidants is virgin coconut oil (VCO). Coconut oil is a source of tocotrienols, capric acid, and lauric acid which consider natural antioxidants. These materials act as free radicals' scavengers that were thought to have a crucial role in aging, cancer, atherosclerosis, and diabetes. The biological effects of VCO have been proven by several studies that it reduces oxidative stress by enhancing the antioxidant defense system, clearing up free radicals and reducing lipid peroxidation. VCO demonstrated with various pharmacological activities such as antioxidant, anti-inflammatory, immunomodulatory, anti-cancerous, antidiabetic and anti-bacterial activities.[13, 14]

From the previous review, rarely reliable studies were found to reveal the protective effect of VCO and propolis against the toxic effect induced by MTX on salivary glands (SG). So, this study aimed to shed light on the individual and combined counteract effect of VCO and propolis against the toxicity induced by MTX on the submandibular SG.

Materials and methods

Materials

- Methotrexate in a concentration of 50 mg/2ml dissolved in 0.9% saline in form of vials, Hikma Pharmaceuticals, Giza Governorate, Egypt.
- Propolis in the proportion of 10 g propolis to 100 ml of solvent (ethanol 80%) prepared in the Beekeeping Research Section, Plant Protection Research Institute, Agriculture Research Centre at Giza governorate, Egypt.
- Natural Virgin Coconut Oil, Imtenan Company, Cairo Governorate, Egypt.

Methods:

Study design: The study was carried out on fifty adult male albino rats with an average of 150-180 grams body weight. Rats were acclimated 7 days before the experimentation.

Animal grouping:

50 rats were randomly and equally divided into 5 groups (n=10) as follows:

- **Group 1** (control): rats were received saline by oral gavage for 17 days.
- **Group 2** (MTX): rats were injected (Ip) MTX (20 mg/kg) on day 14 only.[15]
- **Group 3** (propolis and MTX): rats were administrated propolis by oral gavage with dose of 100 mg/kg/day body weight for 17 days and were injected (Ip) MTX (20 mg/kg) on day 14 only. [16]
- **Group 4** (VCO and MTX): rats were administrated VCO by oral gavage with dose of (5 ml/ kg body weight of rat) for 17 days. MTX (20 mg/kg) was injected (Ip) on day 14 only.[15]
- **Group 5** (VCO, propolis and MTX): rats were administrated propolis by oral gavage with dose of 100 mg/kg/day body weight for 17 days, VCO by oral gavage with dose of (5 ml/ kg body weight of rat) for 17 days and MTX (20 mg/kg) was (Ip) injected on day 14 only.

After the experiment period (17 days), rats were euthanized by extra dose of anesthesia. Their submandibular SG of the right and left sides were dissected out and separated**.** Submandibular glands of the *right side* were processed for histological and immunohistochemical evaluation while the left side was processed for the ultrastructural evaluation.

Statistical analysis

Obtained data was collected and tabulated. The numerical data were analyzed for normality by checking the distribution of data, calculating the mean and median values,

evaluating histograms and normality curves by using Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). One-way Analysis of variance (ANOVA) and Tukey tests were used for comparing data regarding the area percentage of positive reaction to caspase-3. P value less than 0.05 was considered statistically significant.

Ethical consideration

The present research was conducted after the approval of the Research Ethics Committee (REC) of the Faculty of Dentistry, Suez Canal University in approval number (473/2022).

Results

1) Histological results: (Figure 1, A-J)

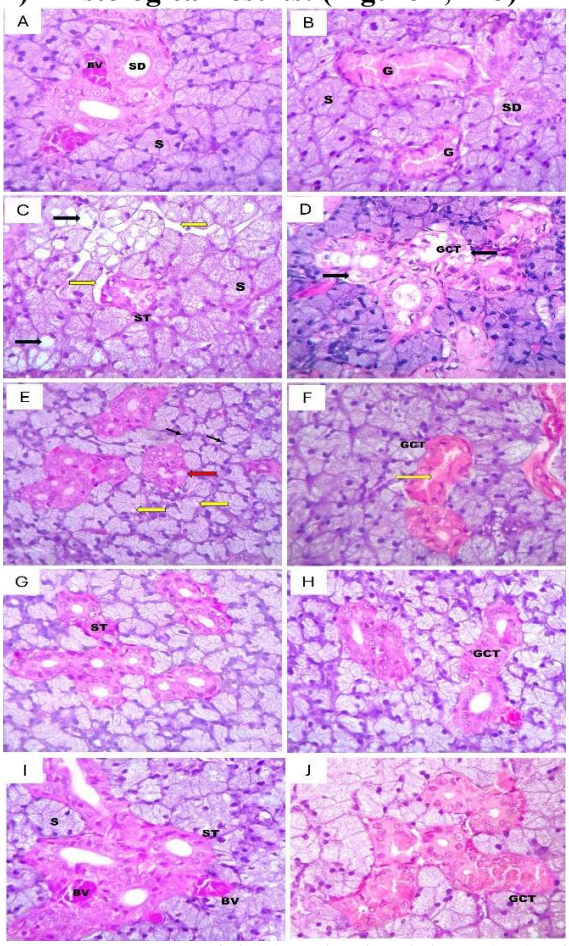

Fig. 1: A: serous acini (S) and striated ducts (SD) of control group. **B:** serous acini (S), straited ducts (SD) and granular convoluted tubules (G) of control group. **C:** Acinar cells (S) and straited duct (ST) of group 2. **D:** The granular convoluted tubules (GCT) of group 2. **E:** The serous acini and straited ducts of group 3. **F:** The granular convoluted tubules (GCT) of group 3. **G:** The serous acini and the straited ducts (ST) of group 4. **H:** The granular convoluted tubules (GCTs) of group 4. **I:** The serous acini (S) and the straited ducts (ST) and congested blood vessels (BV) of group 5. **J:** The granular convoluted tubules (GCT) of group 5.

- **Group 2: (MTX)**

C: The submandibular SG specimens of group 2 revealed marked degeneration and reduction in size of parenchymal elements appeared as spacing surrounding the acini and ducts (yellow arrows) with different size cytoplasmic vacuolization (black arrows) in the acinar and ductal cells. The striated ducts appeared shrunken in size, surrounded by spacing with ill-defined cell lining. There was loss of basal striations and stagnant secretions in their lumens. **D:** The GCTs showed loss of regular configuration, their cells showed deeply stained irregular nuclei and degeneration of cell lining (black arrows) with rupture of cells and leached out their granular content.

- **Group 3: (Propolis and MTX Group**

E: The submandibular SG of group 3 revealed the serous acini with more or less normal appearance regarding size and configuration. Deeply stained nuclei (black arrows) and few cytoplasmic vacuolization (yellow arrows) were seen in some cells**.** The straited ducts showed apparent reduction in height of lining cells with loss of basal striations and few cytoplasmic vacuolization (red arrows). **F:** Some of the GCTs were observed with ill-defined cell boundaries and eosinophilic stained granules at the cells apices which were seen leaching out from the lumen (yellow arrow).

- **Group 4: (VCO and MTX Group)**

G: The submandibular SG of group showed more or less normal histological configuration in the acini cells with minimal reduction in cell height with no obvious

cytoplasmic vacuoles. The straited ducts showed minimal loss of basal striations with no obvious stagnant secretions in their lumens. **H:** The GCTs cell lining showed apical eosinophilic granules while few cells showed leaching of the apical granules into the lumen.

- **Group 5: (VCO, propolis and MTX Group)**

I: The submandibular SG of group 5 showed almost normal gland architecture. The serous acini demonstrated pyramidal cells with basophilic stained granular cytoplasm, basal nucleus and rarely detected cytoplasmic vacuolization. The straited ducts demonstrated columnar cells lining with basal striations while few blood vessel congested with RBCs. **J:** The GCTs lining cells were observed with normal histological configuration and well defined apical eosinophilic granules.

2) Immunohistochemical results: (Figure 2, A-E)

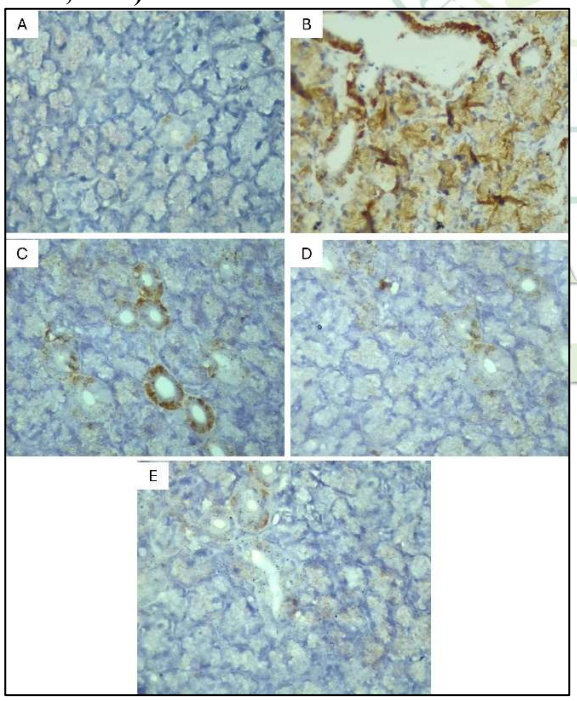

Fig. 2: A: group 1 showing no cytoplasmic staining reaction of caspase-3 in the acini cells and mild cytoplasmic staining reaction in few duct cells. B: group 2 showing strong cytoplasmic staining reaction of caspase-3 in the acini and the duct cell. C: group 3 showing mild cytoplasmic staining reaction of caspase-3 in the acini cells and moderate to strong cytoplasmic reaction in the duct cells. D: group 4 showing mild cytoplasmic staining reaction of caspase-3 in the acini cells and moderate cytoplasmic reaction in the duct cells. E: group 5 showing no cytoplasmic staining reaction of caspase-3 in the acini cells and mild cytoplasmic reaction in the duct cells.

A: The submandibular SG of the control group showed no cytoplasmic staining reaction of caspase-3 in the acini cells and mild cytoplasmic staining reaction in the duct cells of Caspase-3. **B:** for group 2 the immunohistochemical localization of caspase-3 showed strong positive cytoplasmic reaction in acini and duct cells. **C:** for group 3, mild cytoplasmic reaction of acini cells and moderate to strong cytoplasmic reaction of duct cells were detected. **D:** for group 4, mild cytoplasmic reaction of acini cells and moderate cytoplasmic reaction of duct cells were revealed. **E:** for group 5, no cytoplasmic staining reaction of caspase-3 in the acini cells and mild cytoplasmic reaction in the duct cells.

3) Transmission Electron microscopic results: (Figure 3, A-O)

- **Group 2: (MTX group)**

D: Some serous acini showed irregular lysed basally located nuclei, massive degeneration of the cell organelles leaving few strands of dilated RER (red arrows) with accumulation of damaged electron-lucent secretory granules and lysosomes filled with electron dense particles (yellow arrows). **E:** The striated duct cells showed with stunted cell lining and loss of basal striations. The mitochondria were numerous and damaged with loss of cristae (yellow arrow). Cytoplasmic vacuolization (red arrows) and lysosomes filled with electron-dense particles varying in size and shape (blue arrows) were detected. **F:** The GCTs showed marked increase in the lumen width, decrease in size and number of their granular contents and

pleomorphism of the granules and cytoplasmic vacuolization of the duct cells (yellow arrow) were detected.

- **Group 3: (propolis and MTX Group)**

G: Some acinar cells demonstrated irregular lysed basally located nuclei, accumulation of some electron-lucent secretory granules (yellow arrow) and few cytoplasmic vacuolization (red arrows). Dilated RER cisternae (green arrows), damaged mitochondria with loss of mitochondrial cisternae (white arrow) and lysosomes filled with electron dense particles (blue arrows) were detected. **H:** The striated duct demonstrated with loss of some basal striations and moderate number of nearly radially arranged mitochondria. The ductal lumen appeared dilated and irregular. **I:** The GCTs revealed some undulation in the nuclear membrane and pleomorphism of granules with cytoplasmic vacuolization of the duct cells (yellow arrows).

- **Group 4: (VCO and MTX Group)**

J: The serous acinar cells showed pyramidal shaped cells more or less uniform in size with narrow lumen with rounded basally located nuclei and packed with electro-lucent secretory granules with almost normal RER (yellow arrow) surrounding the nucleus. **K:** The striated duct cells showed few basal infoldings with some mitochondria radially arranged while other ones were irregularly arranged. **L:** The GCTs revealed marked ultrastructural improvement in size and number of their granular contents and obvious decrease in pleomorphism of the granules. No obvious cytoplasmic vacuolization of the duct cells was noticed.

- **Group 5: (VCO. Propolis and MTX Group)**

M: The acinar cells appeared pyramidal in shape and apparently uniform in size with rounded basally located nuclei. There was accumulation of secretory granules with different densities (yellow arrows). Almost normal RER (red arrow) was noticed around the nucleus. **N:** The striated duct revealed basal infoldings and almost normal radially arranged mitochondria. **O:** The GCTs demonstrated large rounded basal nuclei and almost normal, well circumscribed apically located membrane bounded secretory electron dense granules.

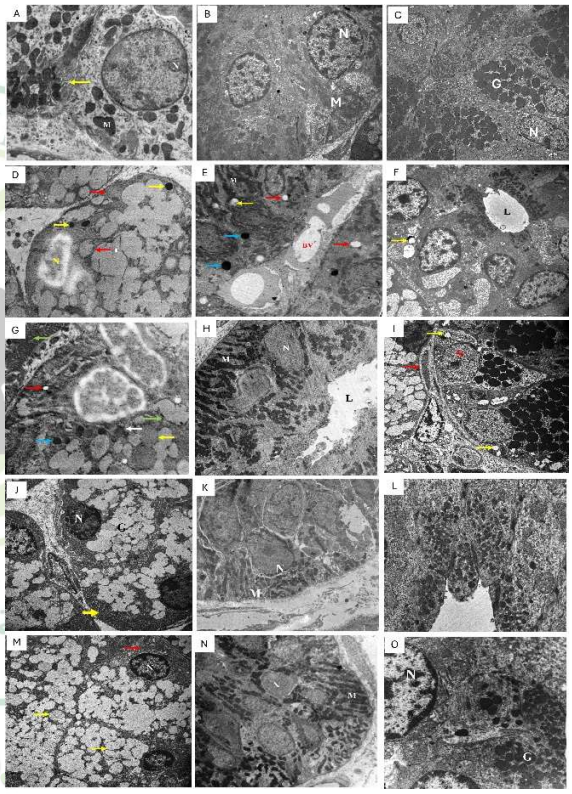

Fig. 3: A*:* serous acinar cell with nuclei (N) and Mitochondria (M) of control group. **B***:* striated duct with nuclei (N) and mitochondria (M) of control group. **C:** The GCTs with basal nuclei (N) and secretory granules (G) of control group. **D***:* serous acinar cells with nuclei (N) of group 2. **E:** striated duct with blood vessel (BV) of group 2. **F**: The GCTs lumen (L) of group 2. **G**: serous acinar cells with nuclei (N) of group 3. **H:** striated duct with mitochondria (M) of group 3. **I:** The GCTs of group 3. **J:** serous acinar cells with nuclei (N) and secretory granules (G) of group 4. **K***:* striated duct with mitochondria (M) and nuclei (N) of group 4. **L**: The GCTs of group 4. **M:** serous acinar cells with nuclei (N) of group 5. **N:** striated duct with mitochondria (M) of group 5. **O:** The GCTs with nuclei (N) and secretory granules (G) of group 5.

Statistical results (Figure 4)

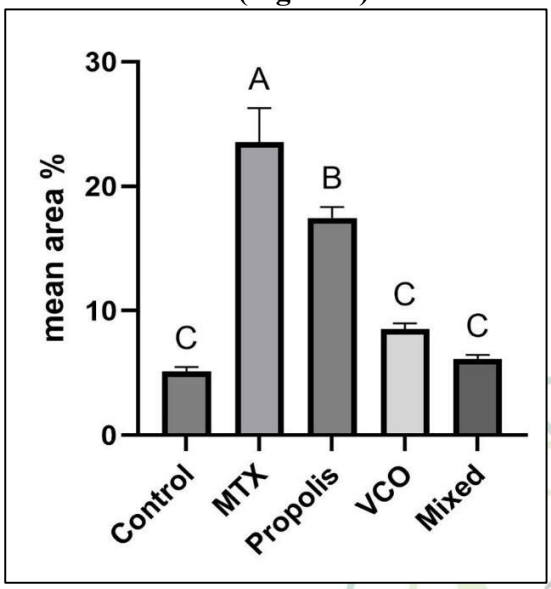

Fig. 4: Bar chart showing mean, standard deviation, and multiple comparison test of Caspase-3 mean area % immunoexpression of the submandibular gland. Different letters indicate significance.

The mean area percent of Caspase-3 immunoexpression in the MTX group was significantly overexpressed than in the control ($p<0.0001$), propolis ($p=0.0017$), VCO group ($p<0.0001$), and a mixed group ($p<0.0001$). A comparison of the mean area percent of Caspase-3 immunoexpression in the propolis group and control revealed a significant increase (<0.0001). Moreover, a significant overexpression was found comparing propolis to VCO ($p<0.0001$) and the mixed group ($p<0.0001$). Comparing VCO and the mixed group to the control showed a non-significant increase ($p=0.0672$) and ($p=0.8895$) in the mean area percent of Caspase-3 immunoexpression. In addition, comparing the mean area percent of Caspase-3 immunoexpression between VCO and mixed group revealed a non-significant difference ($p=0.2554$).

Discussion

Natural antioxidants, including zinc, calcium, vitamins, and polyphenols such as propolis and virgin coconut oil (VCO), have been extensively studied for their protective effects against chemotherapy-induced toxicity. These compounds function primarily by reducing oxidative stress, inhibiting cancer cell proliferation, and mitigating chemotherapy drug-induced damage.[17] In this study, we evaluated the protective effects of VCO and propolis against methotrexate (MTX)-induced toxicity in the submandibular salivary glands using a rat model. Rats were chosen due to their metabolic pathway similarities to humans, ease of breeding, and extensive comparative data available in toxicology research.[18, 19] Chemotherapy-induced salivary dysfunction can significantly impact a patient's quality of life, leading to oral mucositis, dry mouth, rampant caries, difficulty in chewing/swallowing, taste alteration, and oral mucous membrane inflammation.[6, 20]

MTX toxicity, particularly at high doses, leads to oxidative stress, apoptosis, and structural degeneration in salivary glands. In this study, we administered a 20 mg/kg dose of MTX, which aligns with previously reported research on its effects on neurotoxicity and oxidative damage in rats.[15, 21] Histological analysis demonstrated MTX-induced degeneration, with serous acini appearing shrunken and disorganized. These structural alterations, including pyknotic nuclei, cytoplasmic vacuolization, and focal acinar destruction, are consistent with prior findings demonstrating MTX's cytotoxic effects. [22,24] Immunohistochemical analysis further supported these observations, revealing significant caspase-3 overexpression in MTX-treated groups, indicating apoptosis activation.[25]

The protective effects of propolis in mitigating MTX-induced cytotoxicity were evident through histological, ultrastructural, and immunohistochemical findings. Propolis, a natural resinous compound, is known for its

strong antioxidant properties, capable of scavenging free radicals and reducing oxidative stress.[10] The administration of propolis in this study significantly improved the structural integrity of submandibular gland tissues, with notable reductions in cytoplasmic vacuolization and preservation of acinar cell morphology. These results are in line with findings by [16], who reported that propolis administration protected hepatic cells against MTX-induced damage by inhibiting lipid peroxidation and enhancing glutathione levels. Immunohistochemical analysis of the propolis-treated group showed a marked reduction in caspase-3 immunoexpression compared to the MTX group, further confirming its cytoprotective role.[26, 27]

VCO demonstrated comparable protective effects against MTX-induced toxicity through its antioxidative and anti-inflammatory properties. It has been widely recognized for its pharmacological benefits, including neuroprotection and cytoprotection against chemotherapeutic agents. [28] In the present study, histological examination of the VCO-treated group revealed well-preserved glandular architecture, with minimal cytoplasmic vacuolization and improved blood vessel integrity. These findings are consistent with previous studies demonstrating that VCO supplementation mitigates oxidative stress and inflammatory damage induced by chemotherapeutic drugs. [29] Furthermore, caspase-3 immunoexpression was significantly reduced in the VCO-treated group, indicating inhibition of apoptosis and improved cell viability.[30, 31]Clinical studies have also shown VCO's potential in reducing oxidative stress-related conditions, such as radiation-induced mucositis, reinforcing its relevance in managing chemotherapy-induced toxicity. [32] The combined administration of propolis and VCO provided the most substantial cytoprotective effects, surpassing the benefits observed with individual treatments. Histological and ultrastructural analysis showed that submandibular glands in this group closely resembled the control group, with intact acinar structures, normal-sized blood vessels, and absence of cytoplasmic vacuolization. The observed synergistic effect is likely due to the complementary antioxidant mechanisms of both compounds, which include free radical scavenging, lipid peroxidation inhibition, and modulation of inflammatory pathways. [33,35] This is consistent with studies showing that combining natural antioxidants, such as Curcuma and VCO, results in enhanced hepatoprotective effects against chemotherapeutic toxicity. [36]

The ability of combined antioxidant therapy to provide superior protection against chemotherapy-induced toxicity has been extensively documented. [37] reported that propolis, silymarin, and ginger effectively modulated MTX-induced hepatic toxicity, reducing structural alterations and DNA damage. The combination of these natural compounds significantly improved oxidative stress markers and reduced apoptosis, findings that align with the results of the present study. The concurrent use of propolis and VCO in mitigating MTX toxicity likely amplifies their individual antioxidant mechanisms, providing comprehensive protection against oxidative damage and apoptosis.

Mechanistically, propolis and VCO exhibit multiple pathways of cytoprotection. Propolis acts by scavenging reactive oxygen species (ROS), enhancing antioxidant enzyme activity, and modulating inflammatory responses. [16, 34] VCO exerts its protective effects through the inhibition of lipid peroxidation, chelation of metal ions, and enhancement of endogenous antioxidant defenses. [28, 31] These properties make them particularly valuable in counteracting chemotherapy-induced salivary gland

damage, where oxidative stress plays a pivotal role.

This study is among the first to investigate the combined protective effects of propolis and VCO against MTX-induced submandibular gland toxicity. The results suggest that their concurrent administration could be a promising strategy for mitigating chemotherapy-induced cytotoxicity. Further research is needed to explore their clinical applications and the precise molecular pathways through which they exert their protective effects. [38] Future studies should also consider long-term assessments and potential dose optimization to maximize therapeutic benefits while minimizing side effects.

Conclusions

The present study highlights the potential of VCO and propolis as effective protective agents against MTX-induced cytotoxicity in submandibular salivary glands. Their ability to mitigate oxidative stress, inhibit apoptosis, and preserve tissue integrity supports their potential use as adjunctive therapies in chemotherapy regimens. Given their natural origins and strong safety profiles, integrating these antioxidants into clinical practice may offer a viable approach for reducing chemotherapy-induced salivary gland dysfunction and improving patient quality of life.

Ethical consideration

The present research was conducted after the approval of the Research Ethics Committee (REC) of the Faculty of Dentistry, Suez Canal University in approval number (473/2022).

Data avaliablity

Data avaliable upon request.

Funding and Sponsorship

None.

Conflict of Interest

None declared

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