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Evaluating the Therapeutic Effect of Purslane Extract on the Submandibular Salivary Glands of Cyclosporine-A Treated Rats

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EVALUATING THE THERAPEUTIC EFFECT OF PURSLANE EXTRACT ON THE SUBMANDIBULAR SALIVARY GLANDS OF CYCLOSPORINE-A TREATED RATS

Heba M. Abdel Wahab *, Rabab T. Mubarak ** and Iman M. Aboushady***

ABSTRACT

Salivary glands are exocrine glands constituting part of the digestive system. They are affected by various drugs, among which, are the immunosuppressive drugs, like Cyclosporine-A (CsA) which plays a major role in transplant medicine. Thus, this work aimed to study the effects of CsA on the rat submandibular salivary glands and to evaluate the possible therapeutic effect of purslane on CsA induced salivary gland alterations.

Materials & Methods: 30 adult male albino rats, about 3-4 months age and 150-200 gm weight, were equally divided into 3 groups: control group which received a vehicle, CsA group which received 20 mg/kg/day of CsA, and combination group which received CsA combined to 400mg/kg/day of purslane extract. All rats were sacrificed 3 weeks from the beginning of the experiment then the submandibular salivary glands were dissected out, fixed, embedded and tissue sections were prepared for histological staining by hematoxylin and eosin (H&E) as well as immunohistochemical staining through anti-Ki67 antibody. Finally, the area percentage of Ki-67 immunoexpression was measured and statistically compared among the studied groups.

Results: Histological examination of the control group revealed the normal histology of the submandibular salivary gland, while in the CsA group; the acinar cells showed numerous vacuolizations and reduced cytoplasmic basophilia. The granular convoluted tubules (GCTs) revealed decreased apical eosinophilic granular content & cells of the duct system presented vacuolizations. Some excretory ducts retained secretion and marked chronic inflammatory cells infiltrate as well as congested blood vessels were seen. Regarding the combination group, the acini had well defined boundaries and marked reduction occurred in the number of vacuolizations among the acinar and ductal cells. The acini revealed increased cytoplasmic basophilia and the GCTs displayed increased granular eosinophilic content. Excretory ducts rarely retained secretion, some blood vessels were congested and the C.T. septa scarcely displayed chronic inflammatory cell infiltration. Immunohistochemical examination of the Ki-67 stained sections showed a few number of positive nuclei in the control

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group, while the CsA group revealed an apparent increase in the number of positive nuclei. On the other hand, the combination group displayed marked reduction in Ki-67 immunoreactivity than that in the CsA group.

Conclusions: administration of purslane to CsA treated rats resulted in an obvious improvement in the histological picture of the submandibular salivary glands and also it had a potent anti-proliferative effect. Thus, purslane could be beneficial in ameliorating CsA-induced salivary gland alterations.

KEY WORDS: Cyclosporine-A, purslane, submandibular salivary glands, Ki-67 antibody.

INTRODUCTION

Purslane or Portulaca Oleracea (PO) is a well-recognized medical plant in Traditional Iranian Medicine (TIM) and it has been utilized, either alone or in combinations, for many therapeutic purposes. Purslane is broadly used, not solely as an edible plant; but is also used for its curative properties. In TIM, aerial parts and seeds of PO have been used for the cure of uterine pain, fever, aphthous ulcers, diabetes and diarrhea[1]. In addition, **Uddin et al.,**[2] declared that PO is used for the treatment of headache, burns and diseases of the liver, intestine and stomach in addition to shortness of breath, cough and arthritis. Besides, it has been used as a palliative, laxative, muscle relaxant, anti-inflammatory, cardiac tonic and for diuretic treatment. Purslane is registered by the World Health Organization as one of the most commonly used medicinal plants and has been termed "Global Panacea". This plant has various pharmacological activities including: antifungal, analgesic, anti-inflammatory, wound healing and hypoglycemic activities. Recently, researches on purslane have been concerned with detecting phenolic compounds, fatty acids and antioxidant activities[3].

Cyclosporine-A (CsA) is a cyclic undecapeptide having potent immunosuppressive activity and it originated from extracts of one of the Fungi Imperfecti family; the Topocladium Inflatum Gams. Owing to its powerful immunosuppressive characteristics, it has been extensively used to avoid transplanted organs' rejection and to cure autoimmune diseases[4]. There are several recognized side effects of CsA such as: hypertension, nephrotoxicity, hypertrichosis, hyperkalemia, hypomagnesaemia, infections (oral candidiasis) and increased possibility of certain malignancies[5].

Thus, the current study was conducted to investigate the effect of CsA on the rat's submandibular salivary glands and to explore whether the administration of purslane could be beneficial in this case or not.

MATERIALS & METHODS.

Animals

30 adult male albino rats were used, with an average age ranging from 3 to 4 months and weighing about 150 to 200 gm. The rats were obtained from the animal house, Faculty of Medicine, Cairo University. Rats were housed in standard plastic cages and, throughout the experimental period, they were allowed to receive a standard laboratory diet and water *ad libitum*, together with the additional treatment specific for each group. All experimental procedures were conducted according to the recommendations of the ethics committee in the Faculty of Dentistry, Cairo University.

Materials

1-Cyclosporine-A (CsA):

CsA was purchased from Novartis as 50 mg gelatinous capsules (Sandimmun) (Neoral®). The content of the capsule was dissolved in a vehicle (0.9% saline).

2-Purslane: an ethanolic extract of purslane leaves was extracted at the Biochemistry Department, Faculty of Medicine, Cairo University as follows: first, the leaves of the plant were dried in the shade, then the herb was thoroughly extracted with 80% ethanol in a sox let apparatus. Finally, the solvent was removed by evaporation under reduced pressure[6]. The obtained extract was eventually administered to the rats in a vehicle (0.9% saline).

Experimental design.

Group I (Control Group): normal healthy rats which received the vehicle.

Group II (CsA Group): each rat received a daily dose of 20 mg/kg of CsA[7].

Group III (Combination Group): each rat received a daily dose of 20 mg/kg of CsA, in addition to a daily dose of 400 mg/kg of purslane extract[8].

All treatments were administered via gastric gavage and were continued for 3 weeks.

Histological and Immunohistochemical Examination

At the endpoint of the study, all rats were anaesthetized with pentobarbital at a dose of 50 mg/kg[9], the submandibular salivary glands were dissected out and fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections of 4-5 μm thickness were obtained then subjected to:

A-Histological Examination: through H&E stain.

B- Immunohistochemical Examination:

The paraffin sections were mounted over electrically charged slides, then subjected to immunohistochemical staining using the ready to use primary anti-Ki-67 antibody.

Procedures of Staining:

Sections were deparaffinized by incubating them in xylene three times, 5 minutes each. Then, slides were rehydrated in a series of ascending ethanol, washed by distilled water and endogenous peroxidase was blocked by immersing the slides in 3% hydrogen peroxide for 10-15 minutes at room temperature then washed two times with phosphate buffered saline (PBS). Afterwards, the anti-Ki-67 antibody was added, incubated for 30 minutes, then the slides were washed in PBS three times. The secondary antibody, biotinylated goat anti-mouse IgG, was added and incubated for 10-15 minutes at room temperature, then rinsed with PBS three times and Streptavidin peroxidase was added, incubated for 10-15 minutes at room temperature, then rinsed with PBS three times. Finally, the DAB substrate chromogen was applied, incubated for 10 minutes at room temperature, followed by washing in PBS for 3 minutes. All sections were counterstained with hematoxylin, washed, covered and left to dry in air, then mounted in Canada balsam.

Image Analysis

The immunostained sections were examined using Leica Quin 500 analyzer computer system. Immunostaining was measured as area percent in a standard measuring in 10 fields for each group using magnification (x400) by light microscopy transferred to the screen. Mean values and standard deviation were obtained for each specimen.

Statistical Analysis

The obtained data were statistically described in terms of mean ± standard deviation (SD). Comparison between the studied groups was done using Kruskal Wallis test with Mann-Whitney multiple 2-group comparisons. *P*-values less than 0.05 were considered statistically significant. All statistical calculations were done using the computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

RESULTS

Histological Results.

Group I (Control Group):

Light microscopic examination of the rat submandibular salivary gland of the control group revealed roughly circular acinar portions lined with pyramidal cells having spherical basally located nuclei. The smallest ducts, the intercalated ducts, are difficult to be identified under the light microscope. The GCTs were lined with large columnar cells with basal nuclei and apical eosinophilic granules. The striated ducts were lined with columnar cells having rounded central nuclei and prominent basal striations **(Fig. 1a)**. The C.T. septa showed excretory ducts, with pseudostratified columnar epithelial lining, as well as medium sized blood vessels **(Fig. 1b).**

Group II (CsA Group):

After CsA administration, the submandibular salivary gland showed several alterations including: massive intracytoplasmic vacuolizations and reduced cytoplasmic basophilia of the acinar cells. Areas of degeneration and loss of gland architecture were also observed**.** Regarding the duct system, GCTs showed vacuolizations and reduction in their eosinophilic granules. Besides, the striated ducts presented vacuolations and indistinct basal striations **(Fig. 2a)**. Some excretory ducts were dilated and retained secretion, numerous blood vessels were congested and chronic inflammatory cells infiltrate was also detected in the C.T septa **(Fig. 2b)**.

Group III (Combination Group):

Histological examination of the rat submandibular salivary gland revealed an obvious improvement in the histological features of the gland following purslane administration when compared to the CsA group. This was clearly seen as marked reduction of the intracytoplasmic vacuolizations throughout the gland as well as more defined boundaries and increased cytoplasmic basophilia of the acini. As for the duct system, there was an increased eosinophilic granular content of the GCTs **(Fig. 3a)**. Prominent basal striations of the striated duct were observed and the excretory ducts rarely retained secretion. Few blood vessels were congested and chronic inflammatory cells were scarcely detected in the C.T. septa **(Fig. 3b)**.

Immunohistochemical Results.

Group I (Control Group):

Immunohistochemical examination of the rat submandibular salivary gland of the control group showed a few number of positively stained nuclei

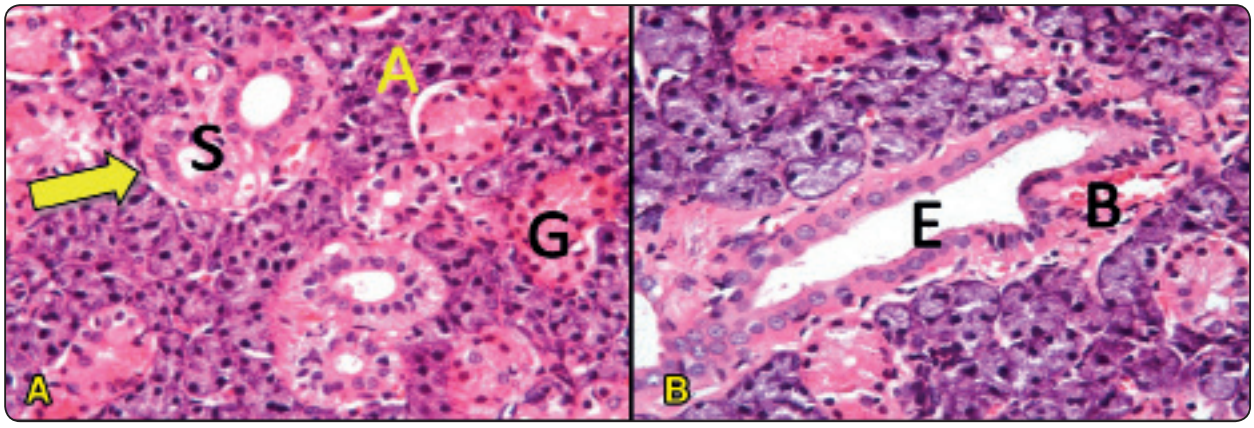

Fig. (1): A photomicrograph of the rat submandibular salivary gland of the control group showing: **a)** Acinar portions (A), granular convoluted tubules (G) and striated duct (S) with prominent basal striations (yellow arrow) (H&E, Orig. Mag.400). **b)** An excretory duct (E), lined with pseudostratified columnar epithelium and a medium sized blood vessel (B) could be seen in the C.T. septa (H & E, Orig. Mag. 400).

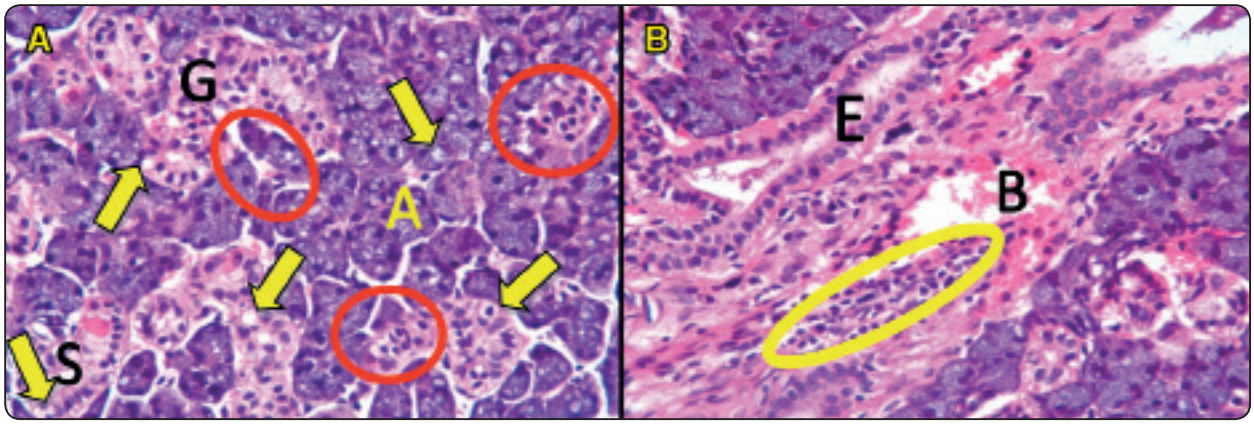

Fig. (2): A photomicrograph of the rat submandibular salivary gland of the CsA group showing: **a)** Areas of degeneration and loss of gland architecture (red circles), decreased cytoplasmic basophilia of the acini (A), decreased eosinophilic granules of the GCTs (G), indistinct basal striations of the striated duct (S) and massive vacuolizations (yellow arrows) throughout the gland parenchyma (H&E, Orig. Mag.400). **b)** A dilated excretory duct (E) which retained secretion, a congested blood vessel (B) and chronic inflammatory cells infiltrate (yellow oval) could be also noticed in the C.T. septa (H&E, Orig. Mag.400).

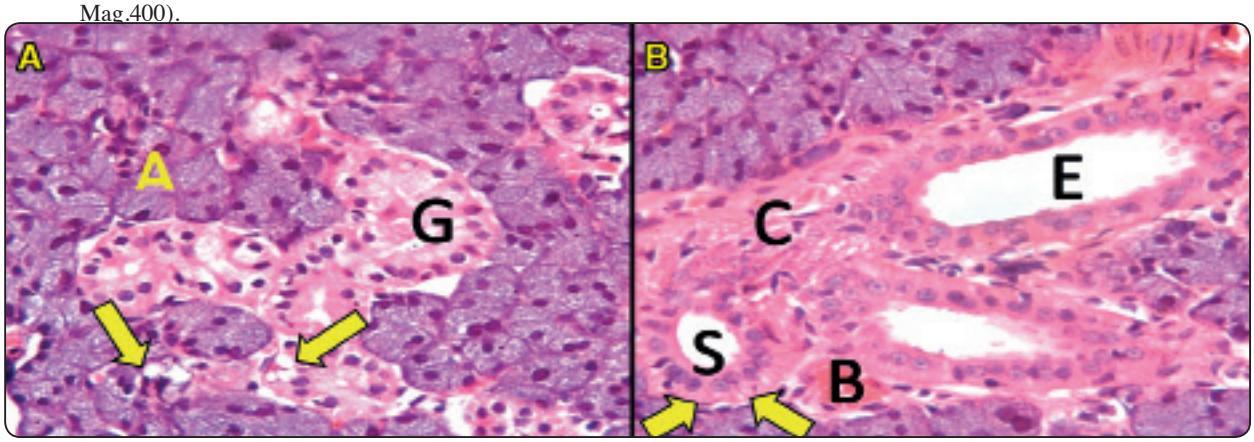

Fig (3): A photomicrograph of the rat submandibular salivary gland of the combination group showing: **a)** Well-defined boundaries and increased cytoplasmic basophilia of the acini (A), increased granules of the GCTs (G) and an obvious reduction in the intracytoplasmic vacuolizations (yellow arrows) among the acini and ducts (H&E, Orig. Mag.400)**. b)** Prominent basal striations (yellow arrows) of the striated duct (S), the excretory duct was dilated without retained secretion (E), congested blood vessel (B) in the C.T. septa (C) which rarely displayed inflammatory cell infiltration (H&E, Orig. Mag.400)**.**

for Ki-67 throughout the gland. The immunoreactivity for Ki-67 ranged from mild to moderate among the stained nuclei **(Fig. 4).**

Group II (CsA Group):

Examining the sections of the rat submandibular salivary gland of the CsA group showed an apparent increase in the number of Ki-67 positively stained nuclei among the acini and ducts when compared to the control group. The nuclear immunostaining ranged from moderate to strong **(Fig. 5).**

Group III (Combination Group):

When the Ki-67 immunostained sections of the rat submandibular salivary gland of the combination group were examined, in comparison to the CsA group, an obvious decrease occurred in the number of positively stained nuclei throughout the gland and the immunoreactivity for Ki-67 ranged from mild to moderate **(Fig. 6)**.

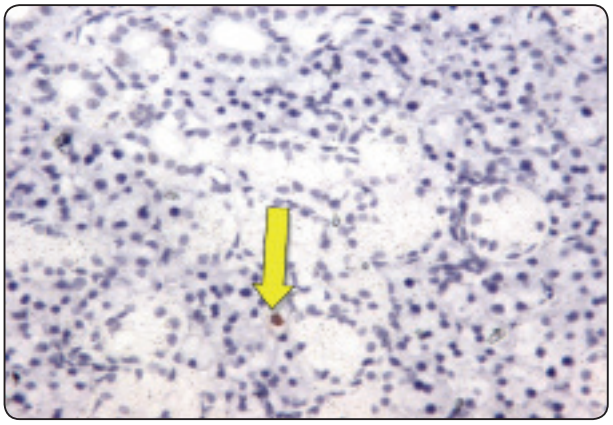

Fig. (4): A photomicrograph of the rat submandibular salivary gland of the control group showing: mild to moderate nuclear immunoreactivity for Ki-67 among few parenchymal cells (yellow arrow) (DAB, Orig. Mag.400).

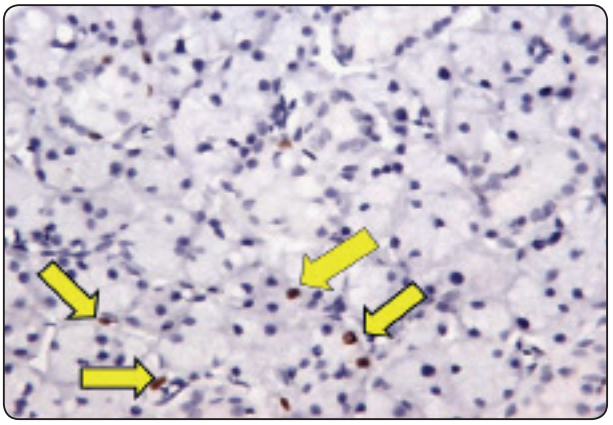

Fig. (6): A photomicrograph of the rat submandibular salivary gland of the combination group showing: mild to moderate nuclear immunoreactivity among fewer number of nuclei (yellow arrows) (DAB, Orig. Mag. 400).

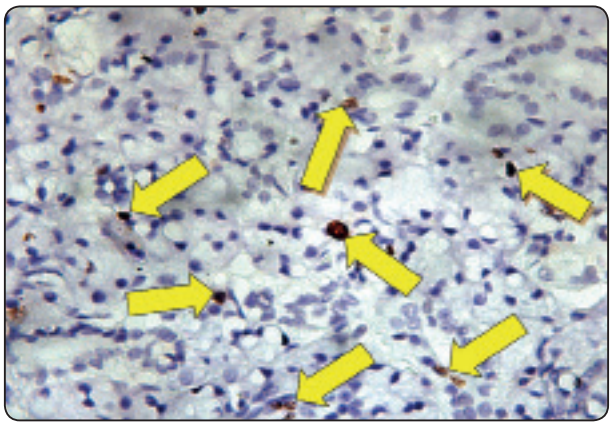

Fig. (5): A photomicrograph of the rat submandibular salivary gland of the CsA group showing: a strong nuclear immunoreactivity for Ki-67, which was widely dispersed throughout the gland parenchyma (yellow arrows) (DAB, Orig. Mag. 400).

Image Analysis and Statistical Results.

The greatest mean area % of Ki-67 immunoexpression was recorded in the CsA group with the least value obtained in the control group. Additionally, comparing the mean area % of Ki-67 between each pair of the studied groups, through Mann-Whitney test, revealed that the mean area % of Ki-67 in the CsA group showed a statistically highly significant increase than that in the control group. On the other hand, the mean area % of Ki-67 in the combination group was significantly decreased than that in the CsA group. However, it was still significantly increased when compared to that of the control group **(Table 1)**.

TABLE (1): Detailed results of Mann-Whitney Test showing the difference in the mean area % of ki-67 immunoexpression among the studied groups.

	Group I (Control Group)	**Group II (CsA Group)**	**Group III (Combination Group)**
Mean ±SD	0.214±0.077	1.210 ± 0.481	0.520 ± 0.215
Group I (Control Group)		0.009*	0.009*
Group II (CsA Group)	0.009*		0.028*
Group III (Combination Group)	0.009*	0.028*	

**** significant at p<0.05.***

DISCUSSION

Cyclosporine-A (CsA), an immunosuppressive drug, is broadly used to avoid transplant rejection as well as to cure numerous autoimmune diseases[10]. Many researchers studied the role of oxidative stress in chronic CsA treatment. Among these, **Korolczuk et al.,** [11] concluded that CsA increases the intra-mitochondrial Ca++, oxidative stress and production of reactive oxygen species (ROS), while it delays glucose metabolism of the mitochondria as well as ATP production. It was originally sold as a conventional oil-based form for oral use; however, this preparation has a little drug bioavailability. Consequently, in order to attain a more steady pharmacokinetic form, another product was formulated which consisted of a micro-emulsion (Sandimmun Neoral; oral solution or soft gelatin capsules) [12], which was therefore the chosen product to be used in the present work.

Oxidative stress takes place when production of free radicals exceeds the ability of the body to counteract them. This imbalance may be owed to either: decreased antioxidants production; or excessive free radicals production leading to injury to cellular organelles and increased lipid peroxidation[13]. One of the most common naturally occurring antioxidants is purslane which is considered an important source of the antioxidant vitamins: α-tocopherol, ascorbic acid and β-carotene as well as glutathione[14]. Antioxidants are compounds that can hinder lipids or other molecules oxidation by inhibiting the oxidizing chain reactions[3]. Purslane has numerous biological properties, for instance, being an: analgesic, skeletal muscle relaxant, anti-inflammatory, bronchodilator, anti-asthma and anti-pyretic[15], antispasmodic, antiseptic and diuretic[16], antibacterial and an enhancer of wound-healing[17]. Thus, the present study was designed to evaluate the possible therapeutic effect of purslane on CsA-induced salivary gland alterations.

Concerning the CsA group, each rat received a daily dose of 20 mg/kg of CsA which is in accordance with that used by **Said et al.,** [7] when they studied the effect of CsA on langerhans cells (LCs) in the rat's gingiva and they demonstrated that CsA triggered a dose dependent decrease in the amount of dendritic LCs in the gingival epithelium of the rat. Histological examination of the CsA group showed several morphologic changes including: massive intracytoplasmic vacuolizations among the acini, GCTs, striated ducts as well as decreased cytoplasmic basophilia of the acinar cells. Degeneration and loss of acinar cell boundaries could also be noticed in some areas of the gland. In addition, reduction in the apical eosinophilic granules of the GCTs was apparent when compared to those in the control group. Besides, dilated excretory ducts with retained secretion were detected, congested blood vessels and chronic inflammatory cells infiltrate were occasionally seen in the C.T. septa.

The intracytoplasmic vacuolizations and decreased cytoplasmic basophilia of the acinar cells could be explained according to **Dehpour et al.,** [18] who reported that cells of the submandibular salivary gland of CsA treated rats showed swollen mitochondria and minor reduction in the amount of secretory granules which suggested that protein synthesis is decreased leading to reduced cytoplasmic basophilia of the acinar cells. Additionally, **Fetouh and Abdelghany**[19] demonstrated that the pancreatic acinar cells of CsA treated rabbits displayed apparent reduction in their zymogen granules and profuse cytoplasmic vacuolations. The rough endoplasmic reticulum (ER) was diminished and vacuolated; consequently, this could explain the presence of cytoplasmic vacuolizations & decreased cytoplasmic basophilia seen in the current study. And more recently, **Ram and Ramakrishna**[20], demonstrated reduced cell viability and marked cytoplasmic vacuolations which seemed to be distention of ER cisternae and alteration of its structure.

Although the duct system was less affected, the CsA induced alterations observed in the present study could be related to changes in the salivary production and components as previously demonstrated by **Dehpour et al.,** [21] upon administration of therapeutic doses of CsA for long duration. And more recently, **Lee et al.,** [22], reported that CsA can affect submandibular gland ductal morphogenesis, thus, it can alter salivation and salivary composition.

In the present study, each rat in the combination group received CsA in addition to a daily dose of 400 mg/kg of ethanolic purslane extract. The same dose was utilized by **Hozayen et al.,** [8] in nephrotoxic rats; and they demonstrated an improvement of renal functions, enhanced antioxidant activities and reduced peroxidation. The ethanolic extract was the type of choice in the current investigation as it was proven by **Mohammed & Soad**[23] to retain the maximum antioxidant activities in comparison to the aqueous extract. This may be attributed to the great amounts of phenolic compounds (coumarins, flavonoids, alkaloids and saponins) in the ethanolic extract as compared to the aqueous one. Examining the H&E stained sections of the rat submandibular salivary gland of the combination group revealed that, in comparison to the CsA group, the acini had more defined cell boundaries, marked reduction in the number of intracytoplasmic vacuolizations and increased basophilia of the acinar cytoplasm. Cells of the GCTs showed increased apical granular eosinophilic content and much less intracytoplasmic vacuolizations. The striated ducts showed an almost normal cell lining with distinct acidophilic basal striations and their cells rarely displayed vacuolizations. Dilated excretory ducts with rarely retained secretion and few congested blood vessels were observed in the C.T. septa which scarcely showed inflammatory cell infiltration.

The apparent improvement in the histological features of the submandibular salivary gland of CsA treated rats after purslane administration is in accordance with several studies confirming the beneficial effects of purslane as a well-known antioxidant. For instance, in a study by **Mohammed and Soad** [23] conducted on hepato-toxic rats, they verified that purslane was capable of decreasing all the elevated biochemical factors, preventing the increase of serum uric acid, hepatic enzyme level, nitric oxide and lipids as well as improving liver functions, suggesting an important hepato-protective effect. In another study by **Hanan et al.,** [24] to investigate the biological & histopathological effects of purslane on hepato-toxic rats, they demonstrated that purslane could have antioxidant activities and could defend the tissues against lipid peroxidation caused by free radicals. The increased acinar basophilia detected in the present work could be attributed to the antioxidant effect of purslane which decreases the unfolded protein reaction & restricts ROS increase and misfolding of proteins which is particularly vital for the function and persistence of cells[25]. On the other hand, reduction of chronic inflammatory cell infiltrate was evident in the current study after administration of purslane which could be attributed to the modulating effect of antioxidants on the tissue cytokine levels as previously reported by **Okunieff et al.,** [26].

Ki-67, a well-known nuclear proliferation marker, is one of multiple cell-cycle regulating proteins which can be identified by immunohistochemistry. It reacts with a nuclear non histone protein which is expressed in all cell cycle phases, except the G0 phase. Several studies demonstrated the correlation between an elevated Ki-67 labeling index and poor tumor differentiation[27]. Regarding the Ki-67 results of the current study, the rat submandibular salivary gland of the control group showed mild to moderate nuclear immunoreactivity among a few number of nuclei. In the CsA group, the acini and duct system displayed a strong nuclear immunoreactivity for Ki 67 in a large number of nuclei; this could be caused

by the oxidative stress induced by CsA which may disturb DNA repair mechanisms and initiate cell proliferation[28]. On the other hand, CsA can hinder apoptosis by blocking the permeability transition pore of the mitochondria and inhibiting the release of cytochrome-c, so immunosuppressive treatment with CsA is often related to cancer progress[20]. In the same year, **Elkhier et al.[29],** suggested that the enhanced expression of Ki-67 may have an important role in the pathogenesis of CsA induced gingival overgrowth and they concluded that patients in this case are at high risk of developing a neoplasm. On the contrary, other studies revealed that the acanthosis noticed in CsA-treated patients is not caused by increased keratinocytes proliferation but is rather due to extended cell life produced by the CsA antiapoptotic effect[30].

In the combination group, an obvious decrease in the number of Ki-67 positively stained nuclei was detected among the gland. This could be related to the established antioxidant and antiproliferative activities of PO. **In 2016, Guo et al.[31]** reported that PO showed a powerful antiproliferative effect as it delays cell growth, so; it has a possible beneficial role in treating breast cancer. On the other hand, dissimilar results were obtained by **Al-refai et al., [32]** who demonstrated that an antioxidant, like green tea, resulted in a non-significant increase in the expression of Ki-67, and a significant increase in the antiapoptotic activity in the submandibular glands of methotrexate treated albino rats. In the present work, statistical analysis confirmed the obtained immunohistochemical results, as the area % of Ki-67 increased significantly in the CsA group when compared to the control one. Concerning the effect of purslane administration on CsA treated rats, the area % of Ki-67 immunoexpression was significantly decreased in the combination group when compared to the CsA one.

Conclusively, the histological, immunohistochemical and image analysis results proved that the ethanolic extract of purslane, as an antioxidant agent, has resulted in an obvious improvement in the histological features of the rat submandibular salivary gland and also reduced the elevated level of Ki-67 immuno-expression in the combination group compared to that in the CsA group. Finally, purslane extract could be intensely recommended as a natural easily available antioxidant source. However, care should be taken regarding unnecessary antioxidant supplementation and further clinical trials are required to accurately study the effect of purslane and to determine the safest dosage for its consumption.

CONCLUSIONS

From the current work, it could be concluded that CsA has damaging effects on the submandibular salivary glands of rats. However, purslane administration obviously attenuated the CsA-induced salivary gland alterations and also, it exerted an apparent antiproliferative effect. Thus, more studies should be conducted to evaluate the possible clinical applications of purslane.

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