RESEARCH ARTICLE

MEASUREMENT OF SALIVA FLOW RATE AND m\textit{i}RNA-132 EXPRESSION IN RELATION TO THE HISTOLOGICAL STRUCTURE OF SUBMANDIBULAR SALIVARY GLAND FOLLOWING ADMINISTRATION OF ANTI-OBESEITY AGENTS (AN ANIMAL STUDY).

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Abstract

Background: Orlistat was among the medications used for treatment of obesity. Recently, natural extracts were used to overcome the adverse effects that might result from antiobesity medications. Thus, our study aimed to compare the effect of orlistat and green coffee extract on submandibular salivary glands regarding their histology, miRNA content, and other salivary constituents. Design: 35 male albino rats were divided into three groups: Group I (Control group): formed of 5 rats. Group II (Orlistat group): 15 rats, each was given a daily oral dose of orlistat (32 mg/kg/day). Group III (Green Coffee Extract group): 15 rats, each received a daily oral dose of aqueous extract of green coffee (93mg/kg/day). Just before scarification, saliva was collected from all rats for measurement of the salivary flow rate and detection of salivary constituents. Rats of groups II and III were sacrificed after 2, 4 and 6 weeks from the beginning of the experiment and glands were prepared for histological examination. Results: Histological results of groups II and III revealed degenerative changes of the glands elements ranging from moderate or even severe changes in group II to minor changes in group III which was confirmed by the statistical analysis results. Conclusion: Orlistat and green coffee extract had variable drastic effects on the submandibular salivary glands’ structure and function. However we were able to prove that natural extracts as green coffee could be considered a better alternative for treatment of obesity.

Introduction:

Obesity is considered an essential health problem influencing both developed and developing societies around the world (Dunn, 2015). In this condition, excess accumulated fat leads to adverse effects on body health (Amin \textit{et al.}, 2014). Obesity was found to be related to increased risk for various diseases such as diabetes type II, hypertension,
cardiovascular diseases and some types of cancers (Haidari et al., 2014 and Finucane et al., 2011). Hence, finding an effective and safe method to control body weight is a matter of importance. Nevertheless, frustration of patients and therapists with alteration of lifestyle made the use of medicines and surgery to be a pleasing choice of treatment (Salem and Rezaeian, 2011).

Orlistat (Xenical) is a lipophilic drug used for treatment of obesity. It prevents triglycerides hydrolysis, consequently decreases monoglycerides and fatty acids absorption resulting in weight loss. Unluckily, several adverse effects has been reported with the use of orlistat as abdominal pain, diarrhea, oily stools, pancreatitis and few documented cases of liver failure and hepatitis (Elbakary and Bayomy, 2011).

Hence, the serious adverse effects and high expenses of most anti-obesity drugs have led overweight patients to shift to natural products which may help in weight loss (Ormsbee et al., 2014). Coffee is a popular beverage with complex structure. Several constituents are found in the grain or manufactured during its processing. Many studies reported the numerous beneficial effects of these components on human health (Florián et al., 2013). Green coffee extract (GCE) is a well-known form of coffee which is processed from unroasted green coffee bean (Andrade et al., 2012). Natural ingredients present in green coffee have been found to increase metabolism of fat in the liver and to prevent fat absorption, thus inhibiting fat accumulation and weight gain (Meng et al., 2013).

It has been proposed that the synergistic effect of caffeine, chlorogenic acid and polyphenolic constituents in GCE help in suppression of weight gain as well as fat accumulation (Revuelta-Iniesta and Al-Dujaili, 2014). However, some studies documented that the effect of GCE on weight loss is related to its content of chlorogenic acid (Song et al., 2014).

MicroRNAs (miRNAs) are a family of small (around 22 bases long), single-stranded, non-coding RNA molecules (Kebschull, and Papapanou, 2015). They are associated with controlling different biological activities in cells, tissues and some pathological processes in infections and autoimmune diseases (Nayar et al., 2016).

Many disorders have been found to be related to miRNA dysregulation. MicroRNAs can be used as markers for diagnosis of several diseases when their biological action in relation to diseases is well understood (Yoshizawa and Wong, 2013). They have the advantages of being measured, evaluated, and obviously stable, which make them more prevalent than other types of markers (Alevizos et al., 2011).

Saliva is a complex fluid which has exosomes (microvesicles) containing miRNAs (Gallo and Alevizos, 2013). Nowadays, salivary miRNAs have been widely utilized in diagnostic and biological applications of salivary researches (Yoshizawa and Wong, 2013).

Accordingly, our study aimed to compare the effect of anti-obesity agents; orlistat and green coffee extract on submandibular salivary glands of albino rats regarding their histology, salivary miRNA content, and other salivary constituents. Moreover, to prove the beneficial effect of natural extracts as a safe alternative for treatment of obesity.

Materials and Methods:-

Animals:-
Thirty five adult male albino rats weighing 150-200 gm were used in this study. The animals were housed in the animal house of biochemistry department, Faculty of Medicine, Cairo University. They were allowed to access freely standard balanced diet and freshwater supply ad-libitum. The study design was approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC) Medical Science Sector.

Experimental design:-
After one week acclimatization period, the animals were randomly divided into three groups as follows:

Group I (Control group): This group included 5 rats, which were allowed to access freely standard balanced diet and freshwater supply.
Group II (Orlistat group): This group consisted of 15 rats. The animals were given a daily oral dose of orlistat (32 mg/kg/day) (Elbakary, R. and Bayomy, 2011) dissolved in fish oil and delivered by gastric tube. Orlistat was purchased as a capsule with a trade name (Xenical) manufactured by Hoffmann La Roche, Germany.

Group III (Green Coffee Extract group): The rats (15 in number) received a daily oral dose of aqueous extract of green coffee (93mg/kg/day) through a gastric tube. The aqueous extract was prepared by adding 15gm green coffee beans to 100mL distilled water and allowed to boil for 15 minutes; then filtered through sterile gauze. The extract was eventually poured into glass jar (Florían et al., 2013).

Just before scarification, saliva was collected from all rats for measurement of the salivary flow rate and detection of salivary constituents.

Total saliva collection:-
Prior to saliva collection, pilocarpine (0.6 mg/kg) was injected intraperitoneally. Small cotton balls were pre-weighed and applied in the rat’s oral cavity (Romero et al., 2012) for nearly 30 minutes. The total saliva collected was calculated as the difference between cotton balls’ weight pre- and post-collection. The salivary flow rate was calculated by dividing the amount of saliva collected (in micro liters) on collection time taken (in minutes).

Salivary total protein:-
Directly after saliva collection, the protein concentration was determined using the Bradford method (Bradford assay; Bio Rad, Hercules, CA, USA)

Fluoride determination:-
Fluoride level in saliva was determined by mixing 1 ml of saliva with the reagent solution of Color-Chart-Comparison-Drop-Count-Test-Kits (ORLAB, India). The sample’s color changed and was compared with the respective color chart. The reading of the matched color on the color chart corresponded to the concentration of the respective parameter in the saliva sample. The test kit was based on bleaching of zirconium xylene orange complex where fluoride ions break the zirconyl xylene orange complex to form colorless zirconium fluoride. Fluoride level in saliva was assessed in mg/ml.

Amylase assessment:-
Determination of amylase level in collected saliva was carried out using ELISA technique, which is based upon in vitro quantitative determination of rat AMY2 concentrations in saliva. The ELISA kit was supplied by E lab science (Catalog number: E-EL-R2545, USA) and the steps were followed according to manufacturer’s instructions. Amylase concentration in saliva was assessed in mg/ml.

Real time Polymerase chain reaction (PCR):-
Specimens of salivary glands’ tissues of all studied groups were first homogenized and total RNA was then isolated using RNA easy Mini Kit (Qiagen) and was further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). For quantitative expression of miRNA 132; 100 μg of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with TaqMan PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95 °C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55 °C and 30 seconds at 72 °C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of 6U as housekeeping gene. We used 1 μM of both primers specific for each target gene. Primers sequence and annealing temperature specific for each gene are demonstrated in Table (1).

The rats of groups II and III were sacrificed at three time intervals; after 2, 4 and 6 weeks from the beginning of the experiment. At each interval, 5 rats from each group were euthanized by an intra-cardiac overdose of sodium thiopental (sodium thiopental 80 mg/kg). Submandibular salivary glands of both sides were dissected and prepared for histological examination.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence from 5’- 3’</th>
<th>Gene bank accession number</th>
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<tbody>
<tr>
<td>MiRNA132</td>
<td>F: gcagtaacagtctacagcca</td>
<td>MIMAT0008381</td>
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<tr>
<td></td>
<td>R: gtccagttttttttttttttcgac</td>
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<td>6U</td>
<td>F: atacagagaagattagcatggcc</td>
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<tr>
<td></td>
<td>R: cgaatttgcgtgtcatcttgt</td>
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Table 1: showing primer sequence of target genes

**Histological examination:**

**Light microscopic examination:**
The dissected salivary glands specimens were fixed immediately in 10% formaldehyde solution for 24 hours, dehydrated, cleared and embedded in paraffin. Serial sections of about 5μm thickness were obtained. For routine investigations, sections were stained with haematoxylin and eosin (H&E) (Bancroft, J. and Gamble, 2012).

**Statistical analysis:**
Statistical analysis was performed using a commercially available software program (SPSS 22; SPSS, Chicago, IL, USA).

As data was parametric, significance of the difference between different groups at each time point and difference within the same group in different times were evaluated using one way analysis of variance (ANOVA) test, followed by Tukey’s post hoc test. The level of significance was set at p < 0.05. Correlations between quantitative variables were done using Pearson correlation coefficient (Chan, 2003).

**Results:**

**Histological results:**

**Group I (Control group):**
The submandibular salivary glands of the control group revealed normal architecture of its elements. The acinar cells were intermediate in structure between serous and mucous cells (seromucous), the intervening striated ducts appeared intact with their basal striations together with the granular convoluted tubules (GCT) which appeared with their basally situated nuclei and dense apical granules. The excretory ducts showed normal ductal outline and lining within the connective tissue septa between the lobules of the gland (Fig.1).

**Group II (Orlistat group):**

**Two weeks interval:**
At two weeks interval, few areas of vacuolation were detected within the acini and striated ducts. Moreover, the acini revealed unclear cellular boundaries together with nuclear degeneration. Extravasated RBCs were evident between the acini. The GCTs appeared with indistinct boundaries (Fig.2a). The excretory ducts showed slight irregularity in their outline. Connective tissue septa were thickened with engorged blood vessels and inflammatory cells infiltration (Fig.2b).

**Four weeks interval:**
Histological findings of this group revealed signs of deterioration and degeneration among the gland's elements. The acini exhibited disrupted cellular boundaries with numerous vacuolation, indistinct outline; yet some appeared collapsed and shrunken. Regarding the nuclei, they were pyknotic and exhibited pleomorphism and hyperchromatism. Moreover, the acini lost their intact arrangement; they were detached and separated further apart. Disfigurement and abnormal configuration was again detected among the striated ducts and GCTs which appeared collapsed with nuclear degeneration and apparent increase in vacuolation compared to the previous interval (Fig.3a). Perinuclear halo was apparent among the lining cells of the excretory ducts which revealed discontinuity in their outline at discrete areas and exhibited signs of nuclear degeneration and pleomorphism. Extravasated RBCs were seen between the acini as well as in the vicinity of the excretory ducts. Increased fibrous content together with and inflammatory cells were evident (Fig.3b).
**Six weeks interval:**
In addition to the massive changes and deterioration detected within the glands in the previous intervals; marked shrinkage was apparent among the acini, striated ducts and GCTs at six weeks. The acini completely lost intimate contact and were widely separated (Fig.4a). The excretory ducts showed evident discontinuity in several areas along their outline and revealed suspended secretion in their lumen. Inflammatory cells infiltration was detected in the vicinity of the ducts (Fig.4b).

**Group III (Green Coffee Extract group):**
**Two weeks interval:**
At two weeks, the acini were apparently normal with uniform intact outline, darkly stained basal nuclei and basophilic cytoplasm. Similarly, the striated ducts assumed nearly normal architecture except for minor discrete vacuolations. However, GCTs were vacuolated with few extravasated RBCs among the acini. (Fig.5a). The excretory ducts revealed intact outline and were associated with distended blood vessels together with few inflammatory cells (Fig.5b). Apparently, thick connective tissue septa were evident inbetween the glandular lobes.

**Four weeks interval:**
Histological findings at this interval revealed minor changes among various elements of the gland. Both, the acini and striated ducts appeared nearly normal with minimal vacuolations. The GCTs showed minimal vacuolations with slight disfigurement. Few extravasated RBCs were detected between the acini and ducts (Fig.6a). Though the excretory ducts revealed intact outline similar to the previous interval; yet slight thickening was noticed along the boundary together with discrete areas of paranuclear vacuolation among the ductal cells. The nearby associated blood vessels showed thickening of their walls and were engorged with RBCs. Thick connective tissue septa were evident with inflammatory cells infiltration (Fig.6b).

**Six weeks interval:**
Minor changes within the acini and striated ducts were detected in relation to four weeks interval and represented by slight shrinkage of the acini with evidence of flattened degenerated nuclei among few of the acinar cells and vacuolated striated ducts. The GCTs appeared with increased vacuolation and distorted cellular boundaries. Extravasated RBCs among the acini were more evident than the previous intervals (Fig.7a). Slight hyperplasia was evident within the excretory ducts which showed discontinuity in their outline at certain areas and were associated with slight increase in inflammatory cells infiltrate (Fig.7b).

![Fig.1](image_url)- Photomicrograph of rats' submandibular salivary glands of group I (control group) showing; normal architecture of its elements: seromucous acini (a), striated ducts (s), GCTs (G) and excretory ducts (E) (H&E, Orig. Mag.X 100)
Fig. 2: Photomicrograph of rats’ submandibular salivary glands of group II (two weeks interval) showing; (a) few vacuolations within the acini and striated ducts (black arrows), unclear cellular boundaries with nuclear degeneration among the acini (asterisks), GCTs with indistinct boundaries (G) and extravasated RBCs (yellow arrows).
(b) slight irregular excretory ducts (black arrow), thickened connective tissue septa (C.T.), engorged blood vessels (yellow arrow) and inflammatory cells infiltration (blue arrow). (H&E, Orig. Mag.x 200)

Fig. 3: Photomicrograph of rats’ submandibular salivary glands of group II (four weeks interval) showing; (a) disrupted cellular boundaries of the acini (a), numerous vacuolation (black arrows), nuclear pyknosis, pleomorphism and hyperchromatism (red arrows), disfigured striated ducts (st) collapsed GCTs with nuclear degeneration and vacuolation (G) and extravasated RBCs (blue arrow).
(b) perinuclear halo among the cells of the excretory ducts (black arrow), discontinuity of ductal outline (asterisk) signs of nuclear degeneration and pleomorphism (arrow heads), extravasated RBCs (blue arrow), increased fibrous content (C.T.) and inflammatory cells (red arrow). (H&E, Orig. Mag.X 200)
Fig. 4: Photomicrograph of rats’ submandibular salivary glands of group II (six weeks interval) showing: (a) marked shrinkage and separation among the acini (a), striated ducts (arrow) and GCTs (G) with complete loss of intimate contact. (b) excretory ducts with evident discontinuity in several areas along their outline (black arrows) suspended secretion in their lumen (asterisk) and inflammatory cells (red arrow). (H&E, Orig. Mag.X 200)

Fig. 5: Photomicrograph of rats’ submandibular salivary glands of group III (two weeks interval) showing: (a) apparently normal acini (a), striated ducts with minor discrete vacuolations (black arrows), moderately vacuolated GCTs (G) and few extravasated RBCs (red arrow). (b) intact excretory ducts (E), distended blood vessels (black arrows), few inflammatory cells (red arrows) and thick connective tissue septa (C.T.). (H&E, Orig. Mag.X 200)
Fig.6:- Photomicrograph of rats’ submandibular salivary glands of group III (four weeks interval) showing: (a) minimal vacuolations among the acini (blue arrow), striated ducts (black arrow) and GCTs (red arrow) with few extravasated RBCs (yellow arrow). (b) slight thickening along the boundary of the excretory ducts with discrete areas of paranuclear vacuolation (black arrow), thickening of the walls of the blood vessels (yellow arrow), thick connective tissue septa (C.T.) and inflammatory cells infiltrates (red arrow). (H&E, Orig. Mag.X 200)

Fig.7:- Photomicrograph of rats’ submandibular salivary glands of group III (six weeks interval) showing: (a) slight shrinkage of the acini (a) with flattened degenerated nuclei among few of the cells (black arrow), vacuolated and distorted GCTs (G), vacuolated striated duct (red arrow), extravasated RBCs (asterisks). (b) slight hyperplasia within the excretory ducts (black arrows), discontinuity in their outline (asterisk) and slight increase in inflammatory cells infiltrate (red arrow). (H&E, Orig. Mag.X 200)

Statistical results:-
Salivary flow:-
There was no significant difference in salivary flow in the control group all throughout all the experimental durations (p = 1).

The salivary flow in the orlistat treated group gradually decreased with significant difference (p= 0.002) when comparing the four weeks duration with the two weeks as well as when comparing the six weeks duration with the two weeks (p= 0.00) and the six weeks duration with the four weeks (p= 0.03).
The salivary flow in the green coffee treated group gradually decreased with significant decrease in the six weeks duration compared to the two weeks (p= 0.006). No significant difference was detected between the four weeks and the two weeks duration (p= 0.17) as well as between the four and six week duration (p= 0.8).

Statistical significant decrease in salivary flow was detected in orlistat treated group compared to green coffee treated group in two weeks duration (p = 0.04), four weeks duration (p = 0.00) and six weeks duration (p = 0.00) (Fig.8)

Amylase
No significant difference was evident in amylase level of the control group during all durations (p = 1).

In the orlistat treated group, the amylase level gradually decreased but with no significant difference all through the experiment when comparing the four weeks and six weeks duration with the two weeks (p= 0.58), (p= 0.08) respectively and when comparing the six weeks duration with the four weeks(p= 0.9).

The amylase level in the green coffee treated group gradually decreased with significant difference in the six weeks duration compared to the two weeks (p=0.005) but no significant difference between the four weeks and the two weeks duration (p=0.16) and between the six weeks and the four weeks duration p=( 0.7)

Statistical significant decrease in amylase level was marked in orlistat treated group when compared to green coffee treated group in two weeks duration (p = 0.00), four weeks duration (p = 0.001) and six weeks duration (p = 0.002) (Fig.9)

Fluoride
No significant difference in salivary flow in the control group (p = 1).

The fluoride level in the orlistat treated group gradually decreased but with significant difference (p= 0.006) when comparing the four weeks duration with the two weeks. Significant decrease in its level when comparing the six weeks duration with the two weeks (p= 0.00) and when comparing the six weeks duration with the four weeks (p= 0.01).

In the green coffee treated group, the fluoride level gradually decreased with significant decrease in the six weeks duration compared to the two weeks (p=0.001) but no significant difference between the four weeks and the two weeks duration (p= 0.47) as well as between the four and six week duration(p=0.1).

No significant difference in flouride was detected in orlistat group compared to green coffee group in two weeks duration (p = 0.14), while significant decrease in orlistat group was obvious in four weeks duration (p = 0.00) and six weeks duration (p = 0.00) compared to the green coffee (Fig.10)

Total protein
No significant difference was detected in the total protein level in the control group throughout the experiment durations (p = 1).

The total protein level in the orlistat treated group gradually decreased but with no significant difference (p= 0.4) when comparing the four weeks duration with the two weeks. There was significant decrease in its level when comparing the six weeks duration with the two weeks (p= 0.007), but no significance when comparing the six weeks duration with the four weeks (p= 0.52).

In the green coffee treated group, the level gradually decreased with significant decrease in the six weeks duration compared to the two weeks (p=0.005) but no significant difference between the four weeks and the two weeks duration (p=0.7) as well as between the four and six week duration(p=0.14).
No statistically significant difference in total protein level in orlistat treated group compared to green coffee treated group in two weeks duration ($p = 0.9$), four weeks duration ($p = 0.64$), or six weeks duration ($p = 0.9$) (Fig.11)

**miRNA 132**

The control group revealed no significant difference in miRNA expression all through the experimental durations ($p = 1$).

In the orlistat treated group, the miRNA expression gradually increased, but no significant difference ($p = 0.52$) when comparing the four weeks duration with the two weeks. Significant increase in its level was detected when comparing the six weeks duration with the two weeks ($p = 0.002$). However, no significant difference when comparing the six weeks duration with the four weeks ($p = 0.16$).

The miRNA expression in the green coffee treated group gradually increased with significant increase in the six weeks duration compared to the two weeks ($p = 0.002$) but no significant difference between the four weeks and the two weeks duration ($p = 0.54$) as well as between the four and six weeks duration ($p = 0.12$). Statistical significant increase in miRNA expression was marked in orlistat treated group when compared to green coffee treated group in two weeks duration ($p = 0.02$), four weeks duration ($p = 0.03$) and six weeks duration ($p = 0.04$) (Fig.12)

**PCR results**

Agarose gel electrophoresis of PCR products of miRNA 132 gene normalized to housekeeping gene 6U revealed significant difference in quantitation between different experimental groups (Control, Orlistat, and Green coffee) in different durations (2, 4, and 6 weeks) (Fig.13).

*Correlations between different salivary constituents relative to miRNA132 and salivary flow rate*

*Correlations between amylase, fluoride, total protein and salivary flow to miRNA132*

Data showed strong negative correlation between fluoride and miRNA ($r = 0.8$), amylase and miRNA ($r = 0.9$), total protein and miRNA ($r = 0.8$), and between salivary flow and miRNA ($r = 0.8$) ($p < 0.01$) (Fig.14).

*Correlations between amylase, fluoride and total protein to salivary flow*

Data showed strong positive correlation between fluoride and salivary flow ($r = 0.9$), amylase and salivary flow ($r = 0.9$), and between total protein and salivary flow ($r = 0.78$) ($p < 0.01$) (Fig.15).
Fig.8: Data were expressed as Mean ± SD, p value <0.05 is considered significant *: statistically significant compared to corresponding value in 2Ws duration #: statistically significant compared to corresponding value in 4Ws duration

Fig.9: Data were expressed as Mean ± SD, p value <0.05 is considered significant *: statistically significant compared to corresponding value in 2Ws duration

Fig.10: Data were expressed as Mean ± SD, p value <0.05 is considered significant *: statistically significant compared to corresponding value in 2Ws duration #: statistically significant compared to corresponding value in 4Ws duration.
Fig. 11: Data were expressed as Mean ± SD, p value < 0.05 is considered significant.
*: statistically significant compared to corresponding value in 2Ws duration

Fig. 12: Data were expressed as Mean ± SD, p value < 0.05 is considered significant.
*: statistically significant compared to corresponding value in 2Ws duration

Fig. 13: PCR products of miRNA 132 gene, G1: Control, G2: Orlistat and G3: Green coffee. A, B and C (2, 4 and 6 weeks respectively)
Fig. 14: Data showing correlation between amylase, fluoride, total protein and salivary flow to miRNA132
Fig.15: Data showing correlation between amylase, fluoride and total protein to salivary flow.

Discussion:
The occurrence of obesity has been obviously increased throughout the world. Many individuals seek to reduce their weight by non-prescribed drug administration as orlistat (Elbakary and Bayomy, 2011). Several researches on overweight patients have shown that orlistat exhibited positive results on serum lipid, glucose and cholesterol levels together with its enhancing effect on weight loss (Lucas et al., 2003). Recently, attention is directed towards the use of natural herbs to overcome adverse effects and high expenses of anti-obesity drugs (Ormsbee et al., 2014).

Accordingly, as some individuals use weight loss drugs and/or natural products to protect themselves from being obese; we thought it would be valuable to know their effect on the oral and paraoral tissues; among which are submandibular salivary glands using albino rats as an animal model.
In our research, the orlistat treated group revealed degenerative changes among the submandibular salivary glands’ acini and ducts. These histological changes were exaggerated throughout the experimental period, starting from the 2nd week, 4th, till the 6th week of treatment. It was reported that patient’s use of orlistat for 8 weeks caused side effects on the liver which was supposed to be due to the cumulative dose effect of the drug. Nevertheless, some previous studies reported that orlistat associated liver failures have occurred immediately with beginning of the treatment (Sall et al., 2014). In accordance with our results, degenerative changes of parotid glands’ elements of rats were found to be exaggerated with the increase of treatment time (El Bolok, 2015).

Deterioration of glands’ elements of the orlistat group was in the form of disrupted cellular boundaries, vacuolation, and collapsed parenchymal structures. The nuclei appeared pyknotic, pleomorphic and degenerated. Moreover, the acini and ducts were detached and separated further apart. These data were concomitant with Caner et al., (2005), who noticed widened intercellular spaces with loss of continuity of the intestinal epithelial brush border of orlistat treated rats. Destruction of the normal architecture of pancreatic tissues was also spotted in orlistat treated rats. Vacuolated and spaced pancreatic acini may be due to increased interstitial edema (Elbakary and Bayomy, 2011). Another study examined the changes in glioblastoma cells in a cell culture after orlistat administration. Signs of apoptosis were manifested in the form of chromatin condensation, nuclear fragmentation, and cellular shrinkage (Grube et al., 2014).

The herein study revealed also thickening of the connective tissue septa with dilated engorged blood vessels and inflammatory cells infiltration of the orlistat treated sections. These findings were parallel to that reported by Nairooz et al., (2010), who documented intense inflammatory cell infiltration in the lamina propria of colonic mucosa of high fat diet rats treated with orlistat. Furthermore, thickening of the connective tissue between pancreatic lobules together with dilated congested blood vessels and leukocytic infiltration were observed in orlistat given rats (Elbakary and Bayomy, 2011). Severely congested blood vessels associated with hemorrhage and inflammatory cells infiltration were also clearly demonstrated in kidney sections of orlistat supplemented rats (Amin et al., 2014), and in liver biopsies of orlistat administered patients (Sall et al., 2014).

The reported degenerative changes and inflammation can be explained by Elbakary and Bayomy (2011), who recognized intense immuno-positive reaction of iNOS (inducible nitric oxide synthase which induces the synthesis of nitric oxide) in pancreatitis in orlistat-treated rats. It was detected that nitric oxide (NO) plus some free radicals result in oxidative stress and consequently share in the pathogenesis of pancreatitis. Reaction of NO and superoxides results in formation of peroxynitrite, which is a potent cytotoxic substance causing cellular injury (Keklikoglu, 2008). Activation of iNOS leads to induction of some apoptotic mediators, as interferon-γ, through a reactive oxygen species (ROS)-mediated mechanism. NO-mediated apoptotic mechanism reveals accumulation of p53 (tumor suppressor protein), variations in expressing some Bcl-2 proteins, destruction of various mitochondrial functions, stimulation of caspase cascade, and DNA disintegration (Ang et al., 2009). These effects may explain the nuclear and cytoplasmic structural alterations that were detected in our study. Dilation and congestion of the blood vessels noticed in this research were clarified by excess production of NO which acts as an endothelial relaxing factor (Jana et al., 2001). This may be an essential cause in systemic and local homodynamic disorders (Panek and Zasada, 2007).

Regarding the effect of green coffee extract, group III samples showed minor degenerative changes compared to the orlistat treated group. These changes appeared in the form of minor discrete vacuolations within the acini and ducts with few extravasated RBCs. In addition, perinuclear vacuolation, degenerated nuclei and slight acinar shrinkage were also detected. On the other hand, the blood vessels appeared distended and engorged together with few inflammatory cells. Thick connective tissue septa were also evident inbetween the glandular lobes.

Up to our knowledge, no data were documented on the effect of green coffee per se on normal tissues. Under this finding, we had to emphasize our discussion on the active components that justify the reported effects.

Green coffee is a rich source of chlorogenic acid (CHA) and caffeine. CHA is prevalent in fruits, vegetables and plants. It is produced by esterification of quinic and caffeic acids (Ruan et al., 2014). Most existing data verified the effect of CHA on induced diabetes, hepatotoxicity, partial nephrectomy, and kidney injury (Pari et al., 2010, Zhao

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et al., 2014, Lou et al., 2016 and Feng et al., 2016). These data revealed that CHA alleviates the histological alterations caused by these disturbances. Previous studies related these valuable effects to its free radical scavenging and antioxidant activity (Lou et al., 2016).

In spite of the reported benefits of the chlorogenic acid, we noticed that green coffee produced minor degenerative changes, congestion of blood vessels and inflammatory cells infiltration among the submandibular salivary glands’ elements. This might be attributed to different reaction of chlorogenic acid when associated with other active ingredients of green coffee as caffeine.

Caffeine is a purine alkaloid present as a natural ingredient in coffee beans (Muriel and Arrauz, 2010). It is one of the most frequently ingested and pharmacologically active constituents (Abd El-Ghany et al., 2012). Caffeine is promptly absorbed from the digestive tract to be distributed to all tissues (Onyeso et al., 2016).

Histological changes observed in our work regarding the green coffee group were consistent with Onyeso et al., (2016). They detected moderate vacuolation and minimal architectural destruction of rats’ liver treated with caffeine. Moreover, the pituitary gland showed scanty cells and clear hollows surrounding the neuronal cell bodies. It was suggested that cytoplasmic vacuolation may be due to destructive changes caused by ingestion of caffeine. On the other hand, some researches demonstrated the effect of energy drinks containing caffeine as a main active ingredient. Extravasated RBCs, thickening of the connective tissue capsule together with dilated engorged blood vessels were noticed in submandibular salivary glands of rats receiving these drinks (Mubarak, 2012). In addition to these observations, degenerated nuclei and perivascular inflammatory cells were obvious in pancreatic islets of rats given Power Horse drink (Ayuob and El Beshbeisy, 2016). It can be speculated that thickening of the connective tissue septa could be due to the effect of caffeine which caused increased fibrin deposition in wound healing of rat gingival connective tissue (Takesue, 1989). Dilatation and congestion of the blood vessels might also be related to the microcirculatory disorders due to caffeine content (Mubarak, 2012).

Here, we have to mention that because of the synergistic effect between caffeine and CHA, green coffee was able to reduce body weight better than chlorogenic acid or caffeine alone (Zheng et al., 2014). But this combination may not be most favorable regarding their mechanism of action on various tissues. It is postulated that caffeine offers a pro-oxidant environment (Ayuob and El Beshbeisy, 2016) which may partially counteract the antioxidant effect of CHA resulting in minor degenerative changes on the salivary glands.

Saliva is a fluid that can be easily collected by stimulation, and its collection is non-invasive. Recently, it has been widely used in the diagnosis of systemic and localized diseases (Kallapur et al., 2013). In dentistry, it has been utilized as a diagnostic method for periodontal diseases and to assess the risk of caries in orthodontic patients. Thus, salivary tests are considered useful, both in planning preventive measures and in the evaluation of the outcomes of treatment (Teixeira et al., 2012). In accordance, we decided to include saliva in our investigation particularly that the salivary glands are the tissue of interest.

It has been found that medications used for treatment of various diseases; among which the anti-obesity drugs might cause adverse effects on the salivary glands (Wolff et al., 2017). In accordance, the statistical results of the ongoing study revealed decrease of salivary flow rate in groups II and III throughout the three experimental periods. The rate of decrease was more detectable in the orlistat treated group. The secretory cells are supplied with muscarinic M1 and M3 receptors, α1- and β1-adrenergic receptors, and certain peptidergic receptors that are involved in the initiation of salivary secretion (Villa et al., 2016). Medications may act on the central nervous system and/or at the neuroglandular junction, resulting in salivary gland dysfunction. Therefore, drugs that have antagonistic actions on the autonomic receptors, but used to treat dysfunctions in various effectors of the autonomic nervous system might also affect the functions of salivary glands and thus cause oral dryness. Among these is orlistat; as an antiobesity drug (Wolff et al., 2017). Moreover, it would be expected that caffeine had a significant, dose-dependent effect on reducing salivary secretion (Barasch and Gordon, 2016).

On contrary, both experimental groups demonstrated increase in miRNA-132 expression which was fairly evident in group II biopsies. Data showed strong negative correlation between salivary flow and miRNA. It should be
highlighted that different miRNA expression patterns are correlated to salivary gland inflammation and dysfunction. The expression levels of miRNA constantly increase with rise of inflammation. Also, miRNA was found to be up regulated when salivary flow rate declines, which indicates salivary dysfunction (Alevizos et al., 2011). These data come in accordance with ours as existence of inflammatory cell infiltrates and dilatation of blood vessels, which are signs of inflammation, were consistently observed along the experimental periods of the treated groups, but more evident in group II. Moreover, Li et al., (2015) confirmed significant elevation of miRNA-132 expression during the inflammatory phase of skin wound healing in humans. The rise of miRNA-132 expression may be an attempt to control the intensity of inflammation. In line with this, it has been reported that expression of miRNA-132 is enhanced by various pro-inflammatory genes, which acts as a negative feedback mechanism to reduce the inflammation (Wanet et al., 2012). It was also documented that different expression levels of miRNA-132 were spotted in monocyte/macrophage inflammatory response to infection, which critically affected resolution of inflammation (Olsen et al., 2017). Furthermore, Fen et al., (2014) verified that induction of inflammation of rat alveolar macrophages resulted in increased expression of miRNA-132 in vitro. This ended in a suggestion that miRNA-132 can be correlated with regulation of rat macrophages inflammatory response. Another study proved that miRNA-132 levels were higher in inflamed intestinal samples compared with quiescent biopsies from individuals with inflammatory bowel disease (Maharshak et al., 2013).

As regards the salivary constituents; our data showed strong positive correlation between fluoride, amylase, total protein and salivary flow. As the salivary flow was significantly decreased throughout the experimental periods; a decrease in the levels of fluoride, amylase, and total protein level was evident.

Concomitantly, Shaila et al., (2013) reported that one of the major factors affecting the protein concentration and composition of whole saliva is the salivary flow rate. As the salivary flow increases, the salivary consistency as well as the cleaning and diluting capacities increase. Therefore, any changes in health that might cause a reduction in salivary flow will be accompanied by an alteration in the level of oral cleaning (Kudva et al., 2010) resulting from the current changes in the salivary constituents. The salivary hypofunction has been characterized by reduced flow rate and alterations in the concentrations of organic and inorganic compounds present in the saliva (Picco et al., 2012).

The presence of fluoride in the saliva depends on its absorption from exogenous sources. A study assumed that since the salivary fluoride concentration is proportional to the fluoride content in the food and as the rats were fed with a standard diet; thus the quantity of fluoride ingested was not different between normal and hypertensive Wister rats at 12 weeks of age (Picco et al., 2012). Unlikely, our statistical results verified a decrease in the fluoride content associated with the reduced salivary flow; a condition which might be related to the adverse effects of medication induced salivary dysfunction.

**Conclusion:**

It is worth to note that orlistat and/or green coffee extract which are considered antiobesity drugs had diverse effects on the submandibular glands of rats resulting in salivary dysfunction and inflammation which was detected histologically and was further confirmed by miRNA-132. Moreover, we were able to prove through collection of saliva, a decrease in salivary flow together with its constituents; a condition which might lead to unfavorable outcomes in the future.

**Disclosure:**
The authors report no conflicts of interest in this work.
References:


