# Evaluation of The effect of Propolis extract on the Tongue mucosa of an Induced toxic rabbit by Fenitrothion

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**Abstract: Objective:** The aim of the present study was to evaluate the effect of propolis as a natural antioxidant in prevention of fenitrothion induced toxicity on rabbit's tongue mucosa. **Methods:** Thirty healthy white New Zealand rabbits weighing between 1800 and 2200 g. were divided randomly into 3 groups, control (group I), fenitrothion administration (group II), fenitrothion administration 1 h after propolis extract administration (groups III) respectively. The rabbits were then sacrificed after 28 days. The tongue sections were examined histologically and immunohistochemically. **Results:** Histopathologically the fenitrothion group showed evidence of hyperplasia, hyperkeratosis with acanthosis. Meanwhile, the basal cell layer revealed basilar hyperplasia, nuclear hyperchromatism and mild dysplasia. The tongue's muscles revealed signs of fatty degeneration. Histological examination of the tongue mucosa of propolis treated group showed a relatively normal appearance. Histomorphometric analysis showed significant increase in the optical density of caspase-3 cleaved activity in experimental group II. **Conclusions**: In a rabbit model the administration of natural antioxidants (propolis) could have beneficial effect on prevention of cytotoxicity induced by organophosphorous compounds (fenitrothion).
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**Key words:** fenitrothion, propolis, tongue, rabbit, caspase-3

## 1. Introduction

Organophosphorus compounds are widely used in agriculture, medicine, and industry. Organophosphorus pesticides, in addition to their intended effects like the control of insects or other pests, are sometimes found to affect nontarget organisms including humans (**Cantelli-Forti *et al.*, 1993**). Exposure to these insecticides can involve a large segment of the population including, agriculture workers and their families, those living in proximity to farms, and the general population who may be exposed through home application of pesticides or via residues on food (**Chaudhuri, 1999**). Organophosphates (Ops) are the term that includes all insecticides containing phosphorus. Fenitrothion[O,O-dimethyl-O-(3-methyl-4-nitrophenyl)phosphorothioate], which is a yellow-brown liquid with an unpleasant odor at room temperature.

Fenitrothion is mainly used in agriculture for controlling chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains and cotton and in forest areas. It is also used for the control of flies, mosquitos, cockroaches and has been successfully used as a vector control agent for malaria in public health programmes and/or indoor use (**IPCS, 1992**). Fenitrothion is a broad-spectrum organophosphorus pesticide that distresses the nervous system by inhibiting acetyl cholinesterase activity (**Sarikaya, 2004**). This inhibition results in the accumulation of acetylcholine (Ach) at the neurone/neurone and neurone/ muscle (neuromuscular) junctions or synapses, causing rapid twitching of voluntary muscles and finally paralysis (**Afshar *et al.*, 2008**). Problems associated with pesticides hazards to man and environment are not confined to the developing countries, but extended to developed nations and still facing some problems in certain locations (**Nuckols *et al.*, 2007 and Suresh, 2007**). Many pesticides in common use can produce some toxic and adverse effects on liver, kidney, thyroid gland and other biological systems when tested on various types of experimental animals (**Poovala *et al.*, 1999**, **Kovacic, 2003).** These toxic effects probably occur through the generation of reactive oxygen species (ROS) causing damage to the various membranous components of cell (**Goel *et al.*, 2005**). These reactive oxygen species (ROS) may be produced as a result of the FNT metabolism by cytochrome P450s or due to the high-energy consumption coupled with the inhibition of oxidative phosphorylation (**Milatovic *et al.*, 2006**). The imbalance between the formation of ROS and mechanism of enzymatic and nonenzymatic antioxidants as a body defense system can lead to oxidative stress. Oxidative stress has been reported to be the primary mechanism of organophosphate

toxicity after prolonged exposure (**Lukaszewicz-Hussain, 2010**).

Antioxidants have proved to be a good defence mechanism against free radical effects, which might be produced from contamination with pesticide and other toxic substances (**Glodfarb, 1999**, **Valko *et al.*, 2007**). The body contains its own antioxidant system, made up of enzymes like catalase, super oxide dismutase and metal binding proteins. Cellular defence mechanism to oxidative damage is activated endogenously by glutathione and other enzymes which convert the oxidised molecules to their reduced from. The endogenous defence mechanism against oxidative damage is complemented by antioxidants.

Propolis (bee glue), a natural product produced by the honeybee (*Apis mellifera*, L.), has been used for thousands of years in folk medicine for several purposes. Its chemical composition is very complex and varies with geographic origin. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other substances, including organic debris (**Viuda-Marcos *et al.*, 2008**). The propolis extract contains amino acids, phenolic acids, Phenolic acid esters, flavonoids, cinnamic acid, terpenes and caffeic acid. Many of the physiological actions of these flavonoids have been attributed to their antioxidant properties, via their reducing capacities (catalysis of electron transport, ability to scavenge free radicals) (**Dimov *et al.*, 1991**, **Amoros *et al.*, 1992**, **Marcucci, 1995, Greenaway *et al.*, 1998, Russo *et al.*, 2002, Mani *et al.*, 2006**). In addition, propolis has been determined to reverse the depletion of liver glutathione, and has radical scavenging activity. Recent reports suggest that propolis may have an important role in balancing antioxidant systems and has an antiperoxidant effect on several tissues, which may account for its beneficial effect in oxidant induced injury **(Bhadauria *et al.*, 2008, Nirala and Bhadauria, 2008).**

Based on the observations that OPI increase the prevalence of cytotoxicity, and that the propolis is an effective antioxidant in the managements of this condition. The current study was carried out to hypothesise that propolis could be used as co-treatment to reduce side effects associated with use of OPI.

## 2. Material and methods

### 2.1. Chemicals:

**Fenitrothion:**

Sumithion (Fenitrothion 50% EC) (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate) was purchased from Kaffer Elzayat Co. for Insecticide Ind. Kaffr Elzayat, Egypt**.**

**Propolis:**

The type of propolis used in this study was Bee Propolis extract (Honey paste) was purchased from ministry of agriculture, el Doky, Egypt.

### 2.2. Experimental procedure

A total of 30 healthy white New Zealand rabbits weighing between 1800 and 2200 g. were used in this study. They were randomly assigned into three groups, 10 rats each and were supervised in a 15 $m^2$ closed balcony system illuminated by a night lamp. Mean temperature was maintained at 22 ± 3 °C. Appropriate nutrition was provided; the main food sources were green vegetables, carrots, and tap water. The experimental procedure was conducted in compliance with ethical principles for animals' research as reviewed and approved by institutional guidelines of Kasr-Elainy animal and experimental laboratory (Faculty of Medicine, Cairo University). Group I (control group) did not receive any medical treatment. Groups II and III were given 1/30 $LD_{50}$ 20 mg/kg body weight of fenitrothion orally once a day for 28 days **(Elhalwagy *et al.*, 2008**). Group (III) were orally administered 100 mg/kg/day body weight propolis extract daily 1 h before fenitrothion administration once a day for 28 days **( Kalogeropoulos *et al.*, 2009**). At the end of 28 days, animals were sacrificed by ketamine over dose. Tongue specimens were dissected and used for the light microscopic, and immunohistochemical examination, forming a total of 30 specimens (10 specimens from each group).

### 2.3. Light microscopic examination

Immediately following death, the tongues were excised, longitudinally bisected and immediately fixed in 10% neutral formalin for 48 h, and then rinsed in distilled water. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin. 30–40 sections of 5 mm thickness were cut. The sections were subjected to haematoxylin and eosin stain according to the conventional method. Histopathologic examination was performed using light microscopy. Histologic diagnoses were rendered using established criteria. Hyperkeratoses were characterized by a thickened keratinized layer, with or without a thickened spinous layer (acanthosis), and an absence of nuclear or cellular atypia. Dysplasias were characterized as lesions that showed various histopathologic alterations, including enlarged nuclei and cells, large and/or prominent nucleoli, increased nuclear to cytoplasmic ratio, hyperchromatic nuclei, dyskeratosis, increased and/or abnormal mitotic figures, bulbous or teardrop-shaped rete ridges, loss of polarity, and loss of typical epithelial cell cohesiveness.

**2.4. Immunohistochemistry (IHC)**

Sections of 5 mm thickness were placed on positive charged slides (SuperFrost Plus-Menzel GmbH). The sections were deparaffinised and endogenous peroxidase activity was blocked with 3% hydrogen peroxide ($H_2O_2$) in PBS for 30 min. Antigen retrieval was performed by microwaving the sections in 0.01 M sodium citrate buffer (pH 6.0). The slides were then rinsed in PBS, blocked with normal goat serum and incubated respectively with the primary antibodies PCNA (diluted 1:2000 in PBS, Sigma–Aldrich, St Louis, MO) and rabbit anti-cleaved caspase- 3 monoclonal antibody (diluted 1:100; Cell Signalling Technology, Boston, MA, USA) over night at 4 8C. They were then rinsed and incubated with secondary antibodies, biotinylated goat anti-mouse and goat anti-rabbit (diluted 1:150 in PBS; Zymed, San Francisco, CA, USA), for 3 h at room temperature. Sections were washed in PBS and incubated with streptavidin-labelled peroxidase complex (diluted 1:150 in PBS; Zymed) for 3 h at room temperature, and the antibody was then visualised with 0.6 mg/ml 3, 30-diaminobenzidine tetrachloride (DAB, Sigma– Aldrich) dissolved in PBS, to which 0.03% $H_2O_2$ was added (brown staining); the sections were subsequently counterstained, blued dehydrated and sealed with Mayer's haematoxylin.

**Caspase-3 staining assessment:**

The histological sections were examined using light microscope to assess the prevalence of positive ones. Caspase-3-labelled cells were identified by brown nuclear, cytoplasmic staining, as caspases translocate from the cytoplasm to the nucleus after activation (**Huo *et al.*, 2010**). The percentage of positive cells was measured in the form of an area and area percent inside a standard measuring frame of area 11434.9 micrometer$^2$ per 10 fields by a magnification (x200) using image analysis software (Leica -Qwin) system. The image analyser consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The Optical density (OD) of caspase-3 was measured using an objective lens of magnification 40, i.e. of a total magnification of 400. Ten fields were measured for each specimen. After grey calibration, the image was transformed into a grey delineated image to choose areas exhibiting positive reactivity with accumulation of all grades of reactivity (minimum, maximum and median grey). Areas of positive reaction were then masked by a blue binary colour.

**2.5. Statistical analysis:**

Quantitative data of the image analyzer were statistically evaluated and presented as means and standard deviation (SD) values. ANOVA (analysis of variance) test was used to compare mean values of caspase-3 immunoexpression related parameters in the studied groups. A probability value (*p* value) less than 0.05 was considered statistically significant.

**3. Results**

**3.1. Histological results**

**3.1.1. The control group (I)**

The tongue of white New Zealand rabbits were formed basically of a typical core of muscles covered by stratified squamous epithelium. The dorsal surface epithelium was stratified and keratinized. It was covered with numerous irregularities and elevations called lingual papillae (Fig. 1).

**3.1.2. The fenitrothion group (II)**

The tongue's sections from rabbits of fenitrothion treated group showed evidence of hyperplasia, hyperkeratosis with the spinous cell layer gradually thickened (acanthosis) and signs of dysplasia in the tongue epithelium. Meanwhile, the basal cell layer revealed basilar hyperplasia, nuclear hyperchromatism, polymorphic nuclei and mild dysplasia. The tongue's muscles revealed signs of fatty degeneration (Figs. 2 a, b, c).

**3.1.3. The propolis extract group (III)**

Histological examination of the tongue mucosa of propolis treated group showed a relatively normal appearance with conical flame shaped filfiorm tongue papillae and normal distribution of tongue muscles (Figs. 3 a, b,c).

**3.2. Immunohistochemical results**

The effect of fenitrothion as well as the prophylactic antioxidant treatment on cell apoptosis was evaluated using caspases – 3. A negative caspase-3 staining was observed in either nuclei or cytoplasm of basal cell layer of the tongue mucosa of group I (Fig. 4). However, group II revealed strong granular cytoplasmic reaction of basal and parabasal layer. However, most of the nuclei escaped this positive reaction (Fig. 5). Immuno-reactivity for caspases-3 was observed mild, faint and scattered in the cytoplasm or in the perinuclear region of basal cells of group III (Fig 6).

**3.3. Statistical analysis**

The optical density of areas occupied by active caspases-3 in the tongue of the control and experimental groups is summarized in Table 1 and Graph 1. An increase in the optical density of caspases-3 was evident in the tongue mucosa of the experimental group II. The increase was statistically evaluated and was found to be highly significant.

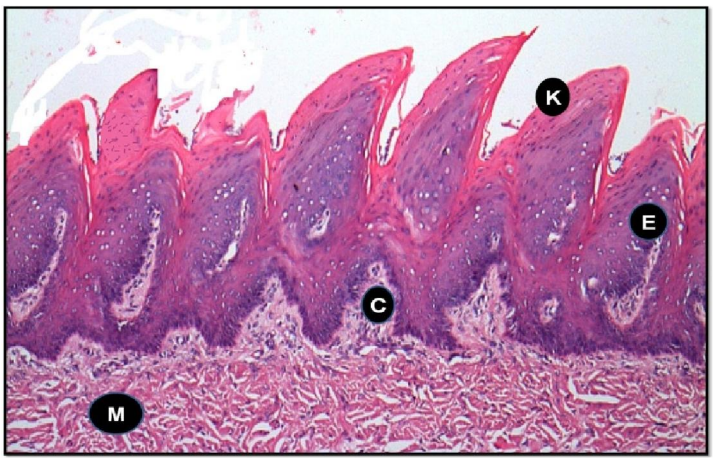



**Fig.1:** A photomicrograph of the rabbit tongue mucosa of the dorsal surface of the control group, showing keratin layer (K), epithelial tissue (E), connective tissue ( C ) and muscle tissue (M) (H&E, x100).

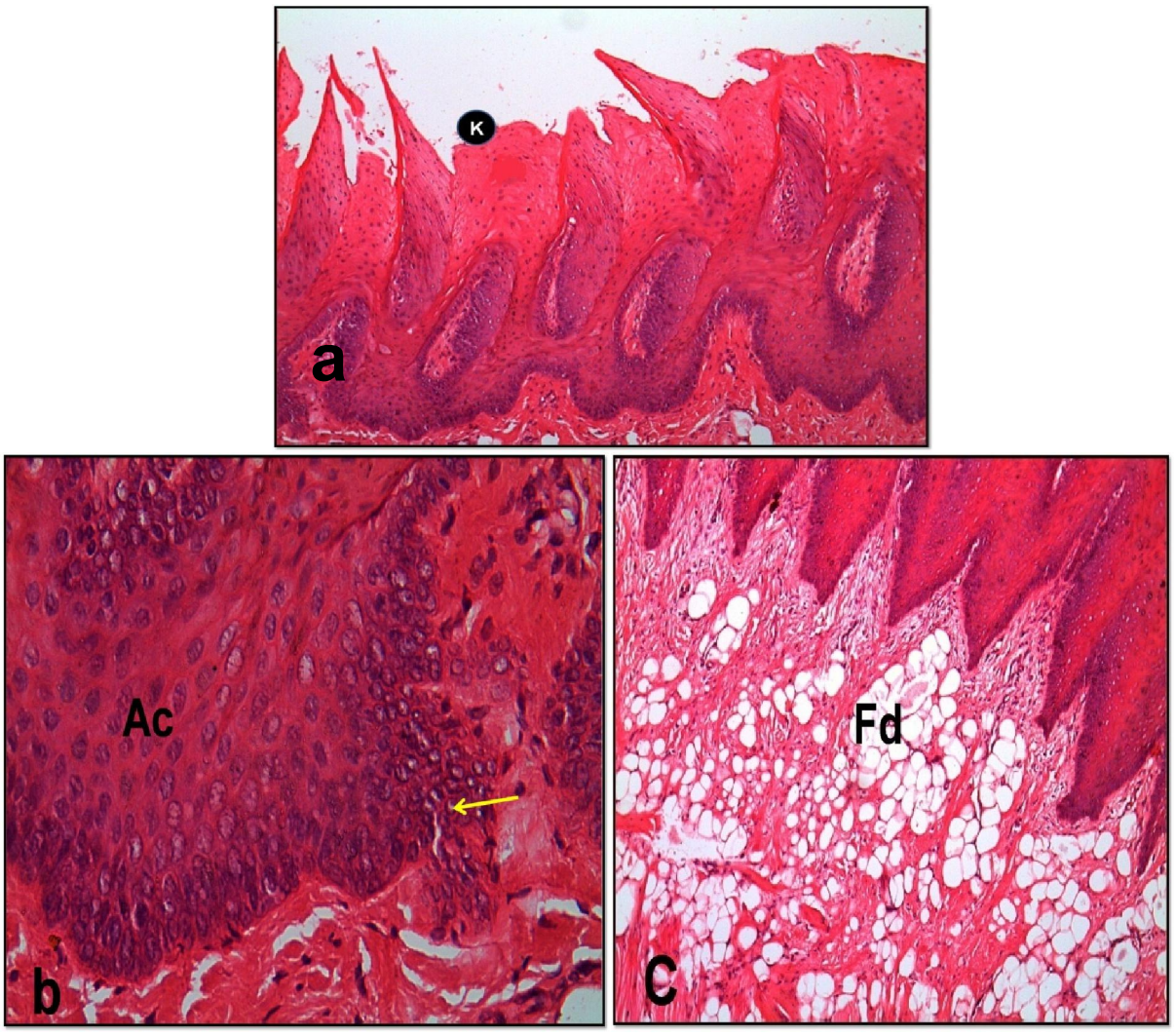



**Fig.2:** A photomicrograph of dorsal surface of the tongue mucosa of group II (fenitrothion treated group) showing: (a) hyperkeratosis (K) (H&E, x100). (b) Acanthosis (AC), hyperchromatism of the basal nuclei and mild dysplasia (arrow) (H&E, x400). (c) Fatty degeneration of the tongue muscles (Fd) (H&E, x100).

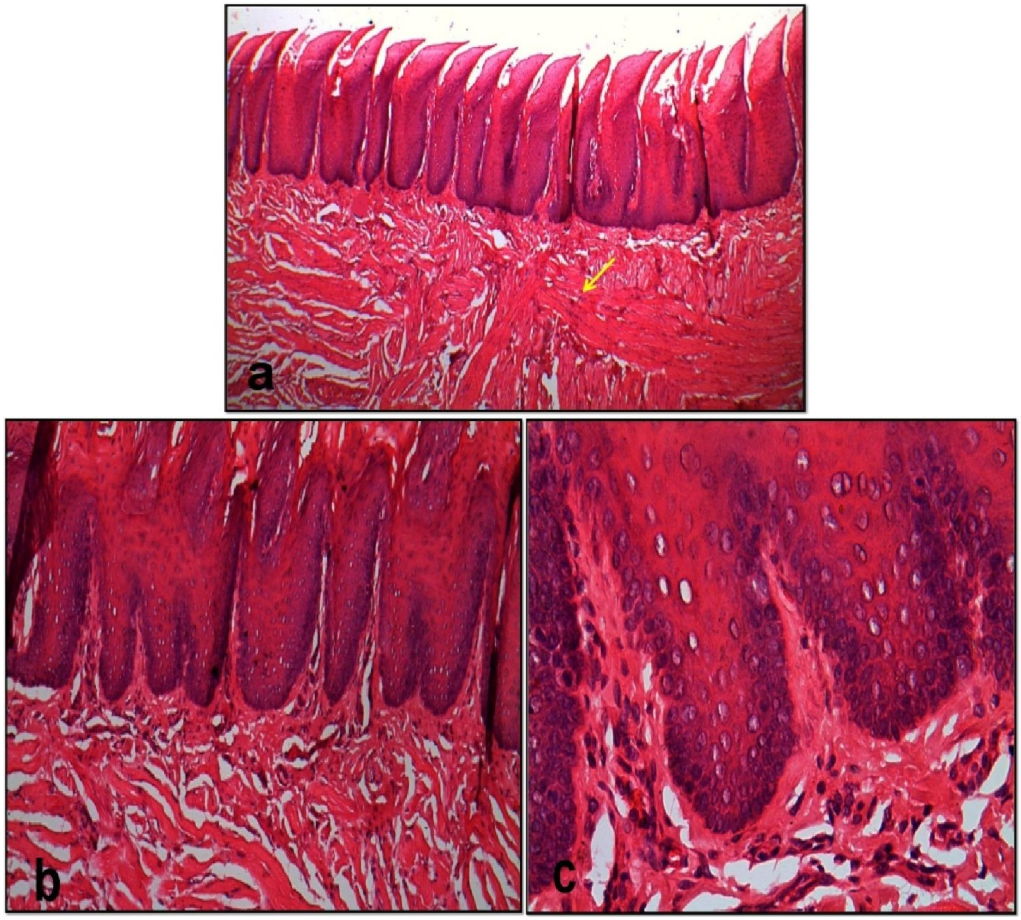



**Fig.3:** A photomicrograph of dorsal surface of the tongue mucosa of group III (propolis treated group) showing: (a) almost normal structure of surface epithelium with conical flame shaped filfiorm tongue papillae and normal distribution of tongue muscles (H&E, x40). (b) Higher magnification with normal appearance of rete pegs and normal distribution of collagen fibers (H&E, x 100). (c) Higher magnification showing normal architecture of basal epithelium and collagen fibers distribution (H&E, x 400).

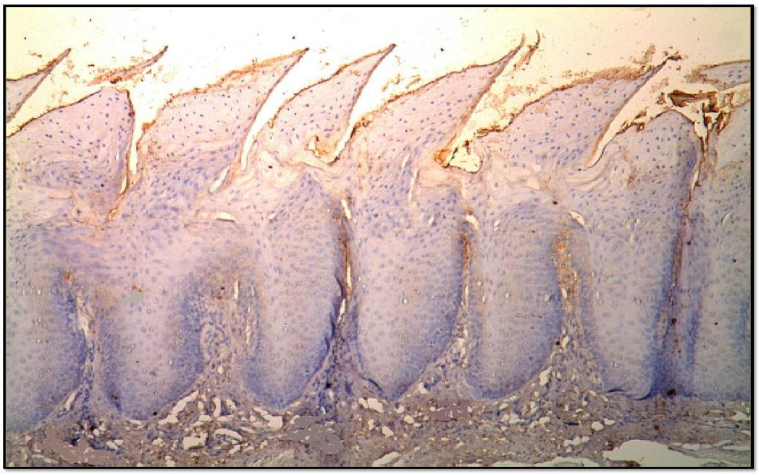


**Fig.4:** A photomicrograph of group I showing negative caspase-3 immunoexpression in either nuclei or cytoplasm of basal cell layer of the tongue mucosa (original magnification, x 100).

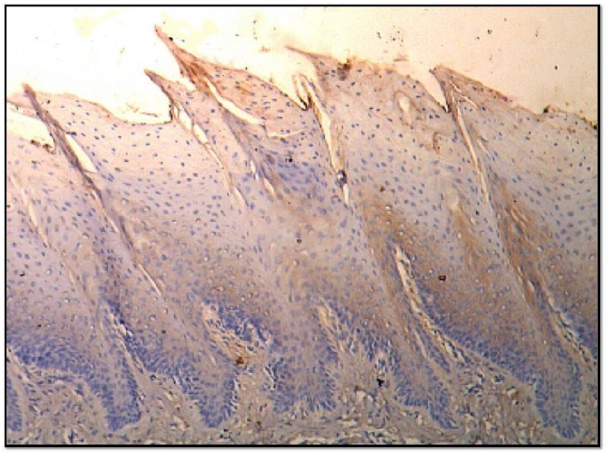


**Fig.5:** A photomicrograph of group II (fenitrothion) showing strong caspase-3 immunoexpression in the cytoplasm of basal and parabasal cell layer of the tongue mucosa. Note: most of the nuclei escaped this positive reaction (original magnification, x 100).

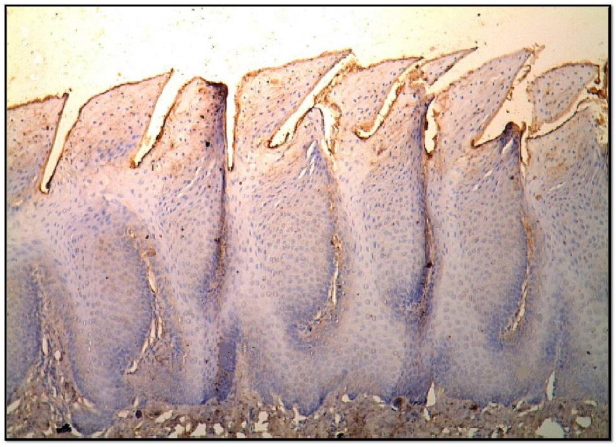
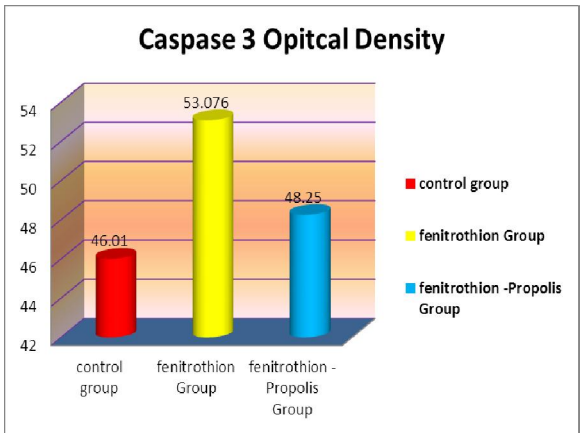


**Fig.6:** A photomicrograph of group III showing mild caspase-3 immunoexpression in the cytoplasm of basal cell layer of the tongue mucosa (original magnification, x 100).

**Table I: the difference in mean Caspase-3 optical density of the different groups using ANOVA Test.**

| Group | Caspase-3 optical density of the tongue | | |
|---|---|---|---|
| | M±SD | F-Value | *p*-Value |
| I-Control group | 46.01±1.25 | 18.25 | <0.0002** |
| II-Fenitrothion treated group | 53.76±2.17 | | |
| III-Fenitrothion-propolis treated group | 48.25±2.60 | | |

** High significant difference at ($p$<0.01).




**Graph I: Represents the difference in mean Caspase-3 optical density between different groups.**

## 4. Discussion

The widespread use of pesticides in agriculture and forestry conservation programs has prompted the need for evaluation of the hazards of such materials to wildlife. Recent reports have emphasized that the probability of exposure exists within the indoor living space, as well as in the agricultural and industrial workplace (**Russell and Overstreet, 1987**). The present work studied the hazardous effects of the organophosphorous insecticides, Fenitrothion (Sumithion) on the tongue mucosa of rabbits.

The present investigation indicated that oral administration of Fenitrothion to rabbits caused significant alterations in the epithelium cells that were demonstrated in the tongue mucosa of rabbits histopathologically, immunohistochemically and histomorphometrically.

In the present study, histopathologically; the Fenitrothion group showed evidence of hyperplasia, hyperkeratosis with acanthosis and signs of dysplasia in the tongue epithelium. The basal cell layer revealed basilar hyperplasia, nuclear hyperchromatism, polymorphic nuclei and mild dysplasia. The tongue's muscles revealed signs of fatty degeneration. On the other hand, the cytotoxic effect was reduced in the other prophylactic experimental group (propolis group). Our findings were in agreement with **Kerem *et al.*, 2007,** who reported that fenitrothion treated rats was accompanied by similar histopathological alterations in liver and kidneys which led to severe effects including the presence of fine subcapsular infiltrations, diffused parenchymatous degeneration of single hepatocytes, and the presence of fine foci constructed of plasmatic cells, and histiocytes located between hepatic plates. In addition, it was demonstrated that Sumithion (fenitrothion) insecticides induced remarkable inhibition of

cytochrome P-450 activity in the liver decreasing drug metabolism and detoxication. This inhibitory effect subjected the body organs to more accumulated toxin in the blood (**Sheweita, 2004**). Our findings in the present study of nuclear hyperchromatism is in agreement with **Rush *et al.*, 2010,** who demonstrated that the chromatin condensation is an important characteristic of apoptosis in primary cortical culture exposed to diazinon organophosphorous insecticides.

The principle mechanism by which fenitrothion induces acute toxicity is probably through the generation of reactive oxygen species (ROS) causing oxidative damage to the various membranous components of cell **(Goel *et al.*, 2005).** The cause of oxidative damage has been reported to be due to the shift in the balance of pro-oxidant (free radicals) and the anti oxidants (scavenging) mediators **(Schulz *et al.*, 2000)**. Antioxidants are compounds that help to inhibit many oxidation reaction caused by free radicals thereby preventing or delaying damage to the cells and tissues (**Kanter; 1998, Jackson; 1998**). Propolis (food products obtained from bees), is important not only for its nutritional properties but also for its functional and biological properties: Antioxidant, anti-inflammatory, antibacterial, antiviral, and anti-ulcerous activities. These activities are mainly attributed to the phenolic compounds such as flavonoids which incorporated in propolis. Recently, Propolis administration reported to improve the activity of hepatic microsomal drug metabolizing enzymes, significantly inhibited lipid peroxidation and markedly enhanced glutathione in liver and kidney through the antioxidant and antiradical activity which were determined to be the phenolic compounds existing in the propolis extract used **(Eraslan; 2007**).

Regarding the results of the present study, it is likely that free radical scavenging and antioxidant properties of propolis protected the tongue against fenitrothion induced injury. Furthermore, it was demonstrated in previous studies that propolis has antibacterial activity and it may have some beneficial effects by accelerating healing ability.

Furthermore, the immunohistochemical results and histomorphometric data of the ongoing study corroborated with the histopathological results. Comparing the optical density between the three studied groups, an increase in the optical density of the areas occupied by active caspases-3 was evident in the experimental groups II (fenitrothion treated rabbits). The increase was statistically highly significant. Caspases are a family of inactive proenzymes that play a crucial role in cell apoptosis, which is the scheduled death of cells. The role of caspase 3 in apoptosis is to cleave and activate caspases 6, 7 and 9 in order to break down the apoptotic cells before removal. After this process, the caspase 3 protein is cleaved and broken down itself by caspase 8 and 10. Activation of caspase-3 was first observed close to the inside surface of the cellular membrane, then transferred to the cytoplasm, and finally translocate to the nuclear region by active transport. During apoptosis, activation of caspase-9 increases permeability of the nuclear pores, which allows cytoplasmic caspases to reach their nuclear substrates and lets soluble proteins that are normally restricted to the nucleus or cytoplasm to distribute throughout the cell **(Faleiro and Lazebnik, 2000).**

## Conclusion

The overall findings of this study clearly demonstrated that oral administration of fenitrothion resulted in some histopathological changes in tongue epithelium in rabbits. We suggest that similar studies should be done on different body tissues of rabbit. It is also recommended that the use of pesticides must be controlled to avoid any hazards to the livings especially humans. Thus it is speculated that a combination of propolis and fenitrothion extract antioxidants, resulting in preventing tissue damage caused by fenitrothion. Therefore, propolis can be used as a protective natural product against the cytotoxicity caused by fenitrothion.

Due to the large number of beneficial effects that propolis presented on the body, these products could be considered as potential ingredients for different foods. In any case, some precautions must be taken for their use in foods to avoid some problems in persons who suffer from allergy by bee related allergens.

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**Conflicts of interest**
No any conflict of interest to declare.

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