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PROTECTIVE EFFECT OF UNCARIA TOMENTOSA EXTRACT AGAINST INDUCED TOXICITY OF FIPRONIL ON ORAL MUCOSA OF RATS. (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Aim: To evaluate the potential effect of Uncaria Tomentosa (UT) extract against fipronil (FIP) induced oral mucositis through histological examination and immunohistochemical analysis as well as determination of certain oxidative stress and inflammatory biomarkers.

Materials & Methods: Forty rats were allocated into four groups (10 rats each); Group I: Untreated rats (control group), Group II: The rats received 120 mg/kg body weight of 10% UT extract orally for 30 days, Group III: The rats received 1/10 LD50 FIP at the dose 9.7mg/kg body weight orally for 30 days, Group IV: The rats received both FIP and UT orally at the same doses of groups II and III for 30 days. All rats were euthanized by cervical dislocation and then the buccal mucosae from both sides were dissected out.

Results: Buccal mucosa specimens that were stained with H&E of FIP treated group showed histological changes in both surface epithelium and lamina propria. However, buccal mucosa of FIP +UT group showed improvement in their histological structures.Buccal mucosa specimens that were incubated with Caspase 3 monoclonal antibody of FIP group exhibited strong positive staining reaction. While those of FIP +UT group expressed weak to moderate staining reaction. Administration of FIP for 30 days resulted in a significant elevation of MDA as an index for lipid peroxidation in tissue specimens and remarked elevation in the expression of inflammatory cytokines, TNF alpha and interleukin 1 beta, was also observed upon the treatment of FIP. Co-administration of Uncaria and FIP counteracted such a rise.

KEY WORDS: Fipronil, Uncaria Tomentosa, Buccal mucosa, Caspase 3.

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INTRODUCTION

Fipronil (FIP,) a second- group phenilpirazol insecticide, is used in both veterinary medicine and agriculture to prevent the presence of many pests such as fleas, ticks, ants and cockroaches. By proceeding as a noncompetitive blocker, the herbicide prevents the chloride channels which are connected to the gamma-aminobutyric acid (GABA) receptors from functioning [1]. By blocking the flow of chloride ions, the antagonistic action of FIP kills the insect by causing excessive brain stimulation [2].

Many species exhibit severe toxicity towards FIP Patients' tonsil samples acquired utilizing the comet assay, a reliable genotoxicity method, also showed a significant genotoxic effect. [3]. The primary signs of acute toxicity of FIP (more than 50 mg/kg b.w.) in experimental mice included tremors, convulsions, altered activity, stooped posture, agitation and alterations to function of nervous system. Few hours following therapy, these symptoms peaked. [4, 5]. Moreover, there have been reports of human cases of unintentional FIP poisoning, which is distinguished by seizures, vomiting and agitation[5].

FIP has been demonstrated to have detrimental effects on the thyroid, liver, and reproductive system in non-target animals despite being less toxic to mammals than to insects. [2]. Long-term exposure to FIP in rats resulted in damage to their thyroid, liver, and kidneys [6].

Moreover, FIP may produce reactive oxygen species (ROS) in cells, which might increase oxidative stress and lipid peroxidation according to some evidence. [7].

Herbal medicine has been used therapeutically because of its stronger beneficial effects and lower potential for harm.Cat's claw is a plant that grows in tropical areas of Central and South America, the Amazon basin, and neighbouring countries. It is also known by the scientific name Uncaria tomentosa (UT). [8]. The plant cat's claw is frequently used as an antioxidant and anti-inflammatory [9].

Alkaloids and flavonoids, two of Uncaria tomentosa's active ingredients, have the power to regulate the immune system and control oxidative radicals. Many ailments are treated with it[10].

So, this study aimed to evaluate the UT extract potential effect against FIP induced oral mucositis in rats through histopathological examination and immunohistochemical localization of caspase 3 (apoptosis indicator) as well as determination of SOD, Catalase, MDA, and GSH (oxidative stress biomarkers) and TNF- α and IL-1β (inflammatory biomarkers).

MATERIAL AND METHODS

Materials:

Materials that were used in the present study were, FIPRONIL (powder), Fipronil technical 95% pure (manufactured by Co.,China Ltd company ,Tianjin Maotian Tech). Cat's Claw extract, Uncaria Tomentosa, 3%Oxindole Alkaloids (capsule, 500mg), Maple life sciences, India.

Methods:

Sample size calculation : Calculation of sample size was performed applying G*Power version 3.1.9.2 [11]. The effect size d was **0.78** utilizing alpha level of 0.05 and Beta level of 0.05, i.e., power = 95%; the estimated sample size (n) should be **40** rats for this study and were divided equally into 4 groups (10 rats each). The sample size in this study was in agreement with many researchers who have published on this point [12].

Study setting:

The present investigation was commenced after the approval of the Research Ethics Committee of the Faculty of Pharmacy, Suez Canal University with ethical code# 202212RA1.

This study was performed on adult male abino rats (average weight 120 g). Rats were housed under standard conditions, environmental temperature (25°C) and controlled lightening in clean metal cages supplying natural diet and drinking water adlibitum.

Samples grouping and study procedures:

The rats were randomly divided into four groups, 10 rats each:

Group I: Untreated rats (control group) only received saline (0.9% NaCl) for 30 days.

Group II: The rats of this group received 120 mg/kg body weight of 10% UT extract dissolved in distilled water orally for 30 days [13].

Group III: The rats of this group received 1/10 LD50 FIP at the dose 9.7mg/kg body weight orally for 30 days. FIP dose was selected according to a previous study [14].

Group IV: The rats of this group received UT & FIP orally at the same doses as both groups II & III respectively for 30 days.

The administration of the daily oral doses was performed using curved metallic oropharyngeal tube throughout the whole experimental period.

At the end of the experiment, euthanization of all rats by cervical dislocation were performed under ketamine anesthesia (80 mg/kg, i.p.). The buccal mucosae of both sides of the rats were dissected out. The left side was processed for histopathological and immunohistochemical studies while the right side was stored at -20°C for biochemical analysis.

Histopathological studies:

Buccal mucosa specimens were immediately fixed in 10 % neutral buffered formalin, processed, dehydrated in ascending grades of alcohol, embedded in paraffin and sectioned (4-6 microns in thickness) to be stained with hematoxylin and eosin (H&E). [15].

Immunohistochemical studies (Detection of Caspase 3)

Buccal sections (4-6-µm thick) from each rat were mounted on poly-L-lysine coated glass slides, deparaffinized in xylene for 20 minutes, rehydrated through descending grades of ethanol and immersed in 10 ml of antigen retrieval. The immersed slides were twice heated by microwave oven at 80°C for 4 minutes then for 7 minutes, 3% hydrogen peroxide was applied on the tissue sections for 5 minutes to inhibit the activity of endogenous peroxidase. The sections were then washed by distilled water followed by phosphate buffered saline (PBS), excess buffer was blotted off the slides except for the tissue sections, One or two drops of the ready to use diluted primary antibody (using rabbit monoclonal antibody for the localization of caspase-3 for detection of any apoptotic changes) were then added on the sections and used as primary antibody. The staining process then established according to True LD, 2008. [16].

Images from the buccal mucosa were obtained by means of a digital camera (Olympus Dp25, Japan) for localization of area percent of apoptotic cells immunostained with caspase 3. The images of the selected parts were analyzed using ImageJ software developed by the National Institute of Health (Bethesda, Maryland, USA).

Biochemical analysis:

1-Determination of SOD, Catalase, MDA, and GSH (oxidative stress biomarkers):

Superoxide dismutase (SOD) and catalase activities were determined using the commercial colorimetric kit (Cat. No. SD2520) and (Cat. No. CA 2516) respectively. They were purchased from Biodiagnostic, Dokki, Egypt. SOD assay depends on

the capability of the SOD to prevent the phenazine methosulphate-arbitrated decrease of nitro-blue tetrazolium dye. Catalase assay was performed using a colorimetric method that relies on the reaction with hydrogen peroxide (H_2O_2) as substrate and the determination of unconverted H_2O_2 using a redox dye. The change in color intensity at 570 nm is reversely proportional to the catalase activity.

The levels of malondialdehyde (MDA) were determined as an index for lipid peroxidation. The method for its colorimetric assay depends on the reaction between MDA and thiobarbituric acid where the resultant color was measured spectrophotometrically at 534 nm. The amount of reduced glutathione (GSH) was determined based on its ability to reduce of 5,5` dithiobis (2 - nitrobenzoic acid). The resultant reduced chromogen is directly proportional to GSH concentration, and its absorbance can be detected spectrophotometrically at 405 nm. [17].

2-Determination of TNF- α and IL-1β (inflammatory biomarkers):

For quantitative measurement of TNF alpha and IL-1 beta in tissue homogenates, rat TNF alpha ELISA Kit (ab236712) and Rat IL-1 beta ELISA Kit (ab100768) were used. Instructions were followed according to the manufactured protocol.

Statistical analysis:

All calculated data were statistically analyzed using suitable statistical tests. A normality test (Shapiro-Wilk) was done to check the normal distribution of the samples. Statistical analysis was carried out using the computer program SPSS software for windows version 26.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp). The differences among the groups were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer test as a post-hoc test. The differences were considered significant at p value less than 0.05. Results are presented as mean ± SD.

RESULTS

Histological results:

Examination of buccal mucosa specimens of group I (control group) showed normal histological features of surface epithelium, underlying lamina propria and submucosa. The surface epithelium was formed of keratinized stratified squamous epithelium with regular, broad, few and short epithelial ridges towards the lamina propria. The lamina propria showed fibroblasts, regular arrangement of collagen fibers and small-sized blood vessels. The submucosa was formed of densely packed collagen fibres and fat cells (Fig. 1 A). Group II (UT group) showed normal histological structure of buccal mucosa as control group, the epithelial surface was formed of four layers; stratum basal, stratum spinosum, stratum granulosum as well as keratin layer (Fig. 1 B). Group III (FIP group) showed an increase in the epithelial thickness of buccal mucosa compared to both control and UT groups. The epithelial ridges became longer and broader. The basal cells showed disturbance in their normal arrangement with marked basilar hyperplasia. The stratum spinous cell layer showed marked acanthosis with oedema and swelling of most of its cells as well as stratum granular cell layer. The nuclei disappeared in some cells of stratum spinosum that showed hyalinized appearance. The stratum granular layer showed more increase in thickness with more rise of their basophilic keratohyaline granules and the keratin layer showed more increase in its thickness. The lamina propria showed marked degeneration of collagen fibers and inflammatory cell infilteration. (Fig. 1 C). Buccal mucosae of FIP& UT treated group (Group IV) rats showed improvement in their structures; the epithelial ridges revealed their normal pattern with few, broad, regular and short epithelial ridges. Furthermore, lamina propria of FIP & UT treated group showed decrease in the extent of degeneration of the collagen fibers (Fig. 1 D).

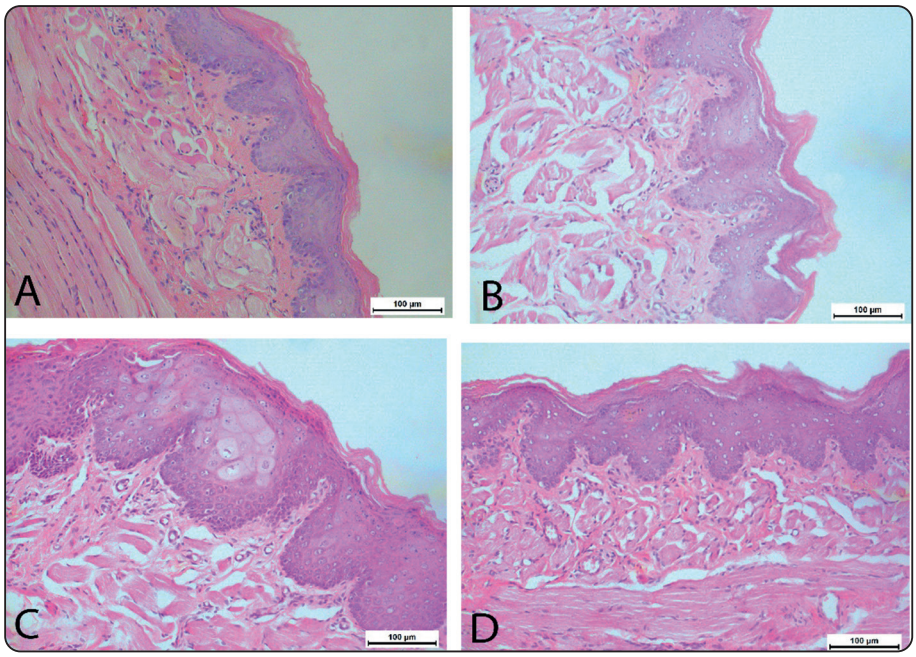

Fig. (1) (A) Photomicrograph of buccal mucosa of Group I (control group) showing normal histological structures of surface epithelium with broad, regular and short epithelial ridges and densely packed collagen fibers of lamina propria, **(B),** Buccal mucosa of UT group showing almost normal structure of buccal mucosa as control group**, (C)** Buccal mucosa of FIP treated group showing swelling of spinous cells and broad epithelial ridges, **(D)** Buccal mucosa of FIP +UT group showing improvement in the surface epithelium with few, regular,and short epithelial ridges. (H&E, orig. mag. 200).

Immunohistochemical localization of caspase 3:

Examination of buccal mucosa slides immunostained with caspase-3 of control group revealed weak positive staining reactivity of cells of the surface epithelium and weak staining reaction of underlying lamina propria (Fig. 2 A). Buccal mucosa of UT group showed also weak staining reaction of cells of the surface epithelium and weak staining of underlying lamina propria (Fig. 2 B). However, strong positive staining reaction of the cells of the spinous and granular cell layers of the epithelial surface of FIP group and strong staining reaction of underlying lamina propria (Fig. 2 C). Meanwhile, Epithelium of FIP & UT group showed weak positive staining reaction and weak staining of lamina propria (Fig. 2 D).

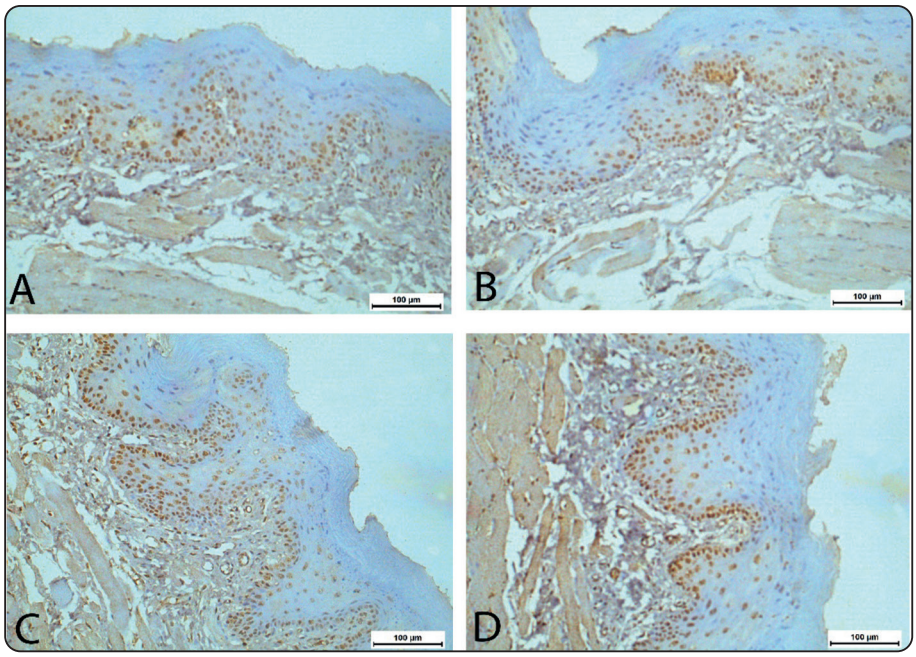

Figure 2 (A) Buccal mucosae of control group showing weak positive staining reaction of Caspase 3 of surface epithelium and lamina propria, (B) UT group showing weak staining reaction of Caspase 3, (C) FIP group showing strong positive staining reaction of Caspase 3 of surface epithelium and lamina propria. (D) FIP+UT group showing moderate staining reaction of basal cell layer and negative to weak reaction of spinous and granular cell layers (Caspase-3, orig. mag. 200).

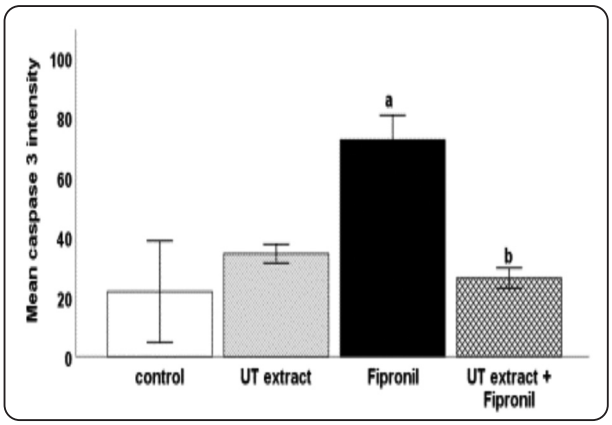

Fig. (3) Mean percentage of immunostained apoptotic cells with caspase 3 in both buccal epithelium & lamina propria was significant; Fipronil treated rats showed an increase in the mean percentage area of apoptotic cells compared to control and uncaria treated group of rats.

FIP and UT effect on SOD, Catalase, MDA, and GSH (oxidative stress biomarkers):

Administration of FIP for 30 days resulted in a significant elevation of MDA as an index for lipid peroxidation in tissue specimens (Figure 4A). This was concomitantly observed with a marked reduction in reduced GSH levels (Figure 4B) and the antioxidant enzymes; SOD and catalase activities (Figure 4C and 4D). On the other hand, co-administration of UT and FIP resulted in the protection against the elevation of MDA (Figure 4A). In addition, co-treatment with UT & FIP significantly raised the levels of reduced GSH (Figure 4B) as well as the activities of SOD and catalase (Figure 4C and 4D).

Results were represented as mean ± SD. Superscript symbols indicate a significant difference at P value less than or equal 0.05 applying one-way ANOVA after that Tukey's test was applied multiple comparisons. [a], represents significant differences of control. [b], represents significant differences of FIP.

No significant difference between control & UT group in the measured oxidative stress biomarkers was found.

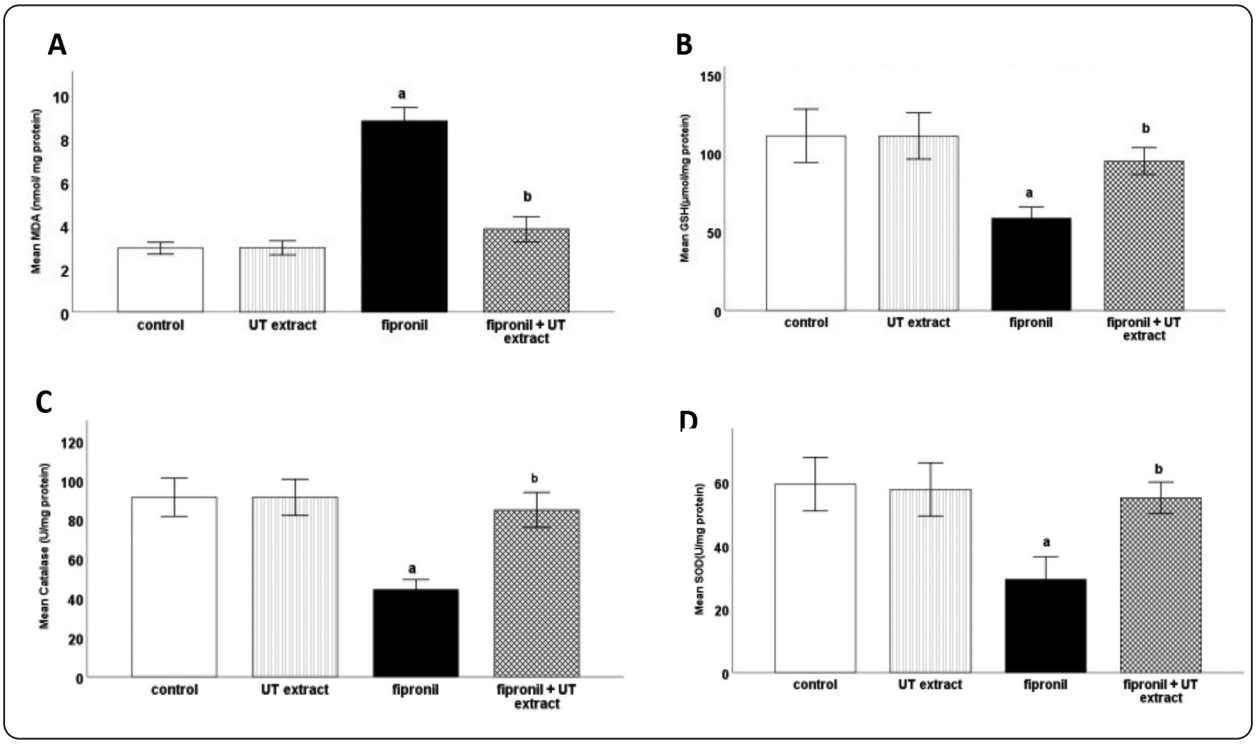

Fig. (4) FIP and UT effect on the measured oxidative stress biomarkers; A) MDA levels, B) Reduced glutathione levels, C) Activities of catalase and D) SOD activities.

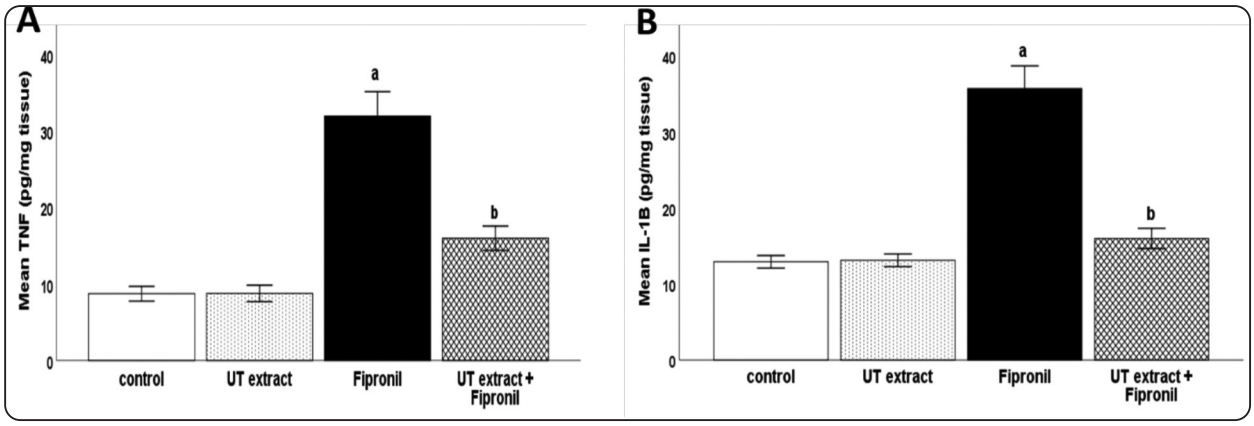

Fig. (5) FIP and UT effect on the measured inflammatory biomarkers levels; (A) TNF-α and (B) IL- 1β.

FIP and UT effect on TNF- α and IL- 1β (inflammatory biomarkers):

Remarked elevation in the expression of TNF- α and IL- 1β that act as inflammatory biomarkers, was observed upon the treatment of FIP (Figure 5). Levels of TNF- α increased by 3 folds upon the administration of FIP for 30 days while the amount of IL-1β raised by 4 folds approximately. Upon using UT and FIP, counteraction to the previously detected elevation in FIP group, was observed

Results were represented as mean±SD. Superscript symbols reveal a significant difference at P value less than or equal 0.05 applying one-way ANOVA after that Tukey's test was applied for multiple comparisons. [a], represents significant differences of control group. [b], shows significant differences of FIP group.

DISCUSSION

Degenerative effects of oral mucosa are common side effects that can occur upon the exposure to insecticides. The present study highlighted the histological changes upon oral administration of FIP for 30 days besides it showed an increase in the inflammatory response and occurrence of oxidative stress in the buccal mucosa isolated form rats treated with FIP.

The histological results in the present study showed a significant increase in the buccal epithelial thickness of rats treated with fipronil in comparison with control and uncaria treated rats. The stratum basal cells showed disturbance of their normal arrangement with marked basilar hyperplasia and more degeneration of areas of the basement membrane with basal cells invasion in the lamina propria. The stratum spinosum layer showed marked acanthosis with oedema and swelling of most cells of the prickle cell and granular cell layers. The stratum granulosum layer showed more increase in thickness with more increase of their basophilic keratohyaline granules and the keratin layer revealed increase in its thickness. Some of the epithelial cells showed cytoplasmic vacuolization. The underlying lamina propria revealed marked dissociation of collagen fibers associated with inflammatory cell infilteration.

Several studies after exposure to insecticides reported similar histological deteriorations in various tissues. [18, 19]. For instance, when mice were given a high dose of FIP, the brain showed remarkable histo-pathological changes, including significant vacuolization in the molecular layer of the brain and neuronal necrosis with concurrent loss of Nissl's substance in the cerebellum. [20].

Reactive oxygen species (ROS) were discovered to be involved in the FIP-induced damage to the

cell's lipid, protein, and DNA constituents.This was demonstrated in our study, where a substantial increase in MDA — a measure of lipid peroxidation — occurred after receiving FIP for 30 days. It has been demonstrated in the past that a rise in lipid peroxidation compromises the integrity of cell membranes since it contributes to polyunsaturated fats' oxidative damage, which is necessary to keep cell membranes functioning normally. [21]. An essential nonenzymatic endogenous antioxidant defence protein called glutathione can defend against harmful chemicals like pesticides. Glutathione needs to be in its reduced state with its -SH group for it to function as an antioxidant. [22]. This was shown in the current study by a decline in the levels of reduced GSH in buccal specimens isolated from rats that had received FIP treatment. Reduced GSH works as a scavenger to ROS produced upon treatment with FIP. Such results matched the previous studies that reported the decline in the amount of reduced GSH upon exposure to different pesticides [23-25].

Catalase and SOD are enzymatic endogenous antioxidants that can scavenge the ROS and thus protect the cell against deleterious toxins. Exposure to FIP resulted in depletion of endogenous enzymatic antioxidants and this matched a previous study that showed the ability of FIP to downregulate the expression of SOD and catalase [26].

Fipronil resulted in marked inflammation evidenced by the increase of inflammatory cytokines comprising the TNF alpha and interleukin 1 beta. FIP was previously shown to induce inflammation in the lung mediated by Toll like receptors [27]. This was concomitant by increasing cell death and this was observed in our study as exposure to FP upregulated the expression of caspase 3.

The generation of ROS led to oxidative stress. [28], and, consequently, resulted in apoptosis [29]. Exposure to FIP may induce apoptosis in living organisms by the ROS generation. After substantial ROS production, male rats were given FIP (5 and 10 mg/kg b.w.), and the percentage of apoptotic cells amplified significantly. This indicates that fipronil triggered apoptosis in vivo via the oxidative stress-related route. [30].

The histological results of the present study are confirmed by the immunohistochemical localization of caspase-3 in the buccal mucosa of fipronil-treated rats immunostained with caspase-3 monoclonal antibody revealed strong positive reaction of the cells of the stratum spinosum and stratum granulosum cell layers of the epithelium and strong positive immunostaining reaction of the lamina propria.

Uncaria tomentosa had shown potential in lessening the oxidation brought on by fipronil. The antioxidant effects of Uncaria tomentosa are attributed to the product's antioxidant content and fractions, which include proto-catechuic acid, chlorogenic acid, syringic acid, caffeic acid, quercetin, kaempferol, rutin, gallic acid, and cateachin. [31].

The histological results of buccal mucosa of FIP& UT treated rats showed improvement in their structures; the epithelial ridges revealed their normal pattern with few, broad, regular and short epithelial ridges. Furthermore, lamina propria of FIP & UT treated group showed decrease in the extent of degeneration of the collagen These results are in accordance with antioxidants and anti-inflammatory properties showed by Uncaria tomentosa.

Thus, it can be proposed that UT can prevent the toxicity arising from insecticides exposure like FIP. This was also confirmed by the downregulation of caspase 3 after administration of UT.

CONCLUSION

Insecticides like fipronil can affect vertebrates directly by being overtly poisonous to them or indirectly by lowering their food source. The treatment of UT extract reduced the oxidative damage and oral mucositis caused by FIP. This action is most likely connected to the phenolic and flavonoid chemicals included in this extract.

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