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Review Article

Protective Effect of Urtica urens L. against Osteoporosis and Hepatotoxicity induced by Imidacloprid in Rats

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Abstract

Aims: Imidacloprid (IMI) has been known to cause osteoporosis and hepatotoxicity. Some reports claim that Urtica urens L. (UU) can reduce toxicity. The present study was undertaken to evaluate the protective effect of UU against this toxicity.

Methods: Rats were divided into control group, 3 groups treated with IMI at 50, 200 or 300 mg/kg/day and three groups injected with IMI (50, 200 or 300 mg/kg/day) + 100 mg/kg/day of UU, for 60 days. Urine and blood samples were collected for dosage of biochemical levels. Livers were removed for oxidative stress and histological examination. The effects of IMI and UU on the bone were analyzed using physicochemical techniques: Scanning electron microscopy (SEM), dispersive X-ray spectroscopic (EDX) and biochemical analyses (Calcium and Phosphorus).

Results: IMI caused an acute renal and liver injury, increased the biochemical and tissular levels of MDA, and decreased the levels of antioxidant enzyme activities. UU injection improved the histological and all biochemical parameters. Results show that IMI caused osteoporosis in female rats.

Conclusions: IMI induced osteoporosis and an acute liver injury accompanied with disturbance of oxidant status. UU injection provided a significant protection thanks to antioxidant properties.

INTRODUCTION

Imidacloprid, 1[(6-chloro-3-pyridinyl) methyl]-N-nitro2-imidazolidinimine, is a neonicotinoid insecticide widely used to fight pests of cereals, fruits, and vegetables due to its low soil persistence and high insecticidal activity at a low application rate [1]. Imidacloprid (IMI) can exaggerate the toxic properties and adverse effects which may be fatal for human and animal health [2,3]. Several studies reported that IMI is causing severe hepatotoxicity, nephrotoxicity, male infertility, and neurological disorders in mice [4,5].

The liver cytochrome P450 (CYPs) is the major enzymes involved in drug metabolism, accounting for ~75% of the total metabolism [6] and activation or detoxification of neonicotinoids [7]. Studies of the metabolites of neonicotinoids have shown that they can be bioactive and act as nAChR agonists or cause secondary toxicity in mammals [8]. Nicotinoids can be formed as metabolites of neonicotinoids with greater selectivity for vertebrate nAChRs than to insect nAChRs [7,8]. Hepatotoxicity of IMI has been reported in mice [9,10]. Imidacloprid act as agonists at the insect nicotinic acetylcholine receptor (nAChR) [7]. It is the fastest growing in sales because of its high selectivity for insect nicotinic acetylcholine receptors [9]. Buckingham et al. showed that imidacloprid affects both AChRs sensitive to -BTX and -BTX-insensitive nicotinic acetylcholine receptors (nAChRs) can act on pharmacologically diverse nAChR subtypes [11]. Oral LD50 of imidacloprid is 131 mg/kg in mice. It is rapidly absorbed from the gastrointestinal tract and eliminated via urine and feces [12].

Urtica urens L. (UU) is a member of Family Urticaceae which includes about 48 genera and 1050 tropical and warm temperate species [13]. It's an annual herbaceous shrub, native of Europe and has become naturalized throughout North America, Africa, Asia, Australia and South America [14]. This plant is a rich source of phenolic compounds [15]. It is widely used as folk medicine [16] and exhibited anti-nociceptive [17], antioxidant [18] and hepatoprotective effects [19]. Beside, afore mentioned data, no previous investigations of Protective effect of UU against hepatotoxicity and osteoporosis were carried out in Tunisia.

Therefore, the present study was undertaken to examine possible effects of IMI on liver and bone by using biochemical and histological techniques in female rats.

The present study aimed also to investigate the preventive in vivo effects of UU EtOH extract on the bone and liver of exposed female rats to IMI

MATERIALS & METHODS

Chemicals

Confidor® 200 OD, 1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine, [CAS No. 138261-41-3], is an insecticide manufactured by Bayer Crop Science (Lyon, France) having as an active ingredient IMI concentration 200 g/l. It was obtained from an agricultural product company in Sfax, Tunisia.

Plant extraction and analysis

Plant extraction. Aerial parts of Urtica urens were collected from Sfax, Tunisia, and identified by Professor Mohamed Chaieb from the Faculty of Sciences of Sfax (Laboratory of Biology and Vegetable Ecophysiology, Faculty of Science, 3038 Sfax, Tunisia). The voucher sample (Pharm-PCT-2562) was deposited at The National Botanical Research Institute Tunisia (INRAT). It was washed with distilled water and then dried at room temperature for 2 days, afterwards, crushed, milled in a knife mill to obtain 50g of Urtica urens powder and subsequently stored in glass bottles at room temperature.

The powder was extracted by using maceration with ethanol. 50g of the powder was macerated in 1 L ethanol (volume fraction is 70%) for 3 days. Later, the solution was filtrated and concentrated in a rotary evaporator at 50°C.

Gas chromatography–mass spectrometry (GC–MS). GC–MS analysis of UU EtOH extract was performed using the equipment Thermo GC-Trace Ultra Version: 5.0, Thermo MS DSQ II. The equipment has a DB 35–MS Capillary Standard non-polar column with dimensions of 30 m × 0.25 mm, film thickness 0.25 µm. The carrier gas used is Helium with at low of 1.0 ml/min. The injector was operated at 250°C and the oven temperature was programmed as follows: 60°C for 15 min, then gradually increased to 280°C at 3 min. The identification of components was based on Willey and NIST libraries as well as comparison of their retention indices. The constituents were identified after comparison with those available in the computer library (NIST and Willey) attached to the GC–MS instrument.

Animals

Sixty-four mature female rats (10–12 weeks old), weighing about 100 g, were purchased from SIPHAT Institute of Tunis. Animals were bred and kept in our animal facility under strict hygienic conditions and were free of major pathogens. Local vivarium conditions were controlled: temperature (24°C), humidity (30–60%), and light (12 h: 12 h light dark cycle). Animals were provided standard laboratory diet (MEDIMIX, Sfax, Tunisia). The experimental protocols were conducted in accordance with the guide for the care and use of laboratory animals issued by the University of Sfax, Tunisia, and approved by the Committee of Animal Ethics (Protocol no. 94-1939). The animals were kept in separate cages to avoid licking or biting of inflammatory areas by other animals.

Experimental design

Adult females rats were divided into 8 groups (n = 8) as follows:

Group 1: the controls received 0 mg/kg BW of IMI;

Group 2: received 50 mg/kg BW of IMI;

Group 3: received 200 mg/kg BW of IMI;

Group 4: received 300 mg/kg BW of IMI;

Group 5: received 100 mg/kg BW of UU;

Group 6: as group 2 and co-treated with 100 mg/kg BW of UU;

Group 7: as group 3 and co-treated with 100 mg/kg BW of UU;

Group 8: as group 4 and co-treated with 100 mg/kg BW of UU.

The period of treatment was 2 months according to Bouafou et al., [20]. The IMI was given by oral gavage. These doses (50, 200 or 300 mg/kg) were chosen according to the works of Eiben and Kaliner [21]. The UU were given intraperionetally at 100 mg/kg BW [22].

Signs of toxicity and mortality. Signs of toxicity such as salivation, diarrhea, paralysis and death were observed once daily throughout the period of exposure.

Preparation of urine, blood and organ samples

- After weighing the animals, they were sacrificed under anesthesia by intramuscular injection with a solution of ketamine 50 mg/ml (200 µl) and a solution of midazolam 5 mg/ml (20 µl) and ether to avoid discharge of hyperglycemic hormones (catecholamines and glucocorticoids). For each rat, blood volume was collected the day of sacrifice by slow aspiration through a syringe placed in the heart (ventricle) of the previously anesthetized animals. The blood obtained was placed in heparinized tubes for biochemical analyses. The samples were centrifuged for 15 min at 4000 rev/min to isolate the plasma erythrocyte pellet and then stored in a freezer at -80°C for further analysis.

- At necropsy, the livers were removed. All livers were removed from adipose tissue from rats. They were rapidly excised, blotted, weighed and processed for histopathology and biochemical assays. Some portions of these organs were rinsed in cold saline buffer, weighed, finely minced and homogenized in the same solution (10%, w/v) and centrifuged at 10.000 g for 10 min. The resulting supernatants were used for immediate lipid peroxidation assays and homogenate aliquots were stored at -80°C for further biochemical assays. The other livers were immediately fixed in 10% formalin solution for histological studies.

- The right distal femurs were shared; their lengths were measured. Subsequently, an incision was made parallel to the long axis of the femur after dividing the fascia and the Musculus biceps femoris. All the experiments were approved by the local Ethical Committee for animal experiments.

The bones of the different groups have been ground to a powder which will be characterized with physico-chemical techniques

Biochemical parameters

Protein quantification.

Protein contents were measured according to Lowry et al. [23], using bovine serum albumin as standard.

Lipid peroxidation: Lipid peroxidation in the tissues was estimated colorimetrically by measuring thiobarbituric acid reactive substances which were expressed in terms of malondialdehyde content according to the method of Draper and Hadley [24].

AOPP levels: Levels of AOPP were determined according to the method of Kayali et al. [25].

Antioxidant enzyme activities

- Catalase activity (CAT).

CAT activity was assayed by the method of Aebi [26].

- Superoxide dismutase activity (SOD).

SOD activity was estimated according to Beauchampand Fridovich [27].

-Glutathione peroxidase activity (GPx).

GPx activity was measured using the procedure of Flohe and Gunzler [28].

Total glutathione level (GSH).

The samples GSH contents were determined by the method of Ellman [29], modified by Jollow et al. [30].

Vitamin E determination.

The extraction of vitamin E was done according to the method described by both Katsanidis and Addis [31].

Biochemical markers in plasma.

The activities of plasma aspartate amino transferase (AST), alanine amino transferase (ALT), and total bilirubin were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Biomaghreb, Ariana, Tunisia, Ref. 20012, 20043, 20047, 20102), respectively.

Histopathological examinations. The liver samples were fixed in 10% buffered formalin, processed through graded alcohols and xylene and embedded in paraffin blocks. Tissue sections were cut at 5 µm at multiple levels and routinely stained with haematoxylin–eosin (H&E). Mounted slides were examined and photographed under a light microscope. All histological evaluations were made twice under blind conditions (without knowledge of treatment).

Physico-chemical parameters

Determination of calcium and phosphorus bone contents. The chemical composition of the bone compound synthesized here was determined from titrations by complexometry for the determination of the calcium content, by spectrophotometry for the total phosphate content (determination of the sum of PO43– and HPO42– ions, using the phospho-vanado-molybdenic method (Gee and Dietz, 1953) and by coulometry (UIC, Inc. CM 5014 coulometer with CM 5130 acidification unit) for the carbonate content.

Scanning electron microscopy (SEM) characterization

Bones were analyzed by SEM, using a JEOL JSM 6301F (Tokyo, Japan). Polymethylmethacrylate embedded specimens obtained from the histological preparations were mounted on the SEM. Quantification for calcium (Ca, mol%), phosphorus (P, mol%) and magnesium (Mg, mol%) was determined by wavelength dispersive X-ray spectroscopic (EDX), the ratio of calcium to phosphorus (Ca/P) was calculated by dividing the values obtained from calcium (mol%) and phosphorous (mol%) at each point.

Statistical analysis. All data are expressed as mean value ± standard errors mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparison tests using statistical software package SPSS (SPSS Inc, Chicago, IL, USA).

Findings

Chemical compositions of plant. GC-MS is a combined technique which is used to identify different substances within the sample. It works on separation of the individual compound by GC according to their RT and the separated compounds were further analyzed at a molecular level by MS. The GC-MS analysis revealed the presence of 31 phytoconstituents from which some are higher hydrocarbon alkanes, terpenes, organic compounds, steroids, and fatty alcohol. The chromatogram in Figure 1 showed distinct peaks on the basis of RT and area %. The phytoconstituents identified along with their chemical structure in the EtOH extract of UU by GC-MS peak report of total ion chromatogram are shown in Table 1.

In UU, heptacosane (13.96%) showed the highest area %, followed by hexacosane (9.45%), heptadecane (5.46%), and hexadecane (5.35%) as major phytoconstituents. These major phytoconstituents are the major volatile compounds which have anti-inflammatory, anti corrosive, antioxidant, antimicrobial and antifungal activities (Dandekar et al. [32]; Rukaiyat et al. [33];

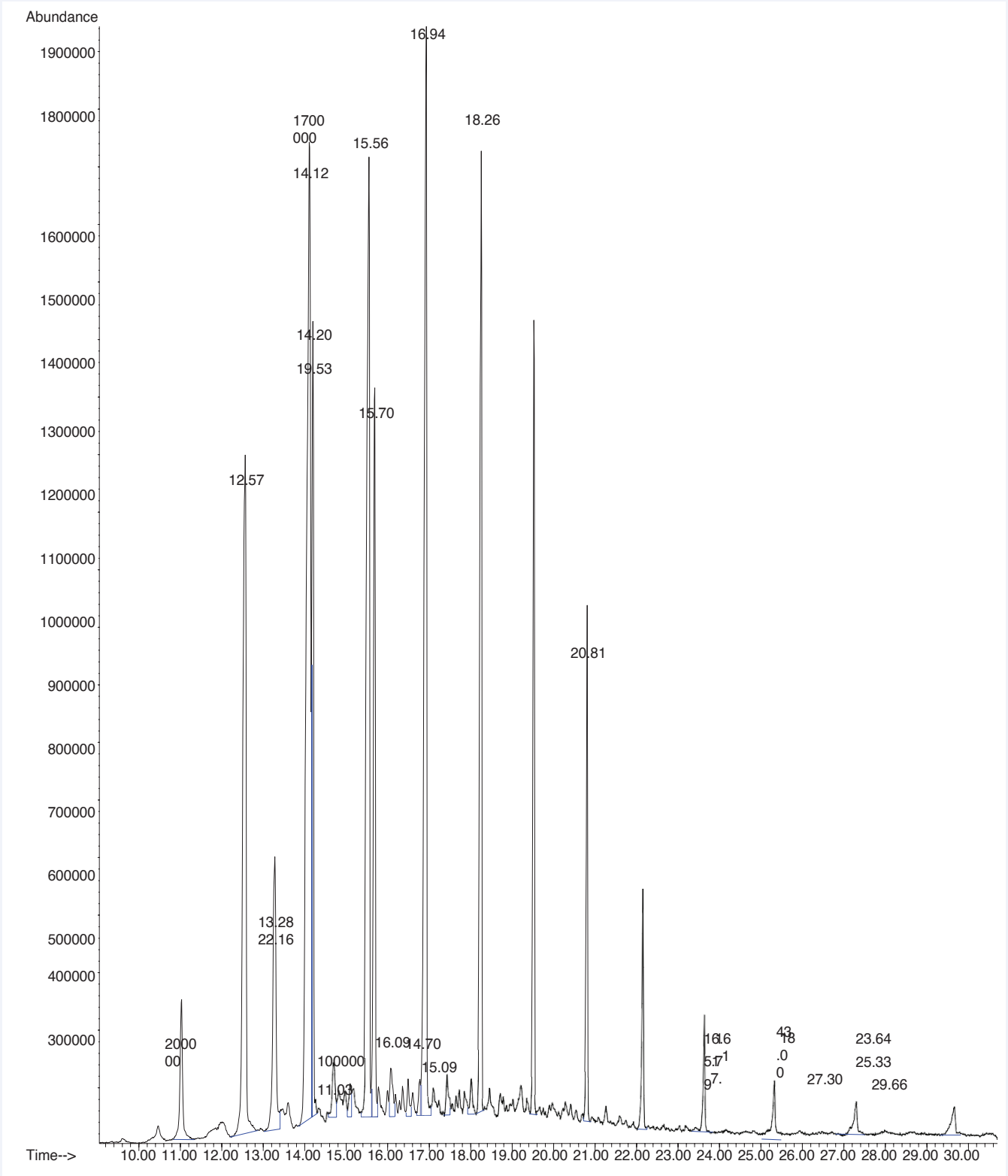

Figure 1 Chromatogram

Table 1: GC–MS spectral analysis of *Urtica urens* L. ethanolic extract.

Peak	RT (min)	Area%	Compound name	Molecular formula	Qual (%)
1	10.71	2.29	3-*Methylnonane*	$C_{10}H_{22}$	43
2	11.20	3.73	Pentadecane	$C_{15}H_{32}$	93
3	12.59	5.35	Hexadecane	$C_{16}H_{34}$	95
4	13.25	1.70	*2-Methylnonane*	$C_{10}H_{22}$	49
5	14.01	5.46	Heptadecane	$C_{17}H_{36}$	96
6	14.09	2.34	Pentadecane	$C_{15}H_{32}$	81
7	15.41	4.19	Octadecane	$C_{18}H_{38}$	93
8	15.56	2.55	*2,9*-Dimethylundecane	$C_{13}H_{28}$	64
9	16.80	3.95	Nonadecane	$C_{19}H_{40}$	96
10	17.39	2.05	*1*-Chlorohexadecane	$C_{16}H_{33}Cl$	58
11	17.45	1.64	1-Hexadecanol	$C_{16}H_{34}O$	43
12	17.54	0.96	*beta-Citronellol*	$C_{10}H_{20}O$	49
13	18.13	4.45	Eicosane	$C_{20}H_{42}$	96
14	18.39	1.83	Tetradecyl Oxirane	$C_{16}H_{32}O$	68
15	18.60	1.56	*1*-Chlorohexadecane	$C_{16}H_{33}Cl$	43
16	19.12	3.50	Tetradecyl Oxirane	$C_{16}H_{32}O$	38
17	19.25	3.42	1-Chlorooctadecane	$C_{18}H_{37}Cl$	38
18	19.32	1.05	6-Nitro-cyclohexadecane-1,3-dione	$C_{16}H_{27}NO_4$	49
19	19.43	4.77	*1*-Chlorohexadecane	$C_{16}H_{33}Cl$	90
20	20.71	3.17	Docosane	$C_{22}H_{46}$	93
21	21.54	2.63	Cetylpyridinium chloride	$C_{21}H_{38}ClN$	58
22	22.07	2.48	*1-Chlorotetradecane*	$C_{14}H_{29}Cl$	93
23	23.55	1.88	1-Chlorooctadecane	$C_{18}H_{37}Cl$	96
24	25.25	2.99	Eicosane	$C_{20}H_{42}$	78
25	27.27	9.45	Hexacosane	$C_{26}H_{54}$	91
26	27.60	1.17	HAHNFETT	N/A	83
27	27.97	1.40	HAHNFETT	N/A	91
28	28.00	0.98	HAHNFETT	N/A	76
29	28.10	0.96	HAHNFETT	N/A	76
30	29.71	13.96	Heptacosane	$C_{27}H_{56}$	86
31	30.50	2.15	Decane	$C_{10}H_{22}$	42

Abubacker and Devi, [34]; Kim et al. [35]; Huguet et al. [36]; Callaghan et al. [37]; Peters and White, [38]).

Relative organ weights

After sacrificing the animals, the kidneys and livers were removed and weighed at the end of the study with a focus on the variation of their absolute weight (AW) and related weight (RW).

The relative organ weight (RW) of each animal was calculated as follows:

$$RW = (AW/BW) \times 100$$

With: AW is the absolute body weight and body weight (BW) is the rate on the day of sacrifice.

During the 60-days IMI treatment, the absolute and relative liver weights decreased significantly (*p<0.05 and **p<0.01). However, when we injected UU, a partial recovery occurred in these parameters [Table 2,3].

Variation of biochemicals

Lipid peroxidation levels (LPO). The level of LPO measured in terms of MDA was altered in the livers of female rats orally administered 50, 200 and 300 mg/kg/day of IMI. Our results revealed an increase of lipid peroxidation in the liver of the IMI-treated group as proven by the enhanced malondialdehyde levels in the liver homogenates of adult rats (+16; +25; +36%) when compared to negative controls. The injection of UU ameliorated lipid peroxidation induced by IMI treatment and significantly modulated the malondialdehyde levels in the liver Table 4.

Markers of protein oxidative damage

Table 4 shows the levels of AOPP indices of protein oxidative damage in the liver tissues of normal and experimental animals. In IMI groups, a significant increase in AOPP levels in the liver (+51.60, +64, +70.33%) when compared with controls. UU injection in IMI+UU-treated rats resulted in a marked decrease in AOPP levels when compared with IMI groups.

Antioxidant enzyme activities in tissues

- In the liver homogenates of IMI-treated rats, GPx, CAT and SOD activities decreased significantly by (-33.56, -37.09, -57.44%) for GPx (-30.89, -9.17, -67.89%) for CAT and by (-15.50, -26.59, -32.56%) for SOD, when compared to controls Tables 5,6.

- The injection of UU regenerated these parameters in (IMI+UU) groups when compared to IMI-groups.

Organs GSH and vitamin E levels

GSH levels: In liver homogenates of IMI treated rats, GSH levels decreased significantly by (-40, -55, -60%) [Table 4] respectively when compared with controls. The injection of UU ameliorated the levels of GSH in the liver of (IMI+UU)-treated groups (4.85, 10.14, 23.99%) when compared with IMI-treated groups.

Vitamin E: The data presented in Table 4 show significant changes in the levels of vitamin E during the treatment of rats with IMI associated or not with UU. The exposure of rats to IMI caused a significant decrease in liver vitamin E levels (-49, -74, -89 %) when compared with controls. In the animals exposed to

Table 2: Evolution of absolute weight AW (g) and relative weight RW (g/100g BW) of liver.

Groups	Liver	
	AW	RW
Control	6.061 ± 0.331	3.927 ± 0.130
50 mg/kg of IMI	5.181 ± 0.399	3.996 ± 0.252
200 mg/kg of IMI	4.756 ± 0.212$^{\alpha*}$	4.261 ± 0.1073
300 mg/kg of IMI	4.435 ± 0.237$^{\alpha**}$	4.558 ± 0.155
100 mg/kg of UU	7.954 ± 0.435$^{\alpha**}$	4.005 ± 0.231
50 mg/kg of IMI + 100 mg/kg of UU	5.945 ± 0.654$^{\beta**}$	3.878 ± 0.187
200 mg/kg of IMI + 100 mg/kg of UU	5.271 ± 0.632$^{£*}$	3.787 ± 0.109
300 mg/kg of IMI + 100 mg/kg of UU	4.422 ± 0.143	3.816 ± 0.132

Values are means ± SEM for eight rats in each group. *p<0.05, **p<0.01 and ***p<0.001

α: compared to controls; β: compared to 50 mg/kg of IMI; £: compared to 200 mg/kg of IMI; €: compared to 300 mg/kg of IMI.

Table 3 Levels of MDA, AOPP and antioxidant enzyme activities CAT and SOD in the liver of the controls and rats treated rats with IMI, IMI + UU or UU.

Groups	*MDA (nmol MDA/mg protein)*	*AOPP (nmol/mg protein)*	*CAT (µmoles CAT/min/mg protein)*	*SOD (Unit SOD/min/mg protein)*
	Liver	*Liver*	*Liver*	*Liver*
Control	42.24 ± 0.66	40.65 ± 0.67	30.45 ± 0.55	0.54 ± 1.78
50 mg/kg IMI	$65.18 \pm 0.58^{\alpha***}$	$35.77 \pm 0.99^{\alpha***}$	$20.62 \pm 0.77^{\alpha***}$	$0.97 \pm 1.09^{\alpha***}$
200 mg/kg IMI	$74.94 \pm 0.53^{\alpha***}$	$25.45 \pm 0.07^{\alpha***}$	$9.44 \pm 0.43^{\alpha***}$	$1.47 \pm 1.08^{\alpha***}$
300 mg/kg IMI	$89.85 \pm 0.67^{\alpha***}$	$21.67 \pm 0.09^{\alpha***}$	$3.22 \pm 0.3^{\alpha***}$	$1.54 \pm 1.66^{\alpha***}$
100 mg/kg UU	$35.3 \pm 1.77^{\alpha**}$	43.88 ± 0.45	$34.12 \pm 0.89^{\alpha*}$	$0.35 \pm 0.65^{\alpha**}$
50 mg/kg IMI + 100 mg/kg UU	$52.9 \pm 0.43^{\beta**}$	$37.89 \pm 0.74^{\beta**}$	$22.89 \pm 0.87^{\beta**}$	$0.67 \pm 0.63^{\beta***}$
200 mg/kg IMI + 100 mg/kg UU	$60.56 \pm 0.22^{£***}$	$27.66 \pm 0.97^{£**}$	$12.87 \pm 0.88^{£**}$	$1.2 \pm 0.89^{£***}$
300 mg/kg IMI + 100 mg/kg UU	$75.77 \pm 0.13^{€***}$	$23.66 \pm 0.43^{€*}$	3.98 ± 0.45	$1.31 \pm 0.55^{€**}$

Values represent means ± SEM (n = 8) in each group. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.
α: compared to controls; β: compared to 50 mg/kg of IMI; £: compared to 200 mg/kg of IMI; €: compared to 300 mg/kg of IMI.
MDA: Malonaldialdehyde. AOPP: advanced oxidation protein product. CAT: catalase. SOD: superoxide dismutase.

Table 4 Levels of GPx, GSH and vitamin E in the liver of the controls and rats treated with IMI, IMI + UU or UU.

Groups	*GPx (nmol of GSH/mn/mg protein)*	*GSH (µg GSH/mg protein)*	*Vitamin E (µg/g)*
	Liver	*Liver*	*Liver*
Control	280.49 ± 0.56	9.43 ± 0.76	100.99 ± 0.77
50 mg/kg of IMI	$259.34 \pm 0.67^{\alpha***}$	$6.31 \pm 0.70^{\alpha***}$	$76.44 \pm 1.67^{\alpha***}$
200 mg/kg of IMI	$220.9 \pm 0.44^{\alpha***}$	$3.56 \pm 0.08^{\alpha***}$	$50.22 \pm 3.88^{\alpha***}$
300 mg/kg of IMI	$214.08 \pm 0.56^{\alpha***}$	$1.64 \pm 0.10^{\alpha***}$	$30.56 \pm 2.89^{\alpha***}$
100 mg/kg of UU	$289.19 \pm 0.09^{\alpha*}$	$11.57 \pm 0.88^{\alpha**}$	$110.56 \pm 1.98^{\alpha*}$
50 mg/kg of IMI + 100 mg/g of UU	$273.21 \pm 1.88^{\beta***}$	$7.81 \pm 0.78^{\beta**}$	$80.34 \pm 0.65^{\beta***}$
200 mg/kg of IMI + 100mg/kg of UU	$233.99 \pm 1.45^{£**}$	$4.16 \pm 0.19^{£**}$	$55.89 \pm 1.09^{£**}$
300 mg/kg of IMI + 100mg/kg of UU	$219.43 \pm 1.89^{€*}$	2.17 ± 0.06	$40.21 \pm 0.67^{€**}$

Values represent means ± SEM (n = 8) in each group. *p<0.05, **p<0.01 and ***p<0.001.
α: compared to controls; β: compared to 50 mg/kg of IMI; £: compared to 200 mg/kg of IMI; €: compared to 300 mg/kg of IMI.
GPx: glutathione peroxidase. GSH : glutathione.

Table 5 Plasma activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin levels of adult female rat controls or treated with IMI and UU, IMI + UU during 60 days.

Groups	*ALT (UI/L)*	*AST (UI/L)*	*Bilirubin (mg/L)*
Control	24.21 ± 2.04	67.96 ± 5.12	0.20 ± 0.01
50 mg/kg of IMI	$37.04 \pm 3.57^{\alpha***}$	$122.50 \pm 5.53^{\alpha***}$	$0.36 \pm 0.03^{\alpha***}$
200 mg/kg of IMI	$51.65 \pm 3.76^{\alpha***,\beta***}$	$140.67 \pm 5.89^{\alpha***,\beta***}$	$0.53 \pm 0.02^{\alpha***,\beta***}$
300 mg/kg of IMI	$60.34 \pm 3.67^{\alpha***,\beta***,£***}$	$155.89 \pm 6.09^{\alpha***,\beta***,£***}$	$0.6 \pm 0.01^{\alpha**,\beta***,£***}$
100 mg/kg of UU	$24.21 \pm 3.40^{\alpha*}$	$68.83 \pm 6.03^{\alpha*}$	$0.21 \pm 0.01^{\alpha*}$
50 mg/kg of IMI + 100 mg/kg of UU	$29.16 \pm 3.78^{\beta***}$	$77.87 \pm 6.52^{\beta***}$	$0.23 \pm 0.01\beta^{***}$
200 mg/kg of IMI + 100mg/kg of UU	$35.87 \pm 2.56^{£***}$	$89.67 \pm 5.45^{£***}$	$0.31 \pm 0.02^{£***}$
300 mg/kg of IMI + 100mg/kg of UU	$40.54 \pm 2.96^{€**}$	$120.34 \pm 6.56^{€**}$	$0.43 \pm 0.03^{€**}$

Values represent means ± SEM (n = 8) in each group. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.
α: compared to controls; β: compared to 50 mg/kg of IMI; £: compared to 200 mg/kg of IMI; €: compared to 300 mg/kg of IMI.

Table 6 Quantitative analysis (atomic%) of elements determined from EDX in the different groups of rats.

	Atomic %							
Groups	*O*	*Na*	*Mg*	*P*	*S*	*Cl*	*Ca*	*Ca/P*
Control	64.29	0.61	0.48	12.18	0.40	-	22.04	1.80952381
50 mg/kg IMI	73.88	0.77	0.24	9.72	0.17	0.17	15.24	1.56790123
300 mg/kg IMI	72.14	0.95	0.26	10.33		0.30	16.03	1.5517909
100 mg/kg UU	64.29	0.61	0.48	12.18	0.40	-	22.04	1.80952381
50 mg/kg IMI+UU	64.29	0.61	0.48	12.18	0.40	-	22.04	1.80952381
300 mg/kg IMI+UU	64.29	0.61	0.48	12.18	0.40	-	22.04	1.80952381

UU and IMI, the vitamin E levels in liver (19.20, 14.42, 24.42%) were ameliorated when compared with IMI-treated groups.

Effects of IMI and UU on biochemical markers in plasma. ALT and AST activities and bilirubin levels in the plasma of the IMI-treated groups increased by 45%, 55% and 54% when compared with those of the controls Table 5. The injection of UU to IMI-treated groups restored all the parameters cited above.

Histopathological findings; Light microscopic examination indicated a normal structure of the liver in the controls Figure 2B1. The exposure of rats to IMI induced degenerative changes

in the organ. IMI caused necrosis, inflammatory processes in the treated rats. Thus, we found in group 50 mg/kg of IMI areas of hepatocyte necrosis and necrotic inflammatory lesions [Figure 2B2] compared with the control. However, we noted necrotic and inflammatory lesions in larger médiolobulair areas in group treated with 200 mg/kg of IMI [Figure 2B3], the lesions were the same that were wide enough in group 300 mg/kg of IMI [Figure 2B4] compared with the other groups. We also noted in this group the presence of small necrotic lesions and inflammatory péricentrolobular regions in the central vein and others in zones 1 and 2 of the hepatic acinus compared with the control group.

Co-administration of UU improved liver histological pictures. These liver damages, observed in IMI groups, significantly decreased in (IMI + UU)-treated groups Figure 2B5-B8. The histological pattern was normal in rats treated only with UU (Figure 2).

Scanning electron microscopy. The results of the SEM analysis were excised at different magnifications, in Figures 3-6.

The SEM results of Figures 3-5 did not reveal the phenomenon of alteration of IMI-treated bone. However, with a grossissement of 1 µm [Figure 6], we can clearly see the phenomenon of textural alteration in the IMI-treated bones (B, C). UU injection shows architecture within the bone resembling that of the control bone.

Bone Study microanalysis-energy spectrometry (EDX). Microanalysis - energy spectroscopy (EDX) is used to obtain the distribution mapping of the different elements by X-ray backscattering. The results obtained for the control group, treated group with 50 mg/kg IMI, treated group with 300 mg/kg IMI, treated group with 100 mg/kg UU, treated group with 50 mg/kg IMI+100 mg/kg UU, and treated group with 300 mg/kg IMI+100 mg/kg UU are shown in Figure 7A-F, respectively.

The composition of femurs of different groups was quantified by measuring the concentration of phosphate and calcium. The quantitative analyzes (atomic%) by EDX of the elements in the different groups were presented in Table 3.

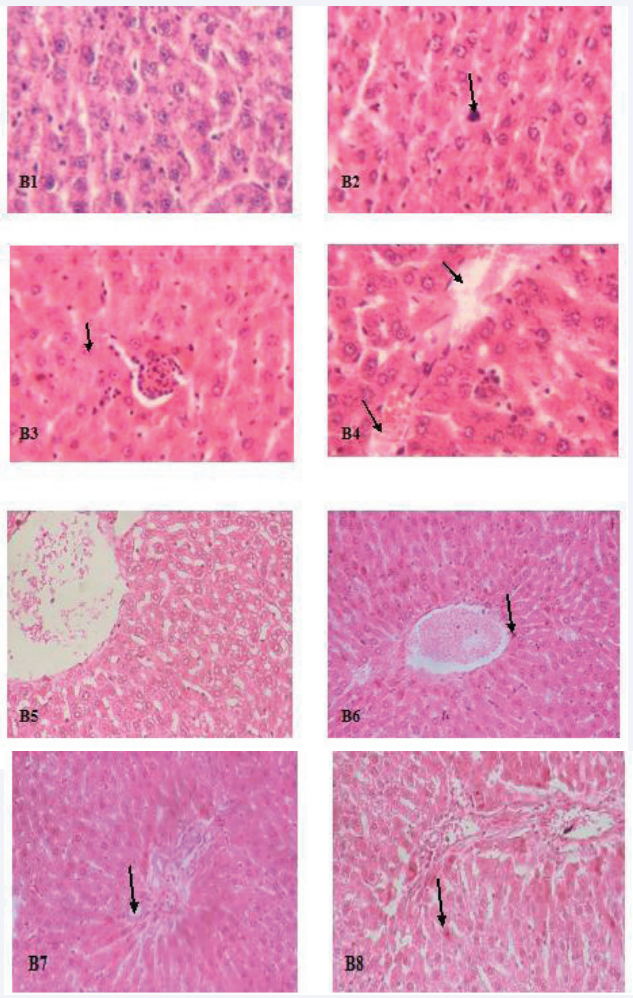

Figure 2 Light microscopic examination

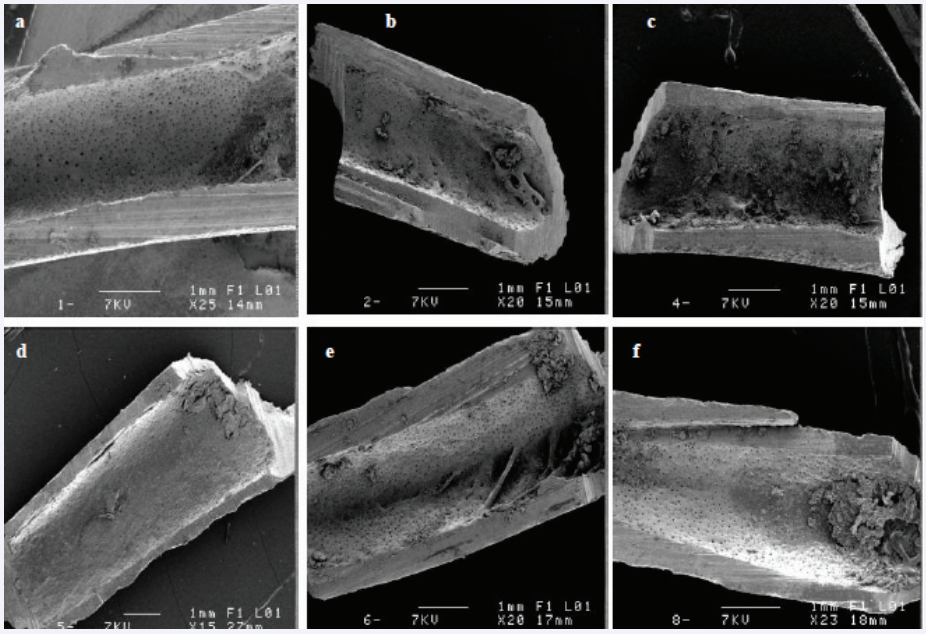

Figure 3 General appearance of the diaphysis of treated rats with IMI, IMI+UU or UU for 60 days.
(a) Femur section of control rats; (b) Femur section of treated rats with 50 mg/kg IMI; (c) Femur section of treated rats with 300 mg/kg IMI; (d) Femur section of treated rats with 100 mg/kg UU; (e) Femur section of treated rats with 50 mg/kg IMI+100 mg/kg UU; (f) Femur section of treated rats with 300 mg/kg IMI+100 mg/kg UU.

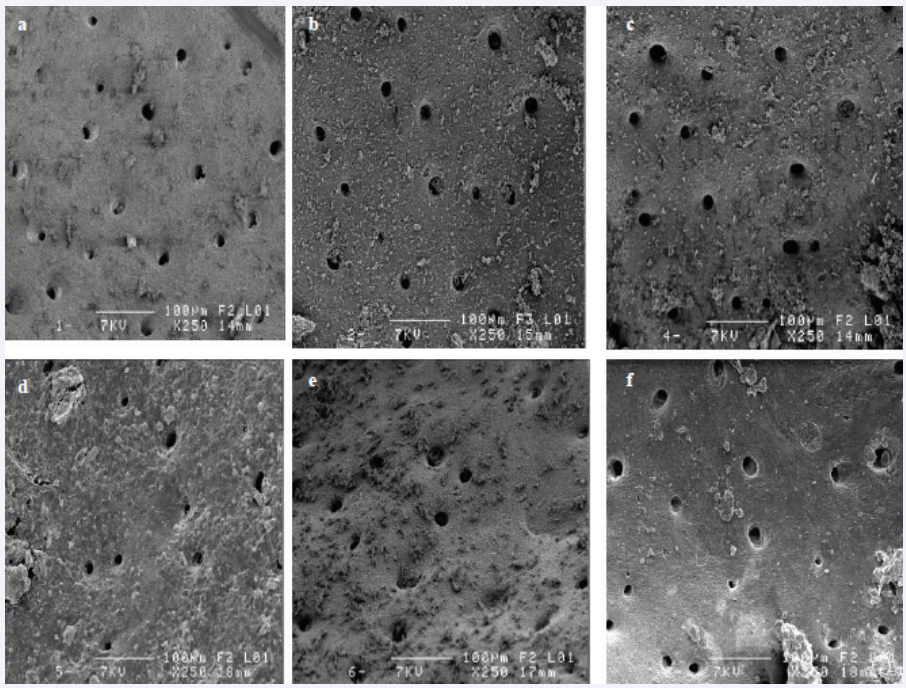

Figure 4 Appearance of endosteum of treated rats with IMI, IMI+UU or UU for 60 days.
a) Femur section of control rats; (b) Femur section of treated rats with 50 mg/kg IMI; (c) Femur section of treated rats with 300 mg/kg IMI; (d) Femur section of treated rats with 100 mg/kg UU; (e) Femur section of treated rats with 50 mg/kg IMI+100 mg/kg UU; (f) Femur section of treated rats with 300 mg/kg IMI+100 mg/kg UU.

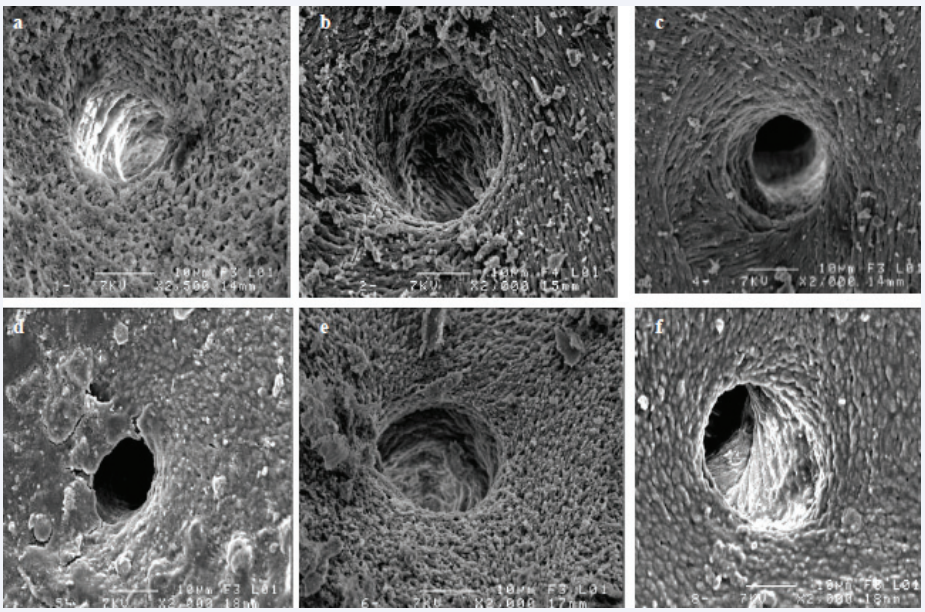

Figure 5 Apparence of the cancellous bone of treated rats with IMI, IMI+UU or UU for 60 days.
(a) Femur section of control rats; (b) Femur section of treated rats with 50 mg/kg IMI; (c) Femur section of treated rats with 300 mg/kg IMI; (d) Femur section of treated rats with 100 mg/kg UU; (e) Femur section of treated rats with 50 mg/kg IMI+100 mg/kg UU; (f) Femur section of treated rats with 300 mg/kg IMI+100 mg/kg UU.

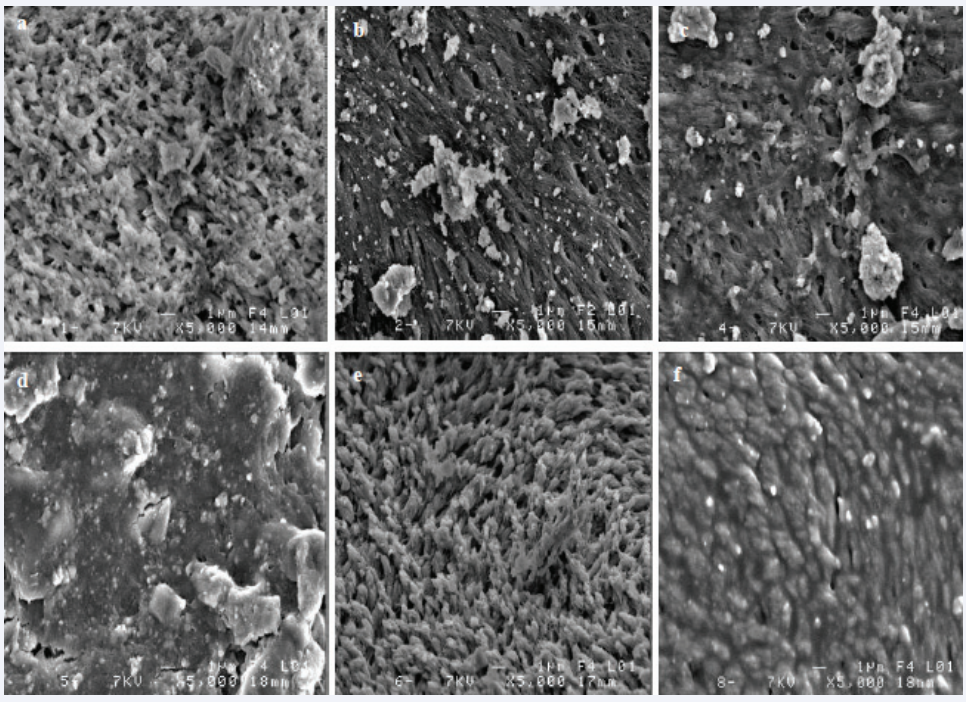

Figure 6 Scanning electron microscopy observation of compact bone.
(a) Femur section of control rats; (b) Femur section of treated rats with 50 mg/kg IMI; (c) Femur section of treated rats with 300 mg/kg IMI; (d) Femur section of treated rats with 100 mg/kg UU; (e) Femur section of treated rats with 50 mg/kg IMI+100 mg/kg UU; (f) Femur section of treated rats with 300 mg/kg IMI+100 mg/kg UU.

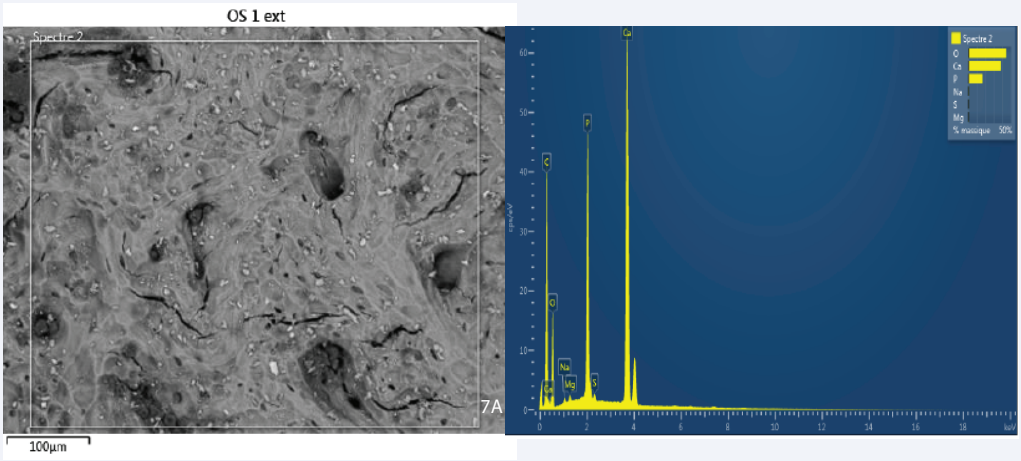

Figure 7 A. EDX spectrum and maps distributions of femoral components of control rats after 60 days.

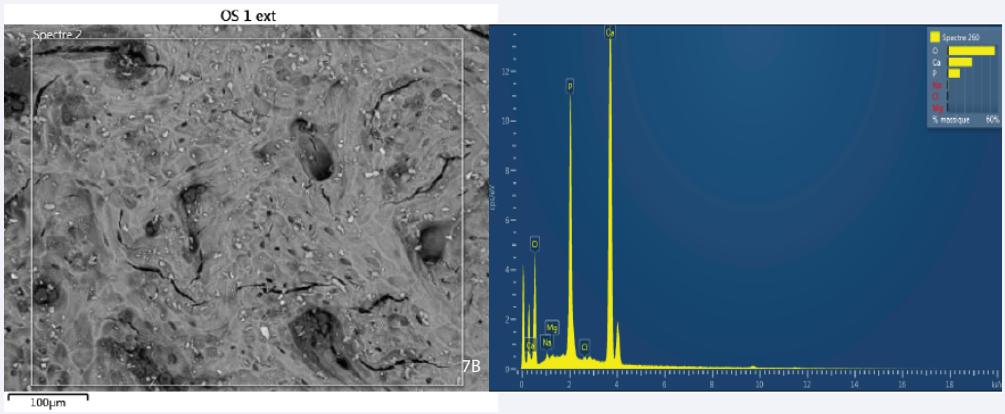

Figure 7 B. EDX spectrum and maps distributions of femoral components of treated rats with 50 mg/kg of IMI

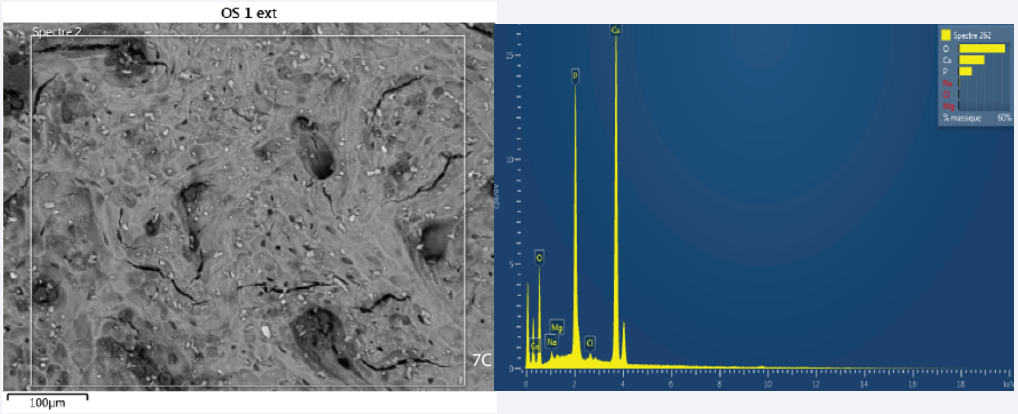

Figure 7 C. EDX spectrum and maps distributions of femoral components of treated rats with 300 mg/kg of IMI.

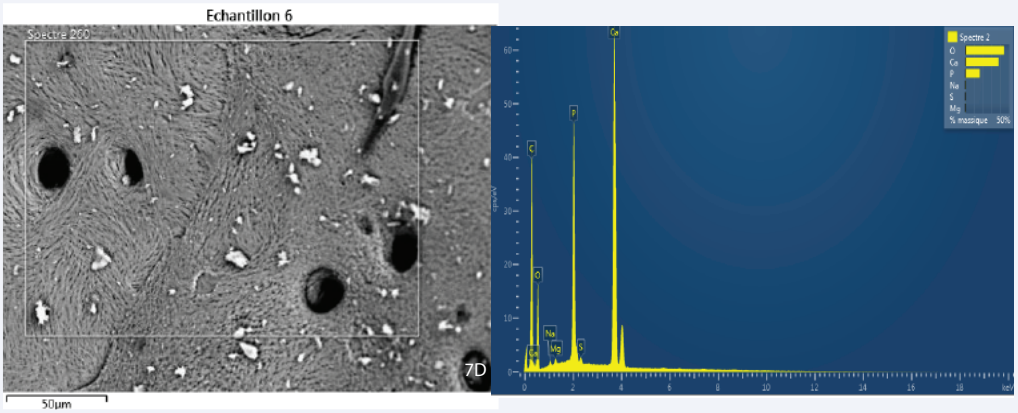

Figure 7 D. EDX spectrum and maps distributions of femoral components of treated rats with UU for 60 days.

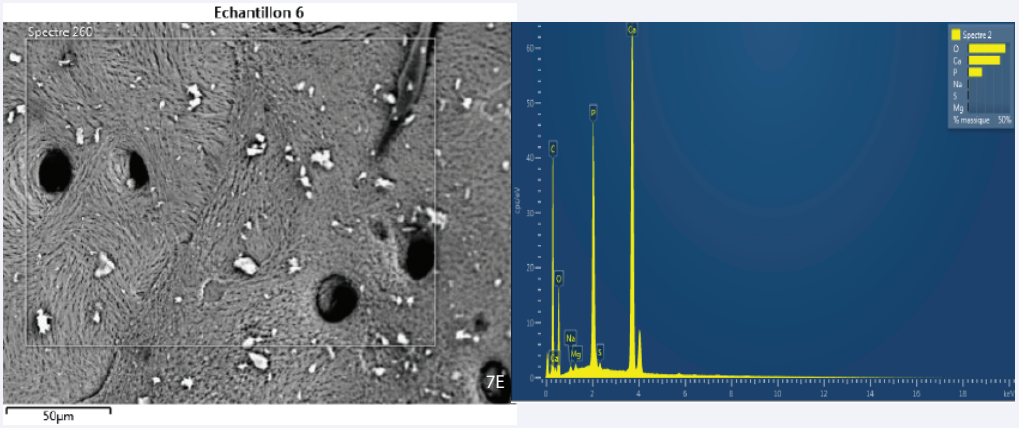

Figure 7 E. EDX spectrum and maps distributions of femoral components of treated rats with 50 mg/kg of IMI+UU.

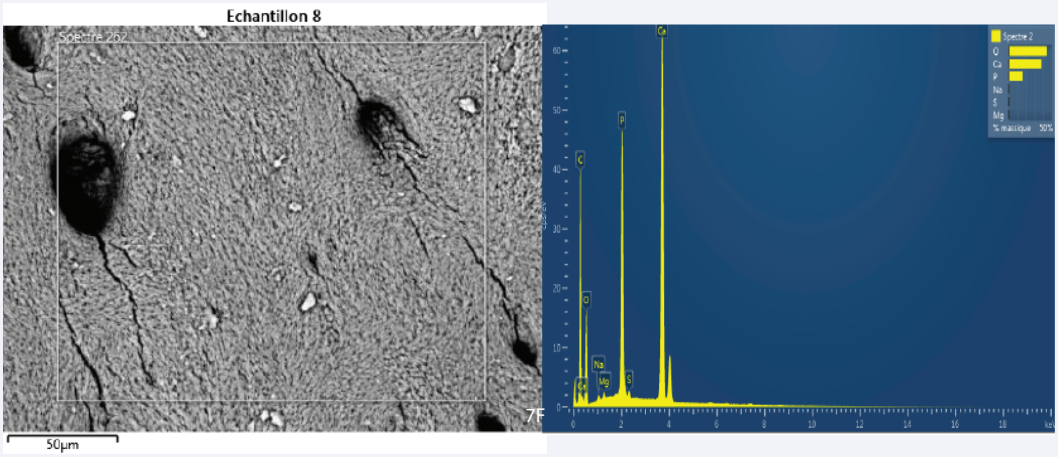

Figure 7 F: EDX spectrum and maps distributions of femoral components of treated rats with 300 mg/kg of IMI+UU.

The results show that the Ca/P ratio is 1.8 (normal between 1.7 and 2) in control femurs and (UU+IMI)-treated femurs. However, this ratio decreases to 1.5 in IMI-treated femurs. Generally, the reduction of this ratio to above 1.67 [39] is due to a structural and microstructural disturbance of the bone, under the action of several phenomena. In our study, that we noted the decrease of this ratio. This ratio increases again to reach the value 1.8 following the addition of UU. This technique confirmed our previous results (obtained through FTIR, XRD, etc), which showed the protective effect of UU against the toxic or disruptive effect of IMI.

DISCUSSION

In this study, we investigated the hepatotoxicity and osteoporosis of IMI in rat after 60 days oral administration. The results demonstrated that treatment with IMI induced a significant toxicity against the bone and liver of rat at 3 doses.

Different mechanisms have been postulated to explain IMI induced liver injury, such as stress oxidant.

The liver is the primary target responsible for metabolism of chemicals, drugs alcohol, food additives, pesticides and natural products [40]. Liver toxicity has also been evaluated based on alterations in the levels of ALT, AST. AST and ALT have been reported as noninvasive methods of evaluating liver fibrosis and cirrhosis [41].

In the present study, we detected changes in the ALT and AST activities. These changes in the activities of these enzymes have been considered as a tool to study varying cell viability and cell membrane permeability [42]. So, ALT is a specific cytosolic enzyme for the liver while AST is localized in cytosol and mitochondria which is in circulation in the early phase of liver injury [43]. In our study, we demonstrated that IMI administered to rats provoked a marked elevation in the activities of plasma ALT and AST, indicating hepatocellular damage. According to Demirbas et al. [44] this elevation could potentially be attributed to the release of these enzymes from the cytoplasm to the blood circulation indicating a necrosis and inflammatory reactions. Also IMI hepatotoxicity is evidenced in the present study by an increase in bilirubin levels. The increase in bilirubin may result from increased bilirubin production resulting the decreased liver uptake, conjugation, as demonstrated by Kumari et al. [45]. According to these authors, the bilirubin may rise after liver injury. Such biochemical changes might account for the occurrence of histopathological alterations in the livers of animals treated with IMI. According to Abdel-Rahman and Zaki [46], there was an infiltration of leukocytes considered as a prominent response of the body facing any injurious effect. Histological modifications were more pronounced in groups 200 mg/kg of IMI-treated rats and 300 mg/kg of IMI-treated rats; this could be explained by the fact that IMI has different mechanisms depending on the dose of insecticide.

The results indicate that they reflected toxicity in the liver of rats treated with three different doses of IMI for 60 days.

We have demonstrated with SEM analysis a phenomenon of textural alteration in the bone treated by different doses of IMI and which increases with the dose. It could be explained by a disturbance of bone remodeling, with delayed ossification and confirms that IMI disturbs well the bone remodeling. Similarly, Badraoui et al. [47] showed that tetradifon (pesticide estrogen-like) disrupted the mineral composition of bone especially calcium and phosphorus which increased in content. However, MEB analysis did not demonstrate the structural alteration in the general organization of the femoral parts in the treated rats. This is probably due to the oestrogen-like effect of tetradifon. Indeed, estrogen inhibits remodeling and enhances bone formation. Tetradifon does not appear to have a deleterious effect on bone remodeling.

In our experimental study, the result of GC-MS analysis specifies that the UU EtOH extract of leaves contains various bioactive compounds which have various medicinal properties that can be useful for the treatment of various diseases. The GC-MS analysis results have shown the presence of the following biomolecules in abundance as shown by their peak value percentages: heptacosane, hexacosane, heptadecane, hexadecane, eicosane, octadecane citronellol [48]. This extract is found to be rich in several bioactive and industrially important compounds. The presence of these compounds make UU EtOH extract as a probable good therapeutic entity against toxicity.

UU pretreatment was shown to prevent the IMI-induced toxicity. In the present study, the parameters of oxidative stress, that is, MDA and AOPP were found markedly increased and the activity of SOD, GPx and CAT, vitamin E were markedly decreased in the tissues of IMI treated rats suggesting that IMI treatment caused oxidative damage to the lipids and proteins in this organs. The rat in the UU + IMI groups had significantly increased SOD, GPx and CAT, vitamin E and C levels and decreased MDA and AOPP levels suggesting that UU protects against the adverse effects of IMI.

SEM results showed that (IMI+UU)-treated groups have normal bones compared to IMI-treated groups. This could be attributed to the presence of bioactive components in the UU EtOH extract (phenolic compounds, flavonoids, minerals (Calcium, Phosphorus, etc)), which stimulates the osteoblastic activity and thanks to the richness of these compounds in OH-.

OH- will react on the treated bone and exactly with Cl- introduced on the senial axis through the IMI. There will be a new substitution between Cl- (treated bone) and OH- (UU) while providing a structure whose tunnels contain only OH- (control bone) and the UU contains both of the ions OH- than of the Cl- ions, then a giant molecule is obtained between IMI and UU which is released from the bone. Very similar results to this study were obtained by Suwalsky et al. [49], SEM analysis demonstrated the highly protective effect of Hilesia Magellanica (Coicopihue) from chilean Patagonia against oxidative damage. Suwalsky et al. [50] also showed the protective effect of Ugni molinae Turcz against oxidative damage of human erythrocytes by scanning electron microscopy (SEM).

In another work, Radhakrishnan et al. [51] investigated that Pedalium murex Linn extract can decreased crystal size and prevented the aggregation of calcium oxalate crystals using SEM and DRX.

Ben Amara et al. [52], have evaluated the ability of selenium, used as a nutritional supplement, to alleviate bone deficiencies in mothers treated with methimazole, an antithyroid drug.

Treatment with methimazole reduced the length and weight of the femur in pups compared to controls. The activities of the antioxidant enzymes of the femur, superoxide dismutase, catalase and glutathione peroxidase have decreased. Lipid peroxidation showed an increase in the high rates of malondialdehyde in the femur. Methimazole also caused a significant decrease in Ca and P levels in bone. In addition, resistant tartrate acid phosphatase was increased, while total alkaline phosphatase was reduced. The administration of selenium in the diet has improved the biochemical parameters cited above. This study suggests that selenium is an important protective element that can be used as a dietary supplement protecting against bone deficiencies.

CONCLUSION

We suggest that IMI has an adverse ecological impact on mammalian animals as it induces female rat bone and liver damages. The dose related IMI intoxication is already causing have concerns. We have found that injection of Urtica urens with IMI causes the toxicity of this insecticide to less in rats, thus confirming a protective role of the plant.

ACKNOWLEDGMENTS

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Ethics Approval and Consent to Participate: The experimental protocols were conducted in accordance with the guide for the care and use of laboratory animals issued by the University of Sfax, Tunisia, and approved by the Committee of Animal Ethics (Protocol no. 94-1939).

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