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The effect of thyme essential oil on duodenal toxicity induced by subacute exposure to voliam targo® insecticide in male rabbits

Hassina Khaldoun [a], Amina Settar [b,*], Yasmine Oularbi [c], Nouara Boudjema [a], Assia Amokrane [a], Nacima Djennane [d], Dalila Tarzaali [e]

[a] Department of Biology, Faculty of Nature and Life Sciences, University of Blida 1, Route de Soumaa, BP270, Blida, Algeria
[b] Department of Agri-food, Faculty of Nature and Life Sciences, University of Blida 1, Route de Soumaa, BP270, Blida, Algeria
[c] Higher National School of Agronomy, Algiers, Algeria
[d] Department of Pathological Anatomy, Centre Hospitalo-Universitaire Bab El Oued, Algiers, Algeria
[e] Institute of Veterinary Sciences, Faculty of Nature and Life Sciences, University of Blida 1, Route de Soumaa, BP270, Blida, Algeria

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ABSTRACT

The increasing use of pesticides has raised concerns about their gastrointestinal toxicity, leading to the search for natural remedies such as thyme essential oil. For that, this study aimed to determine the protective effect of Thymus vulgaris essential oil (TEO) with its chemical composition against Voliam Targo-induced duodenal toxicity. Twenty male rabbits were randomly assigned to four equal groups and treated for 21 consecutive days: Control, VT insecticide group, TEO essential oil group, and VT + TEO group. The main constituent of the essential oil of T. vulgaris was carvacrol 72.9 %. The duodenal injury was assessed using biochemical, histomorphometrical, and immunohistochemical methods. The VT induced an increased number of benign intestinal tissue changes, such as hyperplasia of Brunner glands, disorganization of villi, and infiltration of inflammatory cells. The co-administration of TEO with VT restored the histological organization of the duodenum. In addition, the immunohistochemical examination of the duodenal tissues shows positive immunostaining for the expression of Ki67, P53, and BCL2 proteins in the VT group. Lower expressions were noted in the VT-TEO group compared to the control and TEO groups. The E-cadherin and β-catenin immuno-signals were significantly higher in the essential oil treatment groups' duodenal sections than in the VT group. The study suggested that VT caused duodenal toxicity and that the carvacrol chemotype of TEO could mitigate and alleviate this effect.

1. Introduction

The gastrointestinal mucosa in mammals is a highly dynamic tissue characterized by rapid self-renewal, with its homeostasis maintained through the precise regulation of epithelial cell proliferation, growth inhibition, and apoptosis [1]. The intestinal stem cells (ISCs), located at the base of crypts, produce absorbing cells (enterocytes) and secretory progenitor or transit-amplifying cells (Paneth cells, enteroendocrine, tuft, and goblet) [2]. It is well-established that pesticides and other xenobiotics, which many species are frequently exposed to, significantly impact the small intestine by compromising the gut barrier and triggering systemic inflammation [3]. Extended exposure to these xenobiotics may result in detrimental health effects in humans and animals including the induction of oxidative stress [4],mutagenicity, genotoxicity, carcinogenicity, and multiorgan toxicity including intestinal damage [5].

The VT, an abamectin-based insecticide used in our study, contains abamectin 1.8 % and chlorantraniliprole 4.5 % [6].

Chlorantraniliprole, an insecticide classified as an anthranilic diamide, works by selectively binding to ryanodine receptors in the muscle cell of the insect by releasing calcium that may initiate several complications like paralysis [7,8]. Recently, sub-acute and sub-chronic toxicity studies demonstrated the toxicological effect of chlorantraniliprole on mammals' liver, kidneys, and protein profile [9,10], as well as on various hematological parameters [11] and on developmental and genetic toxicity [12].

Abamectin belongs to the avermectins family, specifically derived from the fermentation of the soil-dwelling actinomycete *Streptomyces avermitilis*. It has been widely used as a pesticide in agriculture, with high efficiency and relatively low toxicity to mammals [13]. However,

* Corresponding author.
E-mail address: settaramina95@gmail.com (A. Settar).

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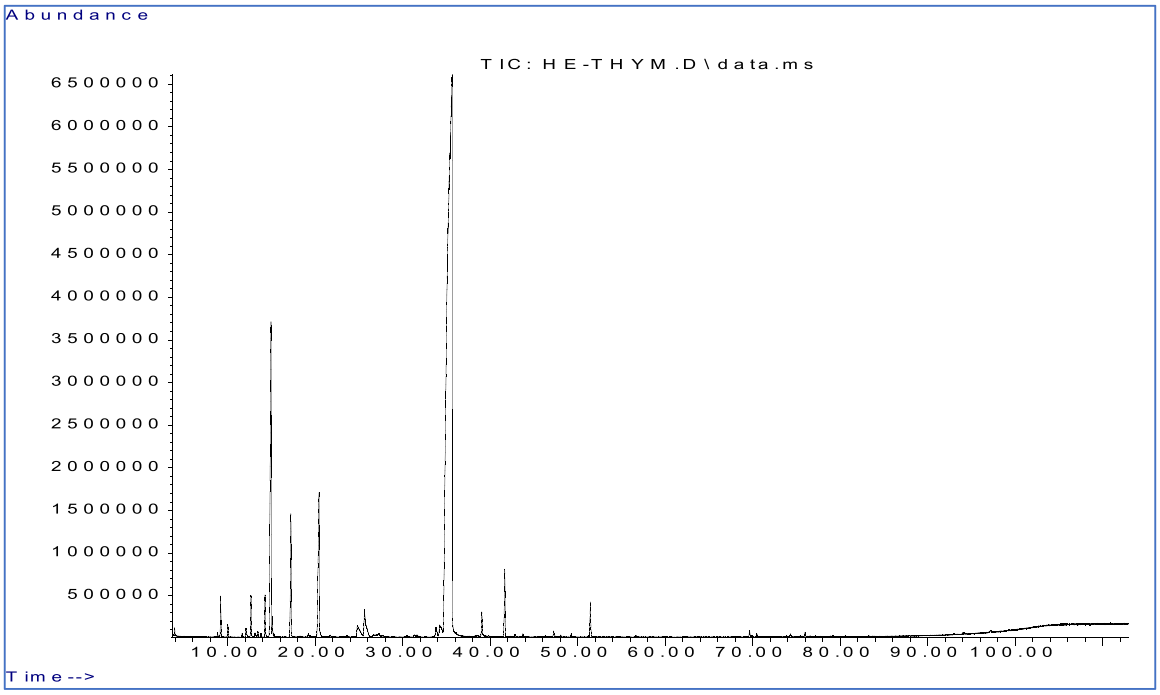

Fig. 1. Gas chromatogram profiles of peak retention of components of *Thymus vulgaris* essential oil.

Table 1
Chemical composition of the fresh leaves of *Thymus vulgaris* essential oil from Blida province (Hammam Melouane) (North-Algeria).

	Components	RT (min)	MC
1	Butanoic acid	3.912	0.08
2	Alpha-Thujene	8.809	0.08
3	Alpha-Pinene	9.168	0.68
4	Camphene	9.98	0.23
5	Beta-Pinene	11.628	0.08
6	1-OCTEN−3-OL	12.052	0.26
7	Beta-Myrcene	12.635	0.82
8	3-Octanol	13.094	0.12
9	l-Phellandrene $	13.418	0.13
10	DELTA.3-Carene	13.771	0.08
11	alpha.-Terpinene	14.242	0.86
12	Para-Cymene	14.913	8.33
13	beta-Phellandrene	15.083	0.37
14	gamma-Terpinene	17.191	2.54
15	Alpha-Terpinolene	19.204	0.08
16	Linalool	20.399	4.35
17	(-)-cis-Myrtanylamine	21.688	0.05
18	Borneol	24.807	0.86
19	4- Terpineol	25.62	1.38
20	2,4,6-Octatrien−1-ol	26.661	0.06
21	Thymol	33.801	1.2
22	Carvacrol	35.626	72.9
23	Carvacryl Acetate	39.022	0.57
24	Trans-Caryophyllene	41.642	1.47
25	Beta-Selinene	43.714	0.06
26	Beta-Bisabolene	47.233	0.12
27	Caryophyllene Oxide	51.436	0.78
	Total		98.54

Components presented in the order of elution
Rt, retention time; MC, mean composition (% area) (aerial parts and leaves samples)

Table 2
Effect of VT and TEO treatment on body weight, Food intake and water consumption on experimental rabbits after subacute exposure of 21 days.

Weight	Control	VT	TEO	VT + TEO
Initial BW (kg)	2.81 ± 0.01^a	2.79 ± 0.06^a	2.86 ± 0.02^a	2.92 ± 0.04^a
Final BW (kg)	3.14 ± 0.03^a	2.94 ± 0.07^b	3.17 ± 0.02^a	3.20 ± 0.05^a
% BWG	11.7^a	5.4^b	10.8^a	9.6^a
Average feed intake (g / rabbit)				
Acclimatation	89.9 ± 4.0^a	79.6 ± 8.6^a	83.6 ± 6.1^a	94.5 ± 4.9^a
Experimentation	135.8 ± 5.4^a	91.8 ± 6.0^b	123.5 ± 3.8^a	119.2 ± 5.7^a
Average water consumption (ml / rabbit)				
Acclimatation	79.6 ± 6.8^a	86.5 ± 4.3^a	102.1 ± 3.5^a	90.38 ± 5.7^a
Experimentation	137.1 ± 2.9^a	115.8 ± 8.4^b	190.1 ± 9.04^c	170.3 ± 9.8^d

Results are given as a mean ± SD for five rabbits in each group. a, b, c, d means within columns with different subscripts are significantly different at $p < 0.05$. VT: Voliam targo; TEO**:** Thyme essential oil; VT + TEO: Voliam targo + Thyme essential oil

several studies reported abamectin organ toxicity including hepatotoxicity [14], nephrotoxicity [13], reproductive toxicity [13,15] and neurotoxicity [16], and provoke gastrointestinal disorders such as mild intoxication symptoms like diarrhea and vomiting after human exposure [17,18].

Numerous plant extracts and their derivatives have demonstrated notable antioxidant proprieties, which are considered a key characteristic of medicinal plants used to treat various diseases including intestinal and gastroenteric disorders [19].

The *Thymus sp.* is an herbal medicine with a variety of biological activities, including antimicrobial and antifungal [20,21], antioxidant, anti-inflammatory, analgesic and antipyretic [22,23], and anti-tumoral activity [24]. Thyme (*Thymus vulgaris)*, locally known as “Himria” in Algeria, holds a prominent place in traditional healing, including its ability to act as expectorant, bronchodilator, antitussive, antispasmodic, anthelmintic, carminative, and diuretic. It is particularly valued in managing common age-related ailments such as stomach and gastrointestinal disorders [25]. The essential oil of *T. vulgaris* has garnered attention also for its biological activities, primarily due to its rich composition of bioactive compounds. The mode of action of these essential oils largely depends on the main constituents present, which include thymol, carvacrol, and p-cymene among others [26]. Essential oils with high levels of the phenolic compound thymol and/or carvacrol

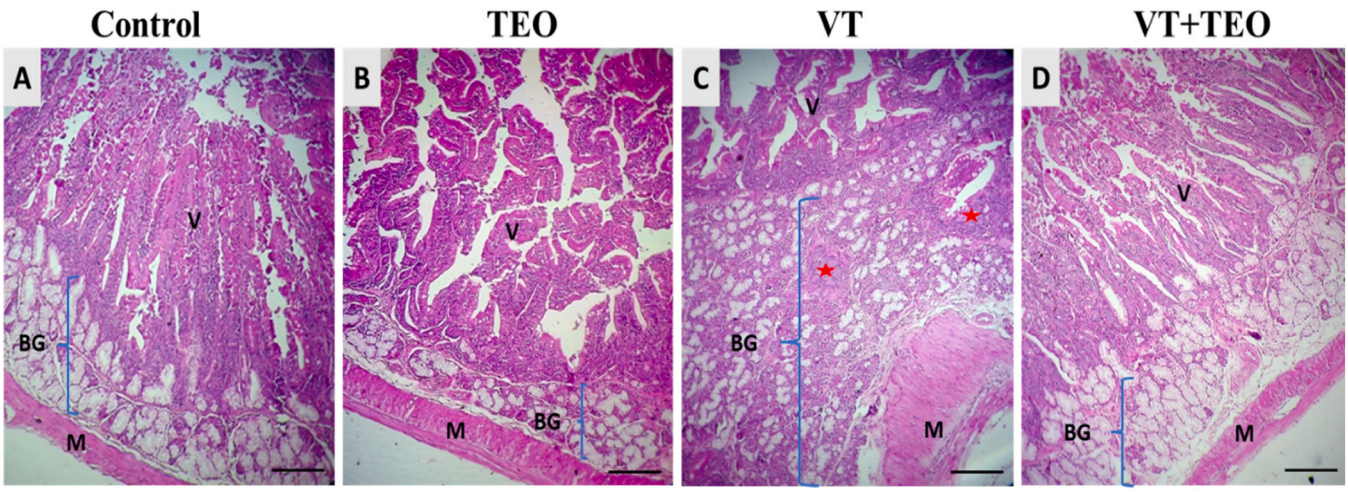

Fig. 2. Light microscopic examination (A, B, C and D; H&E, Gx100): **A and B)** control and TEO groups showing normal duodenal architecture with normal Brunner gland (BG); **C)** VT- group duodenum showing marked cellular infiltration and hyperplasia of Brunner's glands. **D)** Duodenal sections of VT-TEO group showing intestinal villi with a well-preserved epithelium and lamina propria. V: Villi, M: muscularis, red stars: cellular infiltration. Bar = 100 μm.

Table 3
Histomorphometric parameters of duodenum in rabbit (Mean± SD).

DOSE	Muscularis (μm)	Villus height (μm)	Brunner gland depth (μm)
Control	180.53 ± 2.13^{a}	1210.46 ± 11.87^{a}	358.46 ± 25.10^{a}
TEO	177.01 ± 2.47^{a}	1190.94 ± 28.34^{a}	316.27 ± 11.38^{a}
VT	179.22 ± 3.76^{a}	982.31 ± 31.61^{b}	854.13 ± 27.25^{b}
VT + TEO	178.29 ± 2.11^{a}	1066.02 ± 11.89^{b}	390.46 ± 14.50^{c}

a, b, c means within columns with different subscripts are significantly different at $p < 0.05$

are known to have the highest antioxidant activity [27,28]. Combining thymol and carvacrol enhances the plant's antioxidant, anti-inflammatory, and antiapoptotic activities [29]. Therefore, a growing interest in using antioxidants to ameliorate the intestinal toxicity induced by xenobiotics has been recognized [30].

While earlier studies have clarified the harmfulness of each active substance abamectin (ABM) and chlorantraniliprole (CAP), their combined effect on non-target organisms has yet to be proven. This underscores the need to explore these mixtures of insecticides and to identify potentially more effective protective strategies through essential oils. Moreover, the duodenum plays a crucial role in nutrient absorption and serves as the first site of exposure to ingested toxicants, making it highly susceptible to pesticide-induced damage. Its rapid epithelial turnover and complex immune interactions make it a key target for evaluating intestinal toxicity. Therefore, this study aims to examine the subacute toxicity of VT on the histopathological, histomorphometric, and immunohistochemical changes of the duodenum as well as the protective role of the *Thymus vulgaris* essential oil extracted from Algerian origins on male rabbits (*Oryctolagus cuniculus*).

2. Materials and methods

2.1. Insecticide

The commercial Abamectin-Based Insecticide formulation "Voliam Targo® 063SC" (VT) containing abamectin 1.8 % (CAS N° 71751–41–2) and chlorantraniliprole 4.5 % (CAS N° 500008–45–7) was purchased from Syngenta Crop Protection Agrochemicals (Greensboro, USA). All the other chemicals and biochemical reagents were purchased from BIOLABO SA (France).

2.2. Plant material

Flowering *Thymus vulgaris* L. (Lamiaceae*)* plants were collected in June 2020, from Blida province (Hammam Melouane) in North Algeria (36°29' N, 2°50' E, Altitude: 200 m). Aerial parts were used exclusively for extract preparation. The identification and authentication of the plant material was held at the Department of Botany of the National Higher School of Agronomy in Algiers.

2.3. Extraction of essential oil

The thyme essential oil (TEO) was obtained using the hydro distillation process in a Clevenger-type apparatus, then dried using anhydrous sodium sulfate (Na_2SO_4) and preserved in darkness at low temperature (+4 °C) until further use [6].

2.4. Analysis by gas chromatography-mass spectrometry

The composition of TEO was analyzed using gas chromatography coupled with mass spectrometry (GC–MS) on an HP6890 instrument linked to a 5973 A mass spectrometer. Two fused silica-capillary columns with distinct stationary phases were employed: a polar Stabilwax™ column composed of Carbowax™–PEG (60 m × 0.2 mm i.d., 0.25 μm film thickness) and a nonpolar HP5MS™ column (30 m × 0.25 mm i.d., 0.25 μm film thickness). GC–MS spectra were recorded under the following conditions: helium as the carrier gas with a flow rate of 0.3 ml/min; split-less injection mode; injection volume of 1 μL at 250 °C; and an oven temperature program starting at 0 °C for 8 min, increasing by 2 °C/min to 250 °C, and maintained at 250 °C for 15 min. The ionization was performed using electron impact mode at 70 eV. Component identification was achieved by comparing the GC Kováts retention index (RI), calculated relative to a homologous series of n-alkanes (C5–C28), with reference standards available in the laboratory. Additionally, mass spectral fragmentation patterns were matched against data in the National Institute of Standards and Technology (NIST) and Wiley MS libraries, as well as reported literature.

2.5. Gas chromatography-FID quantification

The percentage composition of the identified compounds was determined electronically based on the GC–FID peak areas. Gas chromatography was performed using a Hewlett-Packard 6890 GC–FID system equipped with a fused silica-capillary column containing a non-

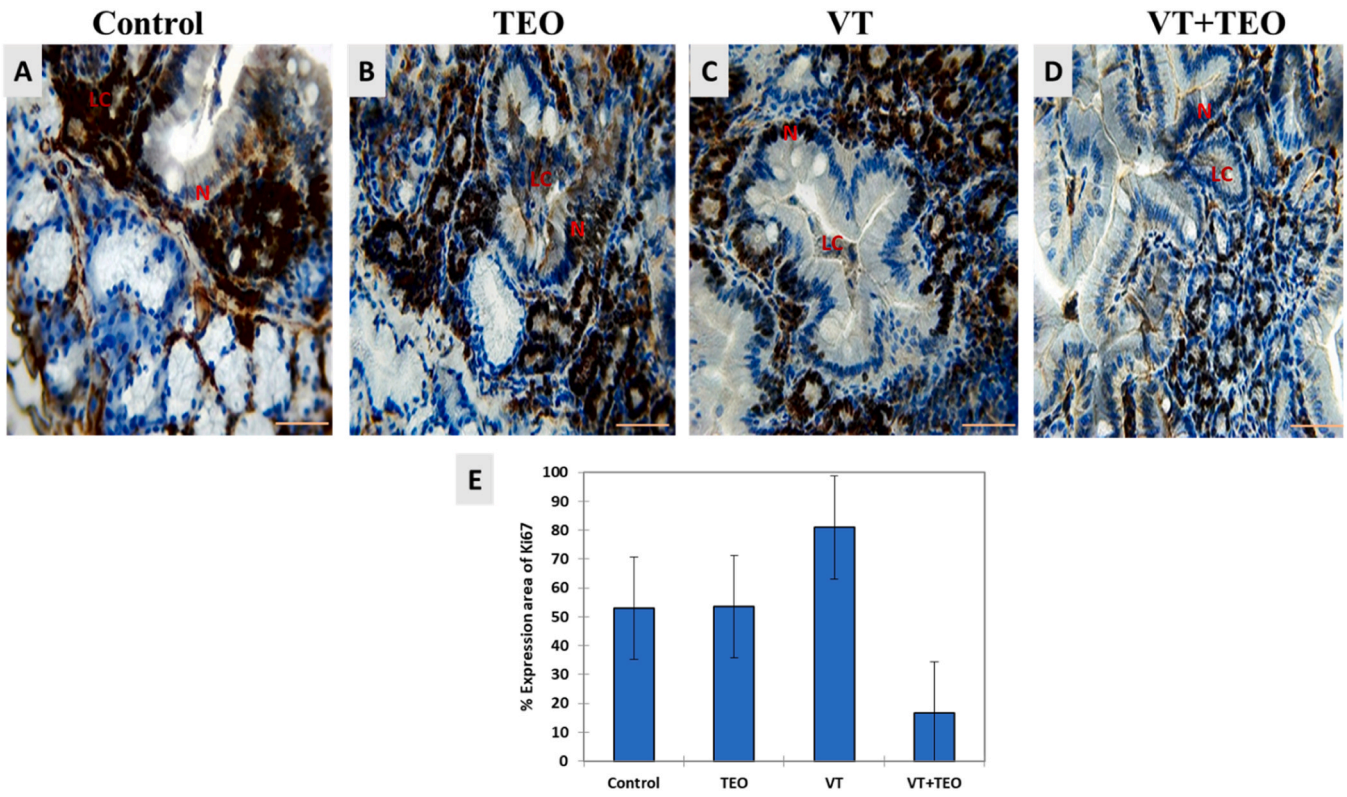

Fig. 3. Ki-67 (cell proliferation marker) expression in control, TEO, VT and VT-TEO treated rabbits. **A** and **C**: Photomicrographs of KI67 immunostaining (x400) showing medium expression of KI67 in the nuclei of the multipotent stem cells of the Lieberkühn crypts in the control (A) and TEO (B) groups.**B**and **D**: photomicrographs of KI67 showing intense immunoreactive expression of KI67 in VT-group (C) and moderate expression of KI67 in nuclei of Lieberkühn crypts in the villus tip in VT-TEO group (D). The number of positive cells was quantified from sections immunohistochemically stained with KI67. Statistically significant difference ($p < 0.05$). LC: Lieberkühn crypts, N: nuclei. Bar = 50 μm.

polar HP5MS™ stationary phase (30 m × 0.25 mm i.d., 0.25 μm film thickness). The mass spectrometer operated in EI mode with an ionization voltage of 70 eV, while the ionization source temperature was set at 250 °C. The column temperature was programmed to start at 60 °C, held for 8 min, then raised at a rate of 2 °C/min to 250 °C, where it was maintained for 10 min. Samples were injected in split-less mode with a volume of 1 μL at an injection temperature of 250 °C. Nitrogen (N6.0) served as the carrier gas at a flow rate of 0.5 ml/min, and flame ionization detection was performed at 320 °C.

2.6. Animals

Twenty healthy adult male rabbits (3–4 months of age, 2.6 ± 0.1 kg of weight) were purchased from the Technical Breeding Institute (ITELV, Baba-Ali) and transferred to the CRD Saidal (Algeria) for experimentation. The rabbits were acclimated for 3 weeks under standard laboratory conditions of temperature (25 ± 3 °C) and 12 h light/dark cycle. A standard pelleted diet and water were available *ad libitum*. A daily weighing of rabbits in the early morning before being fed throughout the acclimation and the experimental periods (3 weeks for each) was done. Food and water intakes were recorded daily.

2.7. Experimental design

Rabbits were equally assigned into four groups and treated daily as follows:

- C-Group (control group): rabbits that received vehicle distilled water.
- VT-Group: rabbits that received VT insecticide 15 mg/ kg body weight dissolved in distilled water.
- TEO-Group: rabbits that received (0.5 mg/ kg body weight) thyme essential oil dissolved in olive oil.
- VT + TEO-Group: rabbits that received 0.5 mg/kg b.w of thyme essential oil dissolved in olive oil + 15 mg/kg body weight of VT dissolved in distilled water as in the VT-treated group.

The treatments were done orally for 21 consecutive days (3 weeks), The subacute toxicity evaluation was performed in compliance with OECD 425 research guidelines [31], also with the ARRIVE guidelines, and approved by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research DE n∘10–90. At the end of the experimental period, rabbits were anesthetized using an intramuscular injection of ketamine and midazolam (5 mg/kg body weight, 1 mg/kg body weight respectively) then humanely sacrificed by decapitation using a sharp guillotine, and intestine tissues were collected for histopathological, histomorphometric, and immunohistochemical analyses. The selective dose of VT is based on its relationship with NOAeLs and LOAELs established for the two active ingredients and on abamectin LD50 (Bokreta et al., 2021; Authority, 2022). The selective dose of TEO is based on the previous study [6].

2.8. Histological examination & histomorphometrical measurements

The three distinct intestinal segments, duodenum, jejunum, and ileum were separated. The duodenal tissue was then processed for histopathological, and histomorphometrical evaluations, as it served as the primary site for assessing subacute toxicity. The duodenum was fixed in 10 % formalin and incorporated in paraffin. Sections were cut to a thickness of 2 μm and stained with, hematoxylin and eosin (HE), for histopathological examination. Then, digital images of duodenal tissue were obtained using a digital camera connected to an Olympus

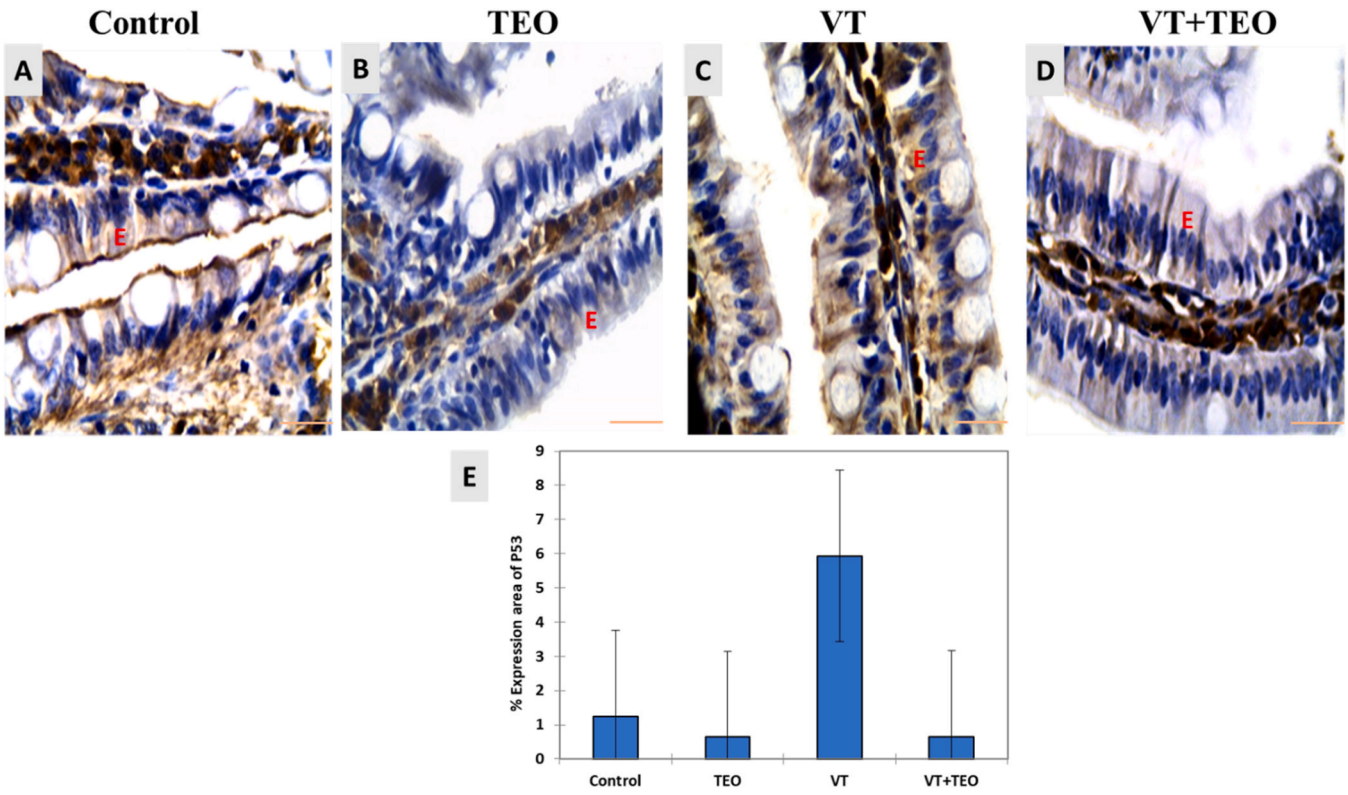

Fig. 4. P53 (cell apoptotic effector) expression in control, TEO, VT and VT-TEO treated rabbits (Gx1000). A, B and D photomicrographs of P53 immunostaining showing slight expression in the cytoplasm of the enterocytes (E) in the control (A) TEO (B) and VT-TEO groups. C strong immunoreactive expression of P53 in the cytoplasm of the duodenum enterocytes in the VT groups. Statistically significant difference ($p < 0.05$). Bar = 50 μm.

microscope (Optika B 183, Italy).

For morphometrical evaluations, the histological glass slides previously stained were randomly selected and photos of duodenal parenchyma were obtained using a digital camera connected to a light microscope (Optika B 183, Italy) via "TS View" software (Microscopes America, Cumming GA, USA). The thickness of mucosa (Villus height), submucosa (Brunner gland depth), and muscularis (Inner Circular layer and Outer longitudinal layer) were measured using measurement tools of "Image View" software (version x64, 4.10.17614.20200822, Russia). A total of 60 measurements for each parameter were taken at magnifications of 100 x and 400 x, then data were statistically expressed.

2.9. Immunohistochemistry

Rabbits' duodenum tissues were sectioned at 1–2 μm, and immunohistochemistry was carried out. The immunostaining process of p53, KI67, Bcl-2, beta-catenin, and E-cadherin was realized using an automated slide stained (Benchmark ULTRA; Ventana Medical Systems, Tucson, AZ) following the manufacturer's protocol.

The primary antibodies used are as follows: Bcl-2 (clone SP66, Ventana, cat# 790–4604); Ki-67 (clone 30–9, Ventana cat# 790–4286); p53 (clone Bp-53–11, Ventana, cat# 760–2542); beta-catenin (clone 14, Ventana, cat#760–4242) and E-cadherin (clone EP700Y, Ventana, cat# 760–4440). Immunostaining was analyzed as a percentage expression area in a standard measuring frame of each animal of the groups, using 40x magnification photo slides processed through QuPath software version 0.3.2. Areas exhibiting brown immunostaining were considered positive for analysis. The percentage expression area was determined using the formula: *Number of positively immunostained cells / Total number of both positive and negative cells in the measurement frame)* × *100*. The resulting data were then statistically analyzed and reported [10].

2.10. Statistical analysis

All data are presented as mean ± standard deviation (SD). Comparisons between groups were made using one-way analysis of variance, followed by Duncan's post-hoc test by Statistica version 10.0 (stat soft Inc., Tulsa, Oklahoma). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Chemical TEO characterization

A total of 27 components were identified in TEO, accounting for 98.54 % of the total composition: α-thujene (8.809 ± 0.08 %), γ-terpinene (17.191 ± 2.54 %), α-pinene (9.168 ± 0.68 %), camphene (9.98 ± 0.23 %), β-.-Selinene (43,714 ± 0.06 %), β-pinene (11.628 ± 0.08 %), Butanoic acid (3912 ± 0,08 %), 1-OCTEN-3-OL (12.052 ± 0.26 %), beta.-Myrcene (12.635 ± 0.82 %), 3-Octanol (13.094 ± 0.12 %), l-Phellandrene (13.418 ± 0.13 %), Delta.3-Carene (13.771 ± 0.08 %), α-terpinene (14.242 ± 0.86 %), carvacrol (35,626 ± 72.9 %), Carvacryl Acetate (39.022 ± 0.57 %), p-Cymene (14.913 ± 8.33 %), β-phellandrene (15.083 ± 0.37 %),α-terpinolene (19.204 ± 0.08 %), linalool (20.399 ± 4.35 %), (-)-cis-Myrtanylamine (21.688 ± 0.05 %), borneol (24.807 ± 0.86 %), 4-Terpineol (25.62 ± 1.38 %), 2,4,6-Octatrien-1-ol (26.661 ± 0.06 %), thymol (33.801 ± 1.2 %), Trans caryophyllene (41.642 ± 1.47 %), β-bisabolene (47.233 ± 0.12 %) and caryophyllene oxide (51.436 ± 0.78 %) (Fig. 1; Table 1). Indeed, the analyzed TEO belongs to the carvacrol chemotype, as indicated by the chemical composition of the essential oil of T. vulgaris from this study.

3.2. Effects of treatments on body weights and feed intake

According to Table 2, during the experimental period, a similar

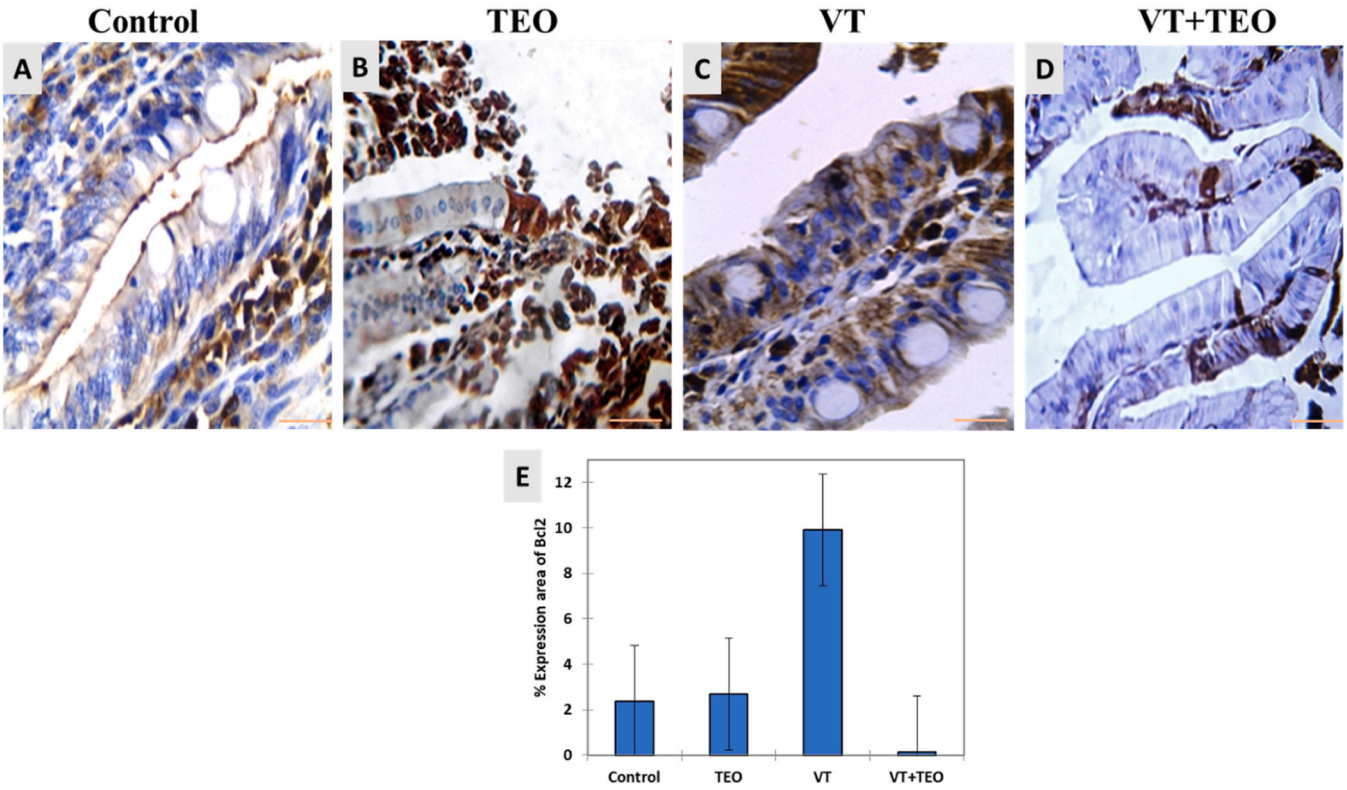

Fig. 5. BCL2 (cell apoptotic effector) expression in control, TEO, VT and VT-TEO treated rabbits (x1000). A and B photomicrographs of BCL2 immunostaining showing moderate expression of BCL2 in the cytoplasm of the intestinal cells of the villus in the control (A) and TEO (C) groups. C photomicrographs of BCL2 immunostaining showing strong expression of BCL2 in the cytoplasm of the duodenum enterocytes in the terminal end of the villus, where they are sloughed off into the lumen and undergo anoikis in the VT group. D photomicrographs of BCL2 immunostaining showing slight immunoreactive expression of BCL2 in the intestinal cells of the villus in the VT-TEO group. Data are expressed as means ± SD in each group. Statistically significant difference ($p < 0.05$). Bar = 50 μm.

increase in body weight in the control (11.7 %) rabbits and in the rabbits treated with TEO (10.8 %) was noticed. However, the lower body weight gain was significant ($p = 0.02$) in the VT treatment group (5.4 %). Interestingly, thyme essential oil co-administered with VT significantly improved the body weight gain of the rabbits (9.6 %).

In the experimentation period, food and water consumption decreased significantly ($p = 0.01$) in rabbits receiving VT 91.8 ± 6.0 g and 115.8 ± 8.4 ml respectively. However, we noticed a significant increase in water consumed by the TEO (190.1 ± 9.04 ml, VT+TEO (170.3 ± 9.8 ml) groups compared to control (137.1 ± 2.9 ml).

3.3. Histological study

The histology data are presented in Fig. 2. Histological observations by light microscopy of H&E duodenal sections from rabbits in the control (Fig. 2A) and TEO groups (Fig. 2B) revealed a normal duodenal architecture composed of four layers: mucosa, submucosa with normal simple tubular Brunner glands, muscularis propria and adventitia. The mucosal layer of the duodenum consisted of several villi composed only of absorptive (enterocytes) and goblet cells. Under the light microscope, Brunner's gland cells are eosinophilic with clear cytoplasm and typically basally oriented nuclei.

Marked cellular infiltration and hyperplasia of Brunner's glands were seen in duodenal sections from the VT group. The hyperplastic Brunner's glands extend above the muscularis mucosae and extend into the mid mucosa, distorting the overlying villous architecture, infiltration of inflammatory cells, and necrotic villi tip (Fig. 2C). Duodenal sections in the VT co-treated with the TEO group showed intact intestinal villi with a well-preserved epithelium and lamina propria (Fig. 2D).

3.4. Histomorphometrical study

No significant changes were detected in the muscularis layer between the four study groups. In contrast, there was a highly significant decrease in mean duodenal villus height (from villus tip to villus crypt junction) in the VT group (982.31 ± 31.61 μm) compared with control (1210.46 ± 11.87 μm). In addition, a highly significant increase in Brunner's gland depth of the duodenal crypts was observed in the VT group (854.13 ± 27.25 μm) compared to the control group (358.46 ± 25.10 μm) (Table 3). However, supplementation of VT with TEO significantly reversed the above changes in villus height and Brunner glands depth. The morphometric study showed increased villus height (1066 ± 11.89 μm) and decreased Brunner glands depth (390.46 ± 14.50 μm) in VT-TEO rabbits. The histological changes of the duodenal wall after VT and TEO treatment are consistent with the morphometric data.

3.5. Immunohistochemical

The detection and distribution of Ki67 immunoreactivity in duodenal sections in the different studied groups are shown in Fig. 3. The intestinal section in the control group (Fig. 3A) and in the TEO group (Fig. 3C) shows a medium positive reaction for Ki67 in the nuclei of the cells within the proliferating crypt compartment (52.9 %; and 53.5 % respectively). In contrast, the Lieberkühn crypt sections in the VT group (Fig. 3B) showed high positive reactions for Ki67 (80.93 %). In contrast, low Ki67 positivity was observed in duodenal sections of VT- TEO (16.7 %) treated rabbits (Fig. 3D).

Furthermore, this study focused on immunohistochemical detection of the expression levels of apoptotic effectors p53 and Bcl-2 (Figs. 4 and 5), together with the evaluation of apoptosis levels in duodenal tissues.

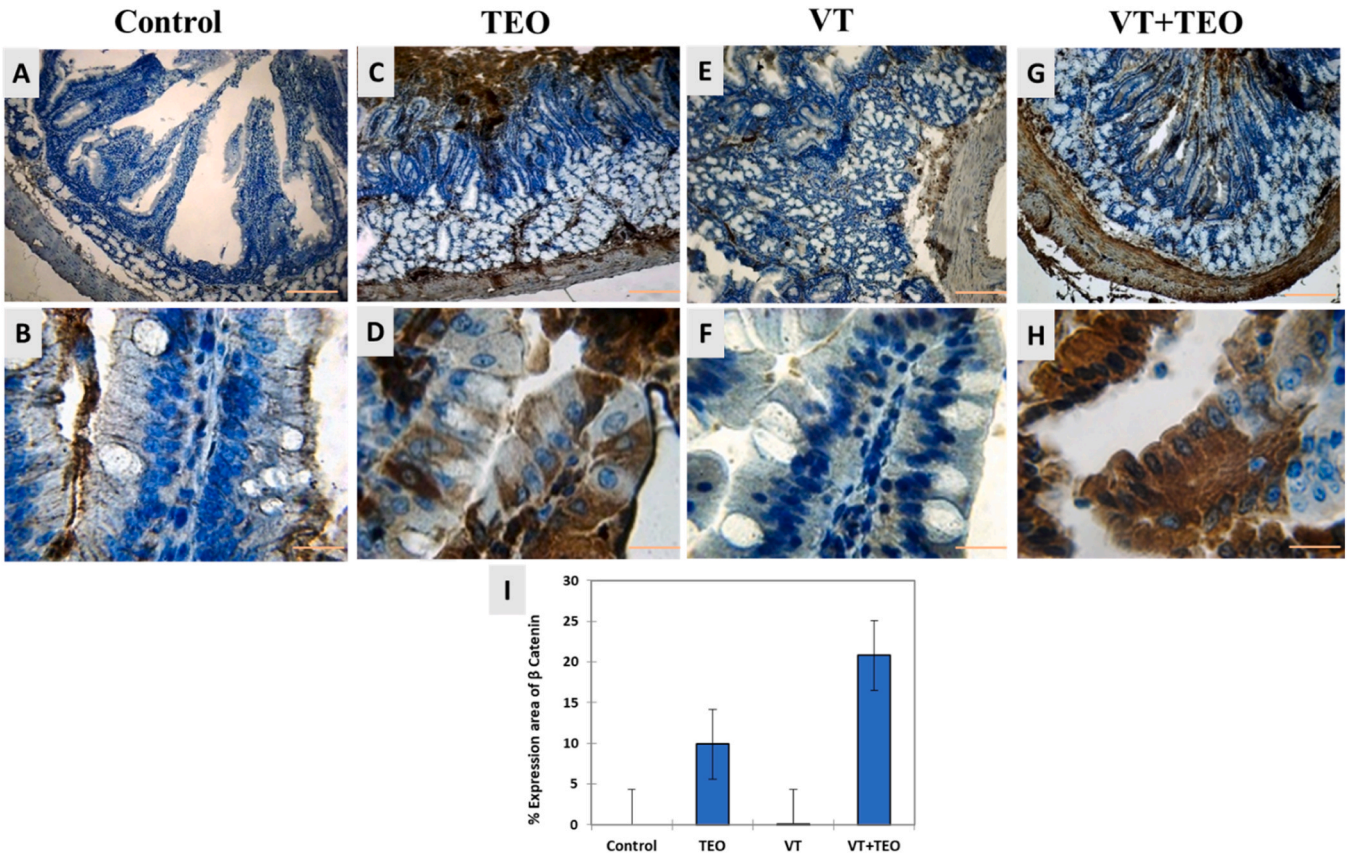

Fig. 6. Beta catenin (cell adhesion marker) expression in the control, TEO, VT and VT-TEO treated rabbits. A, B and E, F photomicrographs of beta catenin immunostaining (x 100 and x1000) showing no expression of beta catenin in the cytoplasm of the enterocytes in the **control (A B)** and **VT** groups **(E F)**. C, D and G, H photomicrographs of beta-catenin immunostaining (x 100 and x1000) showing strong immunoreactive expression of beta-catenin in the cytoplasm of the duodenum enterocytes in the terminal end of the villus, where they are sloughed off into the lumen and undergo anoikis in **TEO-group (C, D)** and **VT +TEO-group (G, H)** groups. The number of positive cells was quantified from sections immunohistochemically stained with Beta catenin. Statistically significant difference ($p < 0.05$). Bar = 50 μm.

The examination of p53 and Bcl-2 immunostained sections from the control group (Figs. 4A and 5A) showed that only slight immunopositive epithelial cells in the villus were expressed as brown cytoplasmic staining (1.24 % for p53; 2.35 % for Bcl-2). However, our results showed an increased expression of P53 and BCL2 in VT (Figs. 4C and 5C) intestinal villi (5.92 %; 9.91 % respectively), and decreased expression of p53 and Bcl-2 in control and TEO groups (0.63 %; 2.68 % respectively) (Figs. 4A, 4B, 4D and 5A, 5B, 5D).

Furthermore, TEO treatment significantly increased the expression of E-cadherin and β-catenin in duodenum cells (Figs. 6C, 6D, 7B and 6G, 6H, 7D). The duodenal epithelium of the TEO and VT-TEO groups of rabbits shows a positive and apparent increase in the number of b-catenin and e-cadherin cell-cell boundary membranous and cytoplasmic immunoreactivity, as well as in the inflammatory cells infiltrating the lamina propria (Figs. 6 and 7).

4. Discussion

The increasing use of pesticides in agriculture has led to rising concern over their detrimental effects on various organs, with the gastrointestinal system being one of the most affected. Pesticide-induced gastrointestinal damage, including inflammation and oxidative stress, has been documented in both humans and animals. In this context, plant-based essential oils, particularly those with antioxidant and anti-inflammatory properties, have gained attention for their potential to mitigate such toxic effects. This study investigated the subacute toxicity of VT, an insecticide formulation of chlorantraniliprole and abamectin, to male rabbits and the eventual protective role of TEO against VT-toxicity. Rabbits exhibit significant similarities to human gastrointestinal function, including structure, digestion, and immune defense mechanisms. These parallels make rabbits an valuable model for studying gastrointestinal diseases, the effects of dietary substances, and the toxicological impacts of various compounds, such as pesticides, providing insights that may be relevant to human health [32–34]. Although CAP is considered non-toxic to mammals by environmental safety guidelines, studies have shown its teratogenic and genotoxic effect in pregnant rats and their fetuses [12].Additionally, ABA has been linked to oxidative stress, inflammation, apoptosis, and autophagy and organ toxicity in animals [13,14,35,36].

Thymus vulgaris, a medicinal plant with multiple curative properties, presents a wide range of potential uses in conventional medicine, food additives, phytopharmaceutical preparations, and the cosmetic industry. This aromatic plant exhibits antioxidative, antimicrobial, antifungal, antispasmodic, antihypertensive, and calming effects [37], as well as anti-carcinogenesis and anti-inflammatory activities [38].

The present subacute toxicity study revealed a significant decrease in body weight gain in VT-treated group (5.4 %) as compared to the control group (11.7 %). The increase in body weight in the VT rabbits supplemented with TEO could be due to the effects of the essential oil which enhances the animal appetite [39]. Furthermore, TEO has been found to increase animal body weight in previous studies, which is in agreement with our findings [40].

T. vulgaris exhibits chemical polymorphism, with variations in its primary volatile components. While most plants and their essential oils show some variability, TEO is notably identified with 20 distinct chemotypes [41]. Numerous studies have evaluated its chemical

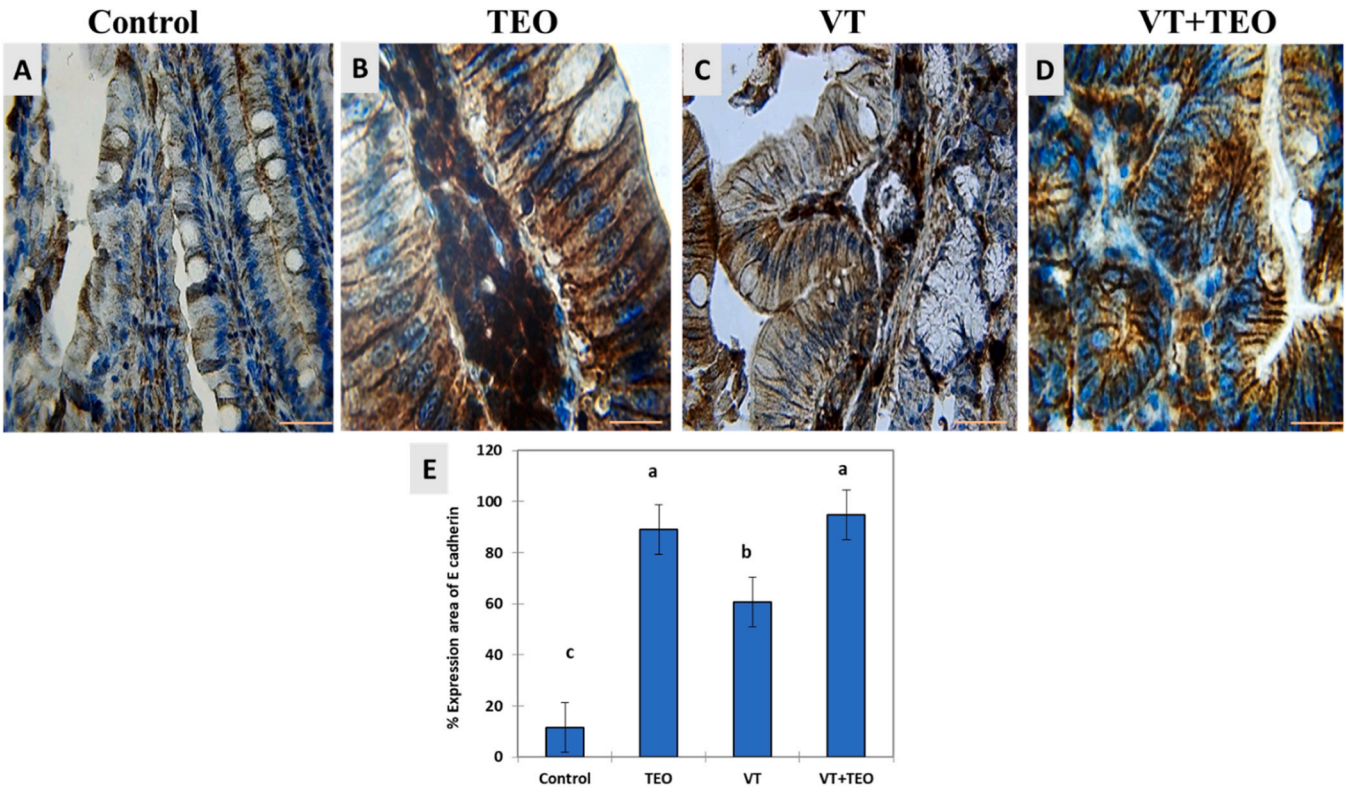

Fig. 7. E-cadherin (cell adhesion marker) expression in the control, TEO, VT and VT-TEO treated rabbits (Gx400). **A** photomicrograph of E-cadherin immunostaining showed moderate expression of E-cadherin in the cell-cell boundary membranous and cytoplasm of the enterocytes of the control rabbits. **B** and **D** Strong immunoreactive expression of E-cadherin in the cell-cell boundary membranous and cytoplasm of the duodenum enterocytes of the villus in TEO and VT-TEO groups. **C** moderate immunoreactive expression of E-cadherin in the membrane and cytoplasm of the duodenum enterocytes of VT-group. Statistically significant difference ($p < 0.05$). Bar = 50 μm.

composition, in particular thymol-dominant types. For instance, thymol was reported as the main constituent at 67.3 % [25], while other studies identified compositions such as thymol (48.1 %) and p-cymene (11.7 %) [42], or γ-terpinene (30.90 %) alongside thymol (47.59 %) [43].

According to the study's analysis of the chemical composition of T. vulgaris essential oil, carvacrol constituted 72.9 % of the total, indicating that the TEO under consideration is a member of the carvacrol chemotype. The chemical constitution of this oil was similar to those of previous studies, which identified carvacrol at 86.25 % [6], 59.29 % [28], and 45 % [44] as the major component of TEO. Carvacrol is a phenolic monoterpenoid that possesses a wide range of bioactivities putatively useful for clinical applications [38] such as antimicrobial, antioxidant, and anticancer activities [45].

The active proliferation of crypt-based stem cells located close to the base of the crypts is an indicator of the intestinal mucosa's continuous regeneration process [46]. Disorders like inflammatory bowel disease are linked to disruption of this balance [47]. The duodenum is the main site through which most food decomposition takes place [48], also, Brunner's glands neutralize chyme entering the duodenum to protect the mucous membrane and ensure the optimum pH for pancreatic enzyme action [49].

In this study, the VT insecticide disrupted the duodenal architecture in treated rabbits. Histology of duodenum of VT-treated rabbits, revealed many disorders in terms of decreased length of villi, increased Brunner gland depth, producing Brunner gland hyperplasia, infiltration of inflammatory cells, and necrotic villi tip. The hyperplastic Brunner's glands extend beyond the muscularis mucosae, infiltrating the mid-level mucosa, and distorting the overlying villous structure. Interestingly, TEO co-treatment reduces VT-induced histopathologic lesions, Brunner gland hyperplasia, and inflammatory responses in the duodenum.

In rabbits, Brunner's glands form a continuous submucosal layer that spans the entire duodenal surface, from the pylorus to the ligament of Treitz, with a length ranging from 20 cm to 25 cm [50]. These glands are classified into two histological types: serous and mucous, with the mucous type being the predominant, constituting more than 90 % of the glands in the proximal duodenum [46].

In human clinics, the term Brunner's gland hyperplasia (BGH) is often used synonymously with "adenoma" and "hamartoma" [51]. Although most cases of BGH are benign and asymptomatic [52], Brunner's gland a benign duodenal tumor, carries a very low risk of progression into adenocarcinoma, though it does possess malignant potential [53]. The exact pathogenesis of Brunner's gland hyperplasia remains unclear. It is hypothesized that in humans, the hyperplasia of these glands may occur as a compensatory response to increased acid production. Steinbach and Kane [54], indicated that the same histological lesions of hyperplasia defined as dilated ducts, intraductal papillary proliferation, and nodular expansion, are observed in sand rats and humans. Several studies have stated the potential role of pesticides in developing cancer in humans [55,56].In fact, pesticides are harmful to humans at high doses, which can lead to acute toxicity, and also at low doses, especially when the exposure is repeated [57,58]. Our study chose a dose of 15 mg/kg bw of VT to evaluate the subacute toxicity and potential gastrointestinal effect on rabbits. This decision was guided by established toxicological data on its active ingredients, chlorantraniliprole and abamectin, and the exploratory nature of this research. Regulatory studies indicate a NOEAL of chlorantraniliprole at 1000 mg/kg/day in male rats during 14-day oral toxicity, and 1188 mg/kg/day during 90-day oral toxicity, with a LOAEL identified at doses causing liver weight increases but no histopathological damage [59,60]. In contrast, abamectin exhibits a significantly lower NOAEL, around 0.5 mg/kg/day, and LOAEL 0.12 mg/kg /day. This value was consistently obtained in several studies, including acute neurotoxicity

studies in rats and subacute toxicity studies in dogs over periods of 18 weeks to one year [61]. The combination of these two active substances in VT necessitates examining their cumulative and potential synergistic effects, particularly at doses beyond their NOAELs but below thresholds associated with acute toxicity. Also, the selected dose of 15 mg/kg/day serves as a model to simulate repeated exposure scenarios relevant to long-term pesticide usage.

Intestinal homeostasis has to be highly controlled, therefore a strict balance between cell death and proliferation has to be maintained, in order to prevent the development of intestinal inflammation or tumor formation [62]. Immunohistochemical analysis of cell-specific antigens is one of the assays used to assess the proliferative activity of cells. For example, antibodies against Ki67 have been used as a prognostic factor to diagnose various types of neoplasms [63].

In the present study, the duodenal epithelium of control rabbits shows a positive Ki-67 immunoreactivity only in intestinal stem cells (ISCs) located at the base of the crypts compartment and low levels of Ki-67 in cells that have recently left this compartment and begun to colonize the villus base. Cells that have migrated to the top of the villus show a decrease in Ki-67 levels. Compared with the control and TEO groups, our results show intense expression of Ki-67 in the nuclei of Lieberkühn's crypt multipotent stem cells in the VT groups. However, low immunoreactive expression of Ki-67 was observed in nuclei of crypts Lieberkühn cells in the VT-TEO group, suggesting that Ki67-negative cells are more differentiated than Ki67-positive cells in the cell population [63]. A reduction in Ki-67 expression signifies a decline in cell proliferation, with lower levels of Ki-67 reflecting the exit from the cell cycle and onset of terminal differentiation.

Furthermore, this study focused on immunohistochemical detection of expression levels of apoptotic effectors Bcl-2 and p53, together with the evaluation of apoptosis levels in duodenal tissues. The examination of p53 and Bcl-2 immunostained sections from the control group showed that only a few immunopositive epithelial cells in the villus were expressed as brown cytoplasmic staining. Our results showed an increased expression of Bcl-2 and p53 in VT intestinal villi, and decreased expression of Bcl-2 and p53 in all other analyzed duodenal tissues.

Produced in the crypts, the cells migrate alongside the crypt-villus axis toward the tip of the villus, where they are shed into the lumen and undergo anionic, a form of apoptosis triggered when epithelial cells lose their attachment to the basement membrane [46].

Carvacrol, the primary component of the tested essential oil, has been shown to induce apoptosis and inhibit cell division, leading to cell death [64]. This suggests that carvacrol could be a promising natural compound for the management of colon cancer [65]. Luo et al.[66] revealed that Carvacrol treatment stops cell proliferation, migration, and invasion. Carvacrol exerted anticancer and anti-proliferative activity [67,68] and exhibited anti-proliferative and antioxidant effects and had therapeutic action in tumor cells without unsuitable effects in healthy cells [69].

Knoop et al. [70] supported that the intestinal epithelium plays a crucial role in isolating luminal antigens from the mucosal immune system. They also suggested that disruptions in the balance between proliferation and apoptosis can lead to immune activation and inflammation, a finding that aligns with our results.

Enterocytes are interconnected through the apical junctional complex which functions as a barrier, defining the apical membrane from the basolateral membrane. The adherent's junction proteins play a main role in maintaining epithelial cellular contact and controlling intracellular signaling pathways and transcriptional regulation [71,72]. Oxidative stress plays a key role in the most toxic effect of insecticides. Numerous studies revealed that oxidative stress induced by insecticides, like chlorantraniliprole [73]; and abamectin [74], can induce disruption of tight junctions and cause intestinal barrier dysfunction [75]. Likewise, TEO treatment significantly increased the expression of E-cadherin and β-catenin in duodenum cells. The duodenal epithelium of the TEO and VT-TEO groups of rabbits shows a positive and apparent increase in the number of b-catenin and e-cadherin cell-cell boundary membranous and cytoplasmic immune-reactivity, as well as inflammatory cells infiltrating the lamina propria. Therefore, in the present study, the co-administration of TEO significantly modulated the expression of the tested markers of proliferation, apoptosis, and cell-cell adhesion levels. This is probably due to the carvacrol chemotype of the tested essential oil.

5. Conclusion

This study provides critical insights into the subacute effect of VT insecticide on male rabbit duodenum, which induced Brunner's gland hyperplasia. Conversely, the carvacrol chemotype of the TEO essential oil could attenuate and ameliorate this effect. The protective effect of TEO was associated with inhibition of cell proliferation and induction of apoptosis in intestinal tissue. Finally, further studies should aim to test a wider range of doses, particularly closer to environmental exposure levels, to refine the relevance of these findings to human health.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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Hassina Khaldoun: Writing – original draft, Methodology, Conceptualization. **Amina Settar:** Writing – review & editing, Validation, Software, Methodology, Conceptualization. **Yasmine Oularbi:** Methodology. **Nouara Boudjema:** Funding acquisition. **Assia Amokrane:** Visualization. **Nacima Djennane:** Resources, Funding acquisition. **Dalila Tarzaali:** Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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