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Effects on Lettuce Yield Parameters and Toxicological Safety Assessment of a Plant-Derived Formulation Based on Rosemary and Eucalyptus Essential Oils

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Abstract: Essential oils from medicinal and aromatic plants are increasingly recognized as a promising class of green molecules for use in crop production. In many cases, the beneficial aspects of a substance are not supported by sufficient toxicological safety testing, even though recent reports suggest that some compounds may be toxic to terrestrial or aquatic non-target species. It is, therefore, essential to investigate the possibility of adverse effects on non-target animals and humans exposed to these substances through the consumption of fruit and/or vegetables. The present study aims to examine the potential effects on yield and quality parameters and investigate the level of in vitro and in vivo toxicity of an Eco-product (EP) based on rosemary and eucalyptus essential oils, to provide a measure for safe use in the agricultural sector. The product was evaluated in lettuce crop production and indicated that one-time application of the EP formula increases yield, activating various secondary metabolism pathways of the plant to cope with oxidative stress. Cytotoxicity assays and in vivo acute oral and dermal toxicity studies suggest that the tested compound does not pose any significant health hazard, and the dissolved product can be classified in Category 5, according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Keywords: medicinal and aromatic plant; biostimulant; oxidative stress; toxicological safety; cytotoxicity; acute oral and dermal toxicity

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1. Introduction

Food security is a major concern for humanity as, according to some population forecasts, the human population will reach 9.5 billion by 2050. The agricultural industry faces a significant problem in ensuring and maximizing crop output under a changing climate [1]. In intensive crop production, fertilizers, pesticides, and resources (soil, energy, water, etc.) are used intensively to increase crop yield, but food safety and quality issues are of great concern. For instance, the European Commission aspires to achieve a 20% reduction in fertilizer use by 2030, as laid out in the "Farm to Fork Strategy".

The quality attributes and storage of fresh produce (vegetables) are affected by multiple parameters, such as environmental (abiotic stresses such as temperature and light conditions), cultivation practices (pest management and harvesting) as well as post-harvest processing and storage conditions [2,3]. Usually, chlorine-based pesticides are used for sanitizing and extending the storage life of fresh produce. However, chlorine and its derivatives have been shown multiple times to exhibit carcinogenic properties in humans [4]. It was also shown that residues of many synthetic pesticides are toxic to wild animals, including

birds and bees, and negatively affect human health [5]. The health and environmental concerns arising from agrochemical use have led to an increase in research interest and the use of biostimulants in agricultural practice [6].

Currently, there is no universally accepted definition nor a scientific consensus on the term biostimulant for agricultural use (see du Jardin, 2015 [7]; Yakhin et al., 2017 [8] and references therein for a detailed review). A relatively recent development has been the inclusion of plant biostimulants in the new EU Fertilizing Products Regulation (FPR), which went into effect in July 2019 (EU 1009/2019) [9] (plant biostimulants are listed in the Product Function Category (PFC) 6). The regulation defines biostimulants as "a fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, (c) quality traits, or (d) availability of confined nutrients in the soil or rhizosphere". The regulation distinguishes plant biostimulants into two main categories: Microbial and non-microbial. Substances influencing plant growth other than as a nutrient or a biostimulant have to be evaluated for registration under the plant protection products Regulation (1107/2009), which requires detailed toxicological assessments.

In principle, biostimulants can be comprised of a wide range of chemicals and/or microorganisms [7,10,11]. We refer our readers to the detailed work edited by Rouphael and Colla (2020) [6] and references therein, covering various aspects of biostimulant use, as well as their impacts on crops. In the current work, we follow the definition of biostimulant as reported in the EU FPR Regulation (1009/2019).

Stimulation is achieved by physical, chemical, and biological factors and can be defined as the biological response triggered by some of these environmental factors that promote the metabolic processes of an organism leading to more efficient growth [12]. Multiple substances have been studied, developed, and produced. However, products containing essential oils (EOs) from medicinal and aromatic plants gained significant attention because they were found to exhibit antioxidant, anti-inflammatory, anti-fungal, and anti-bacterial properties [13]. EOs are commonly used in the food industry of meat products, plant-based products, and dairy products and have been shown to successfully improve food preservation, quality, and safety [13,14]. For example, EOs isolated from *Origanum dictamnus* (dittany) was found to reduce the growth of gray mold (*Botrytis cinerea*) on eggplant fruits while preserving the fruit's attributes [15]. In another study, sage EO (*Salvia officinalis*), in combination with other natural products, was used for the preservation of tomato fruits, and it was also found to reduce gray mold production and fruit decay [16].

Although substances with the potential for use as biostimulants are generally considered beneficial for plant health, their toxicological profile to mammals or other organisms is not commonly studied. For instance, EOs and/or their main components, such as thymol (garden thyme), menthol (mint), and 1,8-cineole (rosemary and eucalyptus), showed acute toxicity in rat animal models at certain concentrations and were categorized as moderately and slightly hazardous by the World Health Organization (WHO) [17]. Toxicity varies greatly between different EO components, with thymol having an acute lethal dose (LD50) of 980 mg kg^{-1} compared to 2480 $mgkg^{-1}$ for 1,8-cineole [17,18]. It is, therefore, important to support the beneficial effects of a product on plants with sufficient pre-clinical toxicological analysis.

Previous studies by our group demonstrated that an Eco-Product (EP formula—based on rosemary and eucalyptus essential oils) shows potential for use as a biostimulant and results in increased tomato fruit antioxidant activity and decreased damage index, signifying that the EP can be used for the preservation of fresh produce [19]. The EP formula was also studied for its effects on plant yield, quality, nutritional, physiological, and enzyme activity parameters on tomato plants, and it was found that the fruits treated with the EP formula had superior quality attributes compared to controls [20]. However, previous work has shown that some of the constituents of the essential oils comprising the EP formula (e.g., 1,8-cineole) exhibit toxicity and were categorized as moderately or

slightly hazardous [17]. This means that in addition to the positive outcomes of the EP formula for tomato plants, its toxicological safety should be assessed.

In fruit vegetables, such as tomatoes, human exposure to the field applications of products used to improve yield parameters via consumption is expected to be lower than for leafy vegetables, as the fruits receive a smaller amount of the sprayed product. In leafy vegetables, such as lettuce, exposure to the product via human consumption is expected to be higher as the whole plant is consumed. Some leafy vegetables are well known for their capacity to accumulate compounds in their biomass, including nitrates [21]. Therefore, the aim of this study was to: (i) examine the potential effects of the EP formula on lettuce yield parameters and (ii) investigate the level of in vitro and in vivo toxicity by using human cell lines and mice animal models to establish the basis for safe use of the EP formula.

2. Materials and Methods

2.1. Test Compound

An Eco-product (EP; named "Agriculture Green-tech E", Meydan Solution Ltd., Larnaca, Cyprus) based on rosemary (*Rosmarinus officinalis* L.) and eucalyptus (*Eucalyptus crabra* L.) essential oils (EOs), was evaluated in this study. The product was a mixture of two essential oils (eucalyptus: rosemary in approximately 2:1 v/v ratio), and it also contained vinegar < 5% w/w as well as emulsifier-treated water (<80%). Individual EOs were analyzed by gas chromatography-mass spectrometry (GC/MS-Shimadzu GC2010 gas chromatograph interfaced Shimadzu GC/MS QP2010 plus mass spectrometer), and *R. officinalis* was found rich in isoborneol (30.29%), α pinene (25.71%), α terpineol (14.89%) and 1,8-cineole (10.81%), while the dominant compounds of the essential oils from *E. crabra* were 1,8-cineole (26.51%), α pinene (24.12%) and δ-3 carene (20.10%) [19].

2.2. Lettuce Cultivation

The lettuce cultivation study took place at the experimental farm of the Cyprus University of Technology, Limassol, Cyprus, during the autumn of 2019. For the evaluation of the EP, the commercial product (CP) Razormin (Atlántica Agrícola, Alicante, Spain) was used as a reference (positive control) in the recommended concentration of 2.5 mL L^{-1}. This product is based on amino acids (7% w/w), while it contains small amounts of micro-nutrients such as iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), and molybdenum (Mo).

Prior to the main study, a preliminary test was conducted to determine the concentration of the EP that would be applied to the lettuce plants to avoid phytotoxicity. The concentration of 2.0% was selected, as it was the highest concentration, not causing any damage to lettuce leaves.

Lettuce (*Lactuca sativa* cv. Nogal) seedlings were purchased from a commercial nursery and were transplanted in pots (5 L) with soil. The soil used was analyzed and was found to have a clay-loam texture, 1.41% organic matter, total $CaCO_3$ at 24.28%, and total nitrogen (N) at 0.40 g kg^{-1}. The pH was measured at 7.71, while the electrical conductivity (EC) was at 0.68 mS cm^{-1}.

The experimental setup consisted of four treatments: (a) water-sprayed plants used as control, (b) CP-sprayed plants, (c) EP-sprayed plants (one application, 1x), and (d) EP-sprayed plants (two applications, 2x). The first spraying took place 10 days after transplanting and the second 12 days after the first application. Each treatment was replicated in six plots, and each plot had 4–5 plants in a completely randomized design. In total, 108 lettuce plants were grown in 4 weeks.

2.3. Plant Physiology and Growth Parameters

A series of plant physiology parameters were assessed right before harvesting the lettuce plants; leaf stomatal conductance (ΔT-Porometer AP4, Delta-T Devices-Cambridge, UK) and chlorophyll fluorescence (OptiSci OS-30p Chlorophyll Fluorometer, CID Inc., Camas, WA, USA) were measured while the concentration of leaf total chlorophylls, a and

b were calculated as previously described [22]. As for the growth parameters, leaf length, leaf number, and plant fresh and dry biomass were assessed in six replicates per treatment.

2.4. Total Phenolics Content and Antioxidant Activity

Fresh lettuce tissue from four replicates per treatment was sampled, and methanolic (50% *v*/*v*) extraction was conducted for the total phenolic content and the estimation of the antioxidant activity. The total phenolic content was measured using the Folin–Ciocalteu method [23]. The extract was additionally used for the determination of the antioxidant activity, of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the ferric-reducing antioxidant power (FRAP) as previously described [24]. The results were expressed as trolox equivalents (mg of trolox g^{-1} fresh weight).

2.5. Hydrogen Peroxide Content, Lipid Peroxidation, and Antioxidant Enzymes Activity

Frozen leaf tissue (~0.2 g) was homogenized with 3 mL of ice-cold 0.1% trichloroacetic acid (TCA), and samples were used for the determination of the hydrogen peroxide content and the lipid peroxidation in terms of malondialdehyde (MDA) content [24]. The enzyme antioxidant activity of superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), and peroxidase (POD) (EC 1.11.1.6) were assayed as previously described [22]. Results were expressed as units of enzyme per mg of protein. The protein content in the enzyme extract was determined using bovine serum albumin (BSA) as a standard. Four replicates were analyzed per treatment.

2.6. Plant Nutrient Content

The nutrient content was determined in dried lettuce tissue. The nitrogen (N) content was determined by the Kjeldahl method (BUCHI, Digest automat K-439, and Distillation Kjelflex K-360, Switzerland). Potassium (K) and sodium (Na) were determined photometrically (Flame photometer, Lasany Model 1832, Lasany International, India), phosphorus (P) was determined spectrophotometrically (Multiskan GO, Thermo Fischer Scientific, Waltham, MA, USA), and magnesium (Mg) and calcium (Ca) by an atomic absorption spectrophotometer (PG Instruments AA500FG, Leicestershire, UK). Data were expressed in g kg^{-1} of dry weight [20].

2.7. Fresh Produce Quality

Harvested lettuce plants were evaluated for their quality at the experiment completion. Six biological replicates were used from each treatment. The color was measured using a Minolta colorimeter model CR400 (Konica Minolta, Osaka, Japan), total soluble solids (TSS in °Brix), titratable acidity (TA in malic acid g kg^{-1}) and ascorbic acid (AA in mg 100 g^{-1} Fw) content were also determined as previously described [14]. Fresh produce marketability, aroma, and appearance were recorded by using a 1–10 scale (1: not marketable quality, i.e., malformation, wounds, infection); 3: low marketable with malformation; 5: marketable with few defects, i.e., small size, decolorization (medium quality); 8: marketable (good quality); 10: marketable with no defects (extra quality)) and results were expressed as a percentage.

2.8. Cytotoxicity Assay

Cell viability and the proliferative effect of the Eco-product were assessed in vitro by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA). The following cell lines, human embryonic kidney cells (HEK-293; ATCC® CRL-1573, ATCC, Manassas, VA, USA) and human umbilical cord-derived mesenchymal stem cells (hUC-MSCs; PCS-500-010™, ATCC, Manassas, VA, USA), were used to set up proliferation assays (1×10^4 cells per well were distributed into 96-well microplates) for the determination of cytotoxic properties of the Eco-product. Serial dilutions (10-fold) of the formula (stock solution; dilution range 10^{-1}–10^{-6}) in PBS were used for sample preparation, and DMSO was used as a negative control. Sample

compounds and controls were added to cells as triplicates and incubated for 2 and 6 days (HEK-293, repeated three times) and for 2, 4, and 7 days (MSC, repeated twice) (at 37 °C, 5% CO_2) under serum-free conditions. Incubation time was kept constant throughout the experiments. The absorbance was measured spectrophotometrically at a wavelength of 570 nm. The IC_{50} (inhibition of cell growth by 50%) was estimated by linear interpolation as compared to the drug-free controls using the GraphPad PRISM software.

2.9. Animals

All the procedures involving animals were approved by the Cyprus Veterinary Services (project license no. CY/EXP/PR.L2/2019) and were conducted in conformity with European and International guidelines (Directive 2010/63/EU of the European Parliament; National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals). Specific pathogen-free CD1 mice weighing 39 ± 3 g (8–10 weeks old) were used. All experimental animals were specifically bred for research and were housed in a controlled environment with constant temperature (22 ± 1 °C), relative humidity (60 ± 10%), and a 12 h light/dark cycle.

2.10. Acute Oral Toxicity

Systemic tolerance following oral administration of the EP formula was investigated in male and female CD1 mice, as per the OECD TG 407 [25], with slight modifications. Thirty-two mice (16 males and 16 females) were randomly selected and divided into four groups of 8 mice/group of equal sex ratio. Given the lack of previous data, a wide dosage range was selected. Three test groups were given doses of the test substance at 500, 1500, and 2500 mg kg^{-1} of body weight per day (b.w./day), introduced at concentrations of 0.5, 1.5, and 2.5% v/v in the 250 mL drinking water supply, and an additional control group received vehicle (free drinking water). Water bottles were shaken daily to ensure the stability of the test substance in the vehicle.

The animals were closely monitored for the 5-week duration of the study for clinical signs of toxicity and discomfort/pain. Body weight, food consumption, heart rate, breath rate, and arterial oxygen saturation (SpO_2) were recorded on a weekly basis. Hematological and biochemical tests and excised organ weight measurements (liver and kidneys) were also performed at the end of the observation period.

2.11. Acute Dermal Toxicity

Dermal toxicity was evaluated following the OECD TG 402 [26]. Female CD1 mice (nulliparous and non-pregnant) were exposed to the test chemical in a stepwise procedure using appropriate fixed doses. A dose-range finding study using single animals in a sequential manner at a starting dose of 200 mg kg^{-1} b.w. (concentrated formula) was performed, followed by the main study conducted with two further animals per dose (1000 and 2000 mg kg^{-1} b.w.; concentrated formula) to confirm the classification outcome. One day prior to dosing, all fur was removed from the dorsal/flank area of the test animals (at least 10% of the total body surface area). Care was taken to avoid abrading the skin, which could alter its permeability. The test chemical was uniformly applied over the exposed area of skin, and the substance was held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-h exposure period. Subsequently, the patches were detached, and the remaining product on the application site was removed by washing the area. Animals were observed immediately after dosing, periodically during the first 24 h, and daily thereafter for a total of 14 days. Observations included evidence of irritation at the application site, such as erythema, edema, atonia, desquamation, necrosis, coriaceousness, fissuring using the Draize criteria, and also somatomotor activity and behavior pattern.

2.12. Statistical Analysis

The statistical analysis for the lettuce experiments was performed using IBM SPSS version 22, comparing data means (±standard error (SE)) with one-way analysis of variance

(ANOVA), and Duncan's multiple range tests were calculated for the significant data at $p < 0.05$. Measurements were performed in four-to-six biological replications/treatments. The statistical analysis of the data of body weight and organ weight was carried out using Student's *t* test. Data for biochemical and hematological parameters were analyzed by ANOVA. $p < 0.05$ was considered statistically significant. The IC_{50} was estimated by linear interpolation, in comparison with drug-free controls, using the GraphPad PRISM software.

3. Results

3.1. Plant Growth and Physiological Parameters

Lettuce plants sprayed either with water (control), or CP had higher leaf lengths compared to plants treated with the EP-2x (Table 1). Fresh plant biomass was stimulated and appeared to increase (up to 26.2%) after the EP-1 application, compared to all other treatments and the control that had similar fresh biomass (average 113.42 g). However, the high dose of EP-2x did not cause any deleterious effects. Foliar application of CP or EP did not affect leaf number produced (average 21 leaves) and plant dried weight (average 7.41 g per plant). Chlorophyll content increased in CP compared to EP-1x, while no differences were found in chlorophylls for CP and control or EP-2x. Leaf stomatal conductance increased in EP-2x compared to the control and CP treatments.

Table 1. Yield, plant growth, and physiological characteristics of lettuce plants in relation to foliar application of different products.

Plant Growth	Control	CP	EP-1x	EP-2x
Leaf length (cm)	27.75 ± 0.64a	27.83 ± 0.38a	26.92 ± 0.47ab	25.83 ± 0.46b
Leaf number	21.00 ± 1.18a	20.83 ± 1.01a	22.67 ± 0.61a	21.00 ± 0.37a
Plant biomass (g)	116.44 ± 9.17b	111.29 ± 7.41b	140.42 ± 6.91a	112.55 ± 6.83b
Plant dry weight (g)	7.16 ± 0.55a	7.38 ± 0.71a	7.76 ± 0.37a	7.32 ± 0.35a
Stomatal conductance (s cm^{-1})	1.22 ± 0.04c	1.34 ± 0.05bc	1.41 ± 0.04ab	1.50 ± 0.07a
Chlorophyll fluorescence (Fv/Fm)	0.812 ± 0.004a	0.813 ± 0.002a	0.803 ± 0.007a	0.812 ± 0.004a
Chlorophyll a (mg g^{-1} Fw)	0.92 ± 0.03ab	0.97 ± 0.02a	0.86 ± 0.05b	0.91 ± 0.02ab
Chlorophyll b (mg g^{-1} Fw)	0.26 ± 0.01ab	0.27 ± 0.01a	0.24 ± 0.01b	0.25 ± 0.01ab
Total Chlorophylls (mg g^{-1} Fw)	1.17 ± 0.04ab	1.24 ± 0.03a	1.10 ± 0.06b	1.16 ± 0.02ab

Control: indicates foliar application with water; CP: indicates the commercial product; EP: indicates the eco-product; 1x: indicates one-time application; 2x: indicates two-times application. Means ± SE in the same row followed by different Latin letters is significantly different according to Duncan's MRT ($p = 0.05$).

3.2. Total Phenolic Content and Antioxidant Activity

Total phenols content and antioxidant activity assayed by DPPH and FRAP were decreased in CP and EP-1x compared to the control and EP-2x treatments (Table 2).

3.3. Damage Index and Antioxidant Enzyme Activity

Lipid peroxidation, as assayed by MDA, was increased in all treatments, compared to the control (Table 2), and H_2O_2 levels were increased in all EP-treated lettuce, with maximum levels at the EP-1x treatment, compared to control and CP-treated plants. SOD activity was increased in CP- and EP-2x -treated plants compared to the water-treated plants. POD activity increased in CP and EP-2x but decreased in EP-1x-treated plants compared to the control treatment. CAT activity increased after the CP application.

3.4. Nutrient Content

The nutrient content in lettuce leaves is presented in Table 2. The content of N and K remained unchanged following foliar applications and averaged 29.35 mg kg^{-1} and 42.01 mg kg^{-1} of dry weight, respectively. Phosphorus levels were found to be significantly

higher after the CP application compared to the EP-1x application. Sodium content was increased in plants after EP-1x application compared to control, CP, and EP-2x treatments. No differences were found for Ca and Mg content in lettuce among the treatments (Table 2).

Table 2. Total phenols content, antioxidant activity (DPPH, FRAP), Damage index (lipid peroxidation-MDA, hydrogen peroxide-H_2O_2), antioxidant enzymes activity (superoxide dismutase-SOD, catalase-CAT, peroxidase-POD) and nutrient content of lettuce plants in relation to foliar application of different products.

	Control	CP	EP-1x	EP-2x
Total phenols (μmol GAE g^{-1} Fw)	1.69 ± 0.06a	1.14 ± 0.11b	1.05 ± 0.04b	1.49 ± 0.12a
DPPH (mg Trolox g^{-1} Fw)	2.55 ± 0.12a	1.78 ± 0.12b	1.86 ± 0.10b	2.45 ± 0.21a
FRAP (mg Trolox g^{-1} Fw)	1.86 ± 0.11a	1.04 ± 0.12b	1.08 ± 0.05b	1.68 ± 0.12a
MDA (nmol g^{-1})	10.91 ± 0.37b	12.30 ± 0.37a	12.43 ± 0.63a	12.38 ± 0.45a
H_2O_2 content (μmol g^{-1})	0.12 ± 0.00c	0.11 ± 0.01c	0.29 ± 0.01a	0.25 ± 0.00b
CAT (unit mg $protein^{-1}$)	30.85 ± 3.71b	37.85 ± 0.78a	29.43 ± 1.37b	29.44 ± 2.89b
SOD (unit mg $protein^{-1}$)	3.19 ± 0.2c	4.52 ± 0.19a	3.77 ± 0.05bc	3.89 ± 0.48ab
POD (unit mg $protein^{-1}$)	0.67 ± 0.01c	1.03 ± 0.01a	0.60 ± 0.02d	0.87 ± 0.02b
N (g kg^{-1} Dw)	29.58 ± 1.41a	28.18 ± 2.44a	32.23 ± 0.88a	27.42 ± 1.46a
K (g kg^{-1} Dw)	42.04 ± 3.54a	42.99 ± 2.38a	40.55 ± 2.84a	42.35 ± 1.08a
P (g kg^{-1} Dw)	3.75 ± 0.06ab	4.08 ± 0.14a	3.60 ± 0.11b	3.70 ± 0.16ab
Ca (g kg^{-1} Dw)	8.66 ± 0.48a	8.23 ± 0.61a	10.75 ± 0.30a	8.44 ± 0.56a
Mg (g kg^{-1} Dw)	6.88 ± 0.14a	6.71 ± 0.31a	6.65 ± 0.11a	6.88 ± 0.14a
Na (g kg^{-1} Dw)	8.66 ± 0.48b	8.23 ± 0.61b	10.75 ± 0.30a	8.44 ± 0.56b

Control: indicates foliar application with water; CP: indicates the commercial product; EP: indicates the eco-product; 1x: indicates one-time application; 2x: indicates two-times applications. Means ± SE followed by different Latin letters are significantly different according to Duncan's MRT (p = 0.05).

3.5. Fresh Produce Quality

Lettuce quality-related attributes in plants subjected to CP or EP foliar applications are presented in Table 3. Ascorbic acid content was decreased with CP, the EP-1x, and EP-2x applications compared to the control treatment. Acidity was found to be increased in EP-1x compared to the CP and EP-2x applications. Lettuce color was affected by the foliar applications, as color L^*, b^*, and chroma parameters increased in CP compared to control and EP applications. The opposite was evidenced for color a^*, as CP-treated plants revealed the lowest values. The hue value decreased, but the color index increased with CP applications compared to the control and EP-1x applications. The CP and EP-2x applications decreased lettuce marketability and appearance compared to the control, while EP-1x performed similarly to the control.

Table 3. Quality-related attributes from lettuce plants in relation to foliar application of different products.

Quality Attributes	Control	CP	EP-1x	EP-2x
TSS (°Brix)	5.51 ± 0.10b	6.16 ± 0.40a	6.40 ± 0.05a	6.30 ± 0.06a
TA (malic acid g kg^{-1})	0.50 ± 0.002ab	0.39 ± 0.062b	0.58 ± 0.031a	0.38 ± 0.033b
AA (mg 100 g^{-1} Fw)	6.33 ± 0.17a	5.35 ± 0.46b	4.50 ± 0.47b	4.84 ± 0.11b
Colour L^*	41.14 ± 0.77b	45.22 ± 0.59a	42.72 ± 0.36b	42.85 ± 0.74b
Colour a^*	−16.52 ± 0.45a	−18.46 ± 0.30b	−16.94 ± 0.12a	−17.00 ± 0.21a
Colour b^*	23.39 ± 1.04b	27.92 ± 0.95a	23.88 ± 0.36b	25.04 ± 0.38b

Table 3. *Cont.*

Quality Attributes	Control	CP	EP-1x	EP-2x
Hue	125.33 ± 0.55a	123.54 ± 0.51b	125.35 ± 0.26a	124.17 ± 0.17ab
Chroma	28.64 ± 1.10b	33.48 ± 0.95a	29.28 ± 0.35b	30.26 ± 0.43b
Colour index	−17.3 ± 0.67c	−14.7 ± 0.45a	−16.62 ± 0.21bc	−15.88 ± 0.34ab
Marketability (1–10)	9.17 ± 0.22a	7.22 ± 0.14c	8.83 ± 0.14a	8.11 ± 0.14b
Appearance (1–10)	8.89 ± 0.21a	7.00 ± 0.24c	8.61 ± 0.13ab	8.28 ± 0.06b

Control: indicates foliar application with water; CP: indicates the commercial product; EP: indicates the eco-product; 1x: indicates one-time application; 2x: indicates two-times applications. Means ± SE in the same row followed by different Latin letters is significantly different according to Duncan's MRT (p = 0.05).

3.6. In Vitro Cytotoxicity Assay

The toxic/non-proliferative effect of the product was evaluated in vitro using two different "healthy/normal" cell lines. The Eco-product showed a proliferative effect at the range of 10^{-3} dilution to 10^{-6} dilution in the HEK-293 cell line without any adverse cytotoxic effect (Figure 1a). The IC50 value was found to be approximately 0, indicating that the product does not cause cytotoxicity at these concentrations (Figure 1c). However, in the MSC cell line, the compound showed increased toxicity at the range of 10^{-2}–10^{-4}, which was rescued with the decline of the product concentration (improved cell viability between 10^{-5}–10^{-7} dilution (Figure 1b). As shown in Figure 1d, the IC50 value for the tested product was found at the concentration of 10^{-5}. This indicates that cell viability in HEK-293 was not interrupted with the addition of the EP; however, when hu-MSC cells were exposed to the formula, viability declined with the increase in the concentration.

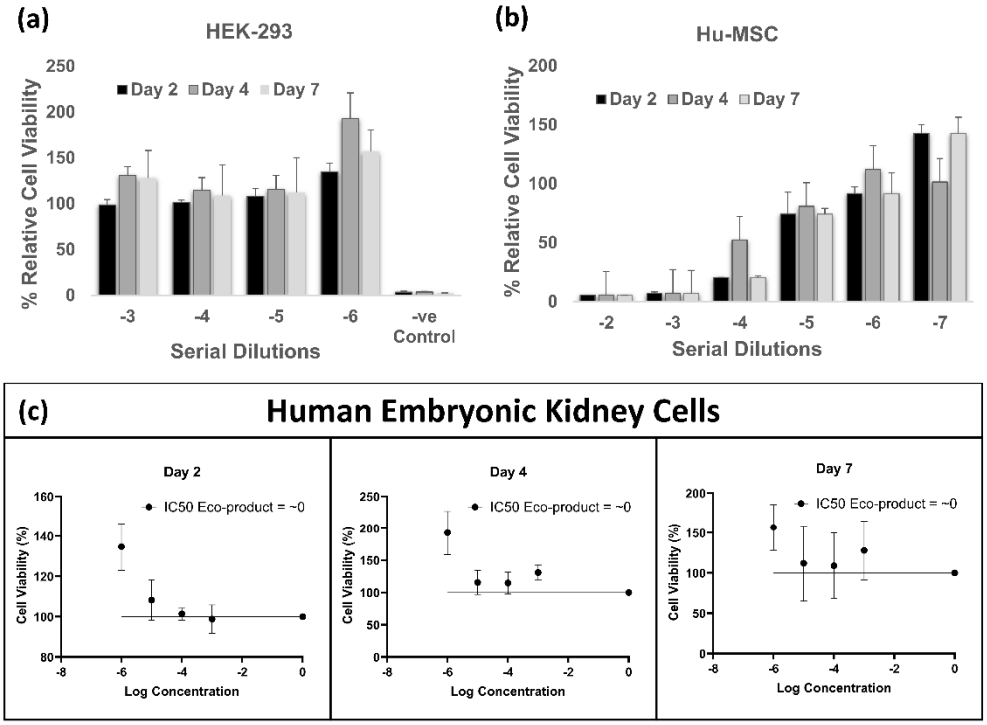

Figure 1. *Cont.*

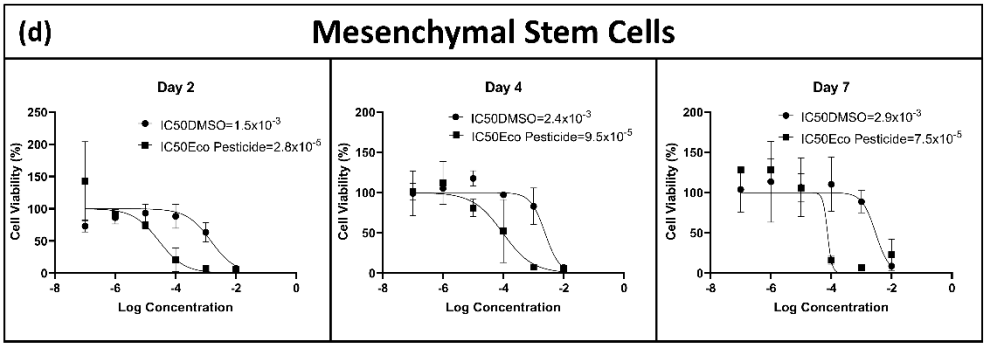

Figure 1. Cell cytotoxicity assessment and dose-response curve. (**a**) Assessment of cell viability in HEK-293 cell line (normalized data). The Eco-product shows a proliferative effect at the range of 10^{-3} dilution to 10^{-6} dilution (incubation period 2, 4, and 7 days) and a non-toxic profile. (**b**) Assessment of cell viability in a hu-MSCs cell line (normalized data). The Eco-product shows reduced cell viability between dilution range 10^{-2}–10^{-5} and a rescued proliferative effect at the range of 10^{-6}–10^{-7} dilution (incubation period 2, 4, and 7 days). Results are expressed as mean ± standard deviation (SD), and statistical significance was considered when $p < 0.05$. (**c**) Dose-response curve of HEK-293 treated with EP for 2, 4, and 7 days. The concentrations range between 10^{-3} and 10^{-6}, and (**d**) Dose-response curve of hu-MSC cells with concentrations ranging between 10^{-2} and 10^{-7}. Results are expressed in percentage of control, and they show the mean of IC50 values ± SD (concentration required to inhibit cell growth by 50%). Data represent the means of two independent experiments, with each concentration tested in triplicate.

3.7. Acute Oral Toxicity

Oral feeding of the EP formula at various doses did not cause any mortality, or gross behavioral changes, either immediately or during the 5-week observation period. All reflexes, food intake, and body weights (with triplicate measurement) were normal and similar to that of the vehicle-treated group (see Figure 2a). Vital signs were recorded using the MouseOx® Plus (Starr Life Sciences Corp., Oakmont, PA, USA), which is a non-invasive pulse oximeter providing real-time and continuous measurements via a thigh sensor. Heart rate, breath rate, and arterial oxygen saturation (SpO_2) values (with triplicate measurement) from all tested groups were within the physiological range [27,28] and comparable to that of the control group (Figure 2b–d). Blood collection was performed via retro-orbital bleeding in EDTA-coated tubes, and the examination showed no statistically significant changes between treated and control groups in hematological and biochemical parameters (Figure 3a–h) and post-mortem organ weight measurements (Figure 3i).

3.8. Acute Dermal toxicity

After dermal application of the test compound to female CD1 mice, no mortality, deleterious effects, or gross behavioral changes were observed, either immediately or during the 14-day observation period. The treatment areas were individually evaluated and characterized according to the Draize dermal irritation scoring system [29] and the Primary Irritation Index (PII) [30].

Signs of erythema and edema were observed at 24, 48, and 72 h after administration of the high dose of 2000 mg kg^{-1} b.w. However, the overall incidence and the severity of irritation decreased with time and were fully reversible within 7 days (see Figure 4). The calculated PII was 1.33, which classifies the test compound as slightly irritating to the skin. Necropsies did not reveal any gross abnormalities in the organ structure, and the relative organ weight of the test groups was also comparable to that of the control group.

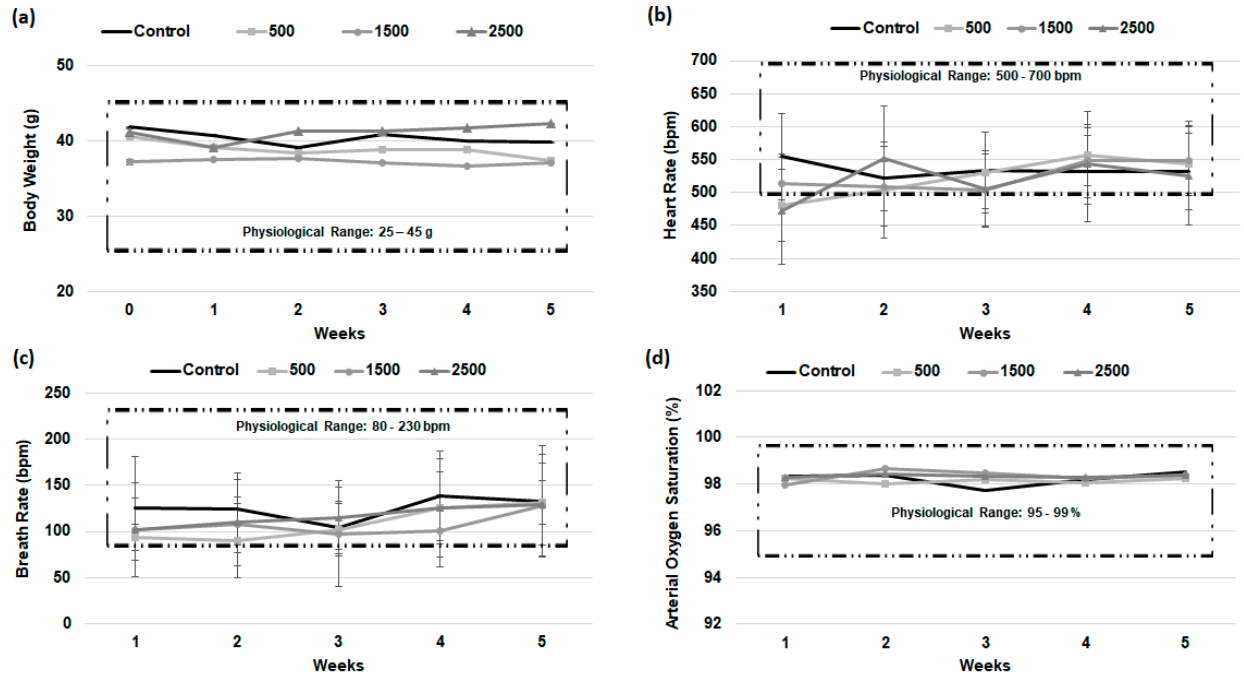

Figure 2. Effect on mouse body weight (**a**) and vital signs (**b**–**d**) after oral administration of the test compound at various doses. All values were within the physiological range and comparable to that of the control group. No statistically significant differences were observed between the two genders. Results are shown as mean ± SD.

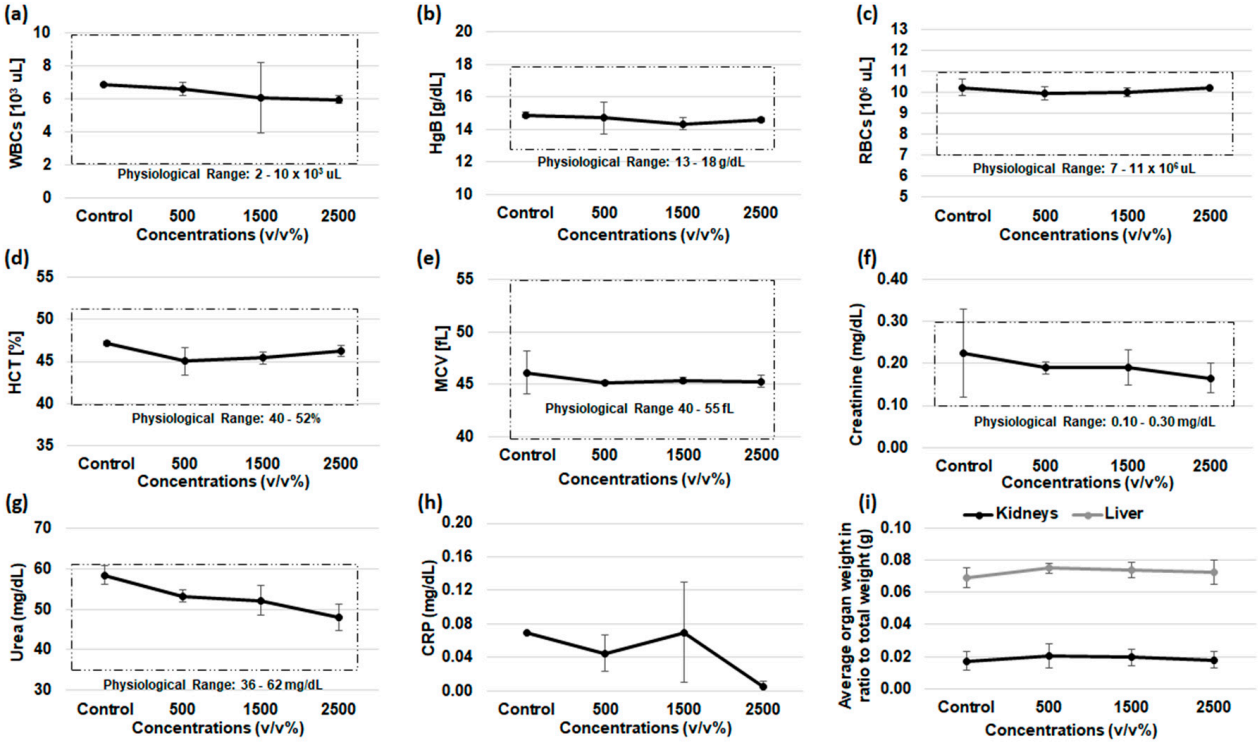

Figure 3. Effect of the test compound on various hematological (**a**–**e**) and biochemical (**f**–**h**) parameters and organ weight (**i**) of mice. All values were within the physiological range and comparable to that of the control group. No statistically significant differences were observed between the two genders. Results are shown as mean ± SD.

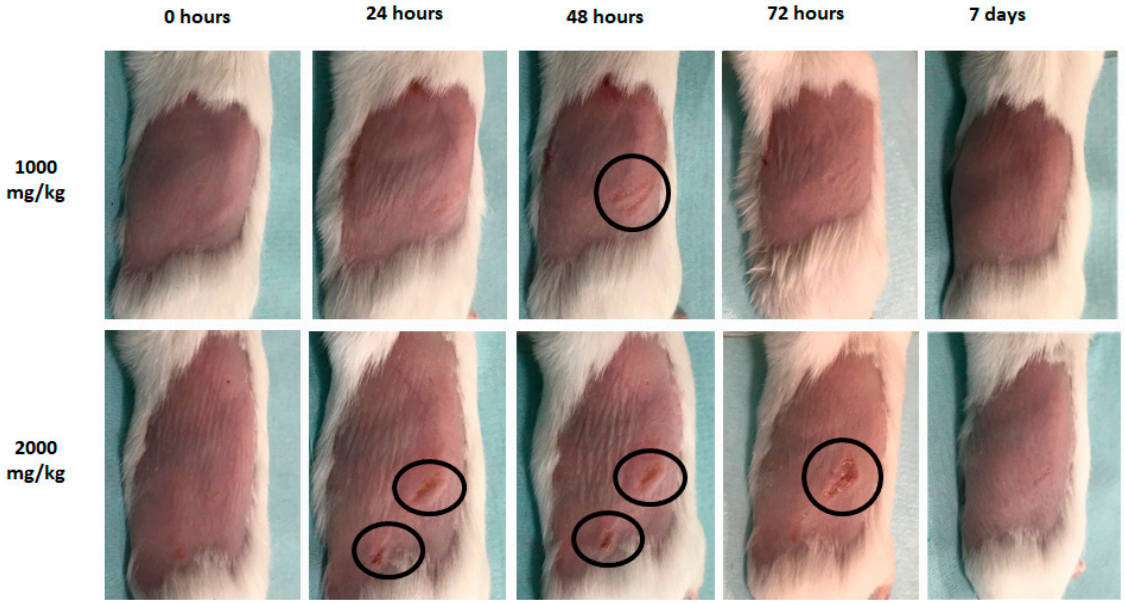

Figure 4. Representative images of mouse skin (of the n number of animals tested) before (t = 0) and at 24, 48, and 72 h and 7 days after dermal application of the test compound.

4. Discussion

There is currently an increased demand for sustainable food with a decrease in the use of synthetic chemicals and an increase in the use of biologicals. Among the practices employed to increase productivity is the use of substances promoting plant growth via various mechanisms. These substances are widely used in various species of plant crops, with different routes and sites of application [31]. The market size of such products was valued at USD 3.2 billion in 2021, and it is projected to grow at a Compound Annual Growth Rate (CAGR) of 12.1% to reach USD 5.6 billion by 2026 [7,32].

The EU included plant biostimulants in the new Fertilizing Products Regulation (FPR—1009/2019) that went into effect in July 2019 [9]. In brief, a substance can be considered a biostimulant if it stimulates plant nutrition independent of its nutrient content leading to better: (a) nutrient use efficiency, (b) tolerance to abiotic stress, or (c) quality traits, (d) availability of confined nutrients in the soil or rhizosphere. The EP Formula in the current study could potentially fall under the biostimulant category if the higher yield in EP-1X (Table 2) resulted from higher nutrient use efficiency. An additional option that requires further work is to investigate whether the activation of enzymatic and non-enzymatic pathways by the EP increases tolerance to abiotic stress conditions. The effects of EP on the quality of lettuce are less clear as application improved, left unchanged, or deteriorated quality attributes (Table 3). The results of the current work provide a baseline for further, more detailed studies that can provide the information required for the evaluation of the product as a biostimulant at the EU level. Biostimulants are specifically excluded from registration as a plant protection product following the provisions in EU Regulation 1107/2009 (Placing of Plant Protection Products on the Market), as modified by the FPR—1009/2019. Yet, if the EP formula does not meet the biostimulant classification, it might have to be evaluated for registration under the plant protection products Regulation (1107/2009) as a substance influencing plant growth other than as a nutrient or a biostimulant, which requires detailed toxicological assessments. There is no biostimulant category under the current legislative framework in Cyprus (Fertilizer Regulations of 2006), therefore, the EP-Product can only be registered as a "Special Formulation" based on the increase in yield—if approved.

The extensive use of products intended to improve plant growth and quality has underestimated the risk of causing environmental harm. Despite the benefits of biostimulants for the agricultural sector and society, studies on the systemic and cellular toxicity of commercially available products are scarce in the literature. Recent reports have shown

that substances in products intended to improve plant growth/quality traits may be toxic to terrestrial or aquatic non-target species [33,34]. It is, therefore, essential to investigate the possibility of adverse effects on non-target animals and humans exposed to these substances through the consumption of fruit and/or vegetables.

The present study investigated the effects on lettuce and the level of in vitro and in vivo toxicity of an Eco-product based on rosemary and eucalyptus essential oils to provide a measure for safe use in the agricultural sector. Experiments with high EP levels (EP-2x) revealed increased antioxidant capacity in lettuce which can be the response of the plant to the increased stress conditions, indicated by the higher damage indexes (MDA and H_2O_2 levels). In that sense, the plant was activating several enzymatic (SOD, POD) and non-enzymatic (phenols) antioxidant tools to defend against oxidative stress [24]. Interestingly, in the lettuce plant, both CP and EP-1x resulted in lower oxidative stress, activating only the enzymatic antioxidant activity and not the non-enzymatic (by having the lower antioxidant capacity and phenolic content). However, when the EP formula was applied to the tomato crop (one time or three times), lower stress conditions were found with decreased MDA, phenols, and antioxidant activity levels [20], highlighting the different responses of fruity and leafy vegetables. This contradicts the findings of other studies with postharvest applications, where EOs based on rosemary and eucalyptus (similar to the EP formula) increased fruit antioxidants, reduced damage index, and maintained fruit quality [19]. Fruit detached from the plant have to activate their antioxidant system to respond to the oxidative challenges, whereas fruit attached to the plant always have the interconnection of their antioxidant responses. Therefore, a balanced EP formula concentration should be considered to achieve the best possible outcomes in terms of plant quality while avoiding any toxic effects on humans and animals.

In that respect, the cytotoxicity studies did not show any signs of toxicity in the HEK-293 cell line and indicated low toxicity in the hu-MSC cell after the addition of the EP formula. The toxicity to the hu-MSCs was evident only at high concentrations of the EP (10^{-2}–10^{-4}), and a positive biological response was found at 10^{-5}–10^{-7}. Subsequently, this resulted in the inhibition of MSC growth at IC50 = 10^{-5} of the EP, which indicates that some of the constituents of the EP formula can cause cytotoxicity, but not all cell types react negatively. Nevertheless, a more in-depth evaluation is required to isolate and characterize each of the EP formula constituents to comprehend their cytotoxic effect in human and animal cells.

In vivo tolerance was also evaluated following oral administration at various concentrations (0.5, 1.5, and 2.5% v/v in the 250 mL drinking water supply). The dosage regime was determined following experiments in which 2.0% was selected as the highest concentration, not causing any damage to lettuce leaves. Even though acute toxicity testing is used to determine the toxicity level of substances (e.g., the LD50 median lethal dose), it also is important to assess the subacute oral toxicity profile to evaluate for possible health hazards likely to arise from repeated exposure. The EP formula did not cause any mortality, or gross behavioral changes, either immediately or during the 5-week observation period on any animals, even though this level of concentration of the individual constituents was shown before to cause mortality at different lethal doses [14]. Oral feeding did not either provoke any clinical signs of toxicity in mice, and food intake, body weight, vital signs, and hematological and biochemical parameters from all tested groups were within the physiological range [35,36] and comparable to that of the control group. According to WHO, this could mean that the EP is classified in the category of "unlikely to present any acute or subacute hazards". In fact, the biochemical analysis demonstrated lower creatinine, urea, and CRP levels with increasing doses (see Figure 3f–h), indicating that the Eco product may exhibit anti-inflammatory properties. Further research is warranted to understand and characterize the possible mechanism of action of the observed effects using different pre-clinical animal models.

Several studies reported therapeutic uses of 1,8-cineole for a wide range of diseases. Previous reports indicate that 1,8-cineole may exhibit antitumor effects by promoting cell

cycle arrest and oxidative stress [37–39] and also therapeutic benefits in inflammatory airway diseases, used as a treatment of respiratory tract diseases due to its antimicrobial, mucolytic, broncholytic, and anti-inflammatory properties [40]. The pharmacological properties of 1,8-cineole (as a common component in both of the examined EOs) have been documented in many studies, including antimicrobial activity [41]. Indeed, the antimicrobial activity of an EO is not always related to the main component of the EO but to the different components that might have such activity. For example, this has been proven in a previous study with the application of eucalyptus (*Eucalyptus globulus* L.) and rosemary (*R. officinalis* L.) EO, their mixture (1:1 v/v), and their main component (1,8-cineole commonly known as eucalyptol) in vitro against *Penicillium expansum* (a common postharvest pathogen). In that study, volatiles of eucalyptus and rosemary EO and their mixture (1:1 v/v) decreased fungal colony growth, while eucalyptol did not affect the fungal growth, and these observations were attributed to the synergistic action of the two EOs various compounds and not from their main common component (eucalyptol) [42]. The synergistic, additive, and/or antagonistic effects of EOs mixtures mainly derive from the interactions between their components (main and less abundant) [43].

Investigating the skin sensitization potential of the EP formula is an explicit need for both hazard and risk assessment. Following dermal application, signs of erythema and edema were observed at the highest dose of 2000 mg kg^{-1} bw. Santos et al. stated that 1,8-cineole may produce different effects based on the route of its application, and they also reported that systemic oral administration provokes an anti-inflammatory effect while local administration produces inflammatory edema [44–46]. The signs of dermal irritation versus the observed oral tolerance may be attributed to the acidic nature of the concentrated formula (pH value of 2.85), amongst other factors. In any case, the overall incidence and the severity of irritation decreased with time and were fully reversible within 7 days.

5. Conclusions

The results and analysis of the current investigation showed that a one-time application of the EP formula increased lettuce yield, activating various secondary metabolism pathways of the plant to cope with the oxidative stress. The single application of the EP can be a possible alternative to commonly used products in agriculture, having an eco-friendly and safe (as documented by the present outcomes) profile for lettuce production. The investigation should further be generalized to assess the effectiveness of the EP in different crops, seasons, and application doses, under the frame of "sustainably and eco-friendly crop production". Additionally, the results of the present study suggest that the tested Eco-product does not pose any significant health hazard. Therefore, it is suggested that the compound is classified in category 4 (codes H302 + H312, if swallowed fully concentrated and in contact with the skin at doses equal or higher of 2000 mg kg^{-1} b.w., respectively) or in category 5 (codes H303 + H313, if swallowed after 0.5% v/v dilution and in contact with the skin at doses equal or lower than 1000 mg kg^{-1} b.w., respectively) of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) [47]. Further research is warranted to test the effectiveness of the EP in different crops, seasons, and application doses but also for a more in-depth evaluation of its toxicological safety to support regulatory compliance and registration.

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