The Journal of Phytopharmacology 2026; 15(1):35-45
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2026; 15(1): 35-45
January- February
Received: 30-09-2025
Accepted: 04-03-2026
Published: 30-03-2026

doi: 10.31254/phyto.2026.15105

Anis Klouz
Faculté de Médecine de Tunis, Universite Tunis El Manar, Tunis, Tunisia

Henda Ferchichi
Faculte des Sciences de Tunis, Universite Tunis El Manar, Tunis, Tunisia

Ayoub Ksouri
Sydney Brenner Institute for Molecular Bioscience, Wits University, Johannesburg, South Africa

Oumayma Abidi
Laboratoire de Modélisation Mathématique, Faculté des Sciences de Tunis, Université Tunis El Manar, Tunis, Tunisia

Asma Louati
Laboratoire de Modélisation Mathématique, Faculté des Sciences de Tunis, Université Tunis El Manar, Tunis, Tunisia

Ouajdi Souilem
Laboratoire de Modélisation Mathématique, Faculté des Sciences de Tunis, Université Tunis El Manar, Tunis, Tunisia

Mounir Bezzarga
1. Laboratoire de Modélisation Mathématique, Faculté des Sciences de Tunis, Université Tunis El Manar, Tunis, Tunisia

2. Institut Préparatoire aux Etudes d'Ingénieur de Tunis, Universite de Tunis, Tunis, Tunisia

Balkiss B. Zahar
Laboratoire des Biomolécules, Venins et Applications Théranostiques, Institut Pasteur de Tunis, Tunis, Tunisia

Zakaria Benlasfar
Ecole Nationale de Médecine Vétérinaire, Sidi Thabet, Tunis

Correspondence:
Dr. Anis Klouz Faculté de Médecine de Tunis, Universite Tunis El Manar, Tunis, Tunisia
Email: anis.klouz@rns.tn

ImmunoDefender: acute safety profile of a novel essential oil antiviral formulation

Anis Klouz, Henda Ferchichi, Ayoub Ksouri, Oumayma Abidi, Asma Louati, Ouajdi Souilem, Mounir Bezzarga, Balkiss B. Zahar, Zakaria Benlasfar

ABSTRACT

Background: Essential oils (EOs) exhibit significant antiviral properties, making them promising candidates for COVID-19 treatment. 'ImmunoDefender' is a novel EO-based formulation designed to inhibit SARS-CoV-2 through *in silico* molecular docking studies. **Objective:** This study evaluates the *In vitro* mutagenicity and *In vivo* acute oral toxicity of 'ImmunoDefender' as a first step in its safety assessment. **Materials and Methods:** Mutagenic potential was assessed using the Ames test with *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537) and *Escherichia coli* WP2 uvrA, both with and without metabolic activation (S9 mix), at concentrations up to 2500 µg/plate (TA98, TA100, TA1535, TA1537) and 316 µg/plate (WP2 uvrA). Acute oral toxicity was evaluated in Wistar rats following OECD 423 guidelines, with single doses up to 10 times the therapeutic level (2000 mg/kg), followed by 14-day observation. **Results:** No mutagenic activity was observed in any bacterial strains. *In vivo*, no mortality, body weight alterations, or significant biochemical and histopathological changes were noted over 14 days post-administration. **Conclusion:** These findings indicate that 'ImmunoDefender' is non-mutagenic and non-toxic under acute exposure conditions, supporting its safety for further preclinical investigations. Future studies should assess long-term toxicity and clinical efficacy in COVID-19 management.

Keywords: Essential Oils, COVID-19, Mutagenicity Tests, Acute Toxicity, *Salmonella typhimurium*, *Escherichia coli*.

INTRODUCTION

Coronaviruses (CoVs) are a diverse family of RNA viruses known to cause significant outbreaks in humans and animals, leading to severe acute respiratory syndromes (SARS). Among them, SARS-CoV-2, first identified in December 2019 in Wuhan, China, triggered the most widespread and severe pandemic in recent history [1]. This novel coronavirus, along with its variants and subvariants of concern (VOCs), has exhibited increased transmissibility and immune evasion, resulting in the disease known as COVID-19. The clinical manifestations of COVID-19 range from mild flu-like symptoms to severe complications affecting the respiratory, neurological, and gastrointestinal systems. Common acute complications include fever, cough, anosmia, pneumonitis, thromboembolic events, cardiogenic shock, renal and hepatic injury, and ischemic strokes [2,3].

Despite the rapid development and deployment of SARS-CoV-2 vaccines, severe forms of the disease and high mortality rates have persisted. By March 10, 2021, the Johns Hopkins Coronavirus Resource Center reported over 117 million infections worldwide, with more than 2.6 million deaths. As of 2022, SARS-CoV-2 infections exceeded 532 million cases globally, with over 6 million confirmed fatalities [3]. The increasing number of breakthrough infections highlights the urgent need for novel antiviral therapeutics to complement existing vaccination strategies and address severe SARS-related diseases.

In response to the World Health Organization (WHO) initiative encouraging drug discovery efforts against COVID-19, research teams and laboratories worldwide have focused on developing and patenting novel formulations. Among the most promising candidates are phytochemicals and essential oils (EOs), which have demonstrated significant biological potential in treating both infectious and non-infectious diseases [4,5].

Several EOs compounds have been extensively studied for their antiviral and anti-inflammatory properties, with *in vitro* evidence supporting their effectiveness against viral pathogens [6]. However, further research is required to identify selective and effective EO-based antimicrobial agents, particularly regarding their optimal dosage and synergistic formulations [7,8].

In Tunisia 'ImmunoDefender' a novel EO-based formulation has been classified as a medicinal product by the Directorate of Pharmacy and Medicines (DPM) of the Ministry of Health, following national regulations (Law 73/55, August 3, 1973).

Protected under patent registration PCT/IB2021/060180, 'ImmunoDefender' has shown promising antiviral effects against SARS-CoV-2. Recent *in silico* structural studies have demonstrated that key compounds within 'ImmunoDefender' exhibit high binding affinity for the main protease (Mpro) of SARS-CoV-2, effectively blocking its catalytic site and potentially inhibiting viral replication [9]. At the molecular level, six bioactive components: Pavetannin C1, Procyanidin C1, Cinnamtannin B1, Cinnamtannin B2, Syzyginin B, and Tenuifolin, form stable interaction complexes with Mpro, exhibiting binding energies ranging from -8 to -11 kcal/mol. These findings strongly suggest that 'ImmunoDefender' may interfere with viral polyprotein processing, thereby impeding SARS-CoV-2 pathogenesis and transmission [9].

Recognized as a new chemical entity (NCE) for COVID-19 treatment, 'ImmunoDefender' isa unique formulation containing 10 naturally derived phytochemicals, with sesame oil serving as an excipient. While EO-based therapeutics hold promise, their potential toxicity remains acritical concern, as plant-derived compounds can exert dose-dependent toxic effects on human organ systems [10,11]. Establishing the safety profile of an NCE-based product is an essential step in drug development, necessitating rigorous *in vitro* and *in vivo* toxicity evaluations. Toxicological assessments are crucial for identifying potential risks associated with plant-based formulations and balancing their therapeutic benefits against possible adverse effects.

This study aims to assess the safety profile of 'ImmunoDefender' through a series of *in vitro* mutagenicity assays and *in vivo* acute oral toxicity studies. The mutagenic potential is evaluated using bacterial gene mutation assays, both in the presence and absence of mammalian metabolic activation systems. Additionally, acute oral toxicity is assessed in Wistar rats, following EU regulatory directives and OECD guidelines. These investigations provide a foundational safety assessment for 'ImmunoDefender', supporting its further development as a potential therapeutic agent for COVID-19 and related infectious diseases.

MATERIAL AND METHODS

Ethical and Ethnopharmacological Considerations

The *in vivo* toxicity study was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines (OECD, 2008). All animal procedures complied with the Guide for the Care and Use of Laboratory Animals (2010), as outlined by the National Academy of Sciences and the National Institutes of Health (NIH Publication 8edition 2010, USA) [12]. The study was performed at SIPHAT Pharmaceutical Industry Core Facilities (Tunis, Tunisia), and the protocol was approved by the Comitéd' Éthique BioMédicale (CEBM) of the Institut Pasteur de Tunis (Approval No. 2021/19/E).

The *in vivo* mutagenicity study was conducted following the Good Laboratory Practice (GLP) regulations of the European Community (EC), as enacted in Germany under the « Chemikaliengesetz » (Chemicals Act). The study adhered to OECD Principles of Good Laboratory Practice (Document No. 1, ENV/MC/CHEM(98)17) and was regulated under Directive 2004/10/EC of the European Parliament and the Council (February 11, 2004).

These principles align with regulatory requirements from major health authorities, including the United States (EPA and FDA) and Japan (MHLW, MAFF, and METI).

Essential Oils and 'ImmunoDefender' Formulation

The essential oils (EOs) used in this study were clove (*Syzygium aromaticum*), menthol crystal, water mint (*Mentha aquatica*), spearmint (*Mentha spicata*), peppermint (*Mentha piperita*), pitch mint (*Mentha pulegium*), eucalyptus (*Eucalyptus globulus*), camphor (*Cinnamomum camphora*), cinnamon (*Cinnamomum zeylanicum*), cajeput (*Melaleuca cajuputii*), and sesame (*Sesamum indicum*). These were sourced from BIODEX-SA Company (Tunis, Tunisia), obtained from certified international suppliers (Table 1).

'ImmunoDefender' is a balanced essential oil formulation designed to enhance the immune response while minimizing potential side effects. Each EO component is incorporated at concentrations not exceeding 1 g per EO. Sesame oil serves as an excipient. The *in vivo* study design incorporated 2D matrix-based risk assessments, considering factors such as LD_{50} values, acceptable daily intake, administration routes, and prior evidence-based EO toxicity reports on laboratory animals (Table 2).

For *in vitro* assays, 'ImmunoDefender' was tested at concentrations of 10, 31.6, 100, 316, 1000, and 2500 µg per plate in the plate incorporation test (without and with metabolic activation), and at 1, 3.16, 10, 31.6, 100, and 316 µg per plate in the preincubation test (without and with metabolic activation).

Bacterial Strains

Five bacterial strains were used to assess mutagenicity:

● *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537)

● *Escherichia coli* (WP2 uvrA) Strains were obtained from Trinova Biochem GmbH (Germany) and tested at LPT Laboratory of Pharmacology and Toxicology GmbH & Co. (Germany). TA98 and TA1537were used to detect frameshift mutations, while TA100 and TA1535 were used for base-pair substitution mutations. The E. coli WP2 uvrA strain was used to assess excisable misreplication damage and DNA strand breaks. Mutagenicity assays were performed using permanent lyophilized copies of the bacterial strains.

Laboratory Animals

Adult male Wistar rats (180-390 g) were procured from SIPHAT Physiology Department Facilities and housed under standard laboratory conditions. The lab animal study protocols were approved by the local Ethics Committee of the InstitutPasteur Tunis (Approval Number: 2021/19/E).

In vitro Bacterial Mutagenic Activity and Gene Mutation Assays

To ensure accurate evaluation, 'ImmunoDefender' was first tested at the maximum technically feasible concentration of 89, 270 µg per plate. Mutagenicity was assessed using bacterial gene mutation assays, following methodologies described by Ames *et al*. (1973, 1975) and Maron & Ames (1983), in compliance with Regulation (EC) No. 440/2008, MethodB.13/14, and OECD Guideline 471. 'ImmunoDefender' was dissolved in acetone, which served as the negative control. The *Salmonella typhimurium* histidine (his) and *E. coli* tryptophan (trp) reversion systems were employed to detect base substitutions, frameshift mutations, and excisable mis-replication damage. In the plate incorporation method, a histidine- or tryptophan-free agar base was overlaid with test organisms and the compound of interest. The presence of a trace histidine or tryptophan allowed limited bacterial replication, enabling assessment of mutation-induced revertant colonies.

In vivo Study Design: Animal Groups and Administration Protocol

A total of 60 Wistar rats were randomly divided into 12 groups (n = 5 per group). Rats were acclimatized for one week before experimental procedures, during which they received mock gavage administrations of physiological serum at 9:00 a.m. daily.

Experimental Groups

Control Group: Sham (untreated) and saline solution (SS) treated group (10,000µl/kg)

● Reference Groups: Rats treated with individual EO components, including sesame oil (excipient), camphor (CM), eucalyptus (EUC), menthol (MT), pitch mint (MP), and cinnamon (CN).

● 'ImmunoDefender' Groups: Rats received doses of 1600 µl/kg (ID1), 6400 µl/kg (ID2), 10,000 µl/kg (ID3), and 32,000 µl/kg (ID4)

All groups received a single oral administration, except the 'ImmunoDefender' trial groups, which were administered in a stepwise cascade at 48-h intervals, with dose escalation contingent on mortality and toxicity observations. The highest dose (32,000 µl/kg) was divided into two administrations (16,000 µl each, spaced 30 minutes apart) to ensure gastric capacity compliance, following OECD Guideline 425.

Animal Observations and Terminal Procedures

Following administration, rats were monitored individually during the first 30 minutes, at regular intervals over 24 h (with emphasis on the first 4 h), and daily for 14 days.

Clinical observations included:

● Behavioral signs (restlessness, dullness, distress)

● Physical condition (skin, eyes, respiratory patterns, motor activity)

● Neurological symptoms (tremors, convulsions, salivation, lethargy, sleep, coma)

● Food and water intake patterns

● Body weight measurements (recorded weekly for two weeks) (National Research Council, 2008) [13].

Biochemical Analysis

At the end of the 14-day observation period, blood samples were collected via retro-orbital venous plexus or facial vein. Serum biochemical parameters were assessed using an automated biochemical analyzer (COBAS C111), focusing on:

● Liver function: Aspartate aminotransferase (AST), alanine aminotransferase (ALT)

● Renal function: Creatinine levels

● Metabolic function: Lipase, creatine phosphokinase (CPK)

Histopathological Analysis

At necropsy, heart, kidney, and liver tissues were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HEstain) for histopathological examination (Pathology Anatomy and Cytology Lab, Tunis, Tunisia).

Statistical Analysis

Statistical analysis was performed using BIOSTAT software. Group comparisons were conducted using one-way analysis of variance (ANOVA). Data are presented as mean ± standard error (SE) or mean ± standard deviation (SD), with statistical significance set at $p<0.05$.

Essential Oil Formulation

'ImmunoDefender' contains 10 EOs with sesame oil as excipient. Details on components and concentrations available in Table 1.

Bacterial Strains

Mutagenicity assays used *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and *E. coli* WP2uvrA.

Animals

60 adult male Wistar rats (180 -390 g) were divided into control and experimental groups.

Testing Procedures

In vitro and *in vivo* toxicity evaluations, with appropriate controls, dosage escalation, and biochemical + histopathological assessments conducted.

RESULTS

Evaluation of ImmunoDefender as an Alternative COVID-19 Therapeutic Approach

This study investigated the safety profile of ImmunoDefender through *In vitro* and *In vivo* toxicity assessments, aiming to establish its potential as an alternative therapeutic strategy against COVID-19. The formulation was developed using a stochastic optimization approach, where the minimum inhibitory concentrations (MICs) of each essential oil were integrated into a weighted model to achieve an optimal balance between therapeutic efficacy and safety.

This data-driven methodology enhances ImmunoDefender's ability to modulate immune responses while ensuring a well-tolerated dosage profile.

Computational docking studies provided strong evidence that the essential oil-based formulation effectively inhibits the main protease (Mpro) of SARS-CoV-2, a key enzyme responsible for viral replication. These findings suggest that, beyond COVID-19, ImmunoDefender could be beneficial for treating respiratory infections such as influenza, acute bronchitis, and inflammation. Given its novel formulation, regulatory requirements necessitated comprehensive mutagenicity and acute oral toxicity evaluations to establish its safety profile.

Preliminary Cytotoxicity Assessment

The cytotoxic potential of ImmunoDefender was assessed using a plate incorporation test in *Salmonella typhimurium* TA100, with concentrations ranging from 312.5 to 89,270 µg/plate, both with and without metabolic activation. Cytotoxic effects were first noted at concentrations of 2500 µg/plate, setting this as the upper limit for the plate incorporation test, while 316 µg/plate was determined as the maximum concentration for the preincubation test [14].

Cytotoxicity and Mutagenicity Assays

The mutagenic potential of ImmunoDefender was evaluated in four *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537) and one Escherichia coli strain (WP2 uvrA) using two independent experiments: a plate incorporation test and a preincubation test, both conducted with and without metabolic activation using a microsomal

fraction derived from Phenobarbital/5,6-Benzoflavone-induced rat liver.

The study was conducted in compliance with Good Laboratory Practice (GLP) regulations, ensuring reliability and reproducibility. The results indicated that ImmunoDefender did not induce gene mutations at concentrations of up to 2500 µg/plate in the plate incorporation test or 316 µg/plate in the preincubation test. There was no significant increase in revertant colony numbers compared with control counts, confirming the absence of mutagenic effects.

Positive control items exhibited at least a 2-fold increase in revertant colonies, validating the test conditions and system sensitivity. The results aligned with historical background values, fulfilling the study's acceptance criteria. These findings indicate that ImmunoDefender does not exhibit mutagenic properties under the tested conditions.

Acute Oral Toxicity Assays in Wistar Rats

Acute oral toxicity assessments were conducted in compliance with OECD guidelines, with the initial dose adjusted to a technically feasible volume that did not exceed 1 mL/100 g bodyweight.

In the first pilot test, a dose of 1.6 mL/kg body weight (40 times the therapeutic dose) was administered by gavage to three Wistar rats. No mortality or adverse effects were observed over a 14-day period, with regular feed and water consumption patterns maintained.

A second pilot test was performed using a dose of 6.4 mL/kg body weight (80 times the therapeutic dose), with no observed mortality. Mild stool softening was noted in two rats but resolved within 24 h, confirming that even at high doses, single oral administrations of ImmunoDefender do not induce significant toxicity.

Cage-Side Observations

Throughout the 14-day observation period, treated rats displayed normal behavioral, motor, and neurological functions, with no signs of distress or toxicity. Skin, fur, eyes, and respiratory patterns remained normal across all groups.

A transient softening of stools was observed in some treated groups on Day 1 post administration, but symptoms resolved spontaneously by the following day. Necropsy findings showed no macroscopic abnormalities in any treated rats, supporting the non-toxic nature of ImmunoDefender.

Food Consumption and Body Weight Monitoring

All groups maintained consistent feeding and hydration patterns throughout the study. No statistically significant changes in body weight were recorded between Day 0 (baseline), Day7 (midpoint), and Day 14 (final measurement), indicating that ImmunoDefender does not adversely affect appetite, metabolism, or weight regulation (Table 3).

Biochemical Analysis

Serum biochemical markers were analyzed at the end of the 14-day period to assess hepatic, renal, and metabolic functions (Table 4).

● Aspartate aminotransferase (AST) levels in the control group reached 259.5 ± 34.25UI, with no significant changes in the treated groups (Figure 1).

● Alanine aminotransferase (ALT) levels in the 1600 µL/kg group were significantly lower (55 ± 11.55 UI, $p = 0.0153$) compared to controls (108 ± 13.47 UI), while no significant differences were observed in other treatment groups (Figure 2).

● Creatinine levels were significantly lower in groups treated with ID1, ID2, and ID4 compared with the control group ($p < 0.05$) (Figure 3).

● Lipase levels remained stable, except for a minor increase in the menthol-treated group (6.6 ± 0.55 IU vs. 5.6 ± 0.55 IU in controls, $p = 0.0527$) (Figure 4).

● CPK activity significantly decreased in the ID1 group ($p = 0.0243$) but showed a significant increase in the eucalyptus-treated group ($p = 0.0103$) (Figure 5).

These findings indicate that ImmunoDefender does not induce hepatotoxicity, nephrotoxicity, or significant metabolic disturbances.

Histopathological Analysis

Gross examination of the heart and kidneys revealed no abnormalities in treated groups compared to controls.

Histological analysis confirmed that ImmunoDefender did not induce major pathological changes in liver tissue, except for minor reversible lesions in two treatment groups. In the camphor essential oil (CM) and *Mentha pulegium* (MP) groups, mild lymphocytic inflammatory infiltrates were observed, along with some eosinophilic polynuclear cells associated with isolated necrotic bodies and mild hepatocyte ballooning degeneration.

These effects were low in intensity and did not indicate severe toxicity. The European Food Safety Authority (EFSA) recommends that camphor concentrations remain below 0.1% in consumer products, aligning with findings that higher camphor exposure may induce mild hepatic responses.

For all other groups, the hepatic parenchyma remained intact, with preserved portal spaces and normal tissue morphology. These findings support the conclusion that ImmunoDefender is non-toxic to the heart (Figure 6a), kidneys (Figure 6b), and liver (Figure 6c) at the tested doses.

Conclusion of Findings

This study demonstrated that ImmunoDefender exhibits no mutagenic activity, as confirmed by the absence of increased revertant colony numbers in *in vitro* assays.

The *in vivo* toxicity assessment in Wistar rats revealed no mortality or significant clinical symptoms at single doses of up to 32,000 µL/kg, confirming the formulation's safety.

Biochemical and histopathological analyses further support the absence of hepatotoxic, nephrotoxic, or metabolic toxicity effects. These findings provide strong preliminary evidence for the safety of ImmunoDefender, supporting its potential for further preclinical development and therapeutic evaluation in COVID-19 and related respiratory diseases.

Table 1: "ImmunoDefender" EO list

	Latin Name
Spearmint Eo	*Mentha spicata*
Menthol (crystals)	Mentha
Cajeput Eo	*Melaleuca cajuputii*
Watermint Eo	*Mentha aquatica*
Cloves Eo	*Syzygium aromaticum*
peppermint Eo	*Mentha piperita*
Pennyroyal Eo	*Mentha poulegium*
Eucalyptus Eo	*Eucalyptus globulus*
Camphor	*Cinnamomum camphora*
Cinnamon Eo	*Cinnamomum zeylenicum*
Sesame Oil	*Sesamum indicum*

Table 2: Administered dose for treated groups (references groups in the acute toxicity study

		Administered dose (mg/kg)
Menthol (crystals)	MT	0.968
Pennyroyal Eo	MP	0.638
Eucalyptus Eo	EUC	1.73
Camphor Eo	CM	2.935
Cinnamon Eo	CN	0.57
Sesame Oil	SE	9.677

EO: Essential Oil

Table 3: Body weight variations for control and treated groups in the acute toxicity study

		Day 0	Day 7	Day 14	P
GROUPS	**Sham**	220 ± 24	216 ± 26	206 ± 30	0.6691
	Saline Solution	221 ± 17	216 ± 16	220 ± 16	0.8725
	Sesame Oil	218 ± 14	216 ± 17	222 ±18	0.8638
	Camphor Essential Oil	221 ± 16	216 ± 16	228 ± 16	0.5144
	Eucalyptus Essential Oil	215 ± 6	208 ± 18	210 ±17	0.7362
	Menthol	219 ± 10	219 ± 10	222 ± 7	0,8778
	Mentha pulegium Essential Oil	215 ± 15	207 ± 13	209 ± 15	0,6966
	Cinnamon Essential Oil	222 ± 19	223 ± 23	219 ± 22	0,9609
	ID1	259 ± 33	273 ± 28	277 ± 29	0,6114
	ID2	349 ± 32	311 ± 54	313 ± 59	0,4131
	ID3	225 ± 12	224 ± 13	221 ± 14	0,9196
	ID4	238 ± 20	232 ± 27	246 ± 22	0,6368

Values expressed as mean ± standard deviations, n = 5; $P < 0.05$

Table 4: Biological parameters in control and treated groups in the acute toxicity study

		AST (UI)	ALT (UI)	Creatinine (µmol/l)	Lipase (UI)	Creatinine phospho-kinase (CPK) (UI)
GROUPS	**Sham**	251.8 ± 34.25	108 ± 13.47	51.6 ± 3.65	5.6 ± 0.55	4630 ± 2356
	Saline Solution	331.6 ± 77.04	121.6 ± 26.75	46 ± 4.24	5.4 ± 0.89	6885.2 ± 1583
	Sesame Oil	166.8 ± 30.8	81.6 ± 5.37	38.4 ± 5.22	5.2 ± 0.84	4285.8 ± 1952
	Camphor Essential Oil	264.6 ± 36.04	102.8 ± 16.57	48 ± 2.12	5.6 ± 0.89	5759.2 ± 1262
	Eucalyptus Essential Oil	382.4 ± 156.62	123.2 ± 23.24	46 ± 7.87	5.8 ± 0.84	8284 ± 3000
	Menthol	359.8 ± 177.65	136.4 ± 63.01	43.6 ± 4.39	6.6 ± 0.55	6954 ± 4082
	***Mentha pulegium* Essential Oil**	203.6 ± 74.14	86.8 ± 15.59	39 ± 2.24	5 ± 0.71	5029.6 ± 3135
	Cinnamon Essential Oil	317.8 ± 192.94	118.8 ± 41.55	47.75 ± 6.39	5.8 ± 0.84	5633.5 ± 1986
	ID1	124 ± 19.63	55 ± 11.55	40 ± 6.93	4.5 ± 0.58	1087 ± 45
	ID2	226.2 ± 80.56	111 ± 60.26	35.6 ± 2.51	4.4 ± 0.55	2905.2 ± 618
	ID3	318.2 ± 120	125.2 ± 27.48	52 ± 3.74	5.8 ± 1.30	4823.25 ± 1961
	ID4	202.6 ± 34.33	97 ± 11.94	34.2 ± 5.81	6 ± 0.71	5484 ± 2531

Values expressed as mean ± standard deviations, n = 5

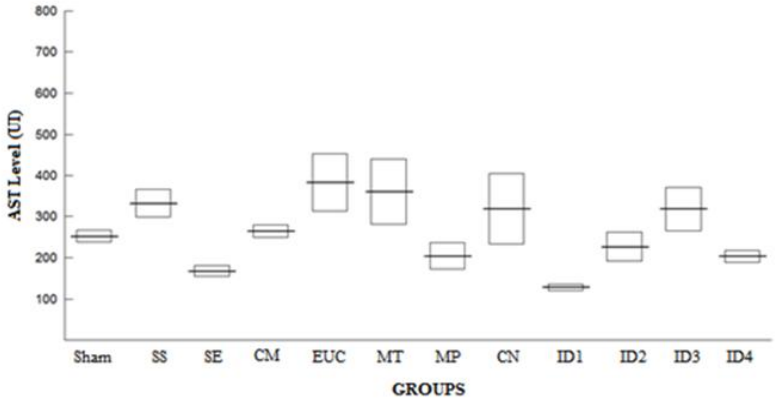

Figure 1: Aspartate aminotransferase (AST) concentrations for each group
Values expressed as mean ± standard error, n = 5
*vs. Sham, P < 0.05
SS**:** Saline Solution;SE: Sesame Oil;
CM: Camphor EO**;** EUC**:** Eucalyptus EO**;** MT**:** Menthol;
MP**:** Menthapulegium EO;CN**:** Cinnamon EO;
ID1**:** 1600 µl/kg of ImmunoDefender; ID2**:** 6400 µl/kg of ImmunoDefender;
ID3**:** 10000 µl/kg of ImmunoDefender; ID 4**:** 32000 µl/kg of ImmunoDefender

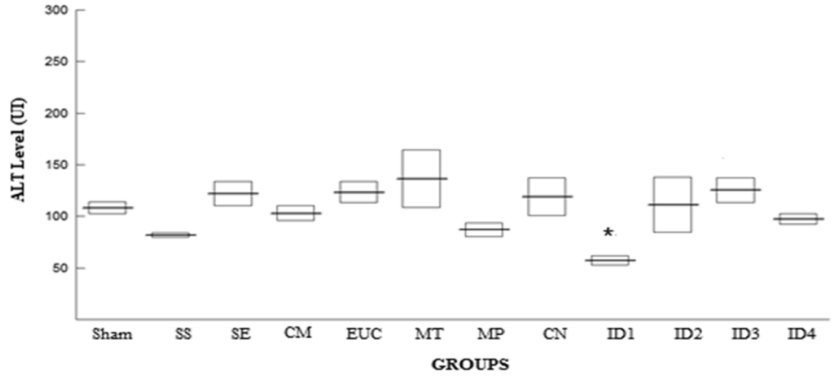

Figure 2: Alanine aminotransferase (ALT) concentrations for each group
Values expressed as mean ± standard error, n = 5
*vs. Sham, P < 0.05
SS**:** Saline Solution;SE: Sesame Oil;
CM: Camphor EO**;** EUC**:** Eucalyptus EO**;** MT**:** Menthol;
MP**:** Menthapulegium EO;CN**:** Cinnamon EO;
ID1**:** 1600 µl/kg of ImmunoDefender; ID2**:** 6400 µl/kg of ImmunoDefender;
ID3**:** 10000 µl/kg of ImmunoDefender; ID 4**:** 32000 µl/kg of ImmunoDefender

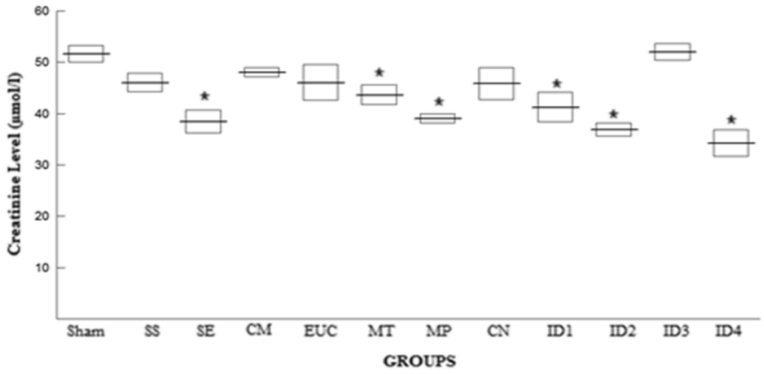

Figure 3: Creatinine concentrations for each group
Values expressed as mean ± standard error, n = 5
*vs. Sham, P < 0.05
SS**:** Saline Solution;SE: Sesame Oil;
CM: Camphor EO**;** EUC**:** Eucalyptus EO**;** MT**:** Menthol;
MP**:** Menthapulegium EO;CN**:** Cinnamon EO;
ID1**:** 1600 µl/kg of ImmunoDefender; ID2**:** 6400 µl/kg of ImmunoDefender;
ID3**:** 10000 µl/kg of ImmunoDefender; ID 4**:** 32000 µl/kg of ImmunoDefender

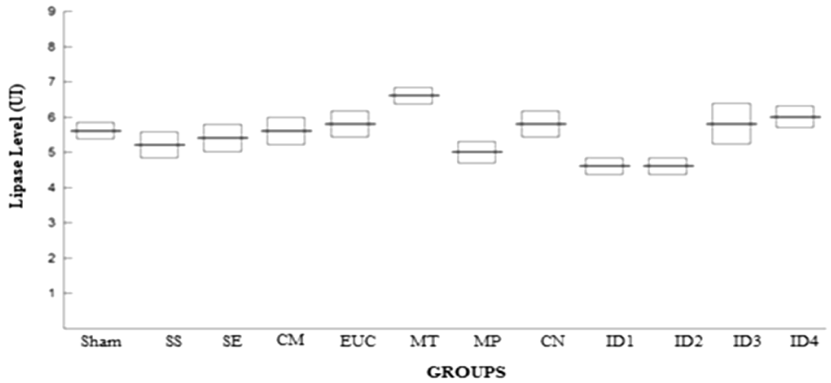

Figure 4: Lipase concentrations for each group
Values expressed as mean ± standard error, n = 5
*vs. Sham, P < 0.05
SS**:** Saline Solution;SE: Sesame Oil;
CM: Camphor EO**;** EUC**:** Eucalyptus EO**;** MT**:** Menthol;
MP**:** Menthapulegium EO;CN**:** Cinnamon EO;
ID1**:** 1600 µl/kg of ImmunoDefender; ID2**:** 6400 µl/kg of ImmunoDefender;
ID3**:** 10000 µl/kg of ImmunoDefender; ID 4**:** 32000 µl/kg of ImmunoDefender

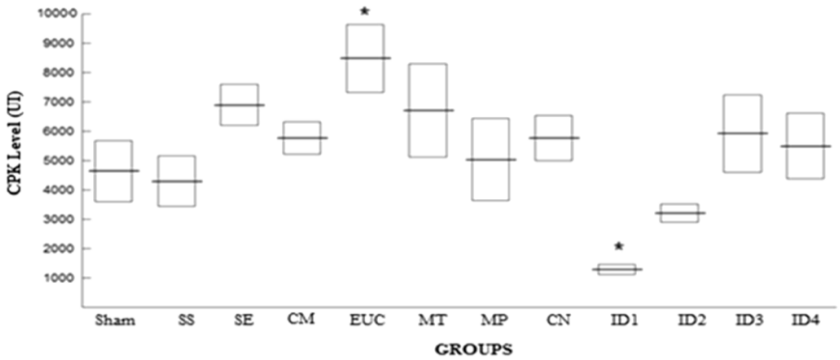

Figure 5: Creatinephospho-kinase (CPK) concentrations for each group
Values expressed as mean ± standard error, n = 5
*vs. Sham, P < 0.05
SS**:** Saline Solution;SE: Sesame Oil;
CM: Camphor EO**;** EUC**:** Eucalyptus EO**;** MT**:** Menthol;
MP**:** Menthapulegium EO;CN**:** Cinnamon EO;
ID1**:** 1600 µl/kg of ImmunoDefender; ID2**:** 6400 µl/kg of ImmunoDefender;
ID3**:** 10000 µl/kg of ImmunoDefender; ID 4**:** 32000 µl/kg of ImmunoDefender

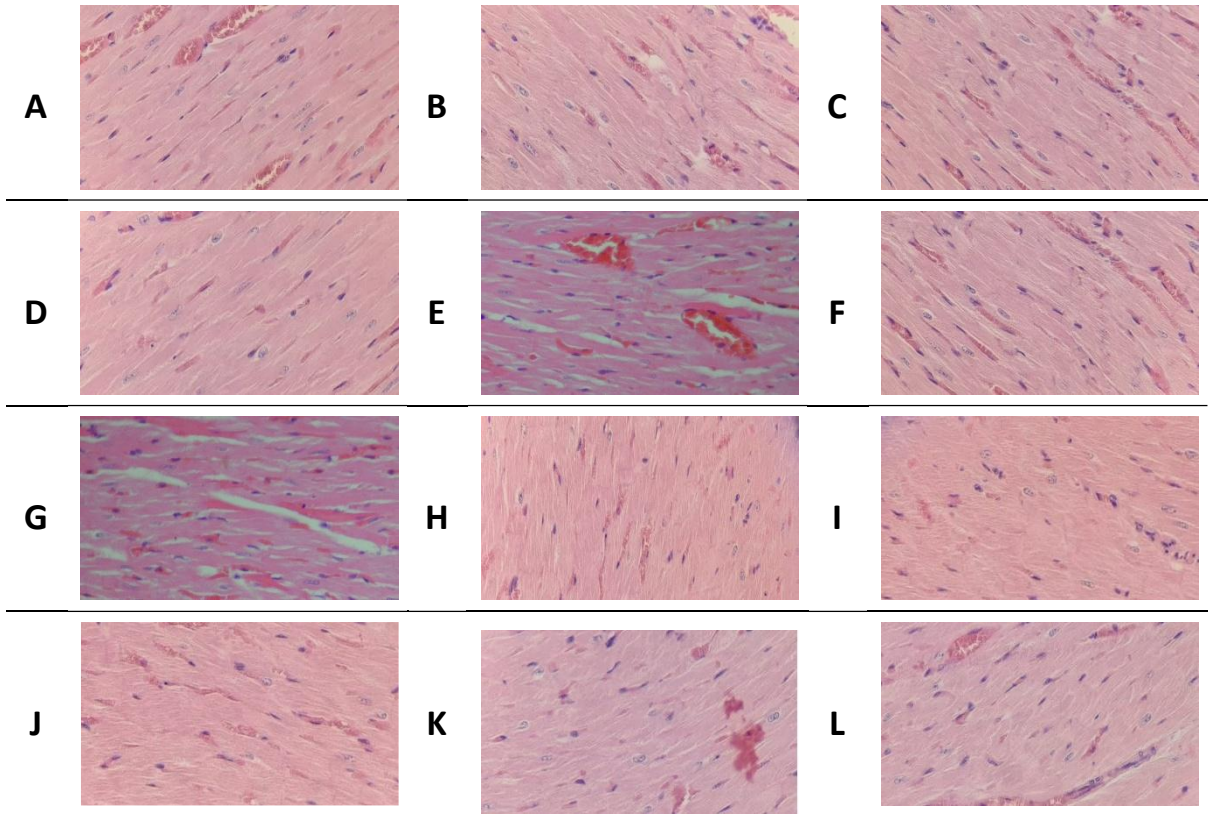

Figure 6a: Histopathologic appearances of Heart tissues among groups

A: Sham (S); B: Saline Solution (SS); C: Sesame O (SE); D: Camphor EO (CM); E: Eucalyptus EO (EUC); F: Menthol (MT); G: Menthapulegium EO (MP); H: Cinnamon EO (CN); I: ID1 (1600 µl/kg of ImmunoDefender); J: ID3 (6400 µl/kg of ImmunoDefender); K: ID3 (10000 µl/kg of ImmunoDefender); L: ID4 (32000 µl/kg of ImmunoDefender)

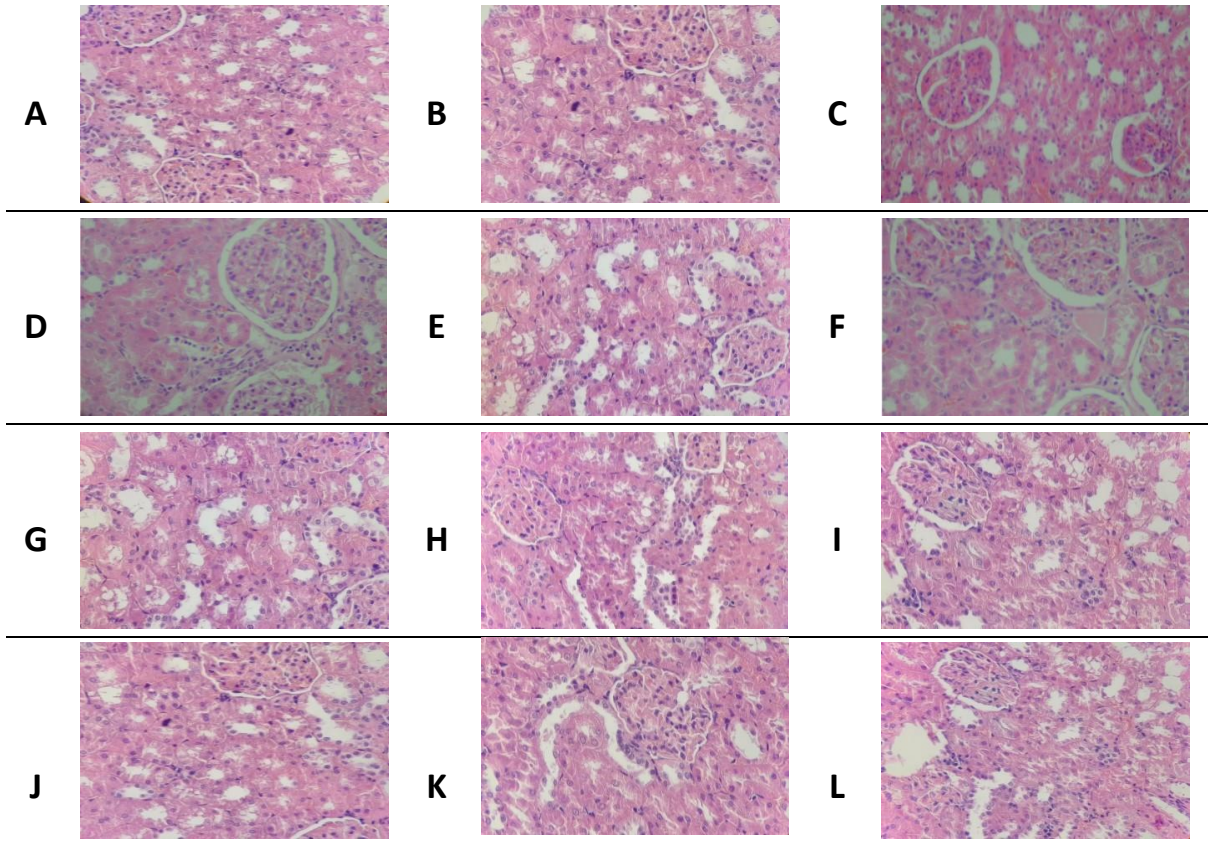

Figure 6b: Histopathologic appearances of Kidney tissues among groups

A: Sham (S); B: Saline Solution (SS); C: Sesame O (SE); D: Camphor EO (CM); E: Eucalyptus EO (EUC); F: Menthol (MT); G: Menthapulegium EO (MP); H: Cinnamon EO (CN); I: ID1 (1600 µl/kg of ImmunoDefender); J: ID3 (6400 µl/kg of ImmunoDefender); K: ID3 (10000 µl/kg of ImmunoDefender); L: ID4 (32000 µl/kg of ImmunoDefender)

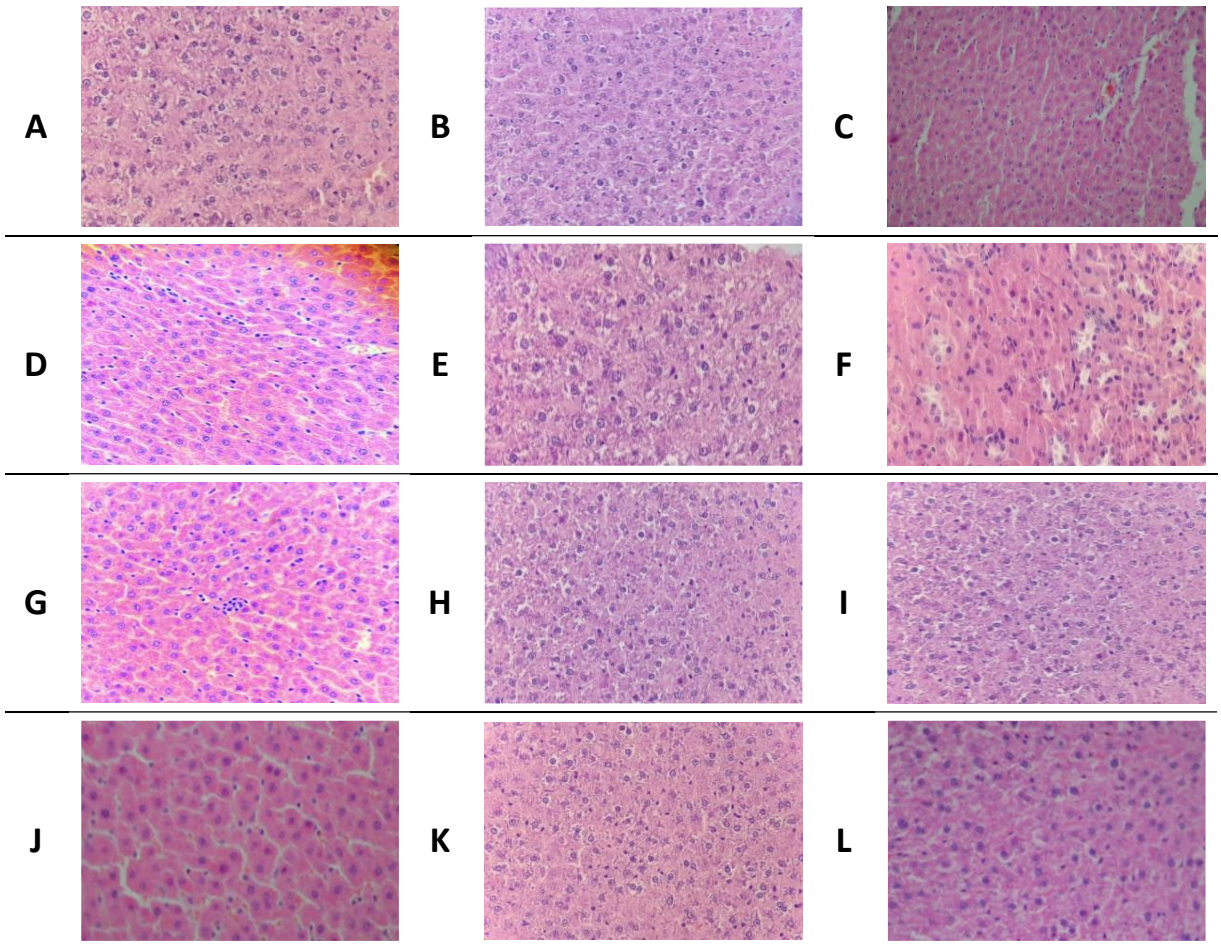

Figure 6c: Histopathologic appearances of Liver tissues among groups

A: Sham (S); B: Saline Solution (SS); C: Sesame O (SE); D1/D2: Camphor EO (CM); E: Eucalyptus EO (EUC); F: Menthol (MT); G1/G2: Menthapulegium EO (MP); H: Cinnamon EO (CN); I: ID1 (1600 µl/kg of ImmunoDefender); J: ID3 (6400 µl/kg of ImmunoDefender); K: ID3 (10000 µl/kg of ImmunoDefender); L: ID4 (32000 µl/kg of ImmunoDefender)

Figure 6: Histopathology of representative internal organs of rats in the acute toxicity study of EPE 40 × magnification (hematoxylin-eosin stain)

The light microscopic observations of heart, kidney and liver tissues in control and treated groups in acute toxicity study sacrificed at the end of 14-day study period (a) Heart section: normal morphology of the myocardium for all groups (no significant histopathological changes were observed) (b) Kidney section: normal general structure with good corticomedular differentiation. There are many glomeruli, none of which is sealed. (no significant histopathological changes were observed) (c) Liver section (no significant histopathological lesions were observed)

DISCUSSION

Emerging viral diseases pose a major global public health challenge, with recent outbreaks including severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, the West African Ebola virus epidemic in 2013, the Zika virus outbreak in the Americas in 2015, and most recently, theCOVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARSCoV-2) in December 2019 [15].

Among the therapeutic targets identified for coronaviruses, 3-chymotrypsin-like protease (3CLpro), also known as the main protease (Mpro), plays a critical role in viral replication and has been widely recognized as a key target for antiviral drug development [16]. The challenge in combating SARS-CoV-2 lies in designing and developing effective Mpro inhibitors as novel anti-coronavirus therapies. Several compounds, including Abacavir and Tenofovir, have demonstrated viral reverse-transcriptase inhibition of Mpro, highlighting the potential of targeting this protease for therapeutic intervention.

ImmunoDefender, a newly formulated bioactive antiviral compound, exhibits a strong binding affinity to the catalytic and allosteric sites of Mpro, suggesting its potential effectiveness in inhibiting SARS-CoV-2 replication. Despite the availability of various natural product formulations with therapeutic effects, many lack comprehensive scientific validation regarding their safety and toxicological profiles. Given the oral administration of ImmunoDefender as a new chemical entity (NCE) for COVID-19 treatment, the present study sought to evaluate its toxicity through acute oral toxicity and mutagenicity assays to ensure its safety for potential clinical application.

Under the conditions of the mutagenicity test, ImmunoDefender was evaluated at concentrations of up to 2500 µg/plate in the plate incorporation test and 316 µg/plate in the preincubation test, both with and without metabolic activation. The results showed no mutagenic effects in *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) or E. coli strain WP2 uvrA. The absence of increased revertant colony numbers in both test conditions confirmed that ImmunoDefender does not induce gene mutations. These findings suggest that the formulation lacks genotoxic potential, supporting its safety for further development.

The *In vivo* acute toxicity study further supported the safety of ImmunoDefender. Six key components of the formulation were administered to Wistar rats at doses ten times higher than their respective concentrations in the final product. All administered doses

remained well below the acceptable daily intake (ADI) and significantly lower than the LD_{50} values of each extract. The acute toxicity study revealed no mortality or observable toxic signs related to body weight, food consumption, or internal organ weight. Feeding and hydration patterns of all rats remained stable throughout the study, with no statistically significant differences in body weight between treated and control groups. Histopathological analysis confirmed the absence of pathological changes in kidney and heart tissues, and no liver lesions were detected.

Sesame oil, used as excipient in ImmunoDefender, is a well-documented bioactive compound known for its antioxidative, anti-arthritic, and hepatoprotective properties. Prior studies have demonstrated that sesame oil attenuates acute kidney injury and prevents creatinine level elevation [17]. In this study, creatinine levels were lower in ImmunoDefender-treated groups compared to controls, suggesting that sesame oil may contribute to renal protection.

ImmunoDefender contains four Mentha-derived extracts, including three essential oils and one crystalline extract. Several studies have demonstrated the anti-inflammatory, antibacterial, antiviral, immunomodulatory, antitumor, neuroprotective, and antioxidant properties of Mentha species [18]. Pharmacological evidence suggests that Mentha extracts exert protective effects on the gastrointestinal, hepatic, renal, respiratory, and nervous systems, in addition to hypoglycemic and hypolipidemic activities.

Given that COVID-19 is a systemic disease with multi-organ involvement, particularly affecting the liver, the potential hepatoprotective effects of ImmunoDefender components were of particular interest in this study.

Liver injury is a frequent complication of COVID-19, with elevated transaminase levels reported in 15% to 53% of infected individuals. The biochemical markers ALT and AST serve as key indicators of hepatocellular injury, with ALT being the most specific. In this study, ImmunoDefender at 1600 µg/kg resulted in a significant decrease in ALT activity (55± 11.55 UI) compared to the control group (108 ± 13.47 UI), suggesting potential hepatoprotective effects. Furthermore, even at the highest administered dose (32,000 µg/kg), ImmunoDefender did not significantly alter total protein levels, a key marker of liver function.

While the present study provides strong preclinical evidence supporting the safety and potential therapeutic benefits of ImmunoDefender, a key limitation is the lack of clinical data confirming its efficacy in humans [19]. Although *In vitro* docking studies and *In vivo* toxicity assessments suggest that ImmunoDefender is a promising candidate for COVID-19 treatment, clinical trials are necessary to establish its pharmacokinetics, optimal dosing, and long-term effects. Natural product-based therapies have historically faced challenges in clinical translation due to variability in bioavailability, metabolism, and patient response. Therefore, further research should focus on conducting randomized controlled trials to evaluate ImmunoDefender in human subjects.

CONCLUSION

This study provides strong preclinical evidence that ImmunoDefender is non-mutagenic and does not induce acute toxicity at the tested doses, supporting its safety profile. However, further research is necessary to determine its long-term effects, pharmacokinetics, and optimal dosing. Future studies should focus on mechanistic investigations to clarify its antiviral mode of action and *In vivo* efficacy assessments to validate its therapeutic potential. Most critically, clinical trials are essential to establish its safety and efficacy in human populations.

Acknowledgements

The authors are grateful to Mrs. Chaima Ghazweni and Dr. Rym Alouini from the National Veterinary University of Sidi Thabet (Tunis) for their valuable technical assistance. The coauthors also thank Dr. Nadia Kourda and Dr. Rania Bouchiba for facilitating theanatomopathological and biochemical study interpretations.

Conflict of interest

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. MB is designated as an inventor in a patent application filed by Biodex SA: Patent filed at INNORPI on Nov. 3, 2020, under registration number 02020/0211; PCT/IB2021/060180 registration Nov. 3, 2021: “Novel therapeutic composition based on essential oils at low doses.” The author MB declares no competing interests.

Financial Support

This work was supported in part by national MESRS research funding under the PAQ6 COLLABORA project.

Abbreviations

ALT: Alanine AminoTransferase;
AST: Aspartate AminoTransferase;
CPK: Creatinephospho-kinase;
EO: Essential Oil;
EPA: Environmental Protection Agency;
FDA: Food and Drug Administration;
MIC: Minimum Inhibitory Concentration;
NCE: New Chemical Entity;
CM: Camphor essential oil;
EUC: Eucalyptus essential oil,
MT: Menthol;
MP: Menthe pulegium essential oil,
CN: Cinnamon essential oil.

REFERENCES

1. Kumar A, Singh R, Kaur J, Pandey S, Sharma V, Thakur L, *et al*. Wuhan to world: the COVID-19 pandemic. Front Cell Infect Microbiol. 2021;11:596201:1-21.
2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, *et al*. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395(10223):497-506.
3. World Health Organization. Clinical management of COVID-19: interim guidance. Geneva: World Health Organization; 2022.
4. De Sousa DP, Damasceno ROS, Amorati R, Elshabrawy HA, de Castro RD, Bezerra DP, *et al*. Essential oils: chemistry and pharmacological activities. Biomolecules. 2023;13(7):1144: 112-32.
5. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. Ind Crops Prod. 2014;62:250-264.
6. Zhao H, Ren S, Yang H, Tang S, Guo C, Liu M, *et al*. Peppermint essential oil: its phytochemistry, biological activity, pharmacological effect and application. Biomed Pharmacother. 2022;154:113559.
7. Freires IA, Denny C, Benso B, de Alencar SM, Rosalen PL. Antibacterial activity of essential oils and their isolated

constituents against cariogenic bacteria: a systematic review. Molecules. 2015;20(4):7329-58.
8. Mieres-Castro D, Ahmar S, Shabbir R, Mora-Poblete F. Antiviral activities of eucalyptus essential oils: their effectiveness as therapeutic targets against human viruses. Pharmaceuticals (Basel). 2021;14(12):1210.
9. Ksouri A, Klouz A, Bouhaouala-Zahar B, Moussa F, Bezzarga M. Docking-based evidence for the potential of ImmunoDefender: a novel formulated essential oil blend incorporating synergistic antiviral bioactive compounds as promising Mpro inhibitors against SARS-CoV-2. Molecules. 2023;28(11):4296.
10. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014;4:177:1-10.
11. Brendler T, Al-Harrasi A, Bauer R, Gafner S, Hardy ML, Heinrich M, *et al*. Botanical drugs and supplements affecting the immune response in the time of COVID-19: implications for research and clinical practice. Phytother Res. 2021;35(6):3013-31.
12. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academies Press (US); 2011.
13. National Research Council (US) Committee on Recognition and Alleviation of Distress in Laboratory Animals. Recognition and alleviation of distress in laboratory animals. Washington (DC): National Academies Press (US); 2008.
14. Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA, Menichini F. Cytotoxic activity of essential oils from Labiatae and Lauraceae families against *In vitro* human tumor models. Anticancer Res. 2007;27(5A):3293-99.
15. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, *et al*. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395(10223):507-13.
16. Zhai T, Zhang F, Haider S, Kraut D, Huang Z. An integrated computational and experimental approach to identifying inhibitors for SARS-CoV-2 3CL protease. Front Mol Biosci. 2021;8:661424.
17. Hsu DZ, Li YH, Chu PY, Periasamy S, Liu MY. Sesame oil prevents acute kidney injury induced by the synergistic action of aminoglycoside and iodinated contrast in rats. Antimicrob Agents Chemother. 2011 Jun;55(6):2532-36.
18. Amorati R, Foti MC, Valgimigli L. Antioxidant activity of essential oils. J Agric Food Chem. 2013;61(46):10835-47.
19. Cristina da Costa Araldi I, Piber de Souza T, de Souza Vencato M, de Andrade Fortes T, Emanuelli Mello CB, Sorraila de Oliveira J, *et al*. Preclinical safety assessment of the crude extract from *Sida rhombifolia* L. aerial parts in experimental models of acute and repeated-dose 28 days toxicity in rats. Regul Toxicol Pharmacol. 2021;124:104974.

HOW TO CITE THIS ARTICLE
Klouz A, Ferchichi H, Ksouri A, Abidi O, Louati A, Souilem O, *et al*. ImmunoDefender: acute safety profile of a novel essential oil antiviral formulation. J Phytopharmacol 2026; 15(1):35-45. doi: 10.31254/phyto.2026.15105