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# Plant-Derived Essential Oils and Aqueous Extract as Potential Ingredients for a Biopesticide: Phytotoxicity in Soybean and Activity against Soybean Mosaic Virus

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**Abstract:** Soybean mosaic disease, caused by the soybean mosaic virus (SMV), is responsible for major losses in yield and seed quality worldwide. Although resistant cultivars are used for its prevention and control, an alternative strategy could consist of applying environmentally friendly antimicrobial agents, such as extracts and essential oils (EOs) of aromatic plants. This study assessed an extract of *Achyrocline satureioides* and EOs of *Minthostachys verticillata*, *Origanum vulgare*, and *Thymus vulgaris* in terms of their phytotoxicity in soybean. Since all the concentrations tested were found to be safe, the activity of each product against SMV was then assayed in vivo, i.e., in experimentally infected soybean plants. The parameters measured were plant height, wet weight, and virus titer. All the treated plants had a greater height and weight than those in the viral control group. The EOs of *M. verticillata* (0.80 mg/mL) and *T. vulgaris* (0.71 mg/mL) inhibited the production of viral antigens, as determined by an ELISA test. These findings could encourage further studies aimed at developing an effective biopesticide against SMV.

**Keywords:** Potyvirus; *Glycine max*; Biocontrol

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## 1. Introduction

Soybean (*Glycine max* (L.) Merry), a legume thought to have originated in northern/central China, is now grown around the world. Argentina is a leading producer: 16.1 hectares were cultivated in the 2021/2022 campaign, during which the average national yield amounted to around 27.7 qq/ha [1]. The seeds are medium-sized and rich in proteins and oils. They have a good balance of essential amino acids, especially lysine and leucine [2], which makes them a valuable addition to human and animal diets.

However, soybean cultivation is often affected by uneven weather, low germination percentages, inadequate growing time and planting space, poor seed irrigation, weeds, and diseases caused by microorganisms (fungi, bacteria, viruses). Among the latter, some of the most important are bacterial blight (the result of infection by *Pseudomonas savastanoi* pv. *glycinea*) [3], bacterial pustules (caused by *Xanthomonas axonopodias* pv. *glycinea*) [4],

brown spot (caused by *Septoria glycines*) [5], root and stem rot (for which *Rhizoctonia solani* is responsible) [6], and soybean leaf blight (whose causal agent is *Cercospora kikuchii*) [7]. Such diseases damage around 8–10% of the annual global production. They not only limit yields but can also deform the seeds and thus reduce their quality [3].

Viral diseases, more specifically, can be responsible for yield losses between 10 and 30%, though these numbers can range from 50 to 100% in the case of serious epidemics. One of the most frequent and destructive pathologies is soybean mosaic disease (SMD), which was first reported in 1915 in the US but is now detected in all soybean-producing countries. Caused by the SMV, it usually reduces yield by between 8 and 35%. Nevertheless, losses amounting to 90% have also been reported, since the damage depends on the soybean genotype and the SMV strain [8].

SMV, a positive-sense, single-stranded RNA virus, belongs to the family *Potyviridae* and to the genus *Potyvirus*. Its hosts are commonly from the Fabaceae family, but it can also infect other plants like *Passiflora* spp., *Pinellia ternata*, *Senna occidentalis*, and *Vigna angularis* [8,9]. It is transmitted by more than 30 types of aphids, including the Asian soybean aphid (*Aphis glycines*). The main source of inoculum is infected seeds. The transmission rates ascribed to seeds (secondary spread) can reach 75% but generally do not exceed 5% [10–14].

The virus enters the plant through natural openings or wounds caused by environmental factors or insect vectors. If the host cannot recognize and counteract its effectors, the virus establishes itself in the cells and tends to cause systemic infection. Its ability to decimate proteins and reduce the host's immune response explains why the disease can be so severe and difficult to control [12,15].

The symptoms vary according to the soybean cultivar, the plant's age at the time of infection, the virus strain, and the weather conditions (infections are more serious in cold temperatures). The leaves become mottled, wrinkled, distorted, and necrotic. They may display vein clearing, chlorotic areas, and shortened internodes. Additionally, the plant may be stunted, and the pods and seeds reduced in size and number [15]. The infected seeds also show mottling.

In general, diseases caused by phytopathogenic viruses are difficult to manage, since viruses evolve quickly, and pests are often involved as vectors. In the case of SMD, prevention and control strategies currently include the following: using cultivars that have been genetically engineered to be resistant or immune, planting virus-free seeds, rotating between alternative crop hosts, interrupting transmission routes (prophylaxis), controlling vectors through pesticides, eliminating the primary source of inoculum and infected plants in the field, and managing weeds. As with any other disease, the success of these practices may vary depending on the aggressiveness of the virus and/or the vector, the vulnerability of the plants, and different environmental factors. Better results are obtained when they are combined as part of integral management programs. Moreover, their appropriate implementation relies heavily on farmers having sufficient knowledge about epidemiology and the disease cycle.

Although resistant cultivars have proven particularly efficient in fighting SMV, they are associated with concerns about the insertion of foreign genes. In addition, several SMV strains can bypass their resistance and infect them [8,12,15]. For this reason, alternative methods for sustainable control are necessary. Natural antimicrobial agents derived from medicinal plants, such as essential oils and extracts, could prove useful as control tools for plant pathogenic viruses [3,16–20].

The strategies used at present for SMD prevention and control include good agricultural practices, breeding and genetic engineering of resistant cultivars, vector control, elimination of the primary source of inoculum and infected plants in the field, rotation of alternative hosts, and weed management. Even though resistant cultivars are a particularly effective and environmentally friendly approach, several SMV strains are still able to infect them [8,12,15]. Alternative methods for sustainable control are thus necessary. This is

where natural antimicrobial agents derived from medicinal plants, such as essential oils and extracts, could come in handy [3,16].

Among such plants, *Achyrocline satureioides* (marcela), *Minthostachys verticillata* (peperina), *Origanum vulgare* (oregano), and *Thymus vulgaris* (thyme) stand out for their antimicrobial, antiviral, antioxidant, and immunomodulatory properties [16,21–32]. The EOs that they synthesize as secondary metabolites are volatile compounds with potential uses in the pharmaceutical, food, agricultural, and cosmetic industries. The same is true for extracts from these plants. The EOs of *T. vulgaris* and *O. vulgare*, for instance, have shown bactericidal and phytotoxin-inhibitory activity against phytopathogens that infect soybean [16]. A study on soybean seeds infected with *Pseudomonas syringae* found that the application of *T. vulgaris* EO reduced the disease caused by the bacterium [3]. On the other hand, the EO of *M. verticillata* and pulegone, one of its main components, inhibited Suid herpesvirus 1 (SuHV-1) [33] and Herpes simplex virus type 1 [23]; and extracts of *A. satureioides* inhibited the Western equine encephalitis virus (WEEV). However, little is known about the effect of these natural products on phytopathogenic viruses.

In earlier studies, we characterized the cold aqueous extract of *A. satureioides* by HPLC-ESI-MS/MS, and detected the presence of quercetin, chlorogenic acid, luteolin, 5,7,8-trimethoxyflavone, 3-O-methylquercetin, and caffeic acid [26,34]. A GC–FID analysis by Escobar et al. (2012) [35] found that the most important compounds in the EO of peperina are pulegone (60.5%), menthone (18.2%), and limonene (3.76%); those in the EO of oregano are γ-terpinene (22.7%), carvacrol (19.7%), and cis-sabinene hydrate (19.7%); and in thyme EO they are carvacrol (29.5%) and p-cymene (31.5%). The last two EOs, moreover, contain low percentages of thymol [36].

The present study assessed the same extract and EOs in terms of their phytotoxicity in soybean and their activity against SMV in vivo, with the aim of finding evidence that may contribute to the ulterior development of a biopesticide against SMD.

## 2. Materials and Methods

### 2.1. Collection of Plant Material

Between 80 and 100 specimens of *Achyrocline satureioides* (Lam) DC were collected in the area of Villa Jorcoricó, in the southern mountains of the province of Córdoba (32°41′26″ S, 64°43′16″ W 800 m ASL). Collection was performed by hand during the flowering stage (in autumn, April–May). Only the shoots were cut; the roots were left in place to allow the plants to regrow. The collected specimens were taxonomically identified by Prof. Luis A. del Vitto at the National University of San Luis. One of them was deposited in that university's herbarium under exsiccate number 6362. Before the preparation of the extract, the plants were washed to remove dirt, insects, and foreign elements, and then left to dry in the open air at room temperature.

The same number of specimens of *Minthosthachys verticillata* (Griseb.) Epling (peperina) (leaves, shoots, and stems) were collected in Santa Rosa, Córdoba (32°04′08″ S, 64°32′10″ W 598 m ASL). They were taxonomically identified at the National University of Río Cuarto. One full specimen was stored in that university's herbarium of vascular plants under exsiccate number 1955. Collection was carried out in the same manner as for A. satureioides, and the plants were also subjected to washing and drying before they were used to obtain the extract.

Finally, 20 kg of leaves and stems of different cultivars of *Origanum vulgare* L. (oregano) and *Thymus vulgaris* L. (thyme) were purchased at a farm called "Los Molles", in the province of San Luis (32°31′34″ S, 64°58′43″ W 1820 m ASL).

### 2.2. Preparation of the Cold Aqueous Extract (CAE) of A. satureioides

The plant material was dried and ground, and 15 g were weighed and extracted with 700 mL of distilled water at room temperature for 2 days. The mixture was filtered through a cloth, and the resulting liquid was labeled "CAE". Then, the extract was lyophilized [37]. Its yield was determined by considering the weight of the initial material vs. the weight of

the final lyophilized powder. After that, the extract was resuspended in phosphate-buffered saline (PBS, pH 7), filtered through Whatman No. 2 paper, and sterilized through 0.22-µm pore cellulose acetate filters.

### 2.3. Obtention of the Essential Oils (EOs) of M. verticillata, O. vulgare, and T. vulgaris

The EOs were extracted from the aromatic plants by hydrodistillation in a Clevenger-type apparatus [38]. Briefly, an extraction column was filled with the plant material and placed on a grid. Water was boiled for 2 h in a 1-L balloon; upon passing through the plant-filled column, the steam carried away the volatile components to a condenser. Two phases were obtained by decantation: EO and water [39,40]. The EO was dried with anhydrous sodium sulfate and stored at 4 °C until it was used.

The concentration of each EO (mg/mL) was calculated based on the density of the oily compound, with the formula $\delta$ = M (g)/V (mL), where M = mass; V = volume. The mass of each EO was obtained from the average weight of five 1 mL fractions.

### 2.4. Phytotoxicity Assay

#### 2.4.1. Soybean Seeds

This assay (as well as the antiviral activity assay, see Section 2.5) was carried out with soybean seeds (cultivar Don Mario 4800 or DM 4800), donated by Dr Rodríguez Pardina from IPAVE-INTA, Córdoba. They were soaked in sodium hypochlorite (3%) for 3 min, rinsed five times with sterile distilled water, and left to dry on absorbent paper for 1 h in a sterile room [3].

#### 2.4.2. Determination of the Phytotoxicity of the Natural Products (NPs) in Soybean

Soybean seedlings (V2) were exposed to different concentrations of each natural product (NP). Different groups of plants (consisting of 10 specimens each) received the following treatments: CAE of *A. satureioides* (0.5, 1, and 1.1 mg/mL); EO of *M. verticillata* (0.4 and 0.8 mg/mL); EO of *O. vulgare* (0.46 and 0.92 mg/mL); or EO of *T. vulgaris* (0.35 and 0.71 mg/mL). Two control groups were included: a negative control (treated with water), and a DMSO control (treated with dimethyl sulfoxide 1/8 in water). The plants were sprayed at the beginning (day 0) and on days 7 and 14. Any observable changes during this time were recorded. When the treatments concluded (on day 21), the plants were evaluated again in terms of morphology, leaf color, height, and wet weight [3].

### 2.5. Antiviral Activity Assay

#### 2.5.1. Source of Soybean Mosaic Virus

The virus used in this assay was an SMV-MJ isolate [41], whose inoculum was recovered from a freeze-dried sample.

#### 2.5.2. Inhibitory Activity of the NPs against SMV Evaluated In Vivo by Applying Koch's Postulates

Soybean plants were grown under greenhouse conditions from DM 4800 seeds (see Section 2.4.1). They were then mechanically inoculated with the virus following Camelo García (2010) [42], with modifications. More precisely, the SMV-MJ isolate was inoculated with potassium phosphate buffer (0.05 M, pH 7.6) in the first emerged trifoliate leaf. Groups of 10 plants were sprayed 7 and 14 days post-inoculation (dpi) with the different NPs: CAE of *A. satureioides* (1.1 mg/mL); EO of *M. verticillata* (0.8 mg/mL); EO of *O. vulgare* (0.92 mg/mL); or EO of *T. vulgaris* (0.71 mg/mL), in independent trials. Symptoms were monitored daily until day 21 after inoculation. The NPs were considered to be effective when symptoms were absent or reduced.

A positive control (10 plants infected with SMV, left untreated), a negative control (10 uninfected plants sprayed with sterile distilled water), and a DMSO control (10 plants infected with SMV and treated with DMSO 1/8) were included.

### 2.6. Determination of Viral Replication Inhibition through Indirect ELISA

Twenty-one dpi, the inhibition of viral replication was indirectly measured by analyzing the virus titer with PTA-ELISA (Plate Trapped Antibody-Enzyme Linked Immunosorbent Assay) [43]. The primary antibody was a rabbit polyclonal anti-SMV serum, produced at IPAVE-INTA (unpublished data). The secondary antibody was a goat anti-rabbit IgG conjugated with alkaline phosphatase (BIO-RAD, Hercules, CA, USA). The reaction was detected by adding 0.75 mg/mL of p-Nitrophenyl Phosphate, Disodium Salt (PNPP) (Agdia Inc, Elkhart, IN, USA). Six healthy samples and one SMV-positive sample per plate were used as controls. The reactions were quantified in a Thermo Labsystem MultisKan MS spectrophotometer. A sample was considered positive when the absorbance at 405 nm ($A_{405}$) was higher than the mean of the healthy controls plus three times the standard deviation (cut-off), or 0.100.

### 2.7. Statistical Analysis

The data were statistically analyzed (ANOVA) on GraphPad Prism v6.0. Values were expressed as the mean ± standard deviation (SD). The data from the phytotoxicity assays and antiviral studies were compared with the parametric *t*-test. The level of significance was established at $p < 0.05$.

## 3. Results

### 3.1. Yield of NPs

The yields of the different products were as follows: CAE of *A. satureioides* 4.76% (*w*/*w*); EO of *M. verticillata* 5.1% (*w*/*v*); EO of *O. vulgare* 0.5% (*w*/*v*); and EO of *T. vulgaris* 1.9% (*w*/*v*).

### 3.2. Phytotoxicity Assay

The following concentrations of each product were tested for their phytotoxicity in soybean: 0.5, 1, and 1.1 mg/mL for the CAE of *A. satureioides;* 0.4 and 0.8 mg/mL for the EO of *M. verticillata;* 0.46 and 0.92 mg/mL for the EO of *O. vulgare;* and 0.35 and 0.71 mg/mL for the EO of *T. vulgaris*. A negative control group was included, which consisted of plants that did not receive any of the NPs. In those experiments where DMSO was used as a diluent, a solvent control was added. The measurements obtained for plant height (including shoots and roots) can be seen in Figure 1a,c,e,g. Those for wet weight are shown in Figure 1b,d,f,h.

Figure 1 shows that there were no statistically significant differences in plant height or wet weight between the negative control group and the treated plants, regardless of the NP concentration tested. This means that these concentrations are not phytotoxic, i.e., they do not affect the normal development of soybean plants. Phytotoxicity of NPs in soybean. (a) Plant height and (b) wet weight after exposure to CAE of *A. satureioides*; (c) plant height and (d) wet weight after exposure to EO of *M. verticillata*; (e) plant height and (f) wet weight after exposure to EO of *O. vulgare*; (g) plant height and (h) wet weight after exposure to EO of *T. vulgaris.* In all cases, significant differences with respect to the control were determined through the *t*-test, ANOVA ($p \leq 0.05$).

### 3.3. In Vivo Evaluation of the Inhibitory Activity of the NPs against SMV

The concentrations ascertained to be safe in the previous step were then assessed in vivo on a greenhouse scale, to determine their inhibitory activity against SMV. Soybean seedlings were experimentally infected with the virus and spray-treated independently with the different NPs. Once again, their total height and wet weight were individually measured for each treatment (Figures 2 and 3).

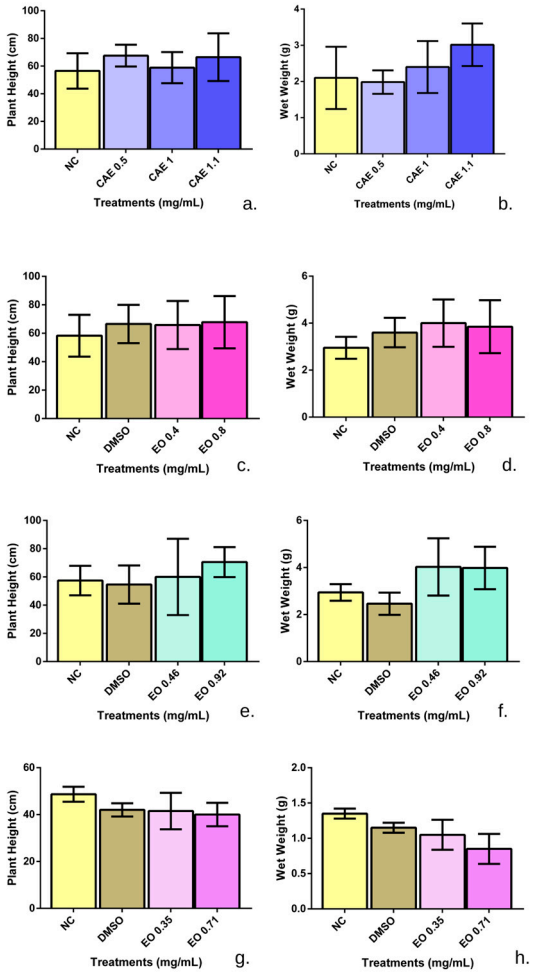


**Figure 1.** Phytotoxicity of NPs in soybean. (**a**) Plant height and (**b**) wet weight after exposure to CAE of *A. satureioides*; (**c**) plant height and (**d**) wet weight after exposure to EO of *M. verticillata*; (**e**) plant height and (**f**) wet weight after exposure to EO of *O. vulgare*; (**g**) plant height and (**h**) wet weight after exposure to EO of *T. vulgaris.* In all cases, significant differences with respect to the control were determined through the *t*-test, ANOVA ($p \leq 0.05$).

As seen in Figure 2, the untreated plants infected with SMV (positive control) were shorter than those in the negative control group ($p < 0.05$). This indicates that the virus significantly affects the normal growth of soybean seedlings. In turn, all the treated plants were taller than those in the positive control group. Statistically significant differences, however, were only registered for those plants treated with the CAE of *A. satureioides* ($p < 0.05$) and the EO of *M. verticillata* ($p < 0.01$), so these NPs seem to protect soybean plants from damage caused by the virus.

Moreover, the plants in the untreated positive control group weighed less than those in the negative control group ($p < 0.01$), which is further evidence of how the virus affects normal growth (Figure 3). The treated plants weighed more than the viral controls, in particular those sprayed with the EO of peperina ($p < 0.001$), which had the highest weight.

The photographs in Figure 4 show the leaves of treated plants and their negative and positive controls. A significant reduction in the spots and other symptoms produced by SMV was observable in the plants treated with the EO of *T. vulgaris*. For their part, the plants treated with the EO of *M. verticillata* did not exhibit any symptoms at all. These results are consistent with the statistical data, according to which the EO of *M. verticillata* is the most efficient among the NPs tested here in counteracting the effects of SMV.

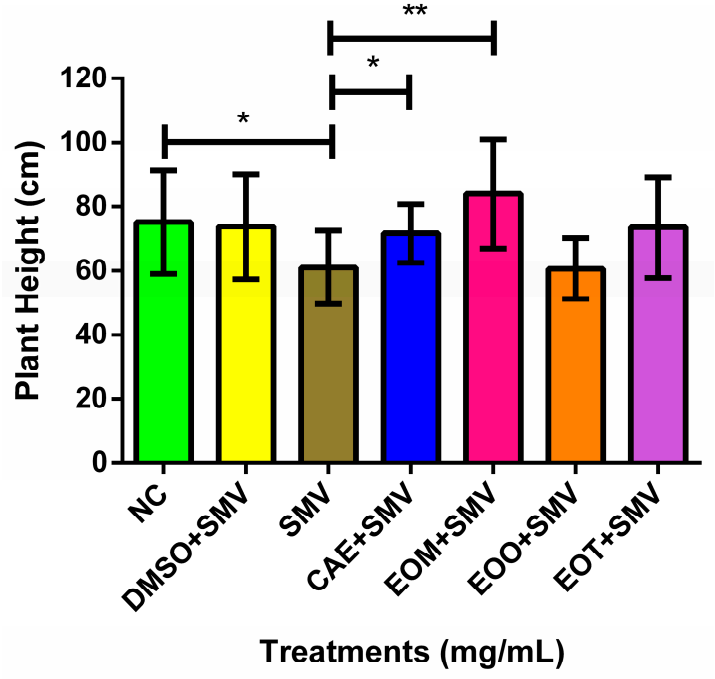


**Figure 2.** Inhibitory activity of NPs against SMV in vivo, as indicated by plant height (cm). NC: negative control; DMSO: dimethyl sulfoxide (solvent control); SMV: soybean mosaic virus; CAE: cold aqueous extract of *A. satureioides*; EOM: essential oil of *M. verticillate;* EOO: essential oil of *O. vulgare;* EOT: essential oil of *T. vulgaris*. Means with different * indicate statistically significant differences ($p < 0.05$). In all cases, significant differences with respect to the control were determined through *t*-test, ANOVA ($p \leq 0.05$). Ref.: * $p \leq 0.05$, ** $p \leq 0.01$.

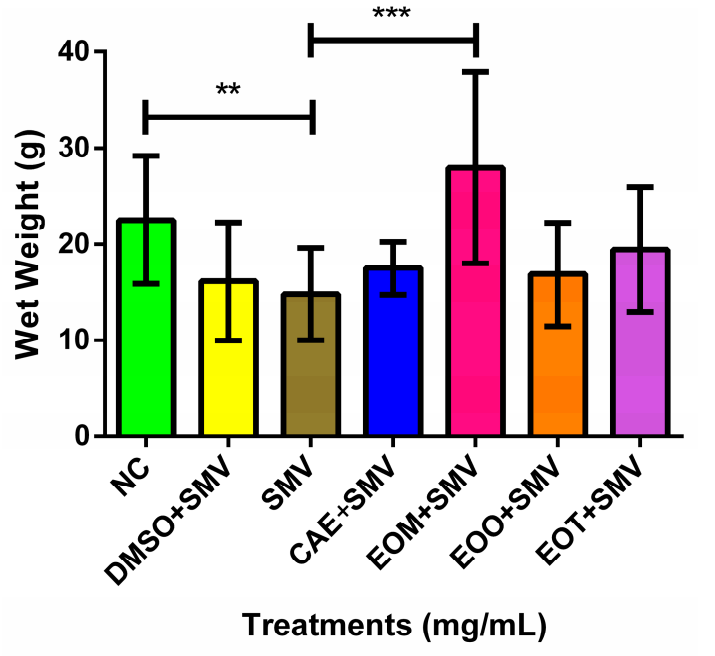


**Figure 3.** Inhibitory activity of NPs against SMV in vivo, as indicated by wet weight (g). NC: negative control; DMSO: dimethyl sulfoxide (solvent control); SMV: soybean mosaic virus; CAE: cold aqueous extract of *A. satureioides*; EOM: essential oil of *M. verticillate;* EOO: essential oil of *O. vulgare;* EOT: essential oil of *T. vulgaris*. Means with different * indicate statistically significant differences ($p < 0.05$). In all cases, significant differences with respect to the control were determined through *t*-test, ANOVA ($p \leq 0.05$). Ref.: ** $p \leq 0.01$, *** $p \leq 0.001$.

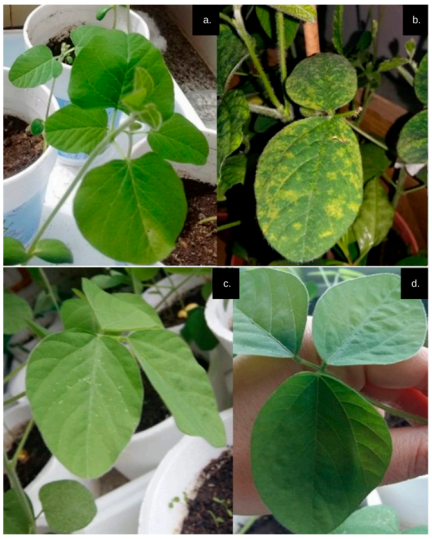


**Figure 4.** (**a**) Negative control; (**b**) positive control (SMV+DMSO); (**c**) SMV+EO of *T. vulgaris*; (**d**) SMV+EO *M. verticillate*.

### *3.4. Inhibition of Viral Replication Determined through Indirect ELISA*

Indirect ELISA, a technique that determines the amount of viral antigen (Ag), was used to assess viral inhibition after the application of the NPs. In other words, it was performed to detect the presence of the virus in the treated plants and the controls. The results of this test are shown in Figure 5.

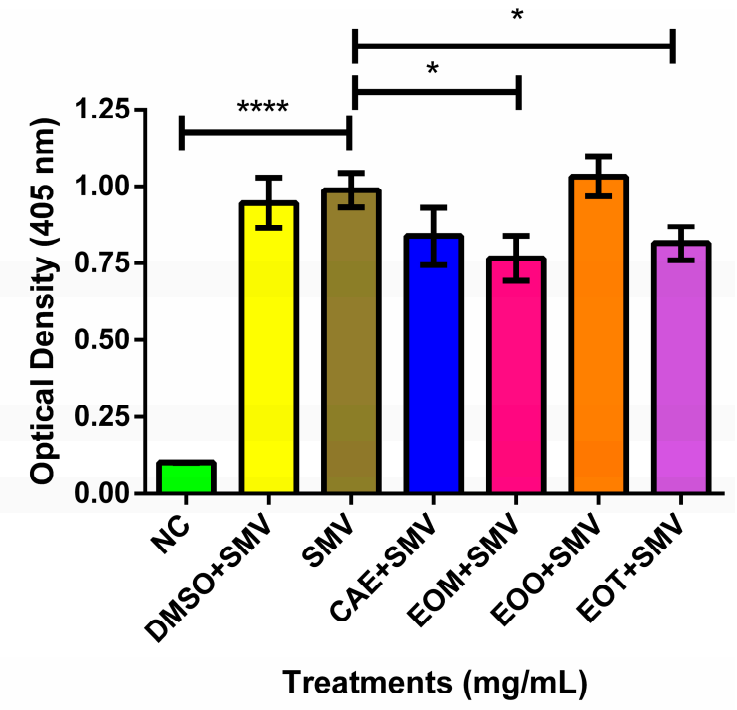


**Figure 5.** Inhibitory activity of NPs against SMV in vivo, as determined by indirect ELISA (405 nm). NC: Negative control; DMSO: dimethyl sulfoxide; SMV: soybean mosaic virus; CAE: cold aqueous extract of *A. satureioides*; EOM: essential oil of *M. verticillate*; EOO: essential oil of *O. vulgare;* EOT: essential oil of *T. vulgaris*. Means with different * indicate statistically significant differences ($p < 0.05$). In all cases, significant differences with respect to the control were determined through *t*-test, ANOVA ($p \leq 0.05$). Ref.: * $p \leq 0.05$, **** $p < 0.0001$.

As expected, a statistically significant difference ($p < 0.0001$) was found between the negative control and the untreated control infected with SMV. With respect to the latter, the EO of *M. verticillata* (0.80 mg/mL) and the EO of *T. vulgaris* (0.71 mg/mL) significantly inhibited viral antigen production or relative virus concentration. This concentration was not significantly altered by the other two products (CAE of *A. satureioides* at 1.1 mg/mL or EO of *O. vulgare* at 0.92 mg/mL).

## 4. Discussion

Among the microbial diseases that affect soybean, one of the most devastating is SMD, which causes significant economic losses around the world [12,13].

SMV, the virus responsible for this disease, spreads rapidly through insect vectors and seeds. Planting virus-free seeds and resistant cultivars, maintaining good agricultural practices, and striving to detect cases early on are some of the control strategies implemented at present [12,44]. Insecticides (which are sometimes applied to control the vectors) are not only toxic to the environment but also quite unsuccessful in reducing the incidence of SMV [13].

Given the demand for sustainable strategies to mitigate the harmful impact of microorganisms on agriculture, a lot of research currently focuses on exploring the antimicrobial properties of natural products. Many medicinal plants have antibacterial, antifungal, and antiviral activity, and can inhibit toxin production, biofilm formation, swarming, swimming, and other virulence factors [16,45–48]. However, any new substance with potential applications in medicine, agriculture, or the food sector must first be assessed in terms of its toxicity.

The present study found that the CAE of *A. satureioides* is not toxic for soybean at the concentrations tested. Although no other research had previously looked into the phytotoxicity of this product, an assay performed on cultures of mouse marrow cells demonstrated it was not genotoxic. In addition, high concentrations of the extract (50 mg/kg p.c.) were safe for mice [34].

When we used this CAE (1.1 mg/mL) to treat soybean plants infected with SMV, both plant height and wet weight improved with respect to the untreated infected specimens, but only the first parameter was significantly different. An aqueous extract of *A. satureioides* was previously reported to act against the Western Equine Encephalitis virus (WEEV) at the intracellular replication stage [26]. This alphavirus, which infects humans and horses and for which there are no other effective antivirals, has a similar genome to that of SMV: the two of them are positive-sense, single-stranded RNA viruses, and may thus be inhibited through similar mechanisms. The antiviral properties of the extract are likely related to the action of quercetin, one of its components, which was effective on its own against other RNA and naked viruses such as poliovirus type 1 [49] and respiratory syncytial virus [50].

Marcela has also been successful against different bacterial species. A hexanic extract showed inhibitory and acidic properties in vitro against mechanisms related to the pathogenicity of *Paenibacillus larvae* (biofilm formation, swarming, synthesis of proteases). *P. larvae* attacks honeybee hives, and the extract was assessed to be safe for larvae and adult bees [32]. Other bacteria which the extract has been observed to antagonize include *Enterococcus faecalis*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar *enteritidis*, and *Staphylococcus aureus* [51]. A decoction of *A. satureiodes* not only inhibited *Staphylococcus* spp. almost completely, but also immunostimulated T CD8+ lymphocytes in humans [23].

The application of EO in aqueous media is limited due to their hydrophobicity and volatile nature, so DMSO is used in low proportion to aid dissolution in water in both in vivo and in vitro experiments. In all our EO assays, emulsions were prepared with this solvent plus sterile water. In our phytotoxicity studies, a solvent control was performed evaluating the possible toxic effect of DMSO: water (at a concentration of 12.5%) on soybean plants, and no toxic damage (in any of the parameters evaluated) was observed in the tested plants.

Regarding previous studies of DMSO toxicity, Kloverpris et al. (2010) [52] exposed in vitro human PBMC to a concentration of 10% for 1 h not affecting the viability. Similar results were obtained in vivo when a dose of 0.22 g DMSO/kg/day was administered in NOD mice not producing diarrhea or weight loss [53]. Other trials demonstrated that minimal side effects were induced during clinical applications [54] only when concentrations of intravenous infusion greater than 40% were tested. They produced intravascular hemolysis [55]. These investigations demonstrated that low concentrations of DMSO in animal cell cultures and in animal species were safe. Therefore, this solvent can be used for a variety of clinical in vitro or in vivo treatments.

Our study is the first report on the toxicity of DMSO in plants; a concentration of 12.5% was shown to be safe for soybean plants. Similarly, antiviral studies included a DMSO control group, infected with SMV and treated with DMSO:water (12.5%) in which no statistically significant difference from the untreated viral control was observed.

On the other hand, EOs and their components have attracted significant scientific interest thanks to their beneficial properties for general health and their lack of toxicity. Their antimicrobial and antioxidant abilities have been explored for their incorporation into food [48], but the present study investigated three of them in relation to their potential benefits for the protection of soybean.

According to our findings, the EO of *M. verticillata* is not toxic for soybean. Earlier research had proven its safety in murine and bovine models [56,57], in human peripheral blood mononuclear cells (PBMC), mouse bone marrow cells [35], porcine and equine blood cells [58], and even brine shrimp (*Artemia salina)* [59,60]. In short, the EO of *M. verticillata* appears to be a safe natural product that could be used on agriculturally important crops destined for human and animal consumption. Several studies compared the toxicity of this EO as a whole against that of some of its components on their own. Rossi et al. (2012) [61] reported that the EO was less toxic than pulegone and menthone for houseflies (*Musca domestica*). Similarly, Vogt et al. (2010) [33] observed that the 50% cytotoxic concentration ($CC_{50}$) values for the oil were higher than for its major components in HEp-2 cells.

In terms of its protective activity, the EO of peperina (0.8 mg/mL) was effective in protecting plants against SMV infection in the present study, as shown by the plants' significantly greater height and weight compared to infected specimens that were not treated. Furthermore, the virus titer or concentration was significantly reduced after this product was applied, and none of the characteristic symptoms of SMD appeared in the plants sprayed with it. Previous studies had confirmed its activity against viruses that infect animal hosts, such as Suid herpesvirus 1 (SuHV1) [33], and Herpes simplex virus type I (HSV-1) [23]. As with *A. satureiodes,* this activity might be due to the action of specific components in *M. verticillata*. Pulegone was observed to inhibit SARS-CoV-2 [62], and limonene, a monoterpene, reduced the infectivity of HSV-1 by 100%, by exerting antiherpetic activity during the early phase of viral multiplication [63].

In addition to inhibiting viruses, this EO showed protective power against the damage caused by aflatoxin B1 (AFB1), a mycotoxin. More precisely, it improved the histomorphometry of intestinal villi and reduced DNA damage in bone marrow cells of male Wistar rats in which AFB1 toxicity had been induced [64]. Evidence of its activity against enteropathogenic bacteria (*S. aureus*, *S. faecalis*, *Bacillus cereus*, *E. coli*, and *Salmonella typhi*) supports its popular use for digestive troubles [35]. Its ability to interfere with growth and biofilm formation in bacteria that cause bovine mastitis has been described as well [47].

The safety of *O. vulgare* EO for soybean was also confirmed in our study. Twenty-one days after being sprayed with 0.46 and 0.92 mg/mL of the oil, none of the plants displayed alterations in their growth parameters. This agrees with the results by Gonçalves et al. (2021) [65], who treated tomato seeds (*Solanum lycopersicum*) with 1.2 mg/mL of oregano EO and recorded no phytotoxicity.

Less is known about its antiviral potential. Polar extracts of *O. vulgare* (infusion, decoction, and hydroalcoholic extract) completely inhibited bovine alphaherpesvirus 1 (BoHV-1), responsible for bovine infectious rhinotracheitis [66]. An aqueous extract was

active against Equine Arteritis Virus (EAV), and aqueous and ethanolic extracts were effective against canine distemper virus (CDV) [67]. Nevertheless, we were unable to reliably demonstrate that the EO of this plant can inhibit SMV at the concentration tested (0.92 mg/mL), since there were no significant differences after treatment in plant height, wet weight, or virus titer. Further studies should perhaps try out other concentrations, or test this EO in combination with other NPs.

As with the other two EOs, that of *T. vulgaris* was not toxic for soybean at 0.35 and 0.71 mg/mL, since there were no significant differences in growth between treated plants and the untreated control. Other researchers similarly recorded no statistically significant modifications in growth after treatment with *T. vulgaris* EO in soybean (at 1.76 mg/mL) [3], and potato (0.170 mg/mL) [68]. In contrast, the oil was deemed highly toxic for brine shrimp and for cancer cell lines in vitro [69]. Its vapors slightly affected the germination of wheat seeds [70], and produced some scalding in apple tissues [71]. This likely means that the toxicity of this EO depends on the concentration and the sensitivity of the species on which it is applied.

We observed that when sprayed at 0.71 mg/mL, it improved height and weight in infected plants with respect to the control. Moreover, it reduced symptoms and viral antigen production in a statistically significant manner. Although several studies have focused on its antimicrobial properties [16,24,72–74], this is the first time it has proven to be effective against a phytopathogenic virus.

Still, there is evidence of its ability to antagonize animal viruses. For instance, it affected the replication of retroviruses like human immunodeficiency virus 1 (HIV-1) [75], and other RNA viruses such as the feline infectious peritonitis virus (*Coronaviridae*). In the last case, a higher concentration than the one tested here (27 µg/mL) reduced virus concentration by 2 log10 TCID50/50 µL [29]. When tested against HSV-1 at 25 µg/mL, it achieved 45% inhibition, while 25 µg/mL of p-cymene (one of its main terpene components) achieved around 70% inhibition [76]. The antiviral activity observed in our assay might therefore be related to the action of this compound. Likewise, an aqueous extract of thyme exerted antiviral activity against HSV-1 and HSV-2 [77].

As far as bacteria are concerned, the EO of *T. vulgaris* has been reported to successfully antagonize *P. syringae* isolated from soybean and reference phytopathogenic strains in vitro [45]. Its effectiveness against *P. syringae* (as well as against *P. savastanoi pv. Glycinea* B076) was then confirmed in vivo in soybean [3] and explained in terms of an ability to interfere with phytotoxin production, biofilm formation [16], swarming, and swimming in these bacteria [31]. Microencapsulated in maltodextrin and hydroxypropyl methylcellulose, thyme EO inhibited *Streptomyces scabiei*, the causative agent of common potato scab [68]. Its antifungal activity, on the other hand, has been documented on *Fusarium avenaceum*, *Botrytis cinerea*, *Penicillium expansum*, *Neofabraea vagabunda* [71], *P. infestans, R. solani* [78], *Drechslera* [70], *Candida albicans,* and *Candida tropicalis* [79]. Finally, it had insecticidal activity on the larvae of the *Aedes aegypti* mosquito, the main vector of urban arboviruses [80], and reduced the survival and longevity of bean weevil adults (*Acanthoscelides obtectus*). It also retracted ovipositioned females of this species [81].

In short, the EO of *T. vulgaris* has a broad spectrum of action, which comprises viruses, bacteria, fungi, and even insects. Nevertheless, more information is necessary about the antiviral capacities of its specific components.

In terms of the specific mechanisms through which these and other NPs fight viruses, several possibilities are feasible. Monoterpenes might bind to viral proteins involved in host cell penetration, and thus prevent the virus from entering the cell [82]. As was mentioned earlier for an aqueous extract of *A. satureioides*, viral replication inside the cell may also be interrupted through molecular interactions between the virus and the natural agents. Lastly, although the present study did not explore this, EOs and extracts may not act directly against the virus, but rather on the host plant by enhancing its immunity. When tomato plants infected with the tobacco mosaic virus were treated with zinc oxide (ZnO)

nanoparticles (synthesized with an aqueous extract of *Mentha spicata*), systemic acquired resistance (SAR) was induced, and disease symptoms were diminished [83–85].

## 5. Conclusions

All the natural products assessed in this study appear to be safe for soybean and had positive effects on plants infected with SMV. The most relevant improvement after the application of the CAE of *A. satureioides* was observed in plant height. The EO of *T. vulgaris* also improved plant height and weight and managed to inhibit the virus, as demonstrated by the statistically significant reduction in the virus titer. The best results, both in terms of plant growth and viral inhibition, were obtained with the EO of *M. verticillata*. Plants sprayed with this product showed no symptoms of disease, i.e., the EO was able to control infection. The only product for which no statistically significant effects could be demonstrated was the EO of *O. vulgare*. Although these findings are conclusive, further research should corroborate them by working with larger sampling sizes and under less or no controlled conditions, e.g., in the greenhouse and in the field. Furthermore, the NPs could also be tested for their ability to inhibit other plant pathogenic viruses. Given the promising performance of the EO of *M. verticillata* in the assays described here, its potential applications may also deserve more in-depth exploration. Finally, a combination of NPs could be assayed, with the ultimate aim of formulating a "biopesticide" to manage SMD sustainably.

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