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In Vitro and *In Vivo* Anti-Tobacco Mosaic Virus Activities of Essential Oils and Individual Compounds

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Essential oils are increasingly of interest for use as novel drugs acting as antimicrobial and antiviral agents. In the present study, we report the *in vitro* antiviral activities of 29 essential oils, extracted from Chinese indigenous aromatic plants, against the tobacco mosaic virus (TMV). Of these essential oils, those oils from ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass effected a more than 50% inhibition of TMV at 100 μg/ml. In addition, the mode of antiviral action of the active essential oils was also determined. Essential oils isolated from artemisia and lemongrass possessed potent inactivation and curative effects *in vivo* and had a directly passivating effect on TMV infection in a dose-dependent manner. However, all other active essential oils exhibited a moderate protective effect *in vivo*. The chemical constitutions of the essential oils from ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass were identified by gas chromatography and gas chromatography-mass spectrometry. The major components of these essential oils were α-zingiberene (35.21%), limonene (76.25%), terpinen-4-ol (41.20%), limonene (80.95%), 1,8-cineole (27.45%), and terpinolene (10.67%). The curative effects of 10 individual compounds from the active essential oils on TMV infection were also examined *in vivo*. The compounds from citronellal, limonene, 1,8-cineole, and α-zingiberene effected a more than 40% inhibition rate for TMV infection, and the other compounds demonstrated moderate activities at 320 μg/ml *in vivo*. There results indicate that the essential oils isolated from artemisia and lemongrass, and the individual compound citronellal, have the potential to be used as an effective alternative for the treatment of tobacco plants infected with TMV under greenhouse conditions.

Key words: Antiviral activity, tobacco mosaic virus, essential oil, individual compound, chemical composition

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Viral plant diseases can be found worldwide and are a serious threat to modern agriculture. The tobacco mosaic virus (TMV), one of the most common viral diseases in plants, is a rod-shaped virus composed of single-stranded RNA encapsulated in a coat protein capsid. TMV can infect members of 9 plant families and at least 125 individual species, including tobacco, tomatoes, peppers, cucumbers, and a number of ornamental flowers [24]. The amount of plant loss due to TMV infection can vary from 5% to 90% depending on the strain of TMV, duration of TMV infection, temperature during disease development, and presence of other pathogens [32]. Most approaches used to control TMV have involved treating the plants with chemical pesticides or using transgenic processes. However, the misuse of non-biodegradable chemicals may inflict severe environmental pollution, health disorders, and toxicity to non-target organisms [22]. Moreover, transgenic technology has not yet been universally accepted. Therefore, there is still a great need for the discovery of novel, more effective, environmentally friendly antiviral methods.

The antimicrobial properties of plant products have been recognized and used as potentially useful products for commercial pesticides or as lead compounds [5, 14]. Among the different groups of plant products, essential oils are especially recommended as one of the most promising groups of natural products for the formulation of safer antimicrobial agents [7, 17]. Many essential oils are classified as "generally regarded as safe" (GRAS) by the United States Food and Drug Administration (FDA), and so are potential targets for developing natural antimicrobial

products owing to their safety with eukaryotic systems [29, 30]. Essential oils are known to induce a wide range of biological effects through their antibacterial, antioxidant, antifungal, and antimutagenic activities [2, 18, 20, 25]. Several studies have demonstrated the antiviral activities and antiviral mechanisms of various types of essential oils and individual compounds against herpes simplex virus type 1 (HSV-1), HSV-2, influenza A/PR/8 virus, infectious bronchitis virus (IBV), and SARS-CoV *in vitro* and *in vivo* [4, 11, 13, 15, 26]. Nevertheless, the efficacy and antiviral mechanisms of essential oils and individual compounds on viral infections, or the viral replication cycles, in plant systems have not been well studied.

In the current study, we have evaluated the antiviral activities of 29 essential oils isolated from Chinese indigenous aromatic plants against TMV *in vitro*; estimated the effects of inactivation, curative properties, and the protective attributes of 6 active essential oils (*i.e.*, ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass) *in vivo*; determined the chemical constituents of these active essential oils by gas chromatography (GC) and GC-mass spectrometry (MS); and further estimated the curative effects of 10 major constituents of the active essential oils *in vivo*. The ultimate goal of our study was to identify potential antiviral agents, or lead compounds, which could be used as commercial chemicals for the protection of tobacco plants from TMV infection under greenhouse conditions.

Materials and Methods

TMV Suspension

The TMV (strain U1) was propagated in *Nicotiana tabacum* cv. K_{326} and purified as described by Gooding and Hebert [12]. The concentration of TMV was 40 mg/ml, determined with an ultraviolet spectrophotometer [virus concentration = (A_{260} × dilution ratio)]/ $E^{0.1\%,\ 260\ nm}$]. The purified virus was stored at −20°C and was diluted to 20 μg/ml with 0.01 mol/l PBS before use.

Plant Essential Oils and Individual Compounds

The plant species used in this study are listed in Table 1. Some of the essential oils were purchased from Shanghai Apple Flavor & Fragrance Company Ltd. (Shanghai, China); the rest of the essential oils were produced in our laboratory by steam-distillation for 3 h using a Clevenger-type apparatus (Ildam, Ankara, Turkey) according to the procedures described in the *European Pharmacopoeia*. The latter plant materials were harvested from the vicinity of Shanghai, China. Identification of these plant materials was initially made using morphological features and then confirmed by Prof. Lei Yao at the School of Agriculture and Biology, Jiao Tong University (Shanghai, China). The obtained essential oils were stored at 4°C in airtight glass vials covered with aluminum foil.

The individual compounds contained in the essential oils were procured as follows: α-zingiberene (≥98%), limonene (≥99%), terpinen-4-ol (≥99%), citral-a (≥95%), and 1,8-cineole (≥99%) (Sinopharm Chemical Reagent Company Ltd., Shanghai, China); γ-terpinene (≥98%), bornyl formate (≥98%), isocaryophyllene oxide (≥97%), α-terpineol (≥98%), and terpinolene (≥95%) (Tokyo Kasei, Tokyo, Japan).

Antiviral Biological Assay of Essential Oils

The antiviral activity of the essential oils was assessed according to the inhibition percentage towards the number of local lesions in *Nicotiana glutinosa* leaves that were cultivated in an insect-free greenhouse. The experiments were conducted when the plants grew to the 5–6 leaf stage. The tested essential oils were dissolved in DMSO [2% (v/v)] and diluted with distilled H_2O to the required concentrations. A solution of an equal concentration of DMSO was used as the negative control. The commercial antiviral agent ningnanmycin, the most successful registered antiviral agent, was used as the positive control [8].

Table 1. Plant essential oils tested.

Essential oil	Plant species	Tissue studied	Essential oil	Plant species	Tissue studied
Artemisia[a]	*Artemisia argyi*	Leaves	Lemon[a]	*Citrus lemon*	Peel of fruits
Basil[a]	*Ocimum basilicum*	Leaves	Lemongrass[a]	*Cymbopogon citratus*	Leaves
Cedar	*Cedrus atlantica*	Fruits	Litsea	*Litsea cubeba*	Fruits
Chamomile	*Matricaria chamomilla*	Flowers	Myrte[a]	*Myrtus communis*	Twigs and leaves
Cinnamomum	*Cinnamomum camphora* L.	Leaves	Nutmeg	*Myristica fragrans*	Seeds
Cinnamon a	*Cinnamomum zeylanicum* L.	Bark	Peppermint[a]	*Mentha piperita*	Whole plant
Clary sage	*Salvia sclarea* L.	Leaves	Pine	*Pinus massoniana*	Twigs and leaves
Corn mint[a]	*Mentha arvensis*	Whole plant	Rosemary[a]	*Rosmarinus officinalis*	Leaves
Cumin	*Cuminum cyminum*	Seeds	Sage	*Salvia officinalis* L.	Leaves
Cypress	*Cupressus sempervirens*	Twigs	Spearmint[a]	*Mentha spicata*	Whole plant
Eucalyptus	*Eucalyptus globulus*	Leaves	Sweet orange	*Citrus sinensis*	Peel of fruits
Fennel	*Foeniculum vulgare*	Seeds	Tangerine peel[a]	*Citrus reticulata blanco*	Peel of fruits
Geranium[a]	*Pelargonium graveolens*	Twigs and leaves	Tea tree[a]	*Melaleuca alternifolia*	Twigs and leaves
Ginger[a]	*Zingiber officinalis*	Fruits	Thyme[a]	*Thymus vulgaris*	Twigs and leaves
Lavender[a]	*Lavandula angustifolia*	Leaves			

[a]Essential oils from these plants were collected by steam-distillation using a Clevenger-type apparatus in our laboratory. The remaining essential oils were purchased from a commercial corporation.

In Vitro Antiviral Screening Assay of 29 Essential Oils

In vitro screening assays to determine the anti-TMV activities of the 29 essential oils were performed using the conventional leaf inoculation method in culture dishes [8, 28]. Fresh leaves, at growth stage 5–6, were inoculated by the juice–leaf rubbing method with TMV suspension (TMV concentration, 20 μg/ml), and then cut in half along the main vein. After 3 h, the halved leaves were immersed in the essential oil solutions (concentrations, 100 μg/ml), as was the negative control, for 30 min, respectively, and then they were taken out and dried. The culture dishes containing treated leaves were cultured at 25°C for 3–4 days in an illuminated incubator (24 h/ daylight). The local lesion numbers in the halved leaves were recorded 3–4 days after inoculation, respectively. Ten leaves were used per treatment, and each experiment was repeated 5 times.

In Vivo Biological Antiviral Assays of Active Essential Oils

The inactivation of TMV by active essential oils *in vivo* was assessed by methods that have been previously described [8, 28]. First, the viral suspension (TMV concentration 40 μg/ml) was inhibited by mixing at a 1:1 ratio with the essential oil solutions (160, 320, and 640 μg/ml) for 1 h. After 1 h, 20 μl of the virus/ essential oil mixtures were then inoculated on the leaves of *N. glutinosa*; a mixture of DMSO and the viral suspension were inoculated as a control. Ten leaves were used per treatment; the local lesion numbers were recorded 3–4 days after the inoculations.

The curative effects of the active essential oils against TMV infection *in vivo* were examined as has been previously described [8, 28]. Growing leaves from age-matched *N. glutinosa* plants were selected. A 20 μl volume of TMV (TMV concentration 20 μg/ml) was pre-inoculated on the surface of the leaves. After 5 h, the essential oil solutions (80, 160, and 320 μg/ml) were smeared on the inoculated leaves; DMSO solution was used as the control. Ten leaves were used per treatment; the local lesion numbers were then counted and recorded 3–4 days after the inoculations.

The ability of the active essential oils to protect against TMV infections *in vivo* was evaluated through methodology previously described [8, 28]. Briefly, the essential oil solutions (80, 160, and 320 μg/ml) were pre-smeared on the growing age-matched leaves of *N. glutinosa*, with DMSO solution acting as the negative control. After 5 h, a 20 μl volume of the TMV inoculums (TMV concentration 20 μg/ml) was applied to the pre-treated surfaces of the leaves using swabs. Ten leaves were used per treatment; the local lesion numbers were counted 3–4 days after the inoculations.

The *in vitro* and *in vivo* inhibition rates of each essential oil were then calculated according to the following formula:

$$\text{Inhibition rate (\%)} = [(C - T)/C] \times 100\%$$

where C is the average number of local lesions in the control sample, and T is the average number of local lesions in the treated sample.

Chemical Analyses of Active Essential Oils

GC-flame ionization detection (FID). Analyses of the components of the essential oils were carried out using an Agilent 6890N chromatography system equipped with an FID and a fused-silica capillary column (HP-5MS: 30 m × 0.25 mm i.d., film thickness 0.25 μm). The operating conditions were as follows: Initial oven temperature of 60°C for 10 min, then raised to 220°C by increments of 4°C/min and held at 220°C for 10 min, and then raised to 240°C by increments of 1°C/min; injector and detector temperatures, 250°C and 270°C, respectively; carrier gas, N_2 (0.8 ml/min); split ratio, 50:1; manual sample injection, 0.2 μl. Retention indices (RI) were determined by a series of *n*-alkanes (C6−C28). Determinations of the relative amounts of the individual components were based on peak areas obtained with FID response factor corrections.

GC-MS. Constituents of the essential oils were confirmed using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N mass spectrometer and a fused-silica capillary column (HP-5MS: 30 m × 0.25 mm i.d., film thickness 0.25 μm). Helium was used as the carrier gas. The mass spectrometer operating conditions were as follows: Ionization voltage, 70 eV; ion source, 250°C. The mass range, from 35 to 500 u, was scanned at 1 scan/s. The GC analyses conditions were as described above.

Identification of constituents. The identification of volatile components was assigned by a comparison of their retention indices (RI) relative to *n*-alkanes (C6−C28) and mass spectra, with those of authentic compounds, by means of the NIST databases and through the literature data [1].

Curative Effects of Individual Compounds Against TMV *In Vivo*

The curative efficacies of the main compounds from active essential oils against TMV infection *in vivo* were examined, at a concentration of 320 μg/ml, by the means that has been described above. A 20 μl volume of TMV (TMV concentration 20 μg/ml) was inoculated on the surface of the leaves. After 5 h, the individual compound solutions were smeared on the inoculated leaves; DMSO solution was used as the control. The local lesion numbers were then counted and recorded 3–4 days after the inoculations. Ten leaves were used per treatment, and each experiment was repeated 5 times.

Data Analyses

All the measurements were performed 5-fold for each treatment, and the data reported as the mean ± standard deviation. The significant differences between mean values were determined by Duncan's Multiple Range test ($p < 0.05$), following one-way analyses of variances (ANOVA). The statistical analyses were performed using SPSS, ver. 18.0 (Chicago, USA).

RESULTS

In Vitro Antiviral Activity Screening of 29 Essential Oils

The *in vitro* inhibitory efficacies of TMV infection by the 29 essential oils from the Chinese indigenous aromatic plants and the commercial antiviral agent ningnanmycin (concentration 100 μg/ml) are shown in Fig. 1. Thirteen essential oils exhibited inhibitory activities against TMV infections *in vitro*, with inhibition rates ranging from 21.0% to 77.9%. Of these, the essential oils isolated from lemongrass, tangerine peel, lemon, tea tree, artemisia, and ginger demonstrated a more than 50% inhibition of TMV at 100 μg/ml, with inhibition rates of 77.9%, 76.0%, 63.5%, 60.7%, 56.5%, and 53.7%, respectively. The essential oils from lemongrass and tangerine peel exhibited higher

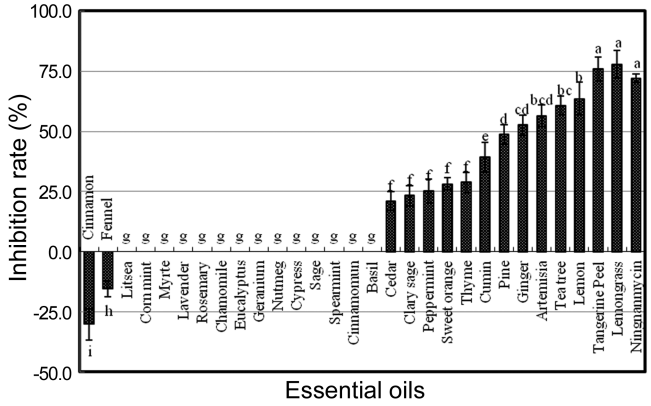

Fig. 1. Anti-TMV activities of 29 essential oils *in vitro*.
Essential oils were evaluated at a concentration of 100 μg/ml. Data are expressed as the mean (column) ± SD (thin bar) of 5 independent experiments. Different letters indicate significant differences between various essential oils ($p < 0.05$).

antiviral activities than the other oils ($p < 0.05$). Compared with ningnanmycin (inhibition rate, 72.1%), no significant differences were found with respect to the antiviral activities of lemongrass and tangerine peel essential oils at 100 μg/ml ($p > 0.05$). In addition, the essential oils isolated from cedar, clary sage, peppermint, sweet orange, thyme, cumin, and pine demonstrated moderate antiviral activities. The remaining essential oils investigated in this study showed no anti-TMV activities.

The inactivating, curative, and protective properties of 6 active essential oils against TMV are shown in Fig. 2. Of the 6 oils examined in detail, as represented in Fig. 2A, artemisia and lemongrass essential oils had a significantly stronger inactivating effect than the essential oils of ginger, lemon, tea tree, and tangerine peel ($p < 0.05$), with inhibition rates of 67.2% and 62.1% at 320 μg/ml, respectively. There were no significant differences between the inactivation effects of artemisia/lemongrass essential oils, and ningnanmycin, at doses of 320, 160, and 80 μg/ml ($p > 0.05$), with results of 66.3%, 46.8%, and 38.1%, respectively. On the other hand, as is shown in Fig. 2B, artemisia and lemongrass essential oils also had a significantly higher curative effect than other applied essential oils ($p < 0.05$), with inhibition rates of 57.1% and 55.3% at 320 μg/ml, respectively. Compared with ningnanmycin, which exhibited 50.2%, 40.3%, and 28.5% inhibitions of TMV at doses of 320, 160, and 80 μg/ml, respectively, these essential oils showed no significant differences at all concentrations ($p > 0.05$; Fig. 2B). By contrast, for protective effect, all active essential oils exhibited a below 40% inhibition rate of TMV infection. At a concentration of 80 μg/ml, only lemon and tangerine peel essential oils possessed weak protective activity against TMV infection (Fig. 2C).

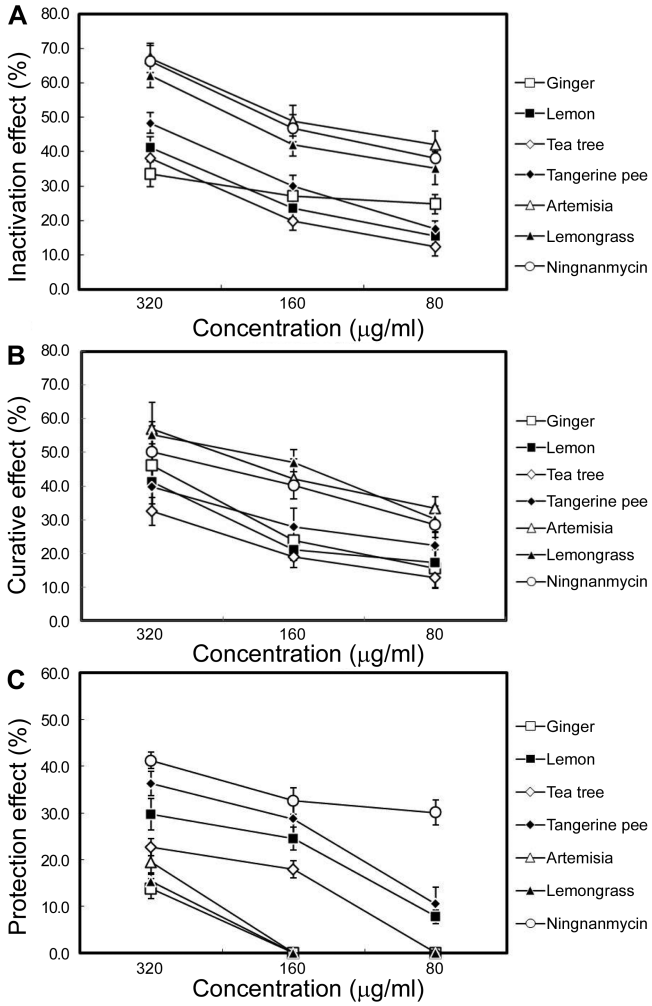

Fig. 2. Inactivation (**A**), curative (**B**), and protective (**C**) effects of essential oils isolated from ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass against TMV infections *in vivo*.
The essential oils were evaluated at concentrations of 320, 160, and 80 μg/ml. Results are expressed as inhibition percentages of local lesions relative to the control. Data are expressed as the mean (column) ± SD (thin bar) of 5 independent experiments.

Chemical Analyses of Active Essential Oils

The chemical compositions of the 6 active essential oils (*i.e.*, ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass) are shown in Table 2. In ginger oil, 17 compounds were identified, representing 93.60% of the total oil; the major constituents were α-zingiberene (35.21%) and β-cedrene (12.42%). When lemon oil was analyzed, 11 compounds were identified, representing 97.88% of the total oil; the major constituent was limonene (76.25%). In tea tree oil, 13 compounds were identified, representing 94.87% of the total oil; the main components were terpinen-4-ol (41.20%) and γ-terpinene (17.33%). In tangerine peel oil, 10 compounds were identified, representing

Table 2. Chemical composition of the essential oils isolated from ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass.

Component	RI [a]	RI[b]	Peak area (%)					
			Ginger	Lemon	Tea tree	Tangerine peel	Artemisia	Lemongrass
α-Pinene	939	932	1.86	5.64	2.09	0.88	-	6.38
Camphene	953	946	7.87	-	-	-	-	3.35
Sabinene	976	969	-	1.79	-	-	-	-
β-Pinene	980	974	-	2.17	0.89	0.52	-	1.36
1-Octen-3-ol	982	974	-	-	-	-	0.32	-
Myrcene	991	988	0.81	2.73	1.42	2.09	-	-
2-Carene	1,004	1,001	-	-	-	-	-	0.26
3-Carene	1,100	1,008	-	-	-	-	-	0.24
α-Terpinene	1,018	1,014	-	-	7.43	-	0.89	2.56
p-Cymene	1,026	1,020	-	1.26	7.93	2.10	0.62	3.62
Limonene	1,031	1,024	1.08	76.25	1.37	80.95	-	9.04
1,8-Cineole	1,033	1,026	2.33	-	5.25	-	27.45	6.88
β-Phellandrene	1,035	1,025	3.96	-	-	0.39	-	-
γ-Terpinene	1,060	1,054	-	1.06	17.33	6.48	1.68	2.84
Artemesia ketone	1,062	1,056	-	-	-	-	0.59	-
p-Tolualdehyde	1,082	1,077	-	-	-	-	4.00	-
Citral diethyl acetal	1,087	1,081	-	2.11	-	-	-	-
Terpinolene	1,092	1,086	-	-	3.74	-	0.42	10.67
α, *p*-Dimethylstyrene	1,105	1,099	-	-	-	-	-	0.22
endo-Fenchol	1,119	1,114	-	-	-	-	-	0.99
trans-Isopulegone	1,123	1,118	-	0.22	-	-	-	-
cis-*p*-Menth-2-en-1-ol	1,125	1,118	-	-	-	-	5.93	0.24
1-Terpineol	1,133	1,130	-	-	-	-	-	1.15
trans-*p*-Menth-2-en-1-ol	1,142	1,136	-	-	-	-	-	0.61
cis-Verbenol	1,143	1,137	-	-	-	-	1.78	-
Camphor	1,147	1,141	-	-	-	-	2.39	-
Menthone	1,153	1,148	-	-	-	0.61	-	-
Citronellal	1,154	1,148	-	-	-	-	-	8.76
β-Terpineol	1,165	1,159	-	-	-	-	-	1.71
Terpinen-4-ol	1,178	1,174	-	-	41.20	-	-	-
α-Terpineol	1,193	1,186	-	-	4.19	0.55	0.43	9.81
Myrtenol	1,200	1,194	-	-	-	-	0.46	-
cis-Piperitol	1,202	1,195	4.75	-	-	-	1.63	-
γ-Terpineol	1,205	1,199	-	-	-	-	-	2.74
trans-Piperitol	1,212	1,207	-	-	-	-	1.13	-
Citronellol	1,229	1,223	-	-	-	-	-	3.53
Nerol	1,230	1,227	-	-	-	-	-	0.71
Carveol	1,233	1,226	-	-	-	-	2.62	-
Pulegone	1,238	1,233	-	-	-	0.48	-	-
Neral	1,241	1,235	-	3.56	-	-	-	-
Geraniol	1,255	1,249	-	-	-	-	-	6.72
Bornyl formate	1,289	1,284	-	-	-	-	19.88	-
p-Menth-1-en-9-ol	1,301	1,294	-	1.09	-	-	-	-
Myrtenyl acetate	1,327	1,324	-	-	-	-	0.52	-
Citronellyl acetate	1,359	1,350	-	-	-	-	-	1.50
Eugenol	1,360	1,356	-	-	-	-	1.92	0.29
α-Copaene	1,378	1,374	2.18	-	-	-	-	-
Geranyl acetate	1,384	1,379	1.10	-	-	-	-	1.32

Table 2. Continued.

Component	RI[a]	RI[b]	Peak area (%)					
			Ginger	Lemon	Tea tree	Tangerine peel	Artemisia	Lemongrass
β-Elemene	1,393	1,389	0.78	-	-	-	-	-
β-Caryophyllene	1,426	1,417	-	-	-	-	3.35	-
β-Cedrene	1,427	1,419	9.42	-	-	-	-	-
β-Farnesene	1,461	1,454	0.62	-	-	-	-	-
α-Zingiberene	1,499	1,493	35.21	-	-	-	-	-
Ledene	1,503	1,496	-	-	1.05	-	-	-
α-Selinene	1,503	1,498	1.03	-	-	-	-	-
β-Bisabolene	1,509	1,505	6.61	-	-	-	-	-
α-Farnesene	1,512	1,505	-	-	0.98	-	-	-
γ-Cadinene	1,520	1,513	2.12	-	-	-	-	-
β-Curcumene	1,521	1,514	8.87	-	-	-	-	-
Elemol	1,553	1,548	-	-	-	-	-	0.85
trans-Nerolidol	1,567	1,561	-	-	-	-	0.61	-
Isocaryophyllene oxide	1,585	1,582	-	-	-	-	13.68	-
Humulene epoxide II	1,615	1,608	-	-	-	-	0.43	-
Total (%)			90.60	97.88	94.87	95.05	92.73	88.35
Essential oil yield (ml/100 g)			2.51	0.32	2.36	0.79	1.32	1.96

[a]Retention index relative to C6–C28 n-alkanes on the HP-5MS capillary column.
[b]RI: Identification by the Kovats index [1].

95.05% of the total oil, and limonene (80.95%) was the main component. GC-MS analyses of artemisia oil identified 23 constituents, representing 93.86% of the total oil; the main components were 1,8-cineole (27.45%), bornyl formate (19.32%), and isocaryophyllene oxide (13.68%).

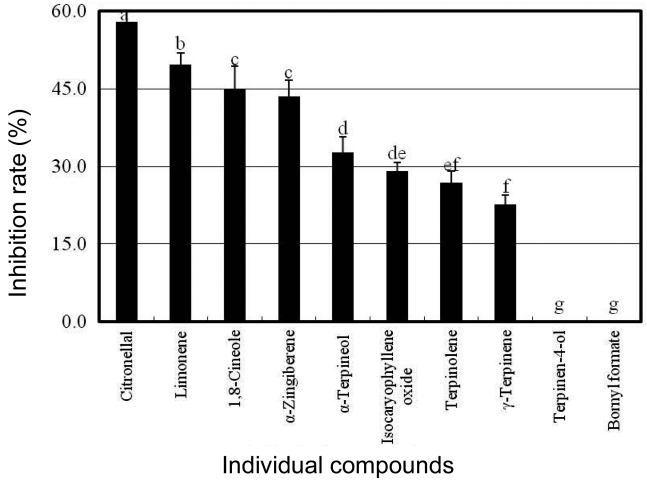

Fig. 3. Curative effects of 10 individual compounds from active essential oils on TMV infection *in vivo*.
N. glutinosa plants were pre-infected with TMV inoculums for 5 h prior to treatment with 320 μg/ml of individual compound solutions. Data are expressed as the mean (column) ± SD (thin bar) of 5 independent experiments. Different letters indicate significant differences between various essential oils ($p < 0.05$).

In lemongrass oil, 27 components were identified, representing 88.35% of the total oil; the main constituents were terpinolene (10.67%), α-terpineol (9.81%), limonene (9.04%), citronellal (8.76%), geraniol (6.72%), 1,8-cineole (6.88%), and α-pinene (6.38%). Table 2 also reports the oil yields from ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass, with yields of 2.51, 0.32, 2.36, 0.79, 1.32, and 1.96 ml/100 g of dry weight, respectively.

Curative Effects of Individual Compounds Against TMV *In Vivo*

The curative effects of 10 individual compounds from the active essential oils against TMV infection are shown in Fig. 3. Of these compounds, citronellal, limonene, 1,8-cineole, and α-zingiberene exhibited a more than 40% inhibition of TMV at 320 μg/ml, with inhibition rates of 57.9%, 49.7%, 45.0%, and 43.5%, respectively. In addition, α-terpineol, isocaryophyllene oxide, terpinolene, and γ-terpinene demonstrated moderate antiviral activities. Terpinen-4-ol and bornyl formate, investigated here, revealed no anti-TMV activities.

Discussion

For many years, plant products have been used for the treatment of many plant diseases, including viral infections. Accordingly, large numbers of plant-derived and synthetic

anti-TMV drugs have been described in several studies [9, 21, 32, 33]. However, until now, plant essential oils and individual compounds had not been systematically analyzed for their anti-TMV potential.

In the present study, we investigated the anti-TMV activities of 29 essential oils and compared these effects with the antiviral potential of ningnanmycin *in vitro* (Fig. 1). We found that the essential oils isolated from lemongrass, tangerine peel, lemon, tea tree, artemisia, and ginger exhibited high antiviral activities at concentrations of 100 μg/ml *in vitro*. However, the remaining essential oils investigated here showed lower activities, or no anti-TMV activities. Differences in the antiviral activities of different essential oils may be attributable to the componential diversity of essential oils, structural diversity of antimicrobial components, and the relative concentrations of these active components contained in the essential oils.

In order to further elucidate the mechanisms of antiviral action(s), the effects of inactivating, curative, and protective qualities of 6 active essential oils were also evaluated *in vivo*. In our investigation of the inactivation of TMV by these oils (Fig. 2A), we found that all active essential oils caused a substantial decrease in viral infectivity, especially artemisia and lemongrass, with inhibition rates of 67.2% and 62.1% at 320 μg/ml, respectively. Meanwhile, artemisia and lemongrass essential oils also had a higher antiviral activity in curative assays, with inhibition rates of 57.1% and 55.3% at 320 μg/ml, respectively (Fig. 2B, $p < 0.05$). However, in assays to investigate the protective effects of plant essential oils, all active essential oils exhibited weaker protective activities than inactivating effects and curative effects (Fig. 2C, $p < 0.05$). These results suggest that the essential oils isolated from artemisia and lemongrass directly inactivate TMV and may interfere with coat proteins or inhibit the formation of capsid proteins, which are necessary for adsorption or entry into the host. In addition, the curative effects of the essential oils from artemisia and lemongrass could also be due to small molecular compounds, contained in the essential oils, which can penetrate into plant cells and exert direct inactivating effects on TMV particles.

Differences in the antiviral mechanisms may be attributable to the componential diversity of the essential oils and the distinction between virus types. Valerija *et al*. [31] reported a direct inactivation of TMV, and the cucumber mosaic virus by *Satureja montana*, through use of essential oils that had the main compounds of thymol and carvacrol, which agrees with our results. The antiviral activities of essential oils isolated from *Melaleuca alternifolia* and *Plectranthus tenuiflorus* against TMV had been previously reported, where the oils were applied to *N. glutinosa* plants as a pre-inoculation spray, with the results being that the number of lesions were significantly inhibited [6, 19]. In addition, De Logu *et al*. [10] reported an inactivation of herpes viruses and the prevention of a cell-to-cell spread by *Santolina insularis* essential oil. However, no antiviral effects were observed during the intracellular replication phase. Pusztai *et al*. [23] reported a specific inhibition of the Cytomegalovirus immediately after early gene expression [23]. Moreover, dissolution of the HSV envelope by treatment with oregano essential oil has been described [27].

In addition, we analyzed the chemical constituents present within the active essential oils by GC and GC-MS (Table 2). The major components of ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass essential oils were α-zingiberene (35.21%), limonene (76.25%), terpinen-4-ol (41.20%), limonene (80.95%), 1,8-cineole (27.45%), and terpinolene (10.67%), respectively. The ratios of these chemicals varied somewhat with respect to other reports of the components of these essential oils, and this could be attributed to the source, cultivation, vegetative stage, and growing season of plants used in these separate investigations [16].

Finally, we investigated the curative effects of 10 individual compounds from active essential oils against TMV infection *in vivo* (Fig. 2C). Of these compounds tested, citronellal, limonene, 1,8-cineole, and α-zingiberene demonstrated higher inhibitory activity against TMV than the other compounds. Several studies have demonstrated the antiviral efficacy of essential oils, containing these individual compounds, against various viruses *in vitro* and *in vivo*. *Melissa officinalis* essential oil was shown to strongly inhibit HSV type-1 and type-2, and the main components in the oils were identified as citral and citronellal [26]. Armaka *et al*. [3] reported that some monoterpenes, such as 1,8-cineole and borneol, completely inhibited viral replication without affecting viral adsorption.

In conclusion, the results of *in vitro* and *in vivo* bioassays support the possibility of using artemisia and lemongrass essential oils, and the individual compound citronellal, as potent natural biofungicides or lead compounds in the treatment of tobacco plants infected with TMV under greenhouse conditions. However, further studies need to be conducted in order to evaluate the cost and applicability of these essential oils and their individual compounds for practical use as particular mechanisms of anti-TMV infections.

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