Table 1 General toxicity of perinatal exposure of chlorpyrifos on dams and offspring.

Dam	0	2.8	14	70	CPF (ppm)
Number of delivery	3	6	6	6	
Body weight (g)	23.8 ± 0.1	24.0 ± 0.9	26.4 ± 1.0**	25.2 ± 0.7	
Liver/BW (%)	7.70 ± 0.36	7.50 ± 0.40	7.63 ± 0.63	8.21 ± 0.41	
Spleen/BW (%)	0.43 ± 0.05	0.47 ± 0.03	0.44 ± 0.02	0.50 ± 0.04	
Thymus/BW (%)	0.17 ± 0.02	0.17 ± 0.04	0.14 ± 0.02	0.14 ± 0.03	
ChE (IU/L)	52.33 ± 6.81	45.75 ± 3.77	31.50 ± 4.43**	18.25 ± 1.50**	
Female offspring (PND21)	0	2.8	14	70	CPF (ppm)
Body weight (g)	9.9 ± 0.5	10.0 ± 0.8	11.0 ± 0.3	10.0 ± 0.8	
Liver/BW (%)	5.57 ± 0.35	5.32 ± 0.69	5.25 ± 0.49	5.56 ± 0.73	
Spleen/BW (%)	0.86 ± 0.14	0.86 ± 0.04	0.80 ± 0.11	0.71 ± 0.13	
Thymus/BW (%)	0.71 ± 0.17	0.67 ± 0.07	0.71 ± 0.04	0.66 ± 0.11	
[a]ChE (IU/L)	31.00 ± 1.87	29.83 ± 2.71	19.17 ± 2.48**	11.50 ± 2.35**	
Male offspring (PND21)	0	2.8	14	70	CPF (ppm)
Body weight (g)	11.0 ± 0.7	10.3 ± 0.7	11.4 ± 0.5	10.2 ± 0.6	
Liver/BW (%)	5.77 ± 0.57	5.45 ± 0.52	5.45 ± 0.28	5.83 ± 0.75	
Spleen/BW (%)	0.84 ± 0.08	0.84 ± 0.07	0.85 ± 0.08	0.77 ± 0.14	
Thymus/BW (%)	0.54 ± 0.11	0.60 ± 0.04	0.57 ± 0.06	0.68 ± 0.06*	
[a]ChE (IU/L)	31.00 ± 1.87	29.83 ± 2.71	19.17 ± 2.48**	11.50 ± 2.35**	
Male offspring (PND77)	0	2.8	14	70	CPF (ppm)
Body weight (g)	23.8 ± 0.2	24.1 ± 0.4	24.1 ± 1.9	24.9 ± 0.9	
Liver/BW (%)	4.70 ± 0.24	4.67 ± 0.52	4.32 ± 0.41	4.5.0 ± 0.24	
Spleen/BW (%)	0.38 ± 0.02	0.38 ± 0.04	0.37 ± 0.04	0.41 ± 0.03	
Thymus/BW (%)	0.14 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	
ChE (IU/L)	35.50 ± 2.08	36.00 ± 2.58	37.00 ± 2.16	34.75 ± 3.95	

Pregnant BALB/c mice (12per group) were exposed to chlorpyrifos (CPF ; 0, 2.8, 14, and 70ppm) in diet, from gestational day 10 to postnatal day (PND) 21. Exposure was ceased by weaning. At PND21 and PND77, mice were sacrificed to determine effects of the compound on body weights (BW), organ weights, and cholinesterase (ChE) activities. [a] Specimens from 2 females and 3-4 males were lumped together for statistics (see Materials and Methods). Values are mean ± SD (n=4). *p<0.05, **p<0.01 (Dunnett's test).

解析を行ったが，統計学的に有意な変化は認められなかった（data not shown）．

3．2．フローサイトメトリー

前述のように，免疫系は各種のリンパ球サブポピュレーションやCD4陽性T細胞サブセットのバランスにより，反応の型が制御されている．そこで，フローサイトメトリーにより一次リンパ器官である胸腺，および二次リンパ器官である脾臓における各種リンパ球サブセットの存在比率への影響を解析した．しかし，CPF暴露は全般的に大きな影響は与えず，NK細胞，CD8陽性T細胞の各サブポピュレーション比率，Th1，Th2の各サブセット比率については有意な影響が認められなかった（data not shown）．

Table 2に，少なくともPND21またはPND77いずれかにおいて有意な変化を示したサブセットの存在比率を挙げた．なお，総細胞数に占めるリンパ球ゲート画分の細胞数には顕著な変化はなかった（data not shown）．

雌については，PND21の脾臓における$CD3^+CD4^+$細胞の増加および胸腺における$CD4^+Foxp3^+$細胞（Treg）の増加が観察された．

雄については，PND21の脾臓における$CD4^+$シングルポジティブ細胞が増加していた．この変化は，PND77でも回復せず持続していた．PND77の脾臓で増加していたのは，$CD4^+CD25^+$細胞（Treg）のみであった．なお，PND21の胸腺で$CD4^+$IL-17A^+細胞（Th17）の減少が認められたが，用量依存性はなく，軽微な影響と考えられた．

CPFの周産期暴露が成熟後のBALB/cマウス脾臓において免疫反応の抑制に関与するTregの存在比率を増加させるという知見は本研究によって初めてもたらされたものである．Tregはほぼ全ての免疫反応に抑制的に働くため，Navarroら[3]がラットにおいて発見したCPFの脾臓T細胞への抑制的影響と考え合わせると，腫瘍免疫などに代表される全身の免疫応答への影響の解析が今後望まれる．

なお，Tregには，胸腺内で分化するFoxp3陽性の内在性Treg（nTreg）と，ナイーブT細胞が抗原提示を受け分化する過程でTGF-β依存的に誘導される誘導性Treg（iTreg）とが存在する[14]．本研究では抗原特異的なiTregの増加は調べていなかったため，現在胸腺依存性抗原（KLH）の免疫実験を遂行中である．

Table 2 Flow cytometry analysis of the effect of chlorpyrifos on lymphocyte subpopulations

Female offspring (PND21)	0	2.8	14	70	CPF (ppm)
Spl: $CD4^+$/Lymph	4.0±0.9	4.0±0.9	4.2±1.1	5.1±0.4	
[a]Spl: $CD4^+$/$CD3^+$	44.3±1.0	45.8±2.3	48.1±2.9	49.9±3.2*	
Spl: $CD4^+CD25^+$/Lymph (Treg)	0.71±0.02	0.69±0.09	0.82±0.08	0.84±0.11	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.42±0.02	0.42±0.04	0.44±0.30	0.85±0.17*	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.14±0.03	0.17±0.05	0.14±0.09	0.09±0.03	
Male offspring (PND21)	0	2.8	14	70	CPF (ppm)
Spl: $CD4^+$/Lymph	4.5±0.4	4.2±0.7	4.0±0.9	5.8±0.2*	
[a]Spl: $CD4^+$/$CD3^+$	48.3±2.0	49.4±2.6	48.9±1.0	52.2±2.6	
Spl: $CD4^+CD25^+$/Lymph (Treg)	0.76±0.08	0.79±0.06	0.82±0.05	0.79±0.17	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.41±0.07	0.71±0.45	0.83±0.26	0.63±0.25	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.14±0.05	0.10±0.03	0.07±0.02*	0.10±0.03	
Male offspring (PND77)	0	2.8	14	70	CPF (ppm)
Spl: $CD4^+$/Lymph	16.3±1.4	16.9±1.8	17.8±0.6	20.0±1.3**	
[a]Spl: $CD4^+$/$CD3^+$	57.4±2.3	57.7±0.9	58.2±3.3	60.5±1.0	
Spl: $CD4^+CD25^+$/Lymph (Treg)	1.93±0.19	2.14±0.21	2.39±0.26*	2.41±0.24*	
Thy: $CD4^+Foxp3^+$/Lymph (Treg)	0.60±0.09	0.61±0.08	0.66±0.23	0.55±0.09	
Thy: $CD4^+IL17A^+$/Lymph (Th17)	0.38±0.09	0.55±0.19	0.38±0.10	0.35±0.10	

Pregnant BALB/c mice were exposed to chlorpyrifos (CPF; 0, 2.8, 14, and 70ppm) in diet from gestational day 10 to postnatal day (PND) 21. Exposure was ceased by weaning. At PND21 and PND77, mice were sacrificed to determine the effects of the compound on the relative proportions of lymphocyte subsets in the spleen (Spl) and thymus (Thy). The percentage of lymphocytes (Lymph) are shown ([a] percent CD3 positive cells). Values are mean±SD (n=4). *$p<0.05$, **$p<0.01$ (Dunnett's test).

4．結　語

本研究では，新生児期におけるCPFへの暴露が成長後の胸腺細胞のレクチン刺激による細胞増殖応答の低下を招くというNavarroらの報告[3]を受け，我々が以前より開発している化学物質の発達期影響の簡便なスクリーニング系[7,8,10]にフローサイトメトリーによるT細胞のサブセット解析を追加し，CPFの潜在的な発達期免疫影響をより詳細に解析することを目指した．

その結果，CPF暴露後のPND21時点で児の血中コリンエステラーゼ活性の抑制が認められたが，PND77には回復した．それにも関わらず，二次リンパ器官である脾臓におけるCD4陽性T細胞の増加がPND21およびPND77で観察され，その中でもPND77のCPF暴露群において増加していた唯一のCD4陽性サブセットは制御性T細胞（$CD4^+CD25^+$）であることを発見した．

なお，緒言で述べた通り免疫系と神経系・内分泌系との間には密接な相互作用があるため，今回観察された*in vivo*での免疫影響が免疫系への直接影響なのか，それとも神経系等を介する間接影響なのかについては明らかでなく，今後の研究が待たれる．

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Antiviral Activities of Diarylheptanoids Isolated from *Alpinia officinarum* against Respiratory Syncytial Virus, Poliovirus, Measles Virus, and Herpes Simplex Virus Type 1 *in vitro*

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Alpinia officinarum has been used as a folk medicine and contains diarylheptanoids that have various biological activities. However, their antiviral activities are less elucidated. We examined the antiviral activities of nine diarylheptanoids isolated from *A. officinarum* against respiratory syncytial virus (RSV), poliovirus, measles virus, and herpes simplex virus type 1 (HSV-1) using a plaque reduction assay. The 50% inhibitory concentrations of seven of the nine diarylheptanoids for RSV were moderately but significantly lower than their 50% cytotoxic concentrations, as determined by a trypan blue exclusion assay. Four diarylheptanoids with anti-RSV activity also showed anti-poliovirus and anti-measles virus activities and three of the four exhibited anti-HSV-1 activity. Thus, seven of the nine diarylheptanoids examined exhibited potential antiviral activity against RSV, and most of the diarylheptanoids with anti-RSV activity, including two diarylheptanoids without anti-RSV activity, were effective against poliovirus, measles virus, and/or HSV-1 *in vitro*. Diarylheptanoids were suggested to have a broad spectrum of antiviral activity.

Keywords: diarylheptanoids, antiviral activity, RSV, HSV-1, measles virus, poliovirus.

Alpinia officinarum (*A. officinarum*), family Zingiberaceae, is known as lesser galangal. This rhizome has been used in various Asian cuisines and as a traditional medicine, such as an antiemetic, stomachic, and analgesic in Asia from ancient times.

Respiratory syncytial virus (RSV) infection is very common in children less than 2 years old and sometimes causes serious bronchilitis and pneumonia [1]. In elderly and high-risk adults, RSV infection is an important illness [2]. Ribavirin, palivizumab, and motavizumab are used for the treatment and prevention of RSV infection [3–6], but there are few clinically specific and effective anti-RSV drugs.

In a series of studies on the development of bioactive components from natural sources, we found that a methanol extract from the rhizome of *A. officinarum* is effective in inhibiting tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in mouse skin [7]. An extract of *A. officinarum* was previously shown to exhibit therapeutic efficacy against herpes simplex virus type 1 (HSV-1) infection in mice [8]. Diarylheptanoids isolated from *A. officinarum* have been shown to exhibit cytotoxic activity [9], suppressive activity of inducible nitric oxide synthase expression [10], inhibitory activity of biosynthesis of prostaglandin and leukotrienes [11,12], and inhibitory activity of proinflammatory mediators [13]. Although a variety of biological activities associated with diarylheptanoids have been demonstrated, antiviral activity of diarylheptanoids has been reported only against influenza virus [14,15]. In the present study, we examined the potential anti-RSV activity of diarylheptanoids *in vitro*. Their anti-RSV activities were compared with antiviral activities against poliovirus, measles virus, and HSV-1 to characterize the anti-RSV activity.

Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were examined for their anti-RSV activity and cytotoxicity *in vitro*. As shown in Table 1, the EC_{50} values of seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7-9**) were significantly lower than their CC_{50} values. DMSO at 1%, the maximum concentration used to dissolve diarylheptanoids in the culture medium, was not cytotoxic. The therapeutic indexes (CC_{50}/EC_{50}) of 7-(4''-hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-2**) and (5*S*)-5-methoxy-1,7-diphenyl-3-heptanone (**AO-7**) were 4.6 and more than 6.1, respectively, and RSV was more

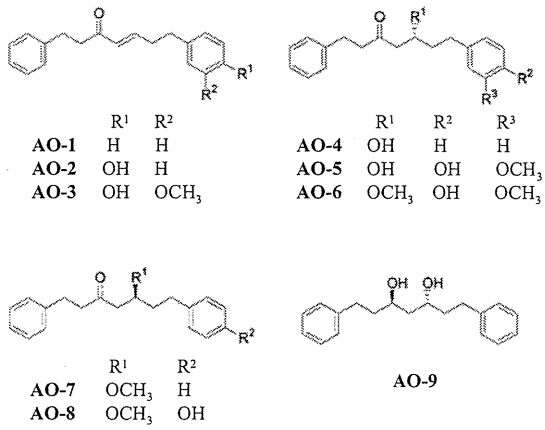

Figure 1: Structures of diarylheptanoids (**AO-1** to **9**) from *A. officinarum*.

susceptible to **AO-2** and **AO-7** than to the other diarylheptanoids examined. In this assay, the EC_{50} value of ribavirin, used as a control, was similar to the results reported previously [16,17]. Thus, seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7-9**) were demonstrated to show moderate, but potential antiviral activity against RSV *in vitro*. This is the first evidence demonstrating the anti-RSV activity of diarylheptanoids *in vitro*.

To evaluate the antiviral spectrum of diarylheptanoids with anti-RSV activity, four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) with anti-RSV activity were examined for anti-poliovirus, -measles virus, and -HSV-1 activities. However, three diarylheptanoids (**AO-2**, **8**, and **9**) with anti-RSV activity were not used, because there were not sufficient amounts to perform a plaque reduction assay. As shown in Table 2, the EC_{50} values of four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) for poliovirus and measles virus were significantly lower than their CC_{50} values. The EC_{50} values of three (**AO-4**, **5**, and **7**) of the four for HSV-1 were also significantly lower than their CC_{50} values. Therefore, among the four diarylheptanoids, three (**AO-4**, **5**, and **7**) with anti-RSV activity exhibited anti-poliovirus, -measles virus, and -HSV-1 activities. **AO-1** exhibited anti-poliovirus and -measles virus activities, but not anti-HSV-1 activity. Because the three diarylheptanoids (**AO-4**, **5**, and **7**) showed antiviral activity against all viruses used in this study, they were suggested to have broad spectrum antiviral activity.

We also examined anti-poliovirus, -measles virus, and -HSV-1 activities of **AO-3** and **6** that did not exhibit anti-RSV activity *in vitro*. As shown in Table 2, **AO-3** was significantly effective for measles virus, but not for poliovirus and HSV-1. However, all three viruses examined were significantly susceptible to **AO-6**. Only measles virus was susceptible to all of the six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) without relation to anti-RSV activity. Diarylheptanoids without anti-RSV activity were also effective against poliovirus, measles virus, and/or HSV-1 *in vitro* and the broad spectrum of antiviral activity was confirmed.

RSV has a different virus structure and replication cycle from poliovirus and HSV-1. However, it has a similar virus structure and replication cycle to measles virus as some paramyxoviruses. In Table 2, six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) exhibited anti-measles virus activity. However, two (**AO-3** and **6**) of them had no anti-RSV activity (Table 1). It is possible that **AO-3** and **6** interfered with a replication step specific to measles virus but not RSV in paramyxoviruses. Although we focused on diarylheptanoids with anti-RSV activity in this study, **AO-3** and **6** were suggested to be potent candidates as anti-measles virus compounds. In our screening of anti-RSV activity *in vitro*, the CC_{50}/EC_{50} value (>6.1) of **AO-7** was highest (Table 1). **AO-7** also exhibited anti-poliovirus, -measles virus, and -HSV-1 activities (Table 2) and may be characterized as a candidate for an anti-RSV compound with a broad antiviral spectrum. Studies of the structure-antiviral activity relationships of many diarylheptanoids isolated from *A. officinarum* [18–20] against various kinds of viruses may be worthwhile to analyze the antiviral actions and to obtain more effective antiviral diarylheptanoids.

RSV was significantly susceptible to seven of the nine diarylheptanoids isolated from *A. officinarum*. Of the nine, six (**AO-1**, **3**, **4**, **5**, **6**, and **7**) with or without anti-RSV activity were effective against poliovirus, measles virus, and/or HSV-1.Thus, diarylheptanoids were suggested to possess a broad spectrum of antiviral activity.

Table 1: Anti-RSV activity and cytotoxicity of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)	CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}
1,7-Diphenyl-4*E*-hepten-3-one(**AO-1**)	36.3 ± 4.2[c]	47.3 ± 1.3	1.3
7-(4"-Hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one(**AO-2**)	5.0 ± 0.0[c]	22.8 ± 2.5	4.6
7-(4"-Hydroxy-3"-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-3**)	42.7 ± 3.5	39.3 ± 6.4	0.9
(5*R*)-5-Hydroxy-1,7-diphenyl-3-heptanone (**AO-4**)	21.7 ± 0.6[c]	38.3 ± 3.4	1.8
(5*R*)-5-Hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone(**AO-5**)	37.0 ± 7.2[c]	84.8 ± 3.8	2.3
(5*R*)-5-Methoxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone (**AO-6**)	13.3 ± 3.8	17.0 ± 0.8	1.3
(5*S*)-5-Methoxy-1,7-diphenyl-3-heptanone (**AO-7**)	16.3 ± 3.5[c]	>100	>6.1
(5*S*)-5-Methoxy-7-(4"-hydroxyphenyl)-1-phenyl-3-heptanone (**AO-8**)	21.7 ± 0.6[c]	31.5 ± 6.6	1.5
(3*R*,5*R*)-1,7-Diphenylheptan-3,5-diol (**AO-9**)	22.3 ± 0.6[c]	56.3 ± 3.1	2.5
Ribavirin	0.67 ± 0.08	NT[d]	NT[d]

The structures of these diaryheptanoids are shown in Figure 1.
[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]$P<0.05$ vs. CC_{50}; [d]Not tested.

Table 2: Anti- poliovirus, -measles virus, and -HSV-1 activities and cytotoxicities of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)			CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}		
	Poliovirus	Measles virus	HSV-1		Poliovirus	Measles virus	HSV-1
AO-1	8.3±2.3[c]	17.3±1.2[c]	53.7±4.7	45.8±1.7	5.5	2.6	0.9
AO-3	64.3±4.9	47.0±4.6[c]	59.7±0.6	63.0±10.4	1.0	1.3	1.1
AO-4	22.7±1.5[c]	17.0±2.0[c]	54.0±5.6[c]	69.5±5.2	3.1	4.1	1.3
AO-5	44.3±4.0[c]	18.3±1.2[c]	58.7±1.5[c]	>100	2.3	5.5	1.7
AO-6	3.7±0.6[c]	6.3±0.6[c]	5.7±0.6[c]	10.8±1.3	2.9	1.7	1.9
AO-7	16.7±2.1[c]	18.0±1.0[c]	18.3±0.6[c]	40.5±5.4	2.4	2.3	2.2
Acyclovir	NT[d]	NT[d]	0.23±0.04	NT[d]	NT[d]	NT[d]	NT[d]

[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]$P<0.05$ vs. CC_{50}; [d]Not tested.

Experimental

Chemicals: Dimethyl sulfoxide (DMSO) and ribavirin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Acyclovir was purchased from GlaxoSmithKline, Tokyo, Japan. Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were isolated from the rhizome of *A. officinarum*, as described previously [9,20].

Cells and viruses: Human epidermoid carcinoma (HEp-2) cells (American Type Culture Collection CCL-23) were purchased from Dainippon Pharmaceutical, Osaka, Japan, and grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% and 2% heat-inactivated fetal calf serum, respectively. Vero E6 cells were provided by Dr K. Shiraki (Toyama University, Japan) and grown and maintained in EMEM supplemented with 8% and 2% heat-inactivated calf serum, respectively. The A2 strain of RSV was obtained from American Type Culture Collection (Rockville, MD) and grown in HEp-2 cell cultures. Poliovirus type 1 (Sabin strain), measles virus (Tanabe strain), and HSV-1 (7401H strain) were provided by Dr K. Shiraki (Toyama University, Japan) and propagated in Vero cells [8].

Antiviral and cytotoxic assays: The anti-RSV activities of 9 diarylheptanoids were examined by a plaque reduction assay using HEp-2 cells [21,22]. Briefly, HEp-2 cells grown in 24-well plates were infected with 100 plaque-forming units (PFU)/0.2 mL of RSV at 37°C for 1 h. The cells were overlaid with 1 mL of maintenance EMEM containing 0.8% methylcellulose and various concentrations of either diarylheptanoids or ribavirin and maintained in a humidified atmosphere containing 5% CO_2 for 4–5 days.

The anti-poliovirus, -measles virus, and -HSV-1 activities were also examined by a plaque reduction assay using Vero cells [8]. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU/0.2 mL of poliovirus, measles virus, or HSV-1 for 1 h. Then the cells were overlaid with 5 mL of nutrient 0.8% methylcellulose medium containing various concentrations of either diarylheptanoids or acyclovir. The virus-infected cultures were incubated for 2–5 days at 37°C. The infected cells were fixed and stained, and the plaques were counted [8]. All diarylheptanoids were dissolved in DMSO and diluted with culture medium to make the various final concentrations. The concentration of DMSO in each medium was less than 1%. Ribavirin and acyclovir were dissolved in distilled water and DMSO, respectively, and used as controls. The 50% effective antiviral concentration (EC_{50}) was the concentration that reduced virus-induced cell destruction by 50%, as described previously [8,21].

The cytotoxicity of diarylheptanoids was assessed by trypan blue exclusion assays using mock-infected HEp-2 or Vero cells. The cells were seeded at a concentration of 5 x 10^4 cells/mL in 24-well plates. After incubation at 37°C for 24 h, the culture medium was replaced with fresh medium containing one of the diarylheptanoids at various concentrations and the cells were further incubated for 48 h. After 48 h, the cells were trypsinized and the number of viable cells was determined by a trypan blue exclusion assay. The 50% cytotoxic concentration (CC_{50}) was determined as the concentration that reduced cell destruction by 50% [21].

Statistical analysis: Statistical significances of differences between the EC_{50} and CC_{50} values were evaluated using Student's *t*-test. A *P* value of 0.05 or less was considered to be significant statistically.

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