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Antiviral Activity of Extracts from Marine Algae†

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Extracts of two species of marine algae, *Constantinea simplex* and *Farlowia mollis*, were tested for antiviral activity in tissue culture and in experimental infections of mice. Treatment of confluent mouse embryo fibroblast cell monolayers with either compound before viral inoculation was effective in inhibiting the replication of herpes simplex virus type 1 and type 2, vaccinia virus, and vesicular stomatitis virus, but not encephalomyocarditis virus, Semliki Forest virus, or murine cytomegalovirus. Prophylactic administration of these extracts was effective in reducing final mortality or prolonging the mean day of death of animals inoculated by the intraperitoneal, intracerebral, or intranasal routes with herpes simplex virus type 2. When therapy was initiated after viral inoculation or at a site other than that of viral inoculation, no significant effect on mortality or on mean day of death was observed. Neither preparation was effective in mice inoculated intraperitoneally with encephalomyocarditis virus, Semliki Forest virus, or murine cytomegalovirus or in animals infected intravaginally with herpes simplex virus type 2. The prophylactic but not therapeutic antiviral activity of these preparations seriously limits their potential use in human herpes simplex virus infections.

Extracts from several species of marine algae collected from the coast of California were reported by Deig and co-workers to possess antiviral properties (1, 2). When tested in tissue culture against 13 mammalian viruses, 8 species inhibited the replication of herpes simplex virus (HSV) type 1 and type 2, and one species inhibited coxsackievirus B5. This action appeared to be somewhat virus specific, as there was no effect on the replication of strains of echovirus, poliovirus, rhinovirus, adenovirus, vaccinia virus, respiratory syncytial virus, vesicular stomatitis virus (VSV), or three togaviruses. Extracts from two of the species showing antiviral activity, *Constantinea simplex* and *Farlowia mollis*, were used in the present study to (i) determine the susceptibility of a representative group of viruses to *C. simplex* and *F. mollis* in tissue culture and (ii) evaluate the prophylactic and therapeutic efficacy of these compounds when administered either parenterally or topically in a series of experimental viral infections of mice.

To evaluate the in vivo effectiveness of these potential antiviral compounds, we utilized four experimental HSV type 2 infections of mice in which the virus was inoculated by the intraperitoneal (i.p.), intracerebral (i.c.), intranasal (i.n.), or intravaginal (i.vg.) routes. In these four model HSV infections, death occurs from encephalitis, but each represents a different site of initial virus replication and pattern of pathogenesis (6, 7, 9). In addition, we evaluated the efficacy of these substances in mice inoculated with encephalomyocarditis virus (EMCV), Semliki Forest virus (SFV), or murine cytomegalovirus (MCMV).

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MATERIALS AND METHODS

Experimental infections. Swiss Webster mice were obtained from Simonsen Laboratories (Gilroy, Calif.). Six- to eight-week-old animals were inoculated i.p. with either 10 plaque-forming units (PFU) of EMCV, 10 PFU of SFV, or 10^5 PFU of HSV type 2 or inoculated i.vg. with 10^4 PFU of HSV type 2. Three-week-old mice were inoculated i.n. with 10^4 PFU of HSV type 2 or inoculated i.p. with 10^6 PFU of MCMV. Two-week-old mice were inoculated i.c. with 2 to 5 PFU of HSV type 2. These experimental infections have all been described previously in detail (3, 7-10). In all experiments the animals were observed for 3 weeks after viral inoculation.

Virus strains. The origins and preparation of pools of EMCV, SFV, MCMV, VSV, vaccinia virus, the McIntyre, Shealey, and Tyler strains of HSV type 1, and the MS, Lovelace, and Alabama strains of HSV

type 2 have been described in previous publications (3, 5, 6, 8). The Wilson, HL-3, and HL-34 strains of HSV type 1 and the Heeter and Turner strains of HSV type 2 were low-passaged isolates obtained from lip or genital lesions. The E-196 strain of HSV type 2 was obtained from H. Haines, University of Miami, Miami, Fla.

Cell cultures and media. Fetal lamb kidney cells were used for HSV titrations, and mouse embryo fibroblast (MEF) cells were used for in vitro virus susceptibility assays. The preparation and maintenance of these cell cultures and the media used have been described previously (4).

Antiviral agents. Extracts of *C. simplex* and *F. mollis* were prepared as previously reported (1, 2) and were provided through the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Extracts were reconstituted in sterile water and administered to mice in 0.1-ml doses of either the primary aqueous extract or a 1:10 dilution of the primary extracts in phosphate-buffered saline (PBS). There was no visible evidence of toxicity to mice in the doses used.

In vitro susceptibility of viruses. The in vitro susceptibilities of six HSV type 1 and six HSV type 2 isolates, EMCV, MCMV, SFV, VSV, and vaccinia virus were determined using a 50% plaque reduction assay in MEF cells as described previously (6). Confluent cell monolayers were treated either 2 or 3 h before or 1 h after viral inoculation. In the pretreatment method, fivefold dilutions of drug were prepared in Eagle minimal essential medium (MEM) and added to confluent MEF cell monolayers. After incubation at 37°C for 2 h, the solutions were aspirated and, without washing, cell monolayers were inoculated with 20 to 70 PFU of the appropriate virus and allowed to incubate at 37°C for 1 h. The monolayers were then overlaid with a 0.5% agarose-MEM solution. For the posttreatment method, fivefold dilutions of drug were prepared in twice-concentrated MEM. Viral inoculation and incubation were performed as above; the drug dilutions were then mixed with an equal volume of a 1.0% agarose solution and added to the cell monolayers. At the appropriate time the cells were stained with neutral red, and the viral plaques were counted. The level of susceptibility is expressed as the reciprocal of the dilution which reduced the plaque count to 50% of the control.

Assay for HSV type 2 in vaginal secretions. Vaginal swabs of treated and untreated mice were obtained on days 1, 3, 5, and 7 after i.vg. inoculation with HSV type 2. The swabs were placed in tubes containing 1.0 ml of MEM and frozen at −70°C until assayed on fetal lamb kidney cells for the presence of virus. Titers of virus are expressed as $\log_{10}$ PFU per milliliter of media in which the swab was placed. Geometric mean titers were used to graphically represent vaginal viral titers.

Statistical evaluation. Differences in final mortality between control and drug-treated mice were evaluated with the Fisher exact test and differences in the mean day of death (MDD) were evaluated with the Mann-Whitney U rank test. A P value of <0.05 was accepted as significant.

RESULTS

In vitro susceptibility of viruses. To confirm the specific inhibition of HSV replication by *C. simplex* and *F. mollis* as reported by Deig et al. (1) and Ehresmann et al. (2), we compared the susceptibility of HSV type 1, HSV type 2, EMCV, SFV, MCMV, VSV, and vaccinia virus in MEF cells. The extract was applied either before or after viral inoculation. The 50% plaque inhibitory levels for each of the viruses tested are listed in Table 1. In almost every instance viral inhibition was greater with pretreatment than with posttreatment of cell monolayers. HSV type 2 was clearly the most susceptible of the viruses to the action of either compound. A 1:5,400 dilution of *C. simplex* or a 1:3,500 dilution of *F. mollis* reduced the HSV type 2 plaque count by 50%, when applied to the monolayers 2 h before viral inoculation. HSV type 1 was six- to sevenfold less susceptible than HSV type 2. When the monolayers were treated 1 h after infection, both compounds were less effective against HSV type 2 challenge, but retained most of their activity against HSV type 1 challenge. HSV type 2 was the most susceptible virus to the action of *C. simplex*. HSV type 1, vaccinia virus, and VSV were inhibited to a lesser degree, whereas EMCV, SFV, and MCMV were not inhibited at the highest concentration used in these assays. A similar relationship in the susceptibility of these viruses to *F. mollis* was observed.

To determine whether the differences in the susceptibility to both compounds observed between HSV type 2 and HSV type 1 were unique to the two isolates tested or were representative of other HSV strains, the 50% inhibitory levels for six type 1 and six type 2 isolates were determined. As Table 2 shows, the type 2 isolates exhibited a 50% reduction with *C. simplex* at dilutions of 1:7,600 to 1:16,900 as compared with a 50% reduction at dilutions of 1:125 to 1:200 for

TABLE 1. *Susceptibility of a representative group of viruses to C. simplex and F. mollis in MEF cells*

Virus	*C. simplex*		*F. mollis*	
	−2 h[a]	+1 h[b]	−2 h	+1 h
HSV type 2	1:5,420[c]	1:100	1:3,565	1:175
HSV type 1	1:720	1:230	1:520	1:870
Vaccinia	1:250	1:25	1:810	1:565
VSV	1:115	<1:10	1:725	1:170
EMCV	<1:10	<1:10	1:25	1:105
SFV	<1:10	<1:10	<1:10	1:75
MCMV	<1:10	<1:10	<1:10	<1:10

[a] Compound applied before virus challenge.
[b] Compound applied after viral inoculation.
[c] Dilution of the primary extracts which reduced the virus plaque count to 50% of the control.

the type 1 isolates. Although the HSV type 1 isolates were more susceptible to the action of *F. mollis* than to the action of *C. simplex*, the type 2 isolates were still 5- to 10-fold more susceptible than the type 1 isolates.

Effect of treatment on mice inoculated i.p. with HSV type 2, EMCV, or SFV. Based on the evidence that the extracts of *C. simplex* and *F. mollis* were effective in vitro, we next evaluated their antiviral activity in vivo. Groups of 15 mice were inoculated i.p. with HSV type 2, EMCV, or SFV. These model infections provide a systemic infection with a virus, HSV type 2, which was susceptible to the extracts and with two viruses which were resistant to the extracts in tissue culture. The results from a single i.p. treatment with 0.1 ml of a 1:10 dilution of *C. simplex* or *F. mollis* administered 24 or 2 h before or 1 h after viral inoculation are shown in Table 3. The HSV type 2 control group treated with PBS had a final mortality of 67%, with an MDD of 10.2 days. Although there was no significant reduction of mortality in any of the treated groups, there was a significant delay in the MDD of mice that received either compound 2 h before viral inoculation. The group of mice inoculated with EMCV and treated with PBS had a final mortality of 67%, with an MDD of 9.0 days. Treatment with either compound was not effective in altering mortality, and only the group that received *C. simplex* 1 h after infection had a prolonged MDD. Mice inoculated with SFV and treated with PBS had a final mortality of 60%, with an MDD of 7.4 days. Treatment failed to alter the mortality but did delay the

TABLE 2. *Susceptibility of HSV type 1 and type 2 strains to C. simplex and F. mollis in MEF cells*[a]

Virus strain	*C. simplex*	*F. mollis*
HSV type 1		
McIntyre	1:145[b]	1:410
Shealey	1:125	1:390
HL-3	1:195	1:435
HL-34	1:170	1:420
Wilson	1:170	1:425
Tyler	1:190	1:410
HSV type 2		
MS	1:15,395	1:2,250
Lovelace	1:12,130	1:3,480
Alabama	1:14,060	1:2,915
E-196	1:16,965	1:2,620
Heeter	1:7,640	1:2,750
Turner	1:7,720	1:2,385

[a] Monolayers treated 3 h before HSV challenge.
[b] Dilution at which virus plaque count was reduced to 50% of the control.

TABLE 3. *Effect of a single treatment of C. simplex or F. mollis on the mortality of mice inoculated i.p. with HSV type 2, EMCV, or SFV*

Virus	Treatment	Time (h)[a]	Mortality		
			No.	%	MDD
HSV type 2	PBS	−2	10/15	67	10.2
	C. simplex	−24[b]	15/15	100	10.0
		−2	5/15	33	13.0[c]
		+1	14/15	93	9.1
	F. mollis	−24[b]	13/15	87	9.8
		−2	9/15	60	12.7[c]
		+1	14/15	93	9.2
EMCV	PBS	−2	10/15	67	9.0
	C. simplex	−24	11/15	73	8.6
		−2	6/15	40	8.0
		+1	10/15	67	10.9[c]
	F. mollis	−24	8/15	53	8.6
		−2	8/15	53	8.9
		+1	8/15	53	7.8
SFV	PBS	−2	9/15	60	7.4
	C. simplex	−24	9/15	60	7.6
		−2	13/15	87	9.8[c]
		+1	10/15	67	8.4
	F. mollis	−24	9/15	60	8.6
		−2	6/15	40	9.8
		+1	8/15	53	8.5

[a] −, Before virus inoculation; +, after virus inoculation.
[b] Treatment with 0.1 ml i.p. of a 1:10 dilution of the primary aqueous extract.
[c] $P < 0.05$.

MDD in the group treated with *C. simplex* at 2 h before infection.

A second experiment was performed to determine whether treatment with multiple doses might be more effective than a single treatment (Table 4). Groups of 15 mice were inoculated i.p. with HSV type 2. The control group treated with PBS had a final mortality of 100%, with an MDD of 10.5 days. Treatment with 0.1 ml of a 1:10 dilution of *C. simplex* or *F. mollis* was initiated 24 or 2 h before or 2 h after inoculation with HSV type 2 and continued once daily for 4 days. Treatment initiated 24 h before or 2 h after viral inoculation failed to alter either mortality or the MDD. In contrast, multiple doses with either preparation beginning 2 h before viral challenge significantly reduced mortality.

Effect of treatment on mice inoculated i.c. with HSV type 2. In our initial series of experiments in vivo, the only evidence of efficacy was in the protection of mice inoculated i.p. with HSV type 2. This model HSV infection has consistently been the most susceptible in response to antiviral therapy (7). To extend these studies, we next examined the effect of treatment in mice inoculated i.c. with HSV type 2. Groups of 15 mice were inoculated with 2 to 5 PFU of virus and treated i.p. with 0.1 ml of a 1:10 dilution of *C. simplex* or *F. mollis* beginning 2 h before or 2 h after viral challenge. As shown in Table 5 (experiment 1), treatment failed to alter either mortality or the MDD.

A second experiment was performed as above, but treatment was administered at the site of viral inoculation by injecting 0.03 ml of primary aqueous extract into the left cerebral cortex 2 h before or 2 h after viral inoculation (Table 5, experiment 2). The PBS-treated control group had a final mortality of 100%, with an MDD of 5.1 days. Treatment with *C. simplex* initiated 2 h before viral inoculation significantly reduced mortality and extended the MDD. *F. mollis* given 2 h before infection did not reduce mortality but did increase the MDD. Treatment 2 h after inoculation with HSV type 2 did not alter mortality or the MDD.

Effect of local treatment on mice inoculated i.n. with HSV type 2. To further evaluate the effectiveness of local treatment at the site of infection, we next utilized groups of 10 mice inoculated i.n. with HSV type 2, a model for disseminated HSV type 2 infection in human newborns. The results of this experiment are summarized in Table 6. A single i.n. treatment

TABLE 4. *Effect of treatment with multiple doses of C. simplex or F. mollis on the mortality of mice infected i.p. with HSV type 2*

Treatment	Time (h)[a]	Mortality		
		No.	%	MDD
PBS	−2	15/15	100	10.5
C. simplex	−24[b]	14/15	93	10.1
	−2	8/15	53[c]	12.5
	+2	13/15	87	10.4
F. mollis	−24[b]	11/15	73	10.3
	−2	8/15	53[c]	11.6
	+2	13/15	87	11.2

[a] −, Before virus inoculation; +, after virus inoculation.

[b] Treatment with 0.1 ml i.p. of a 1:10 dilution of the primary aqueous extract beginning at the time indicated and then once daily for 4 days.

[c] $P < 0.01$.

TABLE 5. *Effect of treatment with C. simplex or F. mollis on the mortality of 2-week-old mice inoculated i.c. with HSV type 2*

Treatment	Time (h)[a]	Mortality		
		No.	%	MDD
Expt 1[b]				
PBS	−2	15/15	100	6.1
C. simplex	−2	13/13	100	5.2
	+2	15/15	100	5.5
F. mollis	−2	15/15	100	5.3
	+2	15/15	100	5.4
Expt 2[c]				
PBS	−2	15/15	100	5.1
C. simplex	−2	10/15	67[d]	8.6[e]
	+2	13/13	100	5.4
F. mollis	−2	14/15	93	7.8[e]
	+2	11/12	92	5.4

[a] −, Before virus inoculation; +, after virus inoculation.

[b] Treatment with 0.1 ml i.p. of a 1:10 dilution of primary aqueous extract given once daily for 4 days.

[c] One treatment with 0.03 ml i.c. of primary aqueous extract.

[d] $P < 0.01$.

[e] $P < 0.05$.

TABLE 6. *Effect of treatment with C. simplex or F. mollis on the mortality of 3-week-old mice inoculated i.n. with HSV type 2*

Treatment[a]	Time (h)[b]	Mortality		
		No.	%	MDD
PBS control	−2	9/10	90	7.2
C. simplex	−2	9/10	90	9.3[c]
	+2	8/10	80	9.4[c]
F. mollis	−2	6/10	60	10.3[c]
	+2	7/10	70	9.4[c]

[a] A single treatment of 0.03 ml i.n. of primary aqueous extract.

[b] −, Before virus inoculation; +, after virus inoculation.

[c] $P < 0.05$.

of 0.03 ml of the primary extracts was administered 2 h before or 2 h after inoculation with HSV type 2. The PBS-treated control group had a final mortality of 90% and an MDD of 7.2 days. Treatment with either compound failed to alter the mortality, but did significantly increase the MDD in all the treated groups.

Effect of local treatment on mice inoculated i.vg. with HSV type 2. The i.vg. route of inoculation offers another model of human disease in which local treatment can be evaluated. In the first experiment a single treatment of 0.05 ml of a 1:10 dilution of primary extracts was administered i.vg. 2 h before (Fig. 1A) or 2 h after viral inoculation. In the PBS-treated control group 15 of 15 mice became infected, with mean titers of $10^{4.6}$ on day 1 to $10^{3.2}$ on day 7. Although there were some mice which did not become infected in the treated groups, there was little difference in titers of virus for the days tested. The mortality of mice in this experiment was the same in treated and untreated animals, and there were no differences in the MDD (data not shown).

In a second experiment mice were inoculated with HSV type 2 as above, and the effect of treatment with multiple doses of *C. simplex* and *F. mollis* was determined. Mice were given 0.05 ml of undiluted extracts i.vg. 2 h before and 2 and 8 h after inoculation with HSV type 2 on day 0, twice on day 1, and once on days 2, 3, and 4. As shown in Fig. 1B, the number of mice that became infected in the PBS-treated control group was similar to that in the first experiment. Mean titers of virus in vaginal secretions in the control group were $10^{3.8}$ on day 1, $10^{4.5}$ on days 3 and 5, and $10^{3.0}$ on day 7. In the group treated with *C. simplex*, 10 of 15 mice became infected, and mean vaginal titers of virus were 1.5 to 2 logs lower than those of the control mice. In the group treated with *F. mollis*, 11 of 15 animals became infected, and mean viral titers were about 1 log lower than the control on each day tested. There were no significant differences in the mortality or the MDD between the control group and either of the treated groups (data not shown).

Effect of treatment on mice inoculated i.p. with MCMV. From the initial experiments it appeared that neither compound was effective against the two RNA viruses utilized, but that prophylactic administration of both algae extracts was somewhat effective against the HSV type 2 infections. MCMV, another member of the herpesvirus group, was highly resistant to the two compounds in tissue culture. Therefore, we would predict it should also not be susceptible to treatment in vivo. To further determine whether there is a correlation between susceptibility in vitro and response to treatment of an experimental infection of mice, groups of 15 animals were inoculated i.p. with MCMV and treated with 0.1 ml of primary aqueous extract of *C. simplex* or of *F. mollis* initiated 2 h before or 2 h after MCMV inoculation and continued twice daily for 5 days. The PBS-treated control

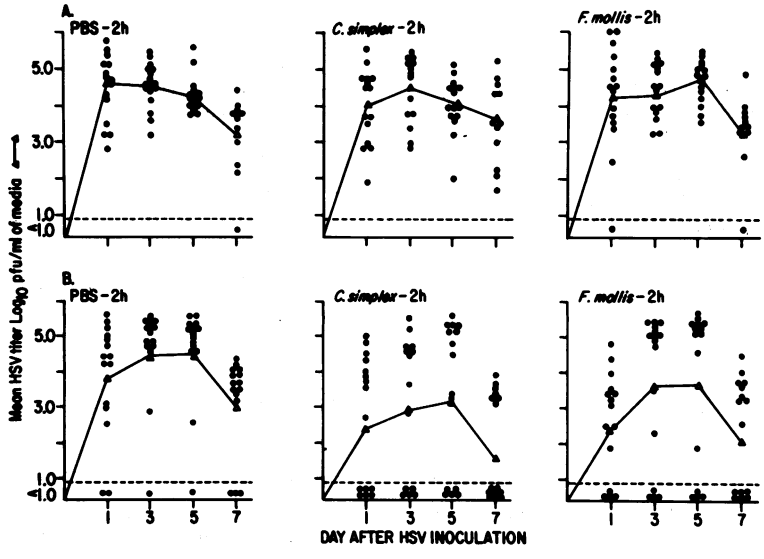

FIG. 1. *Mean titers of virus in vaginal secretions of mice inoculated i.vg. with HSV type 2. (A) Single treatment of a 1:10 dilution of C. simplex or F. mollis administered i.vg. 2 h before viral inoculation. (B) Multiple doses of undiluted C. simplex or F. mollis initiated 2 h before viral inoculation.*

group had a final mortality of 67%, with an MDD of 5.9 days. Therapy with either compound failed to alter either final mortality or the MDD in any of the treated groups (data not shown).

DISCUSSION

Deig and co-workers (1) and Ehresmann et al. (2) reported that extracts of *C. simplex* and *F. mollis* specifically inhibited HSV type 1 and HSV type 2 replication in tissue culture when compared with 11 other viruses. In the present study, using a 50% plaque reduction assay, inhibition of VSV and vaccinia virus in addition to inhibition of HSV type 1 and HSV type 2 was observed. Little or no antiviral effect was observed against MCMV, SFV, or EMCV. Two of the viruses which were resistant in the 50% tissue culture infective dose assay system utilized by Deig and Ehresmann, VSV and vaccinia virus, were found to be susceptible at levels similar to that of HSV type 1. The most striking observation was the eightfold-greater susceptibility of HSV type 2 than HSV type 1 to both extracts. This greater susceptibility of the type 2 strain was verified in a second experiment in which six HSV type 2 isolates were 5- to 10-fold more susceptible than six HSV type 1 isolates. These results suggest that differential susceptibility to *C. simplex* might be used as a biological marker to identify type 1 and type 2 strains of HSV. The MS strain of HSV type 2 utilized in the experimental infections in mice had inhibitory levels similar to the other HSV type 2 isolates tested and appeared to be representative of other type 2 strains.

The active antiviral substance of these compounds appears to be a structural polysaccharide (2). Although the exact mechanism of action is not known, it has been postulated that these substances exert their effect by blocking or coating receptor sites necessary for viral attachment to the cell. This postulate is supported by the evidence that the antiviral effect is lost if treated monolayers are washed before viral inoculation (1) and by the observation that treatment of the monolayers after viral inoculation was in general less effective than treatment before viral challenge (Table 1).

The results obtained from the experimental infections in mice and in tissue culture indicate that these compounds exhibit their greatest antiviral effects when administered before viral inoculation. These effects appear to be of short duration, as mice treated i.p. with either compound 2 h before i.p. inoculation with HSV type 2 had a reduced mortality or an extended MDD, whereas i.p. treatment 24 h before or 2 h after inoculation failed to alter either mortality or the MDD. These results confirm those of a preliminary study reported by Hatch et al. (presented at the Ninth International Seaweed Symposium, Santa Barbara, Calif., 1977) (J. Physiol. **13**(Suppl.):28, 1977). It would also appear that the compound must be given at the site of viral inoculation, as i.c. treatment 2 h before i.c. inoculation with HSV type 2 reduced mortality or prolonged the MDD, whereas treatment administered i.p. failed to alter either mortality or the MDD. These results are compatible with the blocking or coating mechanism of action postulated for these compounds. Only minimal antiviral activity of the algae extracts was observed in mice inoculated i.n. or i.vg. with HSV type 2, and no effect occurred in animals challenged i.p. with EMCV, SFV, or MCMV.

The prophylactic but not therapeutic antiviral efficacy of these compounds seriously limits their potential use in treatment of human HSV infections. The lack of effect when given parenterally or locally after the establishment of an infection suggests that these crude extracts would not be effective in treatment of recurrent HSV infections, generalized infections, or HSV encephalitis in humans. Additional studies involving the identification and purification of the active substance in these extracts is necessary before the potential of these materials as antiviral agents can be fully determined.

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