

**PROANTHOCYANIDIN POLYMERS HAVING ANTIVIRAL
ACTIVITY AND METHODS OF OBTAINING SAME**

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation-in-part of
5 application Serial No. 07/596,893, filed October 12,
1990, which is incorporated herein by reference.

1. FIELD OF THE INVENTION

The present invention relates to the use of
10 proanthocyanidin polymers, having 2 to 30 flavonoid
units, in treating respiratory virus infections.
Additionally, it has been found that the use of the
proanthocyanidin polymers having 6 to 30 flavonoid
15 units is effective in treating virus infections in
general. The chemical characteristics of certain
newly discovered proanthocyanidin polymers are also
encompassed.

2. BACKGROUND OF THE INVENTION

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**2.1 ETHNOBOTANICAL USES OF EXTRACTS FROM
THE CROTON TREE AND FROM CALOPHYLLUM
INOPHYLLUM**

A number of different Croton tree species,
including Croton salutaris, Croton gossypifolius,
25 Croton palanostima, Croton lechleri, Croton
erythrochilus and Croton draconoides, found in South
America, produce a red viscous latex sap called Sangre
de Drago. It is most often utilized by mixed descent
and native people of the Peruvian Amazon for flu and
30 diarrhea. It is taken internally for tonsillitis,
throat infections, tuberculosis, peptic ulcers,
intestinal disorders, rheumatism and to enhance
fertility and is used by both adults and children. It
is also used extensively to stop bleeding, herpes, and
35 for wound healing. The sap is placed directly on open
wounds as an anti-infective and to accelerate the
healing process. It is applied to the gums of

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patients after tooth extractions. It is also utilized as a vaginal wash in cases of excessive bleeding.

It has been shown that Sangre de Drago from *Croton draconoides* and from *Croton lechleri* contains an alkaloid identified as taspine, which exhibits anti-inflammatory activity, Persinos et al., 1979, J. Pharm. Sci., 68:124. Taspine has also been shown to inhibit RNA-directed DNA polymerase activity in the myeloblastosis virus, Rauscher leukemia virus and Simian sarcoma virus (Sethi, 1977, Canadian J. Pharm. Sci., 12:7).

Calophyllum inophyllum is a tree ranging from India to East Africa to Polynesia. Seed oil is used in folk medicine as an antiparasitic in treatment of scabies, ringworm and dermatosis as well as other uses such as for analgesia. In Indo-China the powdered resin is used for ulcers and wound healing. In Indonesia the bark is applied externally to treat swollen glands and internally as a diuretic. The sap is used as an emollient for chest pain as well as for tumors and swelling. Leaf extracts are used as a wash for inflamed eyes. The Cambodians use leaf extracts in inhalations for treatment of vertigo and migraine. The Samoans use the sap as an arrow poison.

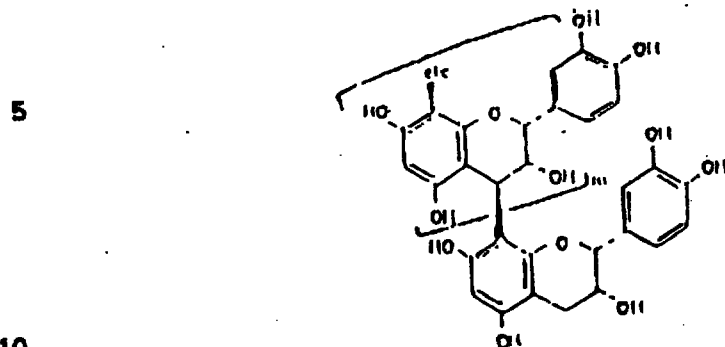
2.2 PROANTHOCYANIDIN MONOMERS AND POLYMERS AND THEIR USES

Proanthocyanidin and proanthocyanidin polymers are found as colorless phenolic substances in a wide variety of many plants, particularly those with a woody habit of growth (e.g., the *Croton* species and *Calophyllum inophyllum*). The general chemical structure of a polymeric proanthocyanidin consists of linear chains of 5, 7, 3', 4' tetrahydroxy or 5, 7, 3', 4', 5' pentahydroxy flavonoid 3-ol units linked

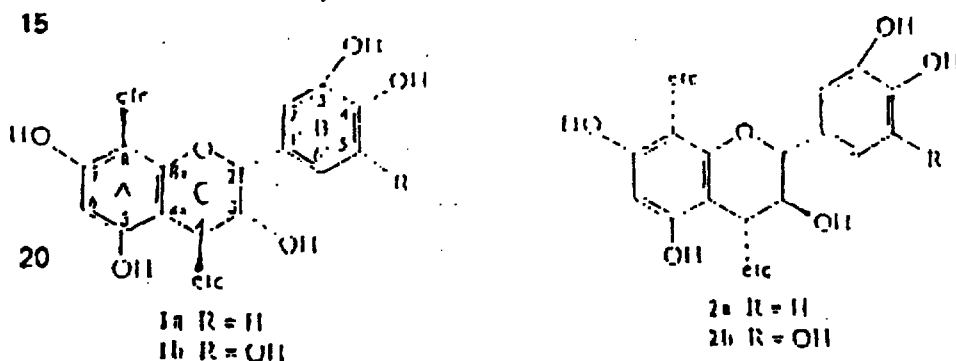


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together through common C(4) - (6) and/or C(4) - C(8) bonds, as shown below.



Biosynthetic studies have indicated that proanthocyanidin polymers consist of monomer units of the type shown below, See Fletcher et al., 1977, *J.C.S. Perkin*, 1:1628.



The monomer unit (generally termed "leucoanthocyanidin") of the polymer chain may be based on either of two stereochemistries of the C-ring, at the 2 and/or 4 position designated *cis* (called epicatechins) or *trans* (called catechin). Therefore, the polymer chains are based on different structural units, which create a wide variation of polymeric proanthocyanidins and a large number of possible isomers (Hemingway et al., 1982, *J.C.S. Perkin*, 1:1217). C13 NMR has been useful to identify the structures of polymeric proanthocyanidins and recent work has elucidated the chemistry of di-, tri- and tetra-meric proanthocyanidins. Larger polymers of the flavonoid 3-ol units are predominant in most plants, and are found with average molecular weights



above 2,000 daltons, containing 6 or more units,
Newman et al., 1987, *Mag. Res. Chem.*, 25:118.

Proanthocyanidins have been reported to
possess protein binding ability and a possible
5 biological role (Newman et al., 1987, *Mag. Res. Chem.*,
25:118). Proanthocyanidin monomers and dimers have
been used in the treatment of diseases associated with
increased capillary fragility and have also been shown
to have anti-inflammatory effects in experimental
10 animals (Beladi et al., 1977, *Ann. N.Y. Acad. Sci.*,
284:358). A procyanidin monomer was found to have
antiviral activity against Herpes Simplex virus in an
in vitro assay. (Beladi, et al., *supra.*) Beladi et
al. tested the viricidal effect of a number of
15 flavonoid monomers including apigenin, pelargonidin,
quercetin and a proanthocyanidin monomer on Herpes
Simplex virus. The proanthocyanidin monomer was found
to be the most effective in virus-inactivating
activity, followed by pelargonidin and quercetin.
20 Apigenin had only a slight effect. The effect of
quercetin and the procyanidin monomer on the
multiplication of Herpes Simplex virus in HEP-2 cells
was also investigated. As concluded by Beladi et al.,
the monomeric flavonoids tested had viricidal
25 activity, but only a slight inhibitory effect on virus
multiplication in the in vitro assays.

Prior to the present invention, there has
been no disclosure regarding the use of
30 proanthocyanidins containing two or more flavonoid
units for treating respiratory virus infections.
Additionally, there has been no disclosure regarding
the use of proanthocyanidins of six or more flavonoid
units for treating virus infections in general.

35 For the purpose of the present application,
a proanthocyanidin polymer isolated from the Croton
species is designated "proanthocyanidin polymer A" and
that isolated from *Calophyllum inophyllum* is designated



"proanthocyanidin polymer B". However, such designation is solely for simplicity of discussion and not necessarily intended to imply that the polymers are different classes of compounds. Both
5 proanthocyanidin polymer A and proanthocyanidin polymer B are considered to be within the term proanthocyanidin polymer as described herein.

2.3 RESPIRATORY SYNCYTIAL VIRUS

10 Respiratory Syncytial Virus (RSV) belongs to the virus family Paramyxoviridae, genus Pneumovirus, and is responsible for causing lower respiratory tract infections such as bronchiolitis and pneumonia in the infant and child. The virus is considered to be in
15 important agent of acute respiratory disease in children and it is expected that up to 50% of infants will suffer from RSV infections during their first winter. In young children with pulmonary or cardiac disease, up to a 37% mortality rate has been reported
20 due to RSV. Although RSV respiratory infection is common in the young, the virus is found in respiratory secretions of infected persons of any age. Outbreaks among the elderly have been associated with serious and fatal illness, and RSV infection can be the source
25 of fever and pulmonary infiltrates in immunosuppressed adults (Englund et al., 1988, Ann. Int. Med., 1:203).

A vaccine of inactivated RSV has proven to be ineffective against RSV infection and leads to an unusual immune and lung inflammation upon subsequent
30 RSV infection. Therefore, recent research has concentrated on developing either potent live vaccines or an effective antiviral compound.

Ribavirin, (1- β -D-ribofuranosyl-1, 2, 4-
35 triazole 3-carboxamide), the current drug of choice, has been found to reduce the severity of illness and amount of virus shed in acute respiratory infections of RSV or influenza virus. Currently ribavirin is



used for the treatment of RSV infections of the respiratory tract in aerosol form. However, because of its toxicity, ribavirin is less desirable for use systemically. Additionally, ribavirin has been found to be teratogenic in animals. This has raised serious concern for female health care personnel administering or exposed to ribavirin (Gladu et al., 1989, *J. Toxic Env. Health*, 28:1).

10 2.4 OTITIS MEDIA AND OTITIS EXTERNA

Otitis media, inflammation of the middle ear, is second only to colds as the most common disease of early childhood. More than 60% of all children will have an episode of otitis media by age
15 six. The greatest risk for the development of acute otitis media lies in infections caused by respiratory syncytial viruses, rhinoviruses, influenza A viruses or adenoviruses (Henderson, et al., *N. Eng. J. Med.*, 1982, 1377; Ruuskanin, et al., *Pediatr. Infect. Dis. J.*, 1989, 94; Sanyai, et al., *J. Pediatr.*, 1980, 11).
20 It has been found that prevention of the respiratory viral infection decreases the incidence of otitis media (Heikkinen, et al., *AJDC*, 445 (1991). Use of a flu vaccine has been found to be effective. However,
25 there are a number of reasons why this is an unsatisfactory approach: it is necessary to administer the vaccine annually, two doses must be given initially, there is a problem of continual antigenic
30 variation of the influenza viruses and the resulting fluctuation in efficacy of the vaccine and the cost.

2.5 INFLUENZA VIRUSES

Influenza viruses are members of the
35 Orthomyxoviridae family and are distributed worldwide. Five pandemics have occurred in the 20th century, with the 1918 outbreak killing at least 21 million. In the United States, over 500,000 deaths



have been attributed to influenza epidemics over the last twenty years. The outbreaks are seasonal, occurring almost every winter, and transmission is primarily through the respiratory route. Amantadine
5 has been shown to shorten the fever duration and respiratory symptoms by only about 50%, but causes some central nervous system side effects. Rimantidine, as yet unlicensed, shows a similar effect, although it is not associated with the central
10 nervous system side-effects seen with amantadine. Primary influenza viral pneumonia, a common complication of influenza infections, has not been shown to be treatable by either rimantidine or amantadine. Prophylactically, influenza has been
15 controlled by the use of inactivated influenza virus vaccines with about 80% protective efficacy.

2.6 HERPES SIMPLEX VIRUS

Herpes simplex virus (HSV) is a member of
20 the Herpoviridae family, and is distributed worldwide. Transmission of the virus occurs via direct contact from person-to-person, with common entry of HSV-1 through the oral cavity and infection of HSV-2
25 through the genital tract. The prevalence of HSV antibody is inversely proportional to socioeconomic status, with close to 100% of HSV-positive adults in underdeveloped countries and 30-50% in developed countries. Acyclovir, idoxuridine (IDU or 2-deoxy-5-iodouridine), trifluridine and vidarabine (adenine
30 arabinoside, Ara-A) are effective in treatment of various HSV infections, with trifluridine and acyclovir the drugs of choice. Vidarabine, the choice for ophthalmic infections, is not effective topically
35 against herpes labialis or other skin/genital infections. Acyclovir is not effective topically when used against recurrent genital infections. However, it is effective when used i.v. and p.o. Acyclovir is



not recommended for routine use in treatment of recurring HSV genital infections as ganglionic latency is still present and outbreaks occur after treatment is stopped.

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3. SUMMARY OF THE INVENTION

The present invention relates to a method of treating respiratory virus infections, comprising administering to a warm-blooded animal, a
10 therapeutically effective amount of an antiviral agent comprising a proanthocyanidin polymer. The proanthocyanidin polymer preferably contains 2 to 30 flavonoid units, more preferably 2 to 15 flavonoid units, and most preferably 2 to 11 flavonoid units.
15 The flavonoid units include but are not limited to catechins, epicatechins, gallocatechins, galloepicatechins, flavanols, flavonols, flavandiols, leucocyanidins, anthocyanidins, or combinations thereof. The flavonoid units can be singly or doubly
20 linked to each other. More specifically, the method of the invention can be used to treat respiratory infections induced by a respiratory syncytial virus as well as Parainfluenza virus 3, Influenza A, Influenza B virus and virus associated with otitis media and
25 otitis externa. The proanthocyanidin polymer can be administered intravenously, intraperitoneally, subcutaneously, intramuscularly, orally, topically, or by inhalation.

30 The proanthocyanidin polymers are effective against most of the viruses implicated in otitis media. They are soluble in aqueous solutions, are readily formulated for parenteral, oral, topical (i.e., into ear and nose) administration and thus are
35 admirably suited for use in the prophylaxis or treatment of otitis media.

The proanthocyanidin polymers are similarly effective against the viruses implicated in otitis



externa. Because such viruses are located outside of the tympanic membrane, the proanthocyanidin polymers can be topically administered into the ear in a suitable formulation so as to come directly into
5 contact with the viruses.

The proanthocyanidins useful for treating respiratory virus infections have a structure selected

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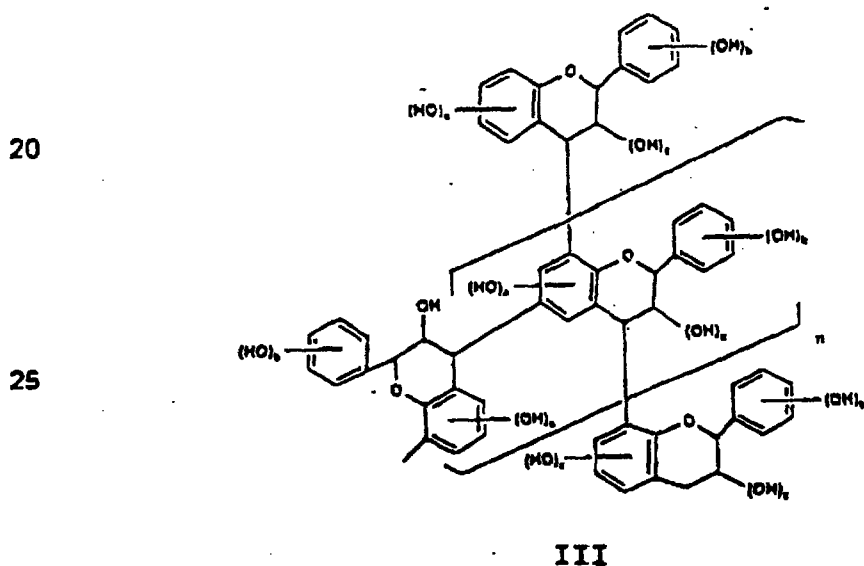
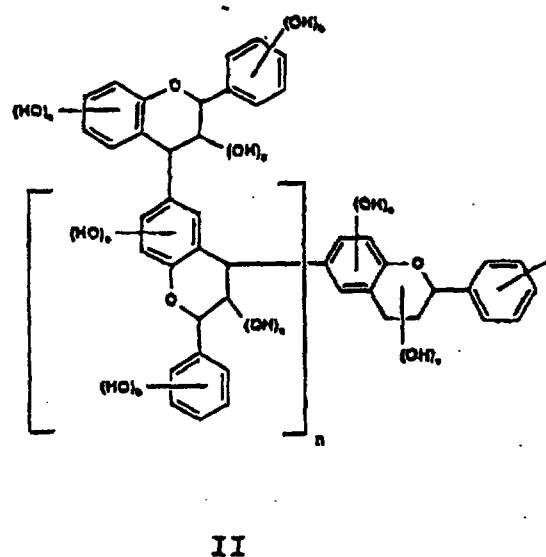
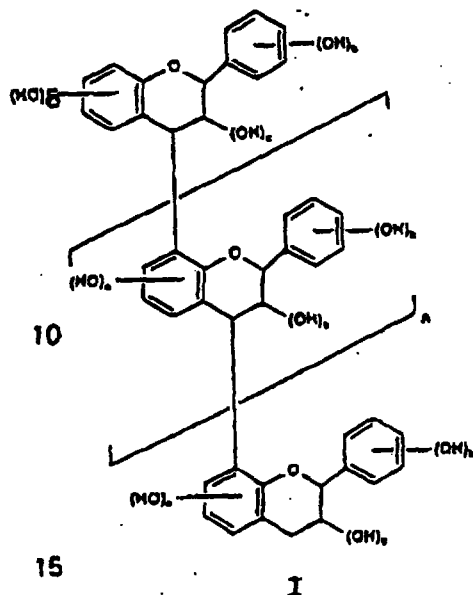
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from I, II, and III, and their esters, or ethers or the corresponding oxonium salts



30 where $a, b = 1$ to 3 , $x = 0$ or 1 , $n = 0$ to 28 , preferably 0 to 13 , more preferably 0 to 9 .

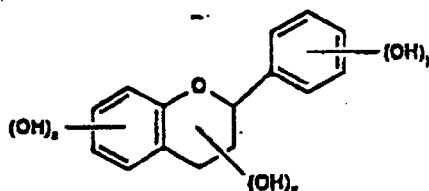
The proanthocyanidin polymer useful for treating a respiratory virus infection can comprise 2 to 30, preferably 2 to 15, most preferably 2 to 11

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monomeric flavanoid units having structure IV, or esters, ethers or corresponding oxonium salts thereof.



IV

where x, y = 1 to 3, z = 1 or 2.

15 Another embodiment of the invention further relates to a method of treating virus infections comprising administering, to a warm-blooded animal, a therapeutically effective amount of antiviral agent

20 comprising a proanthocyanidin polymer containing 6 to 30 flavonoid units, preferably 6 to 15 flavonoid units and more preferably 6 to 11 flavonoid units. The flavonoid units include but are not limited to catechins, epicatechins, gallocatechins,

25 galloepicatechins, flavanols, flavonols, flavandiols, leucocyanidins, anthocyanidins, or combinations thereof. The flavonoid units can be singly or double linked to each other. The method can be used to treat virus infections which are caused by paramyxoviridae,

30 orthomyxoviridae or herpes viruses. The proanthocyanidin polymer can be administered intravenously, intraperitoneally, subcutaneously, intramuscularly, orally, topically, or by inhalation.

The present invention also relates to

35 proanthocyanidins useful for treating virus infections in general having a structure selected from I, II, and III, above, and esters, ethers and corresponding



oxonium salts thereof, where $n = 4$ to 28, preferably 4 to 13, most preferably 4 to 9.

The present invention also relates to novel proanthocyanidins which are obtained from a *Croton* species and from a *Calophyllum inophyllum* species and their esters, ether and oxonium derivatives. Such proanthocyanidin can be isolated from the whole plant, the bark, the leaves, the roots or the latex. In a preferred embodiment, the proanthocyanidin can be obtained from *Croton lechleri* and from *Calophyllum inophyllum*. These novel proanthocyanidin polymers are characterized by IR, UV-visible, and/or ^{13}C NMR spectroscopy.

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4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Fourier-transform infra-red spectrum of proanthocyanidin polymer A isolated from the *Croton* tree.

20 Figure 2 ^1H Nuclear magnetic resonance spectrum of proanthocyanidin polymer A isolated from the *Croton* tree. Sample was in D_2O at 400 MHz.

Figure 3 Broad-band decoupled ^{13}C nuclear magnetic resonance spectrum of proanthocyanidin polymer A in D_2O at 100 MHz.

25 Figure 4 The ^{13}C NMR spectra of proanthocyanidin polymer B isolated from *Calophyllum inophyllum*, obtained at 100 MHz in D_2O .

30 Figure 5 Fourier-transform infra-red spectrum of proanthocyanidin polymer B isolated from *Calophyllum inophyllum*.

Figure 6 The effect of proanthocyanidin polymer A and Amantadine on lung consolidation in mice infected with influenza A (H1N1) virus (early treatment initiation).

35 Figure 7 The effect of proanthocyanidin polymer A and Amantadine on lung virus titers in mice infected with influenza A (H1N1) virus (early treatment initiation).



Figure 8 The effect of proanthocyanidin polymer A and Amantadine on arterial oxygen saturation (SaO_2) in male mice infected with influenza A (H1N1) virus (early initiation).

5 Figure 9 The effect of proanthocyanidin polymer A and Amantadine on arterial oxygen saturation (SaO_2) in female mice infected with influenza A (H1N1) virus (early treatment initiation).

Figure 10 The effect of proanthocyanidin polymer A
10 and Amantadine on lung consolidation in mice infected with influenza A (H1N1) virus (late treatment initiation).

Figure 11 The effect of proanthocyanidin polymer A on lung titers in mice infected with influenza A (H1N1)
15 virus (late treatment initiation).

Figure 12 The effect of proanthocyanidin polymer A and Amantadine on arterial oxygen saturation (SaO_2) in male mice infected with influenza A (H1N1) virus (late treatment initiation).

20 Figure 13 The effect of proanthocyanidin polymer A and Amantadine on arterial oxygen saturation (SaO_2) in female mice infected with influenza A (H1N1) virus (late treatment initiation).

Figure 14 The effect of Ribavirin on lung virus
25 titers in mice infected with influenza A (H1N1) virus.

Figure 15 The effect of intraperitoneal Ribavirin treatment on lung consolidation in influenza A (H1N1) virus infected mice.

30 Figure 16 The effect of intraperitoneal Ribavirin treatment on blood SaO_2 in influenza A (H1N1) virus infected mice.

Figure 17 The effect of proanthocyanidin polymer A and Ganciclovir on HSV-2 vaginal lesions in mice.

35 Figure 18 The effect of proanthocyanidin polymer A in 5% and 10% topical formulations, compared to placebo and acyclovir, in a 5% topical formulation.



5. DETAILED DESCRIPTION OF THE INVENTION

5.1 USEFUL PROANTHOCYANIDIN POLYMERS

Proanthocyanidin oligomers or polymers, useful for the present anti-viral methods are comprised of monomeric units of leucoanthocyanidins. Leucoanthocyanidins are generally monomeric flavonoids which include catechins, epicatechins, gallocatechins, galloepicatechins, flavanols, flavonols, and flavan-3,4-diols leucocyanidins and anthocyanidins. The proanthocyanidin polymers useful for treating respiratory virus infections have 2 to 30 flavonoid units, preferably 2 to 15 flavonoid units, and most preferably 2 to 11 flavonoid units. The proanthocyanidin polymers useful for treating virus infections in general, including but not limited to, infections related to influenza viruses, parainfluenza viruses, viruses of the types known as paramyxoviridae, orthomyxoviridae, and herpes viruses, have 6 to 30 flavonoid units, preferably 2 to 15 flavonoid units, and most preferably 6 to 11 flavonoid units.

Proanthocyanidin polymers having a varying number of flavonoid units are known and have been reported, for example, in W.L. Mattice, et al. *Phytochemistry*, 23, p. 1309-1311 (1984); Z. Czochanska, et al., *J.C.S. Chem. Comm.*, 375 (1979); W.T. Jones, et al., *Photochemistry*, 15, p. 1407-1409 (1976); E. Haslam, *Plant Polyphenols*, p. 75 (1989), which are all incorporated by reference. Those polymers having the recited ranges of flavonoid units and described in these references are useful for the methods of the present invention.



5.2 METHODS FOR OBTAINING USEFUL PROANTHOCYANIDIN POLYMERS

5.2.1 ISOLATION

Useful proanthocyanidin polymers are
5 obtained from various plants including but not limited
to the classes Filices, Coniferae, Monocotyledoneae,
and Dicotyledoneae (J.B. Harborne, THE FLAVONOIDS,
ADVANCES IN RESEARCH SINCE 1980 (1988)). They can be
obtained using the entire tree or plant, the bark,
10 stems, roots or latex.

These plant source materials are extracted
with water and/or a water miscible solvent. The
preferred solvents are alcohol of 1-3 carbon atoms or
acetone. The aqueous extract is used directly or
15 after solvent removal. If the solvent is removed, the
solid residue is redissolved in a solvent, preferably
a lower alcohol or acetone, and insoluble materials
are discarded. The soluble fraction is subjected to
gel filtration (e.g., over a Sephadex), reversed-phase
20 column chromatography (e.g., C-8), or gel-permeation
chromatography (e.g., divinyl benzene cross-linked
gels) such as PL-GEL or membranes (e.g., an Amicon
membrane) using water or water and a water miscible
solvent, with or without a buffer, as the mobile
25 phase. The water miscible solvent is preferably a 1-3
carbon alcohol, acetone or acetonitrile.

The useful proanthocyanidin polymers of this
invention are the fractions detected by ultraviolet
(uv). They have their major UV absorption maxima
30 between about 200-350 nm, with peaks usually at 200-
210 nm, and 275-285 nm.

With regard to the novel proanthocyanidin
polymer compositions which form part of the basis of
this invention, such polymers can be obtained from the
35 Croton tree or Calophyllum species by using the above-
described extraction method. The soluble fraction is
subjected to gel filtration, reversed-phase column



chromatography, gel-permeation chromatography, or membranes. Using water or water and a water miscible solvent, with or without a buffer as the mobile phase and the relevant fractions containing the polymers are detected using uv at a range of about 200-350 nm.

According to a preferred embodiment of the present invention, a novel proanthocyanidin polymer can be prepared as follows: Latex obtained from a Croton tree (e.g., *Croton lechleri*) or from *Calophyllum inophyllum* is used directly or is concentrated by the removal of water (e.g., lyophilized). It is extracted with water, a lower alcohol of about 1-3 carbons, or with acetone to give an aqueous soluble fraction. The aqueous-soluble fraction of the lyophilized or otherwise concentrated material is subjected to partitioning by ethyl acetate/water solution and to gel filtration (e.g., Sephadex) using water and/or water and alcohol and/or water and acetone, with or without a buffer as the mobile phase. The fractions containing a chromophoric band are detected by ultraviolet (uv) (λ_{max} about 200-350 nm) and collected and concentrated and subjected to gel filtration again, if necessary, to yield the proanthocyanidin polymer. Alternatively, the aqueous-soluble fraction described above is lyophilized or concentrated or is used directly and is subjected to reversed-phase column chromatography (RP HPLC), using water and/or water and acetone and/or water and alcohol as the mobile phase. The fractions containing a chromophoric band detected by uv (max about 200-350 nm) are collected and concentrated, and subjected to RP HPLC again, if necessary, to yield the proanthocyanidin polymer. Alternatively, the aqueous-soluble fraction, described above, is lyophilized, concentrated or is directly subjected to gel permeation chromatography (GPC), using water and/or water and alcohol and/or water and acetonitrile, with



or without a buffer, as the mobile phase. The fractions containing a chromophoric band detected by uv at λ_{max} about 195-350 nm are collected and concentrated and subjected to GPC again, if necessary, to yield the proanthocyanidin polymer. See examples in Sections 6.1 - 6.3, *infra*.

As demonstrated in Section 6.2 when chromatographed on a Perkin Elmer LC-620 using Polymer Labs PL-gel 5m 500 A, 300 x 7.5 mm and THF-water (95:5 v/v) system at a flow rate of 1 ml/min, proanthocyanidin A has a retention time of 7.2 min. and proanthocyanidin B has a retention time of 6.5 min. Typical spectra for proanthocyanidin polymers are shown in Figs. 1 and 5.

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5.2.2 SYNTHESIS OF USEFUL PROANTHOCYANIDIN POLYMERS AND DERIVATIVES THEREOF

It is known that leucoanthocyanidins can be condensed in mildly alkaline or acidic solutions to form proanthocyanidins. As an example, L.Y. Foo and R.W. Hemingway, *J. Chem. Soc., Chem. Commun.*, 85 (1984) were able to synthesize a trimer from flavanol monomers. J.A. Delcoun, et al., *J. Chem. Soc. Perkin Trans. I.*, 1711 (1983) teaches that under mild, low pH, conditions and an excess amount of (+)-catechin, condensation occurred to form higher and predominantly linear [4,8]-linked oligomers. Specifically, dimers, trimers and tetramers of the flavonoid analogues were synthesized and characterized by NMR spectroscopy. By use of such condensation reactions proanthocyanidin polymers of up to about 30 flavonoid units can be synthesized for use in the methods of the invention. As an alternative to the above synthesis, enzymatic synthesis can be employed to produce the useful proanthocyanidin polymers. The novel proanthocyanidin polymer compositions of the present invention

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specifically exemplified herein can also be synthesized by such reactions.

Esters of proanthocyanidin polymers can be prepared by standard methods of acylation such as by the reaction of the polymer with acid chlorides or acid anhydrides in the presence of base. Of particular utility is the preparation by reaction with acid anhydrides in pyridine as utilized for the acylation of certain other lower molecular weight flavonoids by Thompson, et al., JCS, 1387 (1972), Fletcher, et al., JCS, 1628 (1977) and Hemingway, JCS, 1299 (1982).

Ethers can be prepared by standard methods of etherification such as by reaction of the polymer with alkyl halides and alkyl tosylates in bases. Methyl ethers are readily prepared by the use of diazomethane as utilized in the etherification of certain other low molecular weight flavonoids by Thompson, JCS, 1387 (1972), Hemingway, JCS, 1299 (1982).

The oxonium salts (sometimes referred to as pyrilium salts or anthocyanidins) may be prepared by the so-called Bate-Smith reaction (Chemistry and Industry, 1953, 377), by warming with aqueous or alcoholic acids (see also, Thompson, et al., JCS, 1387 (1972) and references cited in Haslam, PLANT POLYPHENOLS, 1989, p. 28, Cambridge Press). This reaction can be advantageously catalyzed by iron (Porter et al., Phytochemistry, 25, 223 (1986)).

5.3 CHARACTERIZATION OF NOVEL PROANTHOCYANIDIN POLYMERS

Novel proanthocyanidin polymers prepared according to the present invention have solubility in methanol and water. The polymers are soluble in water and aqueous solution including alcohol solutions to a degree of at least about 10 mg/ml.



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The proanthocyanidin polymers have been analyzed by a number of methods to determine their molecular weight and various other chemical and physical features. Various stereoisomers of proanthocyanidin polymers have been obtained and are within the scope of the present invention.

Column chromatography has been used to isolate water soluble fractions containing novel proanthocyanidin polymers varying in average molecular weight from about 700 daltons to about 3000 daltons, which corresponds to 2-3 to 9-11 average flavonoid units, respectively.

¹³C-NMR spectroscopy indicates that the proanthocyanidin polymers contain flavonoid moieties in which the individual flavonoid ring units possess various stereochemistries.

UV-visible spectroscopy is consistent with the possible presence of a flavylum moiety or moieties within certain of the proanthocyanidin polymers.

The characterization of proanthocyanidin polymer A and proanthocyanidin polymer B is further discussed in the examples at Sections 6.3 and 6.5. Functional derivatives of these polymers can be made and may be useful as intermediate for the preparation of useful proanthocyanidin polymers.

5.4 PROPHYLACTIC AND THERAPEUTIC USES OF PROANTHOCYANIDIN POLYMERS

Proanthocyanidin polymers have been shown to be active *in vitro* and *in vivo* against a wide variety of viruses, and can be advantageously used in prophylactic and therapeutic applications against diseases induced by such viruses.

The proanthocyanidin polymers can be used either alone or in combination with other antiviral or antimicrobial agents to prevent and/or treat diseases



induced by or complicated with viral infections from viruses including, but not limited to: paramyxoviridae such as respiratory syncytial virus, orthomyxoviridae such as influenza A, B and C, and herpes viruses such as Herpes Simplex virus.

Proanthocyanidin polymers have various advantages in the treatment of viral infections including, but not limited to:

- 1) a broad range of antiviral activity;
- 10 2) very low toxicity;
- 3) no teratogenicity; and
- 4) application to both systemic as well as localized (i.e., topical) applications.

15 5.5 ROUTES OF ADMINISTRATION

The proanthocyanidin polymers of the present invention can be administered for prophylactic and therapeutic applications by a number of routes, including but not limited to: oral, injection
20 including but not limited to, intravenous, intraperitoneal, subcutaneous, intramuscular, etc., by topical application such as to nasal, nasopharyngeal linings and into the ear, and by inhalation via aerosolization and application to respiratory tract
25 linings, etc.

When used according to the present invention against viruses, effective dose ranges of the proanthocyanidin polymers are about 5.0 - about 30 mg/kg if given orally (P.O.), about 0.1 - about 10
30 mg/kg if given intraperitoneally (I.P.), and about 5 - about 30 mg/kg/day if given by aerosol. If given topically, the proanthocyanidin polymer is applied in a suitable vehicle at a concentration of about 5 to
35 about 15%.

When administered to warm-blooded animals, including humans, the proanthocyanidins may be

combined with water, an aqueous solution or any physiologically acceptable carrier or vehicle.

The following series of Examples are presented for purposes of illustration and not by way of limitation on the scope of the invention.

6. EXAMPLES

6.1 ISOLATION OF A NOVEL PROANTHOCYANIDIN POLYMER FROM A CROTON LECHLERI SPECIES (PROANTHOCYANIDIN POLYMER A)

10 In one series of experiments, a novel proanthocyanidin polymer was obtained as follows:

C. lechleri trees were tapped and felled near the village of San Pablo de Cuyana on the Nanay River 100 kilometers from Iquitos, Peru. The latex
15 was obtained over a period of 24 hours by scoring the trees.

The latex (11) obtained from the Croton lechleri trees was diluted with isopropanol in the ratio of 1 part latex to 3 parts isopropanol (31) and
20 allowed to stand for 15 hours at 10°C. The resultant isopropanol-diluted latex was centrifuged, and the insoluble material was removed by decantation, leaving the mother liquor (3.7 l). The mother liquor was
25 concentrated to dryness by rotoevaporation at 33°C to give 240 g of a deep-red brown powder. This concentrated material (980 g) was subjected to gel filtration using Sephadex CM-50 with water (20 l) as the mobile phase. The early-eluting fractions
30 containing a red pigment were detected at λ 200-350 nm or by visual detection of a red band eluting through a transparent column. To isolate the proanthocyanidin polymer, the early-eluting fractions were collected and subjected to gel filtration chromatography and/or
35 HPLC as described below.

The HPLC isolation was performed by injecting 5 μ l of concentrated samples onto a Perkin-



Elmer LC Analyst liquid chromatograph system equipped with an IC-200 autosampler, LC 600 pumps and a LC-235 diode array detector using a Waters Ultrahydrogel 500 GPC column with Burdick and Jackson HPLC-grade water at 0.8 ml/min at ambient temperature, with detection at λ 280 and 195 nm. The proanthocyanidin polymer was found to have a retention time of 4.6-5.6 minutes depending on sample concentration, column conditioning and temperature.

10 The red-pigment containing fractions isolated from Sephadex CM-50 were combined and subjected to gel filtration chromatography over Toyopearl HW-40S (2.5 L), using water, 10% acetone/water, 20% acetone/water, and finally 40%
15 acetone/water in a step gradient fashion. The late-eluting red fractions detected by uv-vis as λ_{max} 340 nm, were combined and concentrated for further purification on HPLC as described above. The combined fractions isolated from HPLC were again subjected to
20 gel filtration chromatography over Toyopearl HW-40S to yield a proanthocyanidin polymer designated proanthocyanidin polymer A.

 In another series of experiments, a novel proanthocyanidin polymer was obtained as follows:

25 Four liters of cold crude latex from the *C. lechleri* (or from the entire macerated plant, the bark or the roots) were diluted with twelve liters of isopropanol, stirred and stored at 5°C for 15 hours.
30 The residue was removed by filtration and the solution evaporated to dryness, in vacuo, yielding about 970 g. of solids.

 The solids were added, with stirring, to 6 l of water and 3.6 l of n-butanol. The aqueous phase
35 was separated and concentrated to dryness giving 700 g of material which was added, with stirring, to 2 l methanol, and then 12 l of ethyl acetate was added. The solution was kept at 15° for 15 hrs and the solid



material removed by filtration and discarded. The solution was concentrated to dryness yielding approximately 390 g. of crude proanthocyanidin polymer A.

5 The proanthocyanidin polymer A fraction was concentrated by a combination of cation exchange, adsorption and size exclusion chromatography as follows: 450-600 g of the crude proanthocyanidin polymer A was passed through a CM-Sephadex C-50 pre-
10 column using de-ionized water as the eluent. The orange and dark red fractions were collected and then chromatographed, using de-ionized water, over another Sephadex C-50 column equipped with a UV detector set at 460 nm, and 1-2 l fractions were collected. The
15 initial pink band was discarded and the subsequent effluent passed through a column containing Sephadex G-50 until the broad red-brown band was eluted. 15% aqueous acetone was passed through the G-50 column until the effluent was colorless. Fractions of the
20 effluent were examined by HPLC as described in Section 6.2, and those fractions containing proanthocyanidin polymer A were combined and evaporated to dryness yielding about 210 g. of proanthocyanidin polymer A.

25 **6.2 PURIFICATION OF PROANTHOCYANIDIN
POLYMER A**

 About 150 g. of crude proanthocyanidin polymer A, obtained as described in Section 6.1 above, was dissolved in 300 ml 20% aqueous acetone and was
30 chromatographed over a mixed-mode gel-permeation/absorption column (Toyopearl HW40S, a spherical methyl methacrylate polymer of 40 μ m particle size). 16 l were collected, then the eluting solvent was changed to 40% aqueous acetone and 8 more
35 liters were collected, then the elution was continued with 4 l of 60% aqueous acetone. The fractions containing proanthocyanidin polymer A, as shown by



HPLC (See Section 6.3), were combined and the solvent removed in vacuo yielding approximately 57 g. of solid.

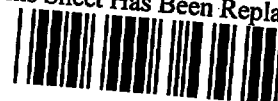
Final purification was accomplished by a combination of adsorption and size-exclusion chromatography: 50-75 g. of the above solid were dissolved in 90% ethanol and introduced onto a column containing Sephadex LH-20 (a cross-linked dextran gel with hydroxypropyl groups attached by ether linkages to glucose units of the dextran chains). Elution was carried out using 10 l of 90% aqueous ethanol, then 15 l of 20% aqueous acetone, 5 l of 40% aqueous acetone, 5 l of 50% aqueous acetone and then 5 l of 60% aqueous acetone. Fractions were collected at 2 l intervals and assayed by HPLC. The fractions containing proanthocyanidin polymer A were combined and evaporated to dryness in vacuo at 35° giving 35 g. of pure proanthocyanidin polymer A.

For the HPLC, a 30 cm gel-permeation column for non-aqueous mobile phases was used in which the stationary phase is Polymer Laboratories PL-Gel 5m 500Å (a divinylbenzene-polystyrene polymer, 5 μ particles, 500Å pore size). The HPLC system was equipped with a diode array detector that generates UV spectra, with the detector set at 280 nm.

Samples are dissolved in 95% aq THF, which is also the developing solvent. At a flow rate of 1 ml/min, the proanthocyanidin polymer A peak maximum has a retention time of 7.2 ± 0.5 min.; proanthocyanidin polymer B has a retention time of 6.5 ± 0.5 min, indicating that proanthocyanidin polymer B has a larger molecular weight than proanthocyanidin polymer A.

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6.3 CHEMICAL IDENTIFICATION AND STRUCTURAL FEATURES OF THE PROANTHOCYANIDIN POLYMER A

In the infrared, the proanthocyanidin polymer A of the invention, obtained as described in Section 6.1 above, shows a very broad intense peak ranging from 3550-2500 and other peaks at 1612, 1449, 1348, 1202, 1144, 1107, 1068 and 1027 cm⁻¹. See Figure 1.

The ¹H NMR spectrum in D₂O (400 MHz 24°C) exhibited very broad peaks at δ 7.1, 6.9, 6.1, 4.7 and 2.8 ppm. See Figure 2.

Ultraviolet-visible spectral analysis in H₂O revealed broad peaks at λ 202, 235, (shoulder), 275, 305 (shoulder), 460 and trailing greater than 600 nm. Although the UV data of the present polymer closely resembles those of other known proanthocyanidins, the visible data are clearly different. The known proanthocyanidin monomers and polymers are colorless (λ 205, 240, 275 nm) and have no absorption in the visible range, whereas certain of the isolated novel proanthocyanidin polymer A of the present invention are colored and have a visible absorption at 460 nm. The color of the proanthocyanidin polymer suggests the presence of a flavylum moiety or moieties within the proanthocyanidin polymer. This is consistent with the visible spectroscopic data reported for closely related monomeric anthocyanins which contain the flavylum moiety (λ 460-560 nm).

The ¹³C NMR spectra of the proanthocyanidin polymer was obtained at 100 MHz in D₂O. The experiments performed were broad-band decoupled. As shown in FIG. 3, the ¹³C NMR spectrum in D₂O exhibited very broad peaks at δ 155(C-5, C-7, C-9), 145(C-3', C-5'), 143(C-3', C-4'), 130(C-1', C'-1'', C-4''), 128(C-1'), 121(C-6), 116(C-2', C-5'), 109(C-8, C-2'', C-6'*), 97(C-6), 82(C-2), 76(C-2), 73(C-3), and 38(C-4) ppm,



for the prodelphinidin B ring. The ^{13}C NMR data suggest some key points of structural differences between the novel antiviral proanthocyanidin polymers of the present invention and known proanthocyanidin polymers. The most significant difference is a substantially larger peak in the region of 109 ppm for the proanthocyanidin polymers of this invention when compared with ^{13}C NMR spectra of other proanthocyanidin polymers known in the literature (compare with spectra in Czochanska et al., 1979, J.C.S. Chem. Comm., p. 375-77; in Harborne, J.B. and Mabry, T.J. ed., *The Flavonoids: Advances in Research*, Chapman and Hall, NY, 1982, pp. 51-132, incorporated herein by reference). The ^{13}C NMR data of the polymer is indicative of the proanthocyanidin class of polymers. In particular, the ^{13}C NMR chemical shift data of the isolated proanthocyanidin polymer (C-6'=132 ppm, C-2'=115 ppm, C-3' & C-4'=145 ppm, C-5'=116 ppm, C-6'-107 ppm, C-3' & C-5' =146 ppm, C-4'-133 ppm) is consistent with a polymer composed of procyanidin B ring moieties with the individual flavonol C ring units possessing both the 2,3-trans and 3,4-trans [similar to (+)-catechin; C-2=83 ppm, C-3=73 ppm, C-4=38ppm] and 2,3-cis-3,4-trans [similar to (-)-epicatechin; C-2=77 ppm, C-3=73 ppm, C-4=73 ppm] stereochemistries. The present experiment data indicates that proanthocyanidin polymer A is comprised of catechin, epicatechin, galocatechin and galloepicatechin. The HPLC data suggest that the average number of flavonoid units is about 7. The HPLC data also suggests that number of flavonoid units vary from 2 to 11.

The proanthocyanidin polymer A is soluble in methanol, water and aqueous solutions. The proanthocyanidin polymer A is soluble in water at concentrations of at least about 10 mg/ml. The polymer has lower solubility in normal saline and



other salt solutions. Mass spectral analysis indicate that proanthocyanidin polymer A has a molecular weight average of about 2,100 daltons.

5 **6.4 ISOLATION OF A PROANTHOCYANIDIN POLYMER
FROM CALOPHYLLUM INOPHYLLUM
(PROANTHOCYANIDIN POLYMER B)**

 A novel proanthocyanidin polymer according to the present invention can be isolated by a method similar to that described above in Section 6.1 from
10 the entire macerated plant, the bark, the leaves, the roots or the latex of *Calophyllum inophyllum*. According to a preferred method, the proanthocyanidin polymer is obtained by the method described in Section 6.1, except that water is used as the preferred
15 extraction solvent.

 In one series of experiments, a novel proanthocyanidin polymer, designated proanthocyanidin polymer B, was obtained from *Calophyllum inophyllum*
20 latex.

 2,849 g of the latex from *Calophyllum inophyllum* was mixed with 12.4 l of a 1:1 mixture of isopropanol and water, stirred and stored at room temperature for 36 hours. The residue was removed by
25 filtration and the solution was evaporated to dryness, in vacuo, giving 133.5 g of solids.

 The solids were added, with stirring, to 30. g of methanol. The solution was then filtered away from the solids, and a 1:1 mixture of water and ethyl
30 acetate was added. The water fraction was separated and n-butyl alcohol was added. The water fraction was separated from the alcohol fraction and concentrated to dryness yielding approximately 10.4 g of crude proanthocyanidin polymer B.

35 The crude proanthocyanidin polymer B was passed through a CM-50 Sephadex CC column using de-ionized water as the eluting solvent. A red band was



collected and fractionated using a LH-20 CC column, eluted with 70% aqueous ethanol solution and 20%, 50% and 70% aqueous acetone solutions to give proanthocyanidin polymer B. At lower concentrations, a solution of proanthocyanidin polymer B is essentially colorless. At higher concentrations, the solution of proanthocyanidin polymer B is tan.

6.3 CHEMICAL IDENTIFICATION AND STRUCTURAL FEATURES OF THE PROANTHOCYANIDIN POLYMER B

The ^{13}C NMR spectrum of proanthocyanidin polymer B (Figure 4) confirms that the polymer is a member of the class of proanthocyanidin polymers and comprises principally catechin and epicatechin monomeric flavonoid units. Gel permeation chromatography (GPC) indicates that proanthocyanidin polymer B has a molecular weight that is larger than proanthocyanidin polymer A. Consistent with the GPC data, HPLC-GPC retention time of proanthocyanidin polymer B is shorter than that for proanthocyanidin polymer A under the same conditions -- again indicating the larger size of proanthocyanidin polymer B. HPLC indicates that polymer B has a molecular weight average of about 3000 daltons, corresponding to an average number of flavonoid units of about 10. The HPLC data also suggests that the number of flavonoid units vary from 5 to 16. Mass spectral analysis indicates that proanthocyanidin polymer A has a molecular weight average of about 2100 daltons.

The Fourier-transform infra-red spectrum of proanthocyanidin polymer B is quite similar to that of proanthocyanidin polymer A (See Figure 5 and Figure 1).

Likewise, the UV-visible spectrum of proanthocyanidin polymer B is similar to that of

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proanthocyanidin polymer A except for the absence of the peak at 460 nm.

7. SCREENING OF PROANTHOCYANIDIN POLYMER A FOR ANTIVIRAL ACTIVITY

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In one series of experiments, proanthocyanidin polymer A was tested for antiviral activity against the following viruses: Respiratory syncytial virus subtype variants, A2-Tracey, A-Long, B-46791, B-47063 and B-18537; parainfluenza virus, type 3 (PIV-3); adenoviruses, type 5 and 7; influenza, A-Taiwan (H1N1); A-Leningrad (H3N2); A-Japan; A-Port Chalmers; A-NWS33; B-USSR; B-Tama; B-RF; measles virus, and Edmonston strain.

15

All viruses were obtained from the Influenza Research Center, Baylor College of Medicine, Houston, Texas, with the exception of measles virus which was obtained from ATCC. For comparison, ribavirin was included in the screening assay.

20

The following procedure was used to assay for antiviral activity. Assays were performed in 96-well tissue culture plates. All dilutions and tissue culture suspensions were prepared in minimal essential medium containing antibiotics penicillin and streptomycin and 2% fetal calf serum (2% FCS-MEM). Test compounds (0.05 ml) were added in quadruplicate to wells of the test plates containing a subconfluent monolayer of HEp2 cells (ca. 3×10^3 cells). The compounds were diluted using serial 2-fold dilutions usually starting with a final concentration of 1 mg/ml. Approximately 100 median tissue culture infectious doses (TCID₅₀) of the appropriate test virus in 0.05 ml was added. Tissue control wells contained medium, without virus or antiviral compound, and antiviral control wells contained antiviral compound without virus. Ribavirin was included in each assay as a positive antiviral control, except for the

25

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adenoviruses where Ribavirin fails to demonstrate antiviral activity. Back titrations of each test virus were also included in each assay. All plates were incubated at 37°C in a 5% CO₂ incubator. Virus control wells were observed daily. When these wells exhibited 80-100% cytopathic effect (CPE), all wells were observed for CPE. In addition to visual and microscopic observance of CPE, inhibition of syncytial formation was used to confirm activity against RSV.

In each antiviral assay, a 50% minimal inhibitory concentration (ED₅₀) was determined. The ED₅₀ was calculated by determining the median minimal concentration of compound tested in wells inhibiting CPE 50% compared to virus control wells. The actual calculation of the ED₅₀ value was done with the aid of the computer program, "Dose-effect analysis with microcomputers" of Chou et al., 1984, Adv. Enz. Regul., 22:27-55.

The effect of each compound on the growth of uninfected tissue culture cells, seeded at low densities to allow rapid growth, was also evaluated. From this assay, a 50% minimal toxic concentration (ID₅₀) was determined. The assay for cell toxicity or inhibition of cell growth involved the following procedure. Test compounds (0.1 ml/well) were serially diluted 2-fold. To the appropriate wells were added 0.1 ml of human HeLa, A549 or HEp2 cells, 0.1 ml of mouse L929 cells, 0.1 ml of monkey Vero cells or 0.1 ml of canine MDCK cells. Approximately 3×10^3 cells were added to each well. Control wells consisted of wells containing a range of cell concentration (e.g., 3×10^3 cells, 1:2 dilution of this number, 1:4, 1:8 and 1:16) in medium without any antiviral compound. A vehicle control consisting of serial dilutions of whatever vehicle was used for a particular compound (e.g., 10% DMSO, 50% methanol) was also included in each assay in



duplicate. All plates were incubated at 37°C in a 5% CO₂ incubator. After control wells containing cells, but no test compound, reached confluency, 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide (MTT) was added to all wells. Three hours later, acid alcohol (0.1 ml of 0.4 N HCl in isopropyl alcohol) was added to each well to solubilize any precipitate formed in each well. Plates were read on a plate reader (UV MAX, Molecular Devices) to determine the optical densities in each well. The median concentration of antiviral compound in the last wells causing a 50% reduction in O.D. was determined and termed "ID₅₀". All wells in the assay were also observed microscopically for inhibition of cell growth. The results are shown in Table 1.



TABLE 1
Effect of In Vitro Screening for Antiviral Activity
with Proanthocyanidin Polymer A

ED ₅₀ (μg/ml)			
<u>Virus</u>	<u>Proanthocyanidin Polymer A</u>	<u>Ribavirin</u>	
Experiment 1			
RSV	8.5	4.0	
Parainfluenza virus 3	>94	15.6	
10 Influenza A	88	15.0	
Influenza B	125	18.0	
Adenovirus	>125	----	
Rhinovirus	>125	125	
Measles	>125	31.3	
Experiment 2			
RSV	17.2	24.2	
Parainfluenza virus	38.3	15.7	
Influenza A	31.3	14.3	
Influenza B	125	18.0	
Adenovirus 5	>250	>1000	
Adenovirus 7	>250	>1000	
20 Rhinovirus	>125	125	
Measles	>125	31.3	
Experiment 3			
RSV	12	31	
Parainfluenza virus 3	5	12	
25 Influenza A	4	14	
Adenovirus 7	>250	>1000	

In the first experiment of Table 1, the ED₅₀ for the proanthocyanidin polymer A was determined to be 8.5 μg/ml for RSV. This activity compares quite closely to ribavirin with a ED₅₀ determined to be 4.0 μg/ml for RSV. The ID₅₀ (an index of cell toxicity) of the proanthocyanidin polymer A was 94 μg/ml. In the second experiment of Table 1, the ED₅₀ for the proanthocyanidin polymer A was 17.2 μg/ml for RSV as compared to 24.2 for ribavirin, and the ID₅₀ was 250 μg/ml. In the third experiment of Table 1 the ED₅₀ for



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the proanthocyanidin polymer A was 12 $\mu\text{g/ml}$ and the ID_{50} was greater than 250 $\mu\text{g/ml}$.

The results of antiviral activity of the proanthocyanidin polymer A compared to ribavirin in each assay was determined by calculating the drug Selective Index (SI), defined as the ratio of $\text{ID}_{50}/\text{ED}_{50}$. The Selective Index of the proanthocyanidin polymer A for RSV was 11 and 14.5 and 21 in the three experiments.

10 Virus rating (VR) was obtained by averaging the sum of the CPE values (0 for no CPE; 4 for 100% CPE) assigned to treated, infected monolayers at each compound concentration. This average is subtracted from the average of the sum of the CPE values in an
15 equal number of virus control wells. A further adjustment was made to reflect any observed cytotoxicity. Values for VR were assigned as follows: VR greater than 1, defined as definite anti-viral activity; a VR of 0.5-0.9, as moderate to questionable
20 activity; a VR of 0.1-0.5, as slight activity. The slight activity could be attributable to cytotoxicity or cytopathic effects of the compound per se. The VR of the proanthocyanidin polymer A against RSV was 1.5, indicating definite anti-viral activity in these in
25 vitro assays. Also, the proanthocyanidin polymer A demonstrated substantial anti-viral activity against parainfluenza and influenza A viruses in all three experiments.

30 In another series of experiments, polymer A was tested for antiviral activity against RSV, types A and B, influenza types A (Flu-A) and B (Flu-B), and parainfluenza types (PIV-1) and 3 (PIV-3).

35 The cell lines used were Human HeLa, A549 and HEp-2 cells, mouse L929 cells, monkey Vero cells, canine MDCK cells.

The effect of the proanthocyanidin polymer A on the viability and growth of cells was determined by



measurement of mitochondrial respiration and expressed as a 50% minimal toxic concentration (ID_{50}). In this assay, as in the ED_{50} assay, the proanthocyanidin polymer A was assayed in serial 2-fold dilutions in quadruplicate across 96-well microtitre plates. Again, wells were seeded at the low density of 3×10^3 cells per well to allow for rapid growth. Vehicle controls which consisted of serial dilutions of vehicle (which, in the case of proanthocyanidin polymer A, was water) were included in each assay in duplicate. Blanks which consisted of media alone and media plus drug were also included. All plates were incubated at $37^\circ C$ in a 5% CO_2 incubator until the tip concentration of cells in control wells reached confluency, usually 3 days. All wells in each assay were observed microscopically for cytotoxicity. Then 0.05 ml of MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diaphenyltetrazolium bromide) (5 mg/ml in PBS) was added to all wells and the incubation continued. Three hours later acid alcohol (0.05 ml of N HCl in isopropyl alcohol) was added to each well. The optical densities (O.D.) of each well were read at 490 nm using a plate reader (UV MAX, Molecular Devices). The percent viability of cells at each concentration of drug was calculated by dividing the mean of the O.D.'s of the drug treated wells (minus the mean of the drug blank) by the mean of the O.D.'s of control wells (minus the mean of the blank), and multiplying by 100. The effect of the vehicle on cell viability was similarly calculated. A dose response curve for the toxicity of proanthocyanidin polymer A was generated and the ID_{50} determined.

The following procedure was used to assay for antiviral activity. Assays were performed in 96-well tissue culture plates. All dilutions and tissue culture suspensions were prepared in minimal essential medium containing antibiotics penicillin and



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streptomycin and 2% fetal calf serum (2% FCS-MEM). Test compounds (0.05 ml) were added in quadruplicate to wells of the test plates containing a subconfluent monolayer of HEp2 cells (ca. 3×10^5 cells). The
5 compounds were diluted using serial 2-fold dilutions usually starting with a final concentration of 1 mg/ml. Approximately 100 median tissue culture infectious doses (TCID₅₀) of the appropriate test virus in 0.05 ml was added. Tissue control wells contained
10 medium, without virus or antiviral compound, and antiviral control wells contained antiviral compound without virus. Ribavirin was included in each assay as a positive antiviral control, except for the
15 adenoviruses where Ribavirin fails to demonstrate antiviral activity. Back titrations of each test virus were also included in each assay. All plates were incubated at 37°C in a 5% CO₂ incubator. Virus control wells were observed daily. When these wells exhibited 80-100% cytopathic effect (CPE), all wells
20 were observed for CPE. In addition to visual and microscopic observance of CPE, inhibition of syncytia formation was used to confirm activity against RSV.

In each antiviral assay, a 50% minimal inhibitory concentration (ED₅₀) was determined. The
25 ED₅₀ was calculated by determining the median minimal concentration of compound tested in wells inhibiting CPE 50% compared to virus control wells. The actual calculation of the ED₅₀ value was done with the aid of
30 the computer program, "Dose-effect analysis with microcomputers" of Chou et al., 1984, Adv. Enz. Regul., 22:27-55.

The selective index for proanthocyanidin polymer A was then calculated as the ratio of the ID₅₀
35 over the ED₅₀.

The results are shown in Tables 2-6. The results indicate that proanthocyanidin polymer A is as



effective as ribavirin for inhibiting growth of RSV-A and B, PIV-1, PIV-3, FLU-A and FLU-B *in vitro*. The selective index values indicate low cytotoxicity for proanthocyanidin polymer A.

Table 2. Effect of Proanthocyanidin Polymer A and Ribavirin Against RSV.

EXP#	PROANTHOCYANIDIN POLYMER A ($\mu\text{g/ml}$)			RIBAVIRIN ($\mu\text{g/ml}$)		
	ED ₅₀	ID ₅₀	S.I.	ED ₅₀	ID ₅₀	S.I.
A-Tracy	9.0	94.0	10.4	4.0	1000.0	250.0
A-Tracy	6.0	750.0	125.0	1.0	1000.0	83.3
A-Tracy	16.0	750.0	46.0	6.0	1000.0	166.7
A-Long	8.0	125.0	15.6	8.0	1000.0	125.0
A-Long	31.0	250.0	8.1	31.0	1000.0	32.3
A-Long	12.0	250.0	20.8	31.0	1000.0	32.3
A-Long	25.0	78.0	3.1	3.0	403.0	134.3
A-Tracy	2.0	94.0	47.0	12.0	1000.0	83.3
B-46791	6.0	100.0	31.0	8.0	1000.0	125.0
B-47063	6.0	98.0	16.3	12.0	1000.0	83.3
B-18537	8.0	100.0	23.5	6.0	1000.0	250.0
MEAN	11.7	260.5	31.6	11.9	945.7	124.1
S.E.M	2.7	75.3	10.3	6.0	54.3	22.5

Table 3. Effect of Proanthocyanidin Polymer A and Ribavirin Against FLU-A.

EXP.	PROANTHOCYANIDIN POLYMER A ($\mu\text{g/ml}$)			RIBAVIRIN ($\mu\text{g/ml}$)		
	ED ₅₀	ID ₅₀	S.I.	ED ₅₀	ID ₅₀	S.I.
A-Taiwan	16.0	750.0	46.9	8.0	1000.0	125.0
A-Taiwan	4.0	750.0	187.5	6.0	1000.0	166.7
A-Taiwan	12.0	750.0	62.5	23.0	1000.0	43.5
A-Taiwan	2.0	63.0	31.5	12.0	1000.0	83.3
A-Taiwan	0.8	250.0	312.5	7.8	1000.0	128.2
A-Taiwan	4.0	25.0	6.3	7.8	1000.0	128.2
A-Shanghai	0.8	187.0	233.8	6.0	1000.0	83.3
A-Leningrad	4.0	750.0	187.5	6.0	1000.0	166.7
B-MWS/33	8.0	41.0	5.1	1.0	100.0	100.0
B-Japan	12.0	24.0	2.0	1.6	78.0	48.8
B-Portchalm	13.0	35.0	2.7	1.0	68.0	68.0
MEAN	7.0	263.5	81.5	8.2	749.6	101.9
S.E.M	1.6	96.7	33.2	2.0	129.3	13.3

Table 4. Effect of Proanthocyanidin Polymer A and Ribivarin Against FLU-B.

EXP#	PROANTHOCYANIDIN POLYMER A			RIBAVIRIN ($\mu\text{g/ml}$)		
	($\mu\text{g/ml}$)					
	ED ₅₀	ID ₅₀	S.I.	ED ₅₀	ID ₅₀	S.I.
B-Yamagata	3.0	187.0	62.3	6.0	1000.0	166.7
B-Yamagata	1.5	187.0	124.7	8.0	1000.0	125.0
B-USSR	62.0	750.0	12.1	4.0	1000.0	250.0
B-USSR	0.8	250.0	312.5	7.8	1000.0	128.2
B-USSR	3.0	250.0	83.3	7.8	1000.0	128.2
B-HongKong	11.0	35.0	3.2	1.0	80.0	80.0
MEAN	13.6	276.5	99.7	5.8	846.7	146.3
S.E.M	9.8	100.0	46.4	1.1	153.3	23.6

Table 5. Effect of Proanthocyanidin Polymer A and Ribavirin Against PIV-1.

EXP#	PROANTHOCYANIDIN POLYMER A			RIBAVIRIN ($\mu\text{g/ml}$)		
	($\mu\text{g/ml}$)					
	ED ₅₀	ID ₅₀	S.I.	ED ₅₀	ID ₅₀	S.I.
PIV-1	0.8	125.0	166.7	12.0	1000.0	83.3
PIV-1	2.0	187.0	93.5	12.0	1000.0	83.3
PIV-1	6.0	750.0	125.0	8.0	1000.0	125.0
MEAN	2.9	354.0	124.9	10.7	1000.0	97.2
S.E.M	1.6	198.8	18.1	1.3	0.0	13.9



Table 6. Effect of Proanthocyanidin Polymer A and Ribavirin Against PIV-3.

EXP.	PROANTHOCYANIDIN POLYMER A (µg/ml)			RIBAVIRIN (µg/ml)		
	ED ₅₀	ID ₅₀	S.I.	ED ₅₀	ID ₅₀	S.I.
PIV-3	94.0	124.0	1.3	16.0	1000.0	62.5
PIV-3	250.0	750.0	3.0	46.0	1000.0	21.7
PIV-3	93.0	125.0	1.3	8.0	1000.0	125.0
PIV-3	16.8	250.0	15.6	16.0	1000.0	62.5
PIV-3	5.0	250.0	50.0	8.0	1000.0	125.0
PIV-3	84.0	78.0	0.9	8.0	1000.0	125.0
PIV-3	8.0	188.0	23.5	6.0	1000.0	166.7
MEAN	78.6	252.1	13.7	15.4	1000.0	98.3
S.E.M	32.4	86.6	6.9	5.3	0.0	19.0

Fractions of proanthocyanidin polymer A have been further purified by HPLC using a GPC column and standards. Specifically, two fractions have been isolated: one fraction having a molecular weight average of 700 daltons (corresponding to 2 to 3 flavonoid monomeric units) and the second fraction having a molecular weight average of 3000 daltons (corresponding to 9 to 11 flavonoid monomeric units). The two fractions show essentially the same chemical shifts in the ^{13}C and ^1H NMR spectra as the unfractionated proanthocyanidin polymer A.

In vitro experiments indicate both fractions are active against RSV. The fraction having an average of 2 to 3 flavonoid units has an in vitro RSV antiviral activity of $\text{ED}_{50}=10 \mu\text{g/ml}$, while the second fraction having an average of 9-11 flavonoid units has an $\text{ED}_{50}=40 \mu\text{g/ml}$.

8. EFFECTIVENESS OF PROANTHOCYANIDIN
POLYMER A FOR TREATMENT OF RSV

In vivo and in vitro assays indicate that the novel proanthocyanidin polymer A of the invention is a useful therapeutic agent for the treatment of RSV infections. The present invention provides a safer therapeutic treatment for RSV infections without the toxic effects associated with Ribavirin, the currently approved composition for treatment of such infections.

Hispid Cotton Rats were inoculated with RSV A2 intranasally on day 1 and given an intraperitoneal dose of 0.1-30.0 mg/kg of the proanthocyanidin polymer prepared in distilled water on day 2, 3 and 4. Control animals received an equal volume of distilled water. In one experiment, as a comparison, a dose of 10-90 mg/kg of Ribavirin was used. All animals were killed on day 5. Lungs were removed and washed via transpleural lavage. The fluid collected was assessed for RSV titer in a microtiter assay using HEp2 cells.



Assays were performed in 96-well tissue culture plates. All dilutions and tissue culture suspensions were prepared in minimal essential medium containing antibiotics and 5% fetal calf serum (5% FCS-MEM). Lung lavage fluid was added in quadruplicate to wells of the test plates containing a subconfluent monolayer of cells (ca. 3×10^5 cells). Approximately 100 median tissue culture infectious doses (TCID₅₀) of the appropriate test virus in 0.05 ml was added. Tissue control wells contained medium, but no virus. All plates were incubated at 37°C in a 5% CO₂ incubator. Virus control wells were observed daily. When these wells exhibited 80-100% CPE, all wells were observed for CPE. In addition to visual CPE, inhibition of syncytia formation is used to confirm activity against RSV. Lung RSV titer log₁₀ was calculated as described by Dubovi et al., 1983; 1984. The results are shown in Tables 7-8.

As shown in Table 7, for each experiment, the proanthocyanidin polymer A demonstrated a dose-dependent decrease in RSV titer compared to controls. This indicates that the proanthocyanidin polymer A has effective antiviral activity against RSV *in vivo*. As shown in Table 8, direct comparison of the antiviral effect of the procyanidin polymer A to ribavirin (intraperitoneal administration) demonstrates that the polymer composition of the invention is significantly more potent than ribavirin, the drug currently used to treat RSV infections.

Hispid Cotton Rats were inoculated with RSV A2 intranasally on day 1 and given an oral dose of 1.0-10.0 mg/kg of the proanthocyanidin polymer on days 2, 3 and 4. All animals were killed on day 5 and lung tissues were collected. Lungs were removed and washed via transpleural lavage. The fluid collected was assessed for RSV titer in a microtiter assay using



HEp2 cells as described above. Results are shown in Table 9.

As shown in Table 9, the proanthocyanidin polymer administered orally significantly lowered RSV titers at 10 mg/kg compared to controls. This indicates that the proanthocyanidin polymer has effective antiviral activity when administered orally against RSV and is more active than ribavirin.

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* * *

TABLE 7

Effect of Proanthocyanidin Polymer A Against RSV Infection of Cotton Rats, Intraperitoneal Dosing

15	Treatment Lung (Mg/Kg) titer,	P
EXPERIMENT #1		
	Control 3.98 ± 0.14 (9549)	---
20	0.1 3.78 ± 0.31 (6025) 36.9% kill	0.08
	0.3 3.21 ± 0.05 (1621) 83.0% kill	0.05
25	1.0 3.28 ± 0.65 (1905) 80.1% kill	0.04
EXPERIMENT #2		
	Control 4.3 ± 0.8 (20,000)	----
30	1.0 3.1 ± 0.5 (1258) 94% kill	0.08
	3.0 3.4 ± 0.5 (2,511) 87% kill	0.05
35	10.0 3.6 ± 0.3 (3,981) 80% kill	0.06



Treatment Lung
(Mg/kg) titer,

P

EXPERIMENT #3

5	Control	3.98 ± 0.14 (9,549)	-----
	3.0	4.00 ± 0.02 n.s. (10,000) 0% kill	
10	10.0	3.88 ± 0.3 n.s. (7,585) 20.6% kill	
	30.0	3.70 ± 0.28 n.s. (5,011) 47.5% kill	

EXPERIMENT #4

15	Control	5.22 ± 0.27 (165,958)	-----
	0.1	4.87 ± 0.32 (74,131) 55.3% kill	n.s.
20	0.3	4.25 ± 0.39 (17,782) 89.3% kill	0.014
	1.0	4.27 ± 0.5 (18,620) 88.8% kill	0.046
25	3.0	4.41 ± 0.19 (25,703) 84.5% kill	0.012

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	Treatment (Mg/kg)	Lung titer,	P
EXPERIMENT #5			
5	Control	4.5 ± 0.2 (31,622)	----
	10	4.5 ± 0.2 (31,622) 0% kill	n.s.
	30	4.0 ± 0.3 (10,000) 68.4% kill	0.03
10	90	2.8 ± 0.3 (631) 98% kill	<0.0001

Lung titer represents: RSV titer log₁₀/g lung
(Mean ± S.D.)

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TABLE 8

**Effect of Proanthocyanidin Polymer A and
Ribavirin Against RSV Infection of
Cotton Rats, Intraperitoneal Dosing**

5 Proanthocyanidin Polymer (Mg/Kg)

		Lung titer,	P
10	Control	5.22 $\pm$ 0.27 (165,958)	---
	0.1	4.87 $\pm$ 0.32 (74,131) 55.33% Kill	n.s.
	0.3	4.25 $\pm$ 0.39 (17,782) 89.29% Kill	0.014
15	1.0	4.27 $\pm$ 0.5 (18,620) 88.78% Kill	0.046
	3.0	4.41 $\pm$ 0.19 (25,703) 84.51% Kill	0.012
Ribavirin (Mg/Kg)			
20	Control	4.5 $\pm$ 0.2 (31,622)	---
	10	4.5 $\pm$ 0.2 (31,622) 0% Kill	n.s.
25	30	4.0 $\pm$ 0.3 (10,000) 68.4% Kill	0.03
	90	2.8 $\pm$ 0.3 (631) 98% Kill	<0.0001

30 Lung titer represents: RSV titer log₁₀/g lung
(Mean $\pm$ S.D.)

* * *



TABLE 9

Effect of Oral Proanthocyanidin Polymer A
Against RSV Infection of Cotton Rats

Treatment Lung (Mg/Kg) titer,		P
5	EXPERIMENT #1	
	Control 4.45 $\pm$ 0.50 (28,183)	---
	1.0 3.62 $\pm$ 0.42 (4168) 85.7% kill	0.045
10	3.0 4.22 $\pm$ 0.23 (16,595) 41.1% kill	n.s.
	10.0 3.95 $\pm$ 0.48 (8912) 68.4% kill	n.s.
15	EXPERIMENT #2	
	Lung titer,	P
	Control 3.86 $\pm$ 0.55 (7,244)	---
20	1.0 3.60 $\pm$ 0.01 (3,981) 45.0% kill	n.s.
	3.0 3.71 $\pm$ 0.50 (5,129) 29.2% kill	n.s.
25	10.0 2.90 $\pm$ 0.57 (794) 89.0% kill	0.06

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EXPERIMENT #3

		Lung titer,	P
5	Control	3.90 $\pm$ 0.3 (7,943)	—
	1.0	3.90 $\pm$ 0.8 (7,943)	n.s. 0.0% Kill
	3.0	4.00 $\pm$ 0.8 (10,000)	n.s. 0.0% Kill
10	10.0	2.90 $\pm$ 0.5 (794)	0.01 92% Kill
	Ribavirin (40 mg/kg)	3.30 $\pm$ 0.5 (1,995)	0.07 80% Kill
15	Lung titer represents RSV titer log ₁₀ /g lung (Mean $\pm$ S.D.)		

An additional series of experiments conducted as described above confirms the in vivo activity of polymer A against RSV.

20 Proanthocyanidin polymer A displayed a mean ED₅₀ value of 1.52 $\pm$ 0.62 mg/kg following 3-day i.p. administration, and 3.6 $\pm$ 1.66 mg/kg following 3-day p.o. administration. See Table 10. The reference antiviral agent, ribavirin, displayed ED₅₀ values of 40 $\pm$ 6, and > 90 mg/kg following i.p. and p.o. administration, respectively. Proanthocyanidin polymer A at a dose of 10 mg/kg p.o. resulted in a 68-92% reduction in lung RSV titers following oral administration, and a 21-80% reduction following i.p. administration. Ribavirin (40 mg/kg) resulted in a >90% reduction in lung titers following oral administration, and a 50% reduction following i.p. administration.

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**Table 10. Effect of Proanthocyanidin Polymer A
Against RSV infection of Hispid Cotton Rats:**

3-Day Intraperitoneal Dosing		ED ₅₀ (mg/kg)	
Exp.#	Proanthocyanidin		Ribavirin
	Polymer A		
5	1	1.0	40
	2	0.3	30
	3	3.0	50
	4	0.3	
	5	3.0	
Mean $\pm$ S.E.M.		1.52 $\pm$ 0.62	40 $\pm$ 5.8
3-Day Oral Dosing		ED ₅₀ (mg/kg)	
Exp.#	Proanthocyanidin		Ribavirin
	Polymer A		
15	1	3.0	>90
	2	1.0	>90
	3	10.0	
	4	3.0	
	5	1.0	
Mean $\pm$ SEM		3.6 $\pm$ 1.66	>90

20 In vivo experiments have also been performed to study the effect of proanthocyanidin polymer A upon course of respiratory syncytial virus infection in African green monkeys.

25 Proanthocyanidin polymer A was weighed out in 25 mg aliquots and refrigerated as the dry powder until use. Glucose was prepared as a 0.5% solution and refrigerated. A 25 mg sample of proanthocyanidin polymer A was dissolved in 50 ml 0.5% glucose daily and the solution sterilized by filtration through a 0.22 μ m membrane filter. Subsequent dilutions were made in 0.5% glucose from the 0.5 mg/ml solution at 1:2 and 1:5 to give concentrations of 0.25 mg/ml and 0.1 mg/ml respectively.

35 Groups of three monkeys were dosed with proanthocyanidin polymer A at 0.5 mg/kg, 0.25 mg/kg or 0.1 mg/kg by administering the drug at 1 ml per kg of



body weight by intravenous injection. The time of administration of the drug infusion to each monkey was approximately 1 minute. A control group of three monkeys received 1 ml of the 0.5% glucose solution.

Four hours after the initial treatment with
5 proanthocyanidin polymer A, each of the monkeys was infected with a 10^2 dilution of stock respiratory syncytial virus. The titer of the stock virus was 1×10^5 TCID₅₀/ml. Each monkey received 1 ml of the 10^2 virus dilution by intratracheal inoculation and 1 ml
10 applied drop-wise to external nares. The total virus dose therefore was 2×10^3 TCID₅₀/monkey. Treatment with proanthocyanidin polymer A was administered again 8 hours later resulting in daily doses of
15 proanthocyanidin polymer A of 1.0, 0.5, and 0.2 mg/kg with three monkeys per group. Twice daily treatment continued at 8 a.m. and 8 p.m. for a total of seven days.

To determine virus shedding throat swabs
20 were taken daily each morning prior to treatment with proanthocyanidin polymer A using a dacron nasal-pharyngeal swab. The swab was placed in 1.0 ml of tissue culture (minimum essential medium with a 10% fetal bovine serum and penicillin, streptomycin and
25 fungizone). Titrations were performed on the throat swab solutions by preparation of ten-fold dilutions which were inoculated into duplicate wells of BSC-40 cells grown in 24-well plates. Following incubation at 37°C in a CO₂ incubator the titer of each specimen
30 was determined by microscopic examination for viral cytopathology and the titer expressed as the log₁₀ of the TCID₅₀ per ml. At the time of each morning specimen collection each monkey was examined for
35 rhinorrhea and other respiratory symptoms including coughing, sneezing and signs of respiratory distress. The animal attendant would also note any coughing or sneezing during routine daily care of the monkeys.



Fourteen days after virus inoculation each of the monkeys was bled and the sera tested for antibody titers in a neutralization assay against approximately 100 TCID₅₀ of respiratory syncytial virus.

The results of intravenous dosing with
5 proanthocyanidin polymer A are summarized in Table 11. Each of the three control monkeys became infected with respiratory syncytial virus, shedding virus in the oropharynx for 7 to 10 days or longer. Virus titers reached five and six logs₁₀ in two of the three control
10 monkeys and 3.5 log₁₀ in the third. In the monkeys receiving proanthocyanidin polymer A at the highest dose of 1 mg/kg/day, the time of virus shedding and titers of virus shed were much reduced. One monkey
15 shed virus for one day, a second for three days, and a third for four days. In one monkey, the maximum virus titer was two logs. The mid dose of 0.5 mg/kg/day was also seen to shorten the time of shedding in two
20 monkeys with the third monkey excreting virus in the oropharynx for nine days. Virus titers were generally lower although maximum titers of four logs were detected in two of the three monkeys and three logs in the third. A minimal effect was seen at the lowest
25 dose of 0.2 mg/kg/day. Virus shedding was seen for six or seven days and a maximum titers of three logs₁₀ were seen in two monkeys and five logs in the third monkey. Antibody titers to respiratory syncytial virus were detected in all monkeys at 14 days with titers of 1:40 to 1:160.

30 Daily mean log₁₀ titers are presented for each treatment group (See Table 12). Mean titers were lower in each of the three treatment groups when compared with the mean titers in the control group on each of the sampling days. The mean titers were
35 considerably reduced in the 1.0 mg/kg/day treatment group, and a dose related reduction is also seen in the other two groups.



Clinical symptoms were more prevalent in the control monkeys than in the monkeys treated with proanthocyanidin polymer A (See Table 13). Rhinorrhea is the most easily observed symptom and was seen in each of the three controls. No signs of toxicity were
5 observed during daily examinations of the monkeys at the time of sampling or during daily inspection during the course of the study.

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Table 11. Effect of Intravenous Dosing With Proanthocyanidin Polymer A Upon the Development of Respiratory Syncytial Virus Infection in African Green Monkeys.

Treatment	Mnky	Log ₁₀ of Titer From Throat Swabs - Days Post-Infection (P.I.)										AB Titer 14 Days P.I.
Group	#	1	2	3	4	5	6	7	8	9	10	
Control	K349	<1.0	<1.0	1.0	2.5	2.0	3.0	3.5	2.5	1.0	<1.0	1:80
0.5% Dex.	K553	<1.0	1.0	3.0	4.0	5.0	5.0	6.0	4.5	2.0	0.5	1:160
b.i.d., i.v.	K340	<1.0	2.5	3.0	4.0	5.0	4.0	4.0	5.0	3.0	3.0	1:160
0.2	K152	<1.0	<1.0	<1.0	2.0	1.5	3.0	2.5	2.5	1.0	2.0	1:40
mg/kg/day	H549	<1.0	<1.0	3.0	3.0	4.0	5.0	4.0	3.0	<1.0	<1.0	1:80
b.i.d., i.v.	K100	<1.0	<1.0	<1.0	1.5	1.0	3.0	2.0	1.0	0.5	<1.0	1:80
0.5	K154	<1.0	<1.0	<1.0	<1.0	2.0	1.5	3.0	4.0	<1.0	<1.0	1:40
mg/kg/day	K332	<1.0	0.5	1.0	2.0	3.0	3.0	3.0	4.0	3.0	1.0	1:80
b.i.d., i.v.	K155	<1.0	<1.0	<1.0	1.5	3.0	2.0	2.0	2.5	<1.0	<1.0	1:80
1.0	K153	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	1.0	<1.0	<1.0	<1.0	1:160
mg/kg/day	K113	<1.0	<1.0	<1.0	<1.0	1.0	1.0	2.0	1.0	<1.0	<1.0	1:160
b.i.d., i.v.	K350	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	0.5	1.5	<1.0	<1.0	1:80



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Table 12. Mean Titers of RSV in Throat Swabs From African Green Monkeys Treated With Proanthocyanidin Polymer A.

Treatment Group	Log ₁₀ Mean Titer of RSV in Throat Swabs (Days P.I.) N=3									
	1	2	3	4	5	6	7	8	9	10
Control	<1.0	1.2	2.3	3.5	4.0	4.0	4.5	4.0	2.0	1.2
0.2 mg/kg/day	<1.0	<1.0	1.0	2.2	2.2	3.7	2.8	2.2	0.5	0.7
0.5 mg/kg/day	<1.0	0.2	0.3	1.2	2.7	2.2	3.0	3.2	1.0	0.3
1.0 mg/kg/day	<1.0	<1.0	<1.0	<1.0	0.3	0.7	1.2	0.8	<1.0	<1.0

Table 13. Symptoms of Respiratory Syncytial Virus Infection in African Green Monkeys Treated With Proanthocyanidin Polymer A.

Treatment Group	Monkey Number	Days of Symptoms During 10 Days		
		Rhinorrhea	Sneezing	Coughing
Control	K349	1	1	1
0.5% Dex	H553	2	3	1
b.i.d., i.v.	K340	2	6	1
0.2 mg/kg/day	K152	2	1	1
b.i.d., i.v.	K549	1	3	1
	K100	-	-	-
0.5 mg/kg/day	K154	-	1	1
b.i.d., i.v.	K132	-	-	-
	K155	-	-	-
1.0 mg/kg/day	K153	2	-	-
b.i.d., i.v.	K113	-	-	-
	K350	-	-	-

-55-

In summary, proanthocyanidin polymer A was seen to have a definite beneficial effect on respiratory syncytial virus infection in African green monkeys. A dose of 1.0 mg/kg given by intravenous
5 injection twice daily resulted in shorter duration of virus shedding from the oropharynx, reduced titers of virus shed and reduced clinical symptoms. A dose of 0.5 mg/kg administered twice daily was also effective in reducing all three parameters of infection although
10 to a lesser degree than was observed at the higher dose.

9. EFFECTIVENESS OF PROANTHOCYANIDIN
POLYMER A
FOR TREATMENT OF INFLUENZA A

15 Male and female BALB/c mice were used in in vivo tests to study the effectiveness of proanthocyanidin polymer A against Influenza A. The mice weighed 13-16 g and were obtained from Simonsen
20 Laboratories (Gilroy, CA). They were quarantined 24 hr prior to use, and maintained on Wayne Lab Blox and tap water. Once the animals were infected, 0.006% oxytetracycline (Pfizer, New York, NY) was added to the drinking water to control possible secondary
25 bacterial infections.

Influenza A/NWS/33 (H1N1) was obtained from K.W. Cochran, University of Michigan (Ann Arbor, MI). A virus pool was prepared by infecting confluent monolayers of Madin Darby canine kidney (MDCK) cells,
30 incubating them at 37°C in 5% CO₂, and harvesting the cells at 3 to 5 days when the viral cytopathic effect was 90 to 100%. The virus stock was ampuled and stored at -80°C until used.

35 The proanthocyanidin polymer A was dissolved in sterile water for injection (WFI) each day at the appropriate concentrations and used immediately. 1-Adamantanamine HCL (amantadine) was purchased from



Sigma (St. Louis, MO) and was dissolved in sterile saline. 1- β -D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and was dissolved in sterile saline.

5 An Ohmeda Blox 3740 pulse oximeter (Ohmeda, Louisville, OH) with ear probe attachment was used.

Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells as described by Sidwell, RW, Huffman JH, 10 Call E.W., Alghamandan H., Cook P.D., Robins, R.K. "Effect of Selenazofurin on Influenza A and B Virus Infections in Mice", Antiviral Res., 6:343-53 (1986).

Two experiments were conducted as follows:

15 Experiment 1: Seventeen male and seventeen female mice were infected intranasally (i.n.) with an approximate 90% lethal dose of virus (0.06 ml). This viral inoculum was equivalent to an approximately 10⁵ cell culture 50% infectious doses in MDCK cells. Treatment with 30, 10 or 3 mg/kg/day of 20 proanthocyanidin polymer A or 125 mg/kg/day of amantadine was begun 2 days pre-virus exposure and continued once daily for 8 days. Pulse oximeter readings of arterial oxygen saturation (SaO₂) were 25 determined on 5 male and 5 female mice daily through 10 days post-virus exposure and again on days 14 and 21. On infection days 2,4,6,8,10,14 and 21, 3 mice (2 male, 1 female or 2 female, 1 male) were killed and their lungs removed, graded for consolidation, and 30 frozen for laser assay of virus titer. Consolidation was scored from 0 (normal) to 4 (100% consolidation). The same animals used for SaO₂ determinations were held for 21 days and deaths noted as they occurred. As toxicity controls, 3 male and 3 female sham-infected 35 mice were treated concomitantly with each dosage level of test compound. These animals together with normal



untreated mice were weighed immediately prior to treatment and 18 hr after the final treatment.

5 Experiment 2: This experiment was identical to Experiment 1, except therapy began 4 hr post-virus exposure.

Statistical Evaluation: Increase in survivor number was evaluated using chi square analysis with Yate's correction. Mean survival time increases, virus titer and SaO_2 value differences were analyzed by t-test.
10 Lung consolidation scores were evaluated by ranked sum analysis. The Exstatix™ program run on a Macintosh II computer was used to determine $\pm$ standard errors (S.E.).

15 An overview of the results of these experiments is shown in Tables 14 and 15. The overview of the two experiments shows mean data compiled from all time points in which assays were made. The toxicity controls for these experiments indicated the 30 mg/kg/day dose of proanthocyanidin
20 polymer A was toxic to the mice.

Experiment 1 (Early treatment initiation): The summary of Experiment 1 (Table 14) indicates
25 significant influenza disease-inhibitory effects of the 30 and 10 mg/kg/day dosages of proanthocyanidin polymer A based on the reduction of lung consolidation. The SaO_2 were significantly increased in the infected female mice receiving the 10 and 3
30 mg/kg/day doses.

Amantadine was intended as a positive control in these experiments. Amantadine did not appear significantly effective in this experiment. However, it has been subsequently learned that this
35 particular influenza virus is resistant to Amantadine.

More detailed examination of these parameters can be seen in Figures 6-9 for Experiment



1. Lung consolidation (Figure 6) was particularly inhibited on the early sampling days; the consolidation gradually increased in all groups through the remainder of the experiment, but never reached the level of the virus controls.

5 Lung virus titers (Figure 10) were not reduced early in the study, but by day 8 and 10, significant reductions were seen in mice treated with the 10 and 3 mg/kg/day dosages of proanthocyanidin polymer A.

10 The SaO_2 data were separated for male and female mice, since 5 animals of each sex were used for these determinations. Essentially no inhibition of SaO_2 decline was seen in male mice treated with either compound (Figure 12). The female mice appeared to
15 respond better, however (Figure 13), with the 10 and 3 mg/kg/day doses of proanthocyanidin polymer A inhibiting by approximately 10% of the SaO_2 decline. It should be noted that in this experiment, therapy
20 ceased on day 6, and by the next day, the SaO_2 in the treated animals began to decrease at a rapid rate. This suggests a need to continue therapy a few additional days.

25 Experiment 2 (Late treatment initiation): The summary results of this experiment are seen in Table 15. It should be noted that in this delayed treatment study all the male toxicity control mice survived the high dose proanthocyanidin polymer A treatment, although
30 they lost considerable weight. These animals were slightly older than those used in Experiment 1, and weighed an average of 1 g more.

As seen in Experiment 1, no significant increase in survivors was seen in the infected,
35 treated groups, although significant mean survival time increases were observed in female mice treated with 10 and 3 mg/kg/day of proanthocyanidin polymer A



(Table 15). Overall mean lung scores were again reduced.

The alternate-day lung consolidation findings are shown in Figure 10. Proanthocyanidin polymer A therapy again caused inhibition of consolidation, although to a lesser extent than seen using earlier-initiated therapy.

Lung virus titers (Figure 11) were only marginally reduced by proanthocyanidin polymer A therapy.

The SaO_2 data for male and female mice are summarized in Figures 12 and 13. As was observed in Experiment 1, the female mice tended to respond better to proanthocyanidin polymer A therapy than the males, with somewhat variable, but dose-responsive inhibition of SaO_2 decline. We conclude this material has a moderate effect against the murine influenza infection.

In view of amantadine's inactivity, an experiment was run using a 75 mg/kg/day dose of ribavirin, which is known to be highly active against the influenza virus infections (Sidwell, R.W., Huffman, J.H., Hare, G.P., Allen, L.B., Witkowski, J.T., Robins, R., "Broad-Spectrum Antiviral Activity of Virazole: 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide", *Science*, 177:705-6 (1972)). The drug was given i.p. twice daily for 5 days beginning 4 hr post-virus inoculation. The same disease parameters as used in the present experiments were employed with the ribavirin study.

The overall results of this ribavirin experiment are shown in Table 16. The drug was not lethally toxic, although it caused moderate host weight loss. All infected, ribavirin-treated mice survived the infection. Lung consolidation and lung virus titers were significantly reduced, and the mean SaO_2 decline was inhibited. The lung consolidation,



virus titer, and SaO_2 data are shown in more detail in Figures 14-16.

Thus, in this experiment, ribavirin exerted significant influenza inhibition.

Proanthocyanidin polymer A when given to
5 influenza A (H1N1)-infected mice i.p. once daily for 8
days, exerted moderate inhibition of the infection.
The material was most efficacious when treatment was
initiated 2 days pre-virus exposure, although
significant inhibition was also seen when therapy was
10 delayed until 4 hr post-virus exposure. The toxicity
controls did not survive a dose of about 30 mg/kg/day.
Disease-inhibiting effects were primarily limited to
10 mg/kg/day. Amantadine was ineffective against this
influenza virus strain. Ribavirin was inhibitory to
15 the virus infection. It is concluded that
proanthocyanidin polymer A has anti-influenza virus
activity.

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Table 14. Effect of i.p. Treatment with Proanthocyanidin Polymer A on Influenza A (H1N1) Virus Infections in Mice (Early Treatment Initiation).

Animals: 13-15g ♂ and ♀ BALB/c Mice.

Virus: Influenza A/NWS/33 virus.

Drug Diluent: Sterile H₂O

Treatment Schedule: qdx8, beginning 2 days pre-virus inoculation.

Treatment Route: i.p.

Experiment Duration: 21 Days.

Toxicity controls						Infected, Treated							
Treatment	Dosage (mg/kg/day)	Surv/ Total		Host Wt. Change ^a (g)		Surv/ Total		MST ^b (days)		Mean Lung Score ^c	Mean Lung Virus Titer ^c (log ₁₀)	Mean SaO ₂ % ^d	
		♂	♀	♂	♀	♂	♀	♂	♀			♂	♀
		♂	♀	♂	♀	♂	♀	♂	♀			♂	♀
Proantho- cyanidin Polymer A	30	0/3	0/3	-	-	0/5	0/5	5.3	4.5	2.3*	7.0	73.1	72.6
	10	3/3	3/3	2.7	0.0	0/5	0/5	8.8	10.4	1.6**	6.9	80.3	83.5*
	3	3/3	3/3	3.5	1.3	0/5	0/5	8.8	8.0	2.9	6.5	80.2	83.4*
Amantadine	125	3/3	3/3	2.9	0.3	0/5	0/5	9.3	9.0	2.4	6.7	79.6	78.1
Water	-	-	-	-	-	0/5	0/5	10.0	9.0	3.6	6.9	82.1	80.1
Normals	-	10/10	10/10	3.4	0.5	-	-	-	-	0.0	-	-	-

^aDifference between initial weight at start of treatment and weight 18 hr following final treatment of toxicity control mice.^bMean survival time of mice dying on or before day 21.^cMean lung scores and virus titers from observations made on days 2,4,6,8. See Figures 6 and 7 for comparisons of results of all these time points.^dMean SaO₂% of readings made daily for 11 days. See Figure 8 for comparisons of daily readings.

*P<0.05 **P<0.01



Table 15. Effect of i.p. Treatment with Proanthocyanidin Polymer A on Influenza A (H1N1) Virus Infections in Mice (Early Treatment Initiation).

Animals: 14-16g ♂ and ♀ BALB/c Mice.

Virus: Influenza A/NWS/33 virus.

Drug Diluent: Sterile H₂O

Treatment Schedule: qdx8, beginning 4 hr post-virus inoculation.

Treatment Route: i.p.

Experiment Duration: 21 Days.

		Toxicity controls				Infected, Treated							
		Surv/		Host Wt.		Surv/		MST ^b		Mean	Mean Lung	Mean	
		Total		Change ^c (g)		Total		(days)		Lung	Virus Titer ^d	SaO ₂ % ^e	
Compound	Dosage (mg/kg/day)	♂	♀	♂	♀	♂	♀	♂	♀	Score ^c	(log ₁₀)	♂	♀
Proantho- cyanidin Polymer A	30	0/3	0/3	-1.2	-	0/5	0/5	3.6	3.4	0.4**	6.3	78.1	78.4
	10	3/3	3/3	3.8	0.4	0/5	0/5	8.4	11.2**	1.5*	6.4	82.8	84.1
	3	3/3	3/3	3.3	0.9	1/5	1/5	9.3	10.3**	1.9	6.7	81.7	83.0
Amantadine	125	3/3	3/3	1.2	0.6	0/5	0/5	7.2	6.8	2.0	6.8	85.4	81.0
Water	-	-	-	-	-	2/5	0/5	8.7	7.0	2.4	6.5	82.5	83.0
Normals	-	10/10	10/10	2.8	0.0	-	-	-	-	0.0	-	86.8	87.0

^aDifference between initial weight at start of treatment and weight 18 hr following final treatment of toxicity control mice.

^bMean survival time of mice dying on or before day 21.

^cMean lung scores and virus titers from observations made on days 2,4,6,8. See Figures 9 and 10 for comparisons of results of all these time points.

^dMean SaO₂% of readings made daily for 11 days. See Figure 11 for comparisons of daily readings.

*P<0.05 **P<0.01

Table 16. Effect of i.p. Ribavirin Treatment on Influenza A (H1N1) Virus Infections in Mice.

Animals: 14-16g ♀ BALB/c Mice.

Virus: Influenza A/NWS/33 virus.

Drug Diluent: Sterile Saline

Treatment Schedule: bid x 5 beg. 4 hr post-virus inoculation.

Treatment Route: i.p.

Experiment Duration: 21 Days.

<u>Compound</u>	<u>Dosage</u> <u>(mg/kg/day)</u>	<u>Toxicity controls</u>		<u>Infected, Treated</u>				
		<u>Surv/</u> <u>Total</u>	<u>Host Wt.</u> <u>Change^a(g)</u>	<u>Surv/</u> <u>Total</u>	<u>MST^b</u> <u>(days)</u>	<u>Mean</u> <u>Score^c</u>	<u>Mean Virus</u> <u>Titer^c(log₁₀)</u>	<u>Mean</u> <u>SaO₂%^d</u>
Ribavirin	75	5/5	0.6	10/10**	>21.0**	0.3**	3.9**	81**
Saline	-	-	-	1/18	5.8	2.3	7.3	71
Normals	-	5/5	0.6	-	-	0.0	0.0	88

^aDifference between initial weight at start of treatment and weight 18 hr following final treatment of toxicity control mice.^bMean survival time of mice dying on or before day 21.^cMean lung scores and virus titers from observations made on days 2,4,6,8. See Figures 14 and 15 for comparisons of results of all these time points.^dMean SaO₂% of readings made daily for 10 days. See Figure 16 for comparisons of daily readings.

**P < 0.01

10. EFFECTIVENESS OF PROANTHOCYANIDIN
POLYMER A
FOR TREATMENT OF PARAINFLUENZA VIRUS

Twelve African green monkeys without
5 antibody to parainfluenzavirus, type 3, (PIV-3) were
used in an experiment to determine antiviral activity
of proanthocyanidin polymer A. The monkeys were
divided randomly into four groups of three monkeys
each. The respective groups received proanthocyanidin
10 polymer A at doses of 10, 3.3, or 1.0 mg/kg/day with
the drug dissolved in 0.5% glucose. A control group
of three monkeys received 0.5% glucose without the
drug. Each of the monkeys received treatment by
15 intravenous bolus injection as divided doses twice
daily at 8 a.m. for 7 days. The injections were made
into the saphenous vein of sedated monkeys. See Table
17.

Treatment was initiated four hours before
virus inoculation. Four hours later, a 10^{-2} dilution
20 of PIV-3 was prepared and inoculated as a 1 ml volume
intratracheally and a 1 ml volume placed on the
external nares. The titer of the inoculated virus was
 $10^{3.5}$ TCID₅₀ per ml.

Throat swabs were taken daily and placed in
25 1 ml of tissue culture medium. Fluid was expressed
from the swab and the tissue culture fluid was
titrated for virus. Tenfold dilutions of the throat
swab specimens were prepared and inoculated into
30 duplicate wells of 24 well tissue culture plates
seeded with Vero cells. Titers were determined by
microscopic examination of the cultures for viral
cytopathology. The monkeys were observed daily for
clinical symptoms including rhinorrhea, sneezing and
35 coughing. At 14 and 21 days post-infection, blood was
drawn for detection of antibody to PIV-3 using a serum
neutralization assay.



Each of the 12 monkeys became infected with PIV-3 with the majority shedding virus within 24 hours after virus inoculation. Virus shedding continued in all monkeys through the eighth day post-infection with some monkeys shedding virus through day 9 and 10. See Table 18.

The mean virus titers for each group are shown in Table 19. Proanthocyanidin polymer A at 10 mg/kg/day appear to reduce the mean titers on all of the days post-infection by one log or better. The 3.3 mg/kg/day dose was seen to have a slightly lesser effect than the 10 mg/kg/day dose but again all titers were less than the controls. No appreciable effect was seen at 1.0 mg/kg/day.

Symptoms of respiratory infection were more common in the control monkeys than in any one of the three proanthocyanidin polymer A treated groups. During the 11 days of observation with 3 monkeys per group, 33 total symptom days are possible. In the group receiving proanthocyanidin polymer A at 10 mg/kg/day only 7 of those 33 days were observed to have monkeys with symptoms. The 3.3 mg/kg/day dose resulted in only 2 days and the 1.0 mg/kg/dose resulted in 7 days. The principal symptom observed was rhinorrhea (See Table 20).

Antibody titers to PIV-3 at 14 and 21 days after virus inoculation are reported. All monkeys were observed to develop antibody with what appeared to be slightly higher titers in the treated monkeys when compared with the untreated controls. See Table 21.

Thus, proanthocyanidin polymer A was seen to have a beneficial effect on the course of parainfluenzavirus, type 3, in African green monkeys treated with 10 and 3.3 mg/kg/day with proanthocyanidin polymer A. Proanthocyanidin polymer A was seen to reduce the titers of virus shed in the



throats of infected monkeys and may have reduced the symptoms seen with this infection.

5 Table 17. Treatment Groups of African Green Monkeys Selected to Evaluate the Antiviral Activity of Proanthocyanidin Polymer A Against Parainfluenzavirus, Type 3

Monkey Number	Sex	Weight (kg)	Treatment with Proanthocyanidin Polymer A
10			
L299	F	2.0	5 mg/kg, b.i.d., i.v.
L341	M	4.6	5 mg/kg, b.i.d., i.v.
L300	M	1.8	5 mg/kg, b.i.d., i.v.
K144	M	5.0	1.67 mg/kg, b.i.d., i.v.
L302	M	2.4	1.67 mg/kg, b.i.d., i.v.
15			
K143	M	4.6	1.67 mg/kg, b.i.d., i.v.
L301	M	2.8	0.5 mg/kg, b.i.d., i.v.
K347	M	4.8	0.5 mg/kg, b.i.d., i.v.
K342	F	2.8	0.5 mg/kg, b.i.d., i.v.
K337	F	2.8	0.5% glucose, b.i.d., iv
K346	F	3.0	0.5% glucose, b.i.d., iv
K345	F	3.2	0.5% glucose, b.i.d., iv
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Table 18. Effect of Proanthocyanidin Polymer A' Treatment Upon Virus Shedding From African Green Monkeys Infected With Parainfluenzavirus, Type 3

Treatment	Monkey Number	Log of Virus Titer on Days Post-Infection									
		1	2	3	4	5	6	7	8	9	10
Control ^a	K337	1.0	4.0	5.0	5.0	6.0	5.0	4.0	3.0	1.5	1.0
0.5% glucose iv.	K346	2.0	3.5	5.0	4.5	5.5	6.0	5.0	4.0	2.0	1.0
	K345	2.0	3.0	4.5	4.5	6.0	5.0	5.0	3.0	1.0	<1.0
P.Pol. A;	L299	1.0	2.0	3.0	4.0	4.0	5.0	2.5	1.5	<1.0	<1.0
10mg/kg/dy	K341	<1.0	2.0	3.0	4.5	3.0	3.5	3.0	2.0	1.5	<1.0
i.v., BID	L300	1.0	2.0	3.0	4.0	3.5	4.0	3.0	1.0	<1.0	<1.0
P.Pol. A;	K144	<1.0	1.0	3.0	4.0	4.0	4.5	5.0	4.0	2.0	1.0
3.3mg/kg/dy	L302	2.0	3.0	2.0	4.5	4.0	5.0	3.5	3.0	1.0	<1.0
i.v., BID	K143	1.0	3.0	2.5	3.5	4.5	5.0	3.5	2.0	1.0	<1.0
P. Pol. A;	L301	2.0	4.0	4.5	5.0	4.5	4.5	3.5	2.0	1.0	<1.0
1.0mg/kg/dy	K347	2.0	3.0	3.0	5.0	5.0	6.0	4.0	1.0	<1.0	<1.0
i.v., BID	K342	2.0	3.0	4.5	5.0	5.0	6.0	5.0	3.0	1.5	1.0



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Table 19. Mean Daily Titers of Parainfluenzavirus, Type 3 in African Green Monkeys Treated With Proanthocyanidin Polymer A

Treatment	Mean Log of Virus Titer on Days Post-Infection									
	1	2	3	4	5	6	7	8	9	10
Glucose	1.7	3.5	4.8	4.7	5.8	5.5	4.7	3.3	1.5	0.7
Control										
P. Polymer A; 10 mg/kg/day	0.7	2.0	3.0	4.2	3.5	4.2	2.8	1.5	0.5	<1.0
P. Polymer A; 3.3 mg/kg/day	1.0	2.3	2.5	4.0	4.2	4.8	4.0	3.0	1.3	0.3
P. Polymer A; 1.0 mg/kg/day	2.0	3.3	4.0	5.0	4.8	5.5	4.2	2.0	0.8	0.3

Table 20. Total Days in Which Monkeys Showed Symptoms to Parainfluenzavirus, Type 3 Infection

5	Treatment	Symptom
	Days/Total	
	Control, Glucose	13/33
	P. Pol.A, 10 mg/kg/day	7/33
	P. Pol.A, 3.3 mg/kg/day	2/33
	P. Pol.A, 1.0 mg/kg/day	7/33

Table 21. Antibody Titers to Parainfluenzavirus, Type 3, in African Green Monkeys Infected With the Virus and Treated With Proanthocyanidin Polymer A

10	P.I.	Monkey	Antibody Titer at Days	
	Treatment Days	Number	14 Days	21
15	Control	K337	1:40	1:160
	0.5%	K346	1:20	1:80
	glucose, i.v.	K345	1:80	1:160
20	P.Polymer A, 10 mg/kg/day, i.v.	L299	1:160	1:320
		K341	1:160	1:320
		L300	1:160	1:320
25	P.Polymer A, 3.3 mg/kg/day, i.v.	K144	1:80	1:80
		L302	1:160	1:320
		K143	1:160	1:160
25	P. Polymer A, 1.0 mg/kg/day i.v.	L301	1:160	1:320
		K347	1:80	1:160
		K342	1:40	1:180

10.1 EFFECTIVENESS OF PROANTHOCYANIDIN POLYMER A FOR TREATMENT OF HERPES SIMPLEX VIRUS TYPE 2 VAGINITIS

In vivo experiments were performed to study the effectiveness of proanthocyanidin polymer A in comparison with ganciclovir against herpes simplex virus type 2. Ganciclovir is known to be effective against herpes simplex virus type 2.

In one series of experiments, both compositions were formulated for intraperitoneal injection by dissolving in physiological saline.



Swiss Webster female mice (Simonsen Labs, Gilroy, CA) weighing approximately 20 grams each at the start of the experiment were infected intravaginally with herpes simplex virus type 2 (HSV-2), E194 strain. This was accomplished in a 3-step process. First, the vagina of each mouse was swabbed for 5 seconds with a cotton tip applicator dipped in 0.1 N NaOH. This treatment irritates the vaginal area so that the infection takes better. Approximately 1 hour later each vagina was dry swabbed for 5 seconds. Then an applicator dipped in virus medium was used to swab each mouse for 20 seconds. The swabs were gently and slowly twisted back and forth during the time they were in place.

Six hours after virus infection, intraperitoneal (i.p.) injections were administered using proanthocyanidin polymer A, ganciclovir, or placebo. Treatments were also given i.p. twice daily on days 1 through 7 after virus challenge. The daily dose of each compound was 30 mg/kg/day, with 15 mg/kg given at each injection. Since on day 0 of the infection only one dose was given, the daily dose for that day was 15 mg/kg/day.

Groups of 5 mice were sham-infected using the process described above for virus infection, except that no virus was present for the final step. These mice were treated the same way and at the same times as above. Mice were checked daily for survival, and weights were recorded before the first (day 0) and after the last (day 8) treatment.

Lesion scores in infected mice were determined daily on days 3-14 of the infection. A score of 1+ indicates redness immediately around the vagina. 2+ indicates spread of the lesion toward the anus. 3+ indicates a lesion (usually with swelling) from the vagina to the anus. There are variations to this since some mice may have a vaginal lesion plus a



lesion on the tail. Because many of the mice go on to die, the lesion score near the time of death is carried through to the end of the 14 days. If this were not done, lesion scores in the placebo group would appear to go down as the most affected mice die off. Some animals developed hind limb paralysis (and later died). This condition did not add to the lesion score.

Deaths were recorded daily for 21 days. The mean day of death calculation took into account only mice that died. Vaginal virus titers were made by titration of virus obtained from vaginal swabs 3 days after virus inoculation. These titrations were conducted in Vero cells in 96-well plates. Calculation of virus titer was made by the 50% endpoint dilution method of Reed, L.J. and Meunch, M., *Am. J. Hyg.*, 27:493-498 (1938).

Statistical interpretations of survival (Fisher exact test), mean day to death (Student's t test) and virus titer (Student's t test) were made by two-tailed analyses.

Table 22 below shows the average lesion scores with standard deviations and statistical analyses for the experiment.



Table 22. Effect of Intraperitoneal Proanthocyanidin Polymer A Treatment on Herpes Simplex Virus Type 2 (HSV-2) Vaginitis in Mice.

Day	Average Lesion Score		
	Proanth. Pol. A (30 mg/kg/day)	Ganciclovir (30 mg/kg/day)	Placebo
3	0.2±0.2 ^b	0.1±0.2	0.3±0.3
4	0.2±0.3	0.2±0.2**	1.1±1.0
5	0.7±0.9**	0.2±0.2**	1.9±1.2
6	1.3±1.4**	0.2±0.2***	3.1±1.4
7	1.5±1.3**	0.2±0.3***	3.1±1.6
10 8	1.8±1.5*	0.3±0.4***	3.0±1.6
9	2.1±1.4	0.4±0.3***	3.2±1.4
10 10	2.3±1.4	0.5±0.5***	3.2±1.3
11	2.3±1.5	0.5±0.4***	3.2±1.3
12	2.2±1.4	0.9±0.2***	3.2±1.3
13	1.8±1.5*	0.6±0.6***	3.2±1.4
14	1.8±1.5*	0.6±0.6***	3.1±1.4
15 Grand Avg. (Days 3-14)	1.5±0.8**	0.4±0.2***	2.6±1.0

* After virus challenge.
^b Standard deviation.
 * p < 0.05, ** p < 0.01, *** p < 0.001 (two-tailed Student's t test).

Proanthocyanidin polymer A showed a statistically significant decrease in average lesion score compared to the placebo control. Ganciclovir was also effective. Figure 17 gives a visual impression of the degree of lesion inhibition exhibited by 30 mg proanthocyanidin polymer A per kg relative to the placebo control and ganciclovir. Table 23 shows survival of the treated mice.



Table 23. Effect of Intraperitoneal Proanthocyanidin Polymer A Treatment on Survival and Vaginal Virus Titer in Mice Infected Intravaginally with Herpes Simplex Virus Type 2 (HSV-2).

5	Compound	Dose	Survivors/ Total (%)	Mean Day to Death	Virus Titer ^a (Day 3)
	<u>Virus-Infected</u>				
	Pro. Pol. A	30	4/10(40)	9.5±3.2	2.9±1.1
	Ganciclovir	30	10/10(100)**	>21	1.7±1.0*
	Placebo	-	3/20(15)	8.2±2.9	3.8±1.2
	<u>Uninfected Toxicity Controls</u>				
					<u>Host Wt. Change^c</u>
	Pro. Pol. A	30	5/5(100)	>21	+2.8
10	Ganciclovir	30	5/5(100)	>21	+1.0
	Placebo	-	5/5(100)	>21	+2.5

^aLog₁₀50% cell culture infectious dose per ml.

^bDetermined on day 8. Represents the difference in grams between day 0 and day 8 weights. *P<0.002, two-tailed Fisher exact test (survival) or two-tailed Student's *t* test (mean day to death and virus titer parameters.)

15 Proanthocyanidin polymer A showed an increased survival (40% survival compared to 15% in the placebo group). The ganciclovir group had 100% survival. Table 23 also shows that proanthocyanidin polymer A treated mice had less virus than the placebo control, 20 but the results were not statistically significant. Ganciclovir caused a significant reduction in virus titer.

Results in toxicity control mice showed proanthocyanidin polymer A was well tolerated and 25 caused no adverse effects at the doses used. Ganciclovir treated mice gained less weight than placebo controls.

Thus, proanthocyanidin polymer A 30 administered intraperitoneally at a dose of 30 mg/kg/day had moderate antiviral activity in this model. There was no apparent toxicity of the proanthocyanidin polymer A formulation to mice.

Another series of preliminary experiments, 35 in which the proanthocyanidin polymer A composition is administered orally, showed some anti-HSV-2 activity but only at the highest dose administered, i.e., 270 mg/kg/day.



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but only at the highest dose administered, i.e., 270 mg/kg/day.

In another series of experiments, the proanthocyanidin polymer A composition was formulated for topical application against HSV-2. The efficacy of proanthocyanidin polymer A is compared with ganciclovir and acyclovir. Both ganciclovir powder and acyclovir cream were obtained commercially. Squibb cream base #8, which served as a vehicle and as the topical placebo control, was also commercially obtained.

The methods of infecting the mice, setting toxicity control and the parameters measured to evaluate the infection were the same as described above for the intraperitoneal tests. Topical treatments on the day of infection were given at +6 hours. They were then given twice daily for 7 more days.

Treatment for 7.5 days with proanthocyanidin polymer A at two doses resulted in a statistically significant reduction in lesion scores of the period of evaluation for the 10% dose. This is presented graphically in Figure 18 and in Table 24. The 5% dose also reduced lesion development relative to the placebo control, but the results were not statistically significant. Acyclovir cream nearly completely prevented lesion formation in infected animals. More mice treated with proanthocyanidin polymer A at 5% and 10% doses survived the infection than placebo controls (Table 25) and vaginal virus titers were reduced at the 10% dose. In addition, the numbers of mice without lesions on day 14 were greater than in the placebo group. None of the uninfected toxicity control mice lost weight or died as a result of treatment with any of the cream formulations. (Table 25).

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It can be concluded that topically applied proanthocyanidin polymer A as a 10% cream was active. Only three mice developed vaginal lesions, but the lesions all progressed to maximum severity. The 5 proanthocyanidin polymer A cream formulations were somewhat dry to the touch, since the composition hydrated and absorbed some of the water from the Squibb cream base. This may have affected the performance of the proanthocyanidin polymer A cream.

10 Partial hydration of the material in water prior to mixing it with the cream base may provide a more aqueous and bioavailable product for topical application.

15 Table 24. Effect of topical Proanthocyanidin Polymer A Treatment on Herpes Simplex Virus Type 2 (HSV-2) Vaginitis in Mice.

Average Lesion Score				
Day ^a	P. Pol. A 5%	P. Pol. A 10%	Acyclovir 5%	Placebo
20				
3	0.3 ± 0.3 ^b	0.2 ± 0.3	0.2 ± 0.2	0.3 ± 0.3
4	0.3 ± 0.5	0.6 ± 1.0	0.1 ± 0.2	0.5 ± 0.9
5	0.7 ± 1.1	0.6 ± 1.1	0.0 ± 0.0 ^{***}	0.8 ± 0.9
6	1.0 ± 1.6	1.3 ± 1.7	0.0 ± 0.0 ^{***}	1.7 ± 1.5
7	1.7 ± 1.8	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.3 ± 1.7
8	1.9 ± 2.0	1.1 ± 1.8	0.1 ± 0.2 ^{***}	2.5 ± 1.8
9	1.9 ± 1.8	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
10	1.6 ± 1.1	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
25				
11	1.6 ± 1.1	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
12	1.6 ± 1.1	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
13	1.6 ± 1.1	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
14	1.6 ± 1.1	1.1 ± 1.8	0.0 ± 0.0 ^{***}	2.6 ± 1.8
Avg	1.4 ± 0.6	1.0 ± 0.3 ^{**}	0.1 ± 0.1 ^{***}	2.0 ± 0.9
(Days 3-14)				

30 ^aAfter virus challenge.

^bStandard deviation.

^cp < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 (two-tailed Student's ttest).

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Table 25. Effect of Topical Proanthocyanidin Polymer A Treatment on Survival, Vaginal Virus Titers, and Numbers Without Lesions on Mice Infected Intravaginally with Herpes Simplex Virus Type 2 (HSV-2).

Compound	Dose ^a	Survivors/ Total (%)	Mean Day to Death	Virus Titer ^b (Day 2)	No. of Ml W/O Lesio
<i>Virus-Infected</i>					
Placebo	--	6/20 (30)	9.6±2.6 ^c	2.8±2.2	6/20
Acyclovir	5	10/10 (100)*	> 21	0.6±0.2*	10/10
P. Poly A	5	5/10 (50)	9.4±1.1	2.6±2.5	5/10
P. Poly A	10	7/10 (70)	7.7±1.5	1.3±1.2	7/10
<i>Uninfected Toxicity Controls</i>					
					<u>Host Wt. Ch</u>
Placebo	--	5/5 (100)	> 21		+ 1.3
Acyclovir	5	5/5 (100)	> 21		+ 2.0
P. Poly A	10	5/5 (100)	> 21		+ 1.6

- ^a Percent of compound in a polyethylene glycol cream base.
^b Log₁₀ 50% cell culture infectious dose per ml.
^c On day 14 of the infection.
^d Standard deviation.
^e Determined on day 8. Represents the difference in grams between day 0 and day 8 weigh
^f P<0.05, two-tailed Fisher exact test (survival and numbers without lesions) or two-tai
Student's t test (mean day to death and virus titer parameters).

**11. EFFECTIVENESS OF PROANTHOCYANIDIN
POLYMER B FOR TREATMENT OF RSV**

Results from *in vitro* tests for RSV
antiviral activity, performed under the same
5 procedures as the tests with proanthocyanidin polymer
A, indicate that proanthocyanidin polymer B is
effective and has an ED₅₀ of 6 µg/ml.

The invention described and claimed herein
is not to be limited in scope by the specific
10 embodiments herein disclosed, since these embodiments
are intended as illustrations of several aspects of
the invention. Any equivalent embodiments are
intended to be within the scope of this invention.
Indeed, various modifications of the invention in
15 addition to those shown and described herein will
become apparent to those skilled in the art from the
foregoing description. Such modifications are also
intended to fall within the scope of the appended
20 claims.

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**WHAT IS CLAIMED IS:**

1. A method of treating a respiratory virus infection, comprising administering to a warm-blooded animal a therapeutically effective amount of an
5 antiviral agent comprising a proanthocyanidin polymer.
2. The method of claim 1 in which the proanthocyanidin polymer contains 2 to 30 flavonoid units.
- 10 3. The method of claim 1 in which the proanthocyanidin polymer contains 2 to 15 flavonoid units.
- 15 4. The method of claim 1 in which the proanthocyanidin polymer contains 2 to 11 flavonoid units.
- 20 5. The method of claim 2, 3, or 4 in which the flavonoid units are catechins, epicatechins, gallocatechins, galloepicatechins, flavanols, flavonols, flavandiols, leucocyanidins, anthocyanidins, or combinations thereof.
- 25 6. The method of claim 1 in which the respiratory virus is a respiratory syncytial virus.
- 30 7. The method of claim 1 in which the respiratory virus is Parainfluenza virus 3, Influenza A virus, Influenza B virus, or a virus associated with otitis media or otitis externa.
- 35 8. A method of treating a virus infection comprising administering to a warm-blooded animal a therapeutically effective amount of an antiviral agent comprising a proanthocyanidin polymer containing 6 to 30 flavonoid units.





9. A method of treating a virus infection,
comprising administering to a warm-blooded animal a
therapeutically effective amount of an antiviral agent
comprising a proanthocyanidin polymer containing 6 to
5 15 flavonoid units.

10. A method of treating a virus infection,
comprising administering to a warm-blooded animal a
therapeutically effective amount of an antiviral agent
10 comprising a proanthocyanidin polymer containing 6 to
11 flavonoid units.

11. The method of claim 8, 9 or 10 in which the
flavonoid units are catechins, epicatechins,
15 gallocatechins, galloepicatechins, flavanols,
flavonols, flavandiols, leucocyanidins,
anthocyanidins, or combinations thereof.

12. The method of claim 8 in which the virus is
20 a paramyxoviridae, orthomyxoviridae or herpes virus.

13. The method according to claim 1 or 8 wherein
the proanthocyanidin polymer is administered
intravenously.

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14. The method according to claim 1 or 8 wherein
the proanthocyanidin polymer is administered
intraperitoneally.

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15. The method according to claim 1 or 8 wherein
the proanthocyanidin polymer is administered
subcutaneously.

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16. The method according to claim 1 or 8 wherein
the proanthocyanidin polymer is administered
intramuscularly.





17. The method according to claim 1 or 8 wherein the proanthocyanidin polymer is administered orally.

18. The method according to claim 1 or 8 wherein the proanthocyanidin polymer is administered topically.

19. The method according to claim 1 or 8 wherein the proanthocyanidin polymer is administered by aerosol.

20. The method according to claim 14 wherein the proanthocyanidin polymer is administered intraperitoneally in a range of about 0.1 to about 10 mg/kg.

21. The method according to claim 17 wherein the proanthocyanidin polymer is administered orally in a range of about 5.0 to about 30 mg/kg.

22. The method according to claim 18 wherein the proanthocyanidin polymer is administered topically in a suitable vehicle in a concentration range of about 5 to about 15%.

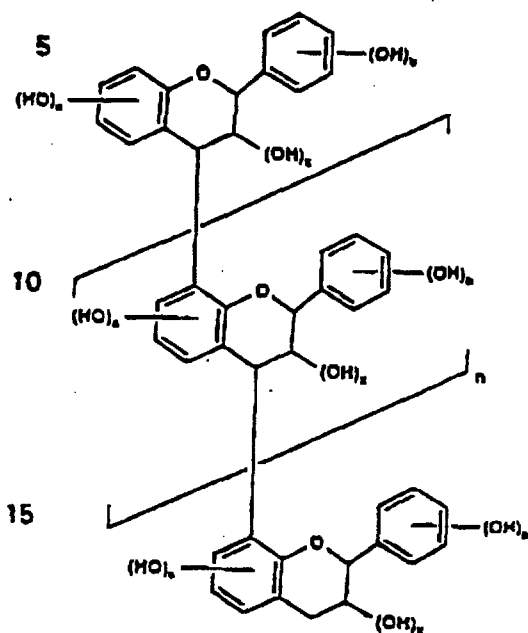
23. The method according to claim 19 wherein the proanthocyanidin polymer is administered in a range of about 5 to about 30 mg/kg/day.

24. The method of claim 2 or 8 in which the flavonoid units are singly or doubly linked to each other.

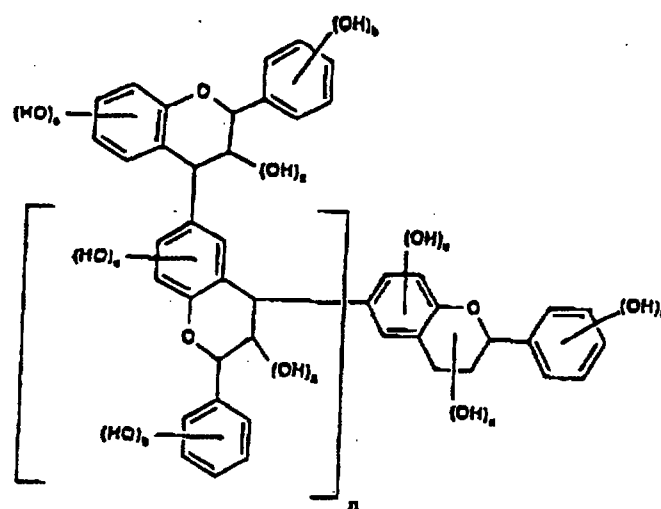




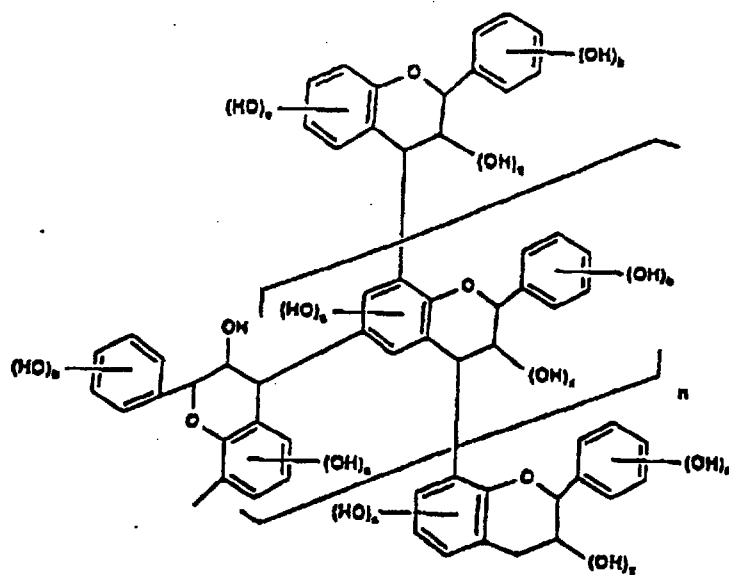
25. The method of claim 1 in which the proanthocyanidin has a structure selected from I, II and III



I



II



III

where $a, b = 1$ to 3 , $x = 0$ or 1 , $n = 0$ to 28 .

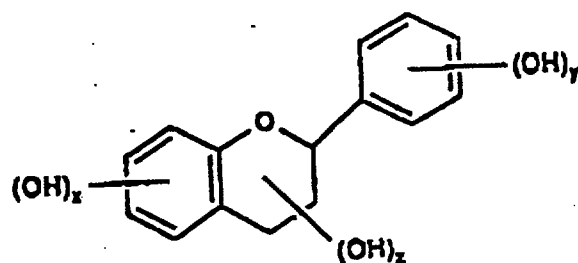
26. The method of claim 25 wherein $n = 0$ to 13 .

27. The method of claim 25 wherein $n = 0$ to 9 .





28. The method of claim 1 in which the proanthocyanidin polymer comprises 2 to 30 monomeric flavonoid units having structure IV



IV

where $x, y = 1$ to 3 , $z = 1$ or 2 .

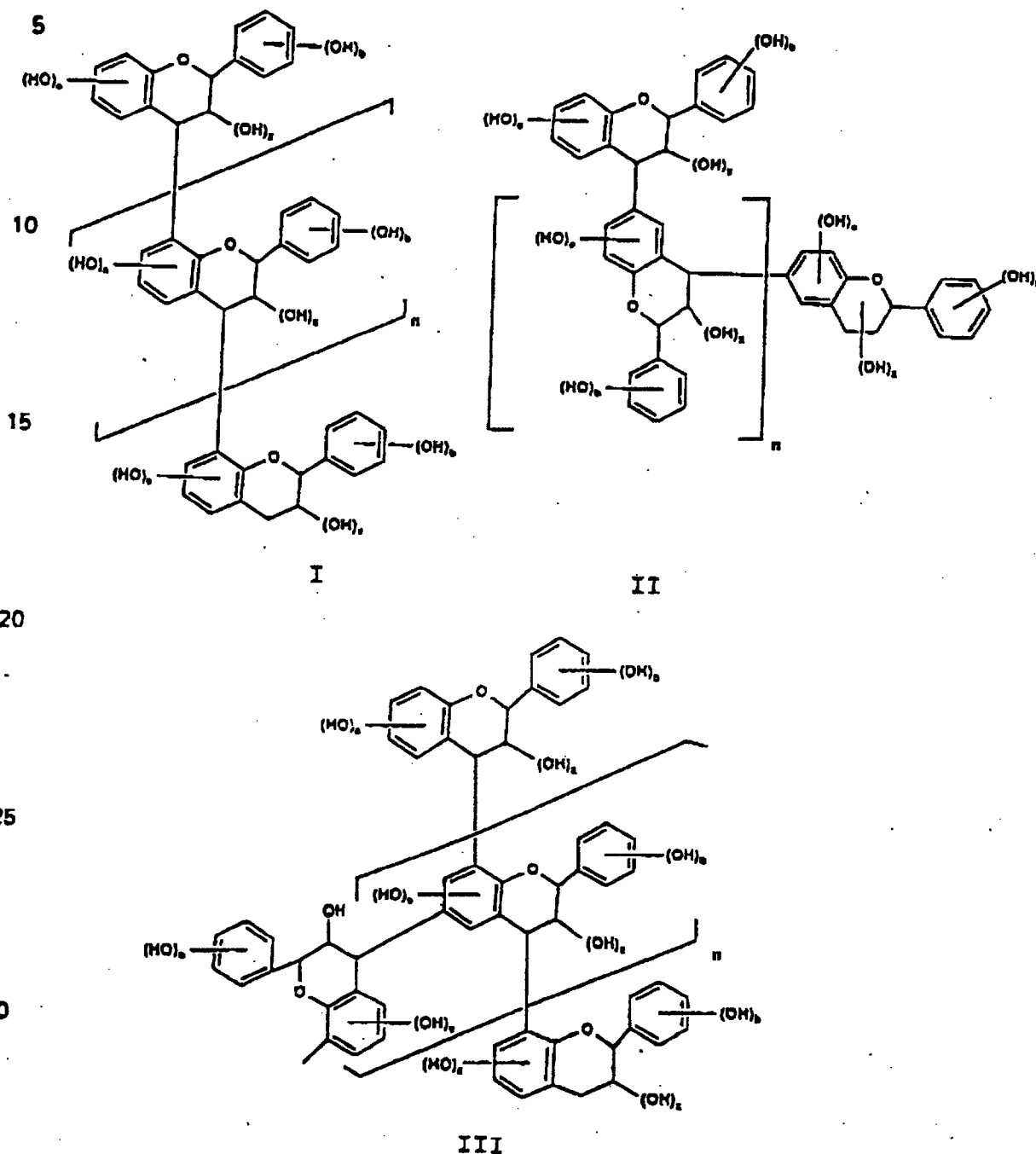
29. The method of claim 28 in which the proanthocyanidin polymer comprises 2 to 15 monomeric flavonoid units.

30. The method of claim 28 in which the proanthocyanidin polymer comprises 2 to 11 monomeric flavonoid units.





31. The method of claim 8 in which the proanthocyanidin has a structure selected from I, II, and III



where $a, b = 1$ to 3 , $x = 0$ or 1 , $n = 4$ to 28 .

32. The method of claim 31 wherein $n = 4$ to 13 .

33. The method of claim 31 wherein $n = 4$ to 9 .



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34. A proanthocyanidin polymer, obtained from a Croton species, and ester, ether and oxonium derivatives thereof, characterized by:

- 5 (a) the capability of dissolving in water and/or an aqueous solution;
- (b) the capability of exerting a pronounced antiviral effect when administered in vivo to an animal or a human; and
- 10 (c) having a structure comprising flavonoid units selected from the group consisting of catechins, epicatechins, gallocatechins, galloepicatechins and combinations thereof.

35. The proanthocyanidin polymer of claim 34
15 having from about 2 to about 11 flavonoid units.

36. The proanthocyanidin polymer of claim 34 having an average of about 7 flavonoid units.

20 37. The proanthocyanidin polymer of claim 34 obtained from *Croton lechleri*.

38. A proanthocyanidin polymer, obtained from a Croton species and ester, ether and oxonium
25 derivatives thereof, characterized by:

- (a) infrared spectral analysis of intense peaks ranging from 3350-2500 and other peaks at 1612, 1449, 1348, 1202, 1144, 1107, 1068 and 1027 cm⁻¹; and
- 30 (b) an ultraviolet spectral analysis of broad peaks at wavelength 202, 235 (shoulder), 275, and 305 (shoulder).

39. A proanthocyanidin polymer, obtained from a Croton species and ester, ether and oxonium derivatives thereof, characterized by:



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- (a) infrared spectra substantially as illustrated in Fig. 1; and
- (b) an ultraviolet spectral analysis of broad peaks at wavelength 202, 235 (shoulder), 275, and 305 (shoulder).

40. The proanthocyanidin polymer of claim 38 further characterized by:

- (c) visible spectral absorption at wavelength of about 460 nm.

41. The proanthocyanidin polymer of claim 39 further characterized by:

- (c) visible spectral absorption at wavelength of about 460 nm.

42. The proanthocyanidin polymer of claim 38 further characterized by:

- (c) ¹³C NMR spectra substantially as illustrated in Fig. 3.

43. The proanthocyanidin polymer of claim 39 further characterized by:

- (c) ¹³C NMR spectra substantially as illustrated in Fig. 3.

44. The proanthocyanidin polymer of claim 40 further characterized by:

- (d) ¹³C NMR spectra substantially as illustrated in Fig. 3.

45. A proanthocyanidin polymer, which is obtained from *Calophyllum inophyllum*, and ester, ether and oxonium derivatives thereof, characterized by

- (a) the capability of dissolving in water and/or an aqueous solution;



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- (b) the capability of exerting an antiviral effect; and
- (c) flavonoid units comprising catechins and epicatechins.

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46. The proanthocyanidin of claim 45 which is characterized by infrared spectra substantially as illustrated in Fig. 5.

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47. The proanthocyanidin of claim 46 which is further characterized by spectra having absorption peaks at 202-204 and 275-280 nm.

48. The proanthocyanidin of 47 which is further characterized by ¹³C NMR spectra substantially as illustrated in Fig. 4.

49. A method for treating a respiratory syncytial virus infection, comprising administering, to a warm-blooded animal, an effective amount of an antiviral agent comprising a proanthocyanidin polymer wherein said proanthocyanidin polymer is obtained from a Croton tree by a method which comprises:

- (a) extracting the whole plant, the bark, the stems, the roots or the latex of the Croton tree with a lower alcohol of about 1-3 carbons, acetone, water or other water miscible solvent or combinations thereof to obtain an aqueous soluble fraction;
- (b) subjecting the aqueous soluble fraction to gel filtration using water and/or water and a water miscible solvent with or without a buffer as the mobile phase; or to reversed-phase column chromatography using water, and/or water and a water miscible solvent as the mobile-phase; or to gel permeation chromatography using water and/or water and





water miscible solvent and acetonitrile with or without a buffer as the mobile phase; or combination thereof and

- 5 (c) collecting the fractions detectable by ultra violet spectroscopy having a λ maximum at about 200-350 nm,

said proanthocyanidin polymer being administered parenterally at a dose range of about 0.1-10 mg/kg body weight, orally at a dose range of about 5.0-30.0
10 mg/kg body weight, or by inhalation at a dose range of 5-30 mg/kg/day.

50. The method of claim 49 in which the Croton tree is *Croton lechleri*.

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51. A proanthocyanidin polymer composition obtained from a Croton tree by a method which comprises:

- 20 (a) extracting the whole plant, the bark, the stems, the roots or the latex of the Croton tree with a lower alcohol of about 1-3 carbons, acetone, water or other water miscible solvent or combinations thereof to obtain an aqueous soluble fraction;

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- (b) 25 subjecting the aqueous soluble fraction to gel filtration using water and/or water and a water miscible solvent with or without a buffer as the mobile phase; or to reversed-phase column chromatography using water,
30 and/or water and a water miscible solvent as the mobile-phase; or to gel permeation chromatography using water and/or water and water miscible solvent and acetonitrile with
35 or without a buffer as the mobile phase; or combination thereof and





- (c) collecting the fractions detectable by ultra violet spectroscopy having a λ maximum at about 200-350 nm.

5 52. The composition according to claim 46, in which the Croton tree is *Croton lechleri*.

53. The composition according to claim 51, further characterized by:

- 10 (a) infrared spectral analysis of intense peaks ranging from 3350-2500 and other peaks at 1612, 1449, 1348, 1202, 1144, 1107, 1068 and 1027 cm^{-1} ; and
- 15 (b) an ultraviolet spectral analysis of broad peaks at wavelength 202, 235 (shoulder), 275, and 305 (shoulder).

54. The proanthocyanidin polymer of claim 53 further characterized by: a ^{13}C NMR spectrum

20 substantially as illustrated in FIG. 3.

55. The composition according to claim 54 further characterized by: visible spectral absorption

25 at wavelength 460 nm.

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