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[Continued on next page]

(54) Title: COMPOSITION PREPARED FROM SAIKOSAPONIN, THE USE AND THE PREPARATION METHOD THEREOF

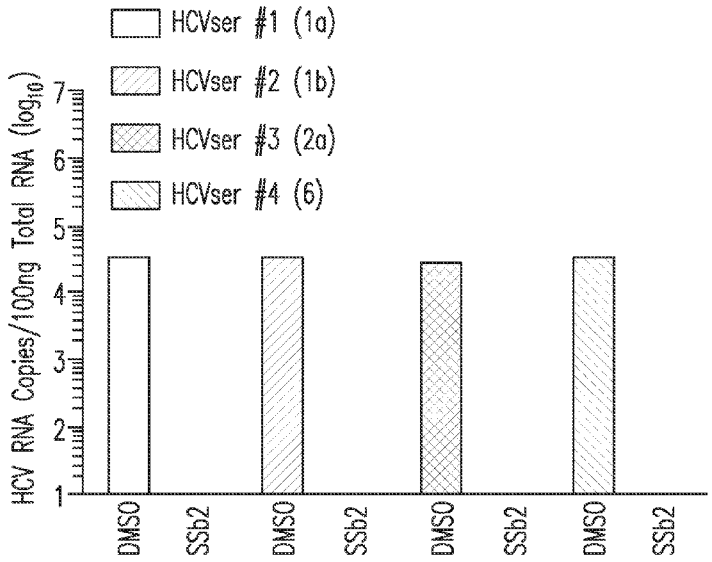

Fig. 7

(57) Abstract: This invention relates to a pharmaceutical composition having saikosaponin as a main component, the use of the pharmaceutical composition, as well as the preparation method thereof, wherein the pharmaceutical composition is used to prevent or treat the infection of one virus selected from the hepatitis C virus, the measles virus, the respiratory syncytial virus, the vesicular stomatitis virus, the dengue virus, and the non-enveloped viruses.

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COMPOSITION PREPARED FROM SAIKOSAPONIN, THE USE AND THE PREPARATION METHOD THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefits under 35 U.S.C. §119 and §365 of Taiwan Application No. 102119835, filed June 4, 2013, at the Taiwan Intellectual Property Office, the contents of which are incorporated by reference as if fully set forth.

FIELD OF THE INVENTION

[0001] The present invention relates to the use of saikosaponin in treating and preventing a virus infection, a pharmaceutical composition including saikosaponin, and a method for preparing the pharmaceutical composition.

BACKGROUND OF THE INVENTION

[0002] Currently, there are about 170 million people in the world (2% of world population) infected with the hepatitis C virus (HCV) and entangled in the hepatic diseases (hepatitis, liver cirrhosis, and liver cancer) which are one of the most serious health and medical burdens in the world. There is no preventive vaccine for HCV, and the existing clinical drugs, interferon-alpha (IFN-α) + ribavirin, do not have an ideal therapeutic effect on the most popular genotype I of HCV (about 50% cure rate) and have many side effects. Further, sustained medical cost for the existing clinical drugs is incurred, which causes many patients to be unable to maintain the medical course due to the financial burden. Although the US Food and Drug Administration (FDA) approved two new drugs, boceprevir and telaprevir, for treating patients infected by genotype I virus in May 2011, these two drugs can only be used in combination with IFN-α and ribavirin rather than being used alone.

Therefore, it is an essential issue to develop a therapeutic drug for HCV and the hepatic diseases that HCV causes.

[0003] During the past decade, virologists endeavored to develop more effective HCV non-structural protein inhibitors to affect the replication of viruses and hope to replace the existing clinical drugs (IFN-α + ribavirin) or to reinforce the combination treatment thereof. Although such an antivirus strategy is advantageous to many HCV patients, many problems such as two main issues, side effects and expensive price, remain to be solved.

[0004] To investigate an economical and effective HPC therapeutic drug with fewer side effects, the present invention uses the endemic herb, *Bupleurum kaoi*, utilizes saikosaponin extracted therefrom to treat or prevent a virus infection, and prepare a pharmaceutical composition having the aforementioned uses that solves the problem of poor solubility of the saikosaponin, which not only provides another choice for drug therapy for HCV but develops native biomedical resources in Taiwan.

[0005] In view of the drawbacks of the prior art, the inventors developed a pharmaceutical composition prepared from saikosaponin, its use, and the preparation method thereof. The summary of the present invention is below.

SUMMARY OF THE INVENTION

[0006] In the method for preventing or treating an infection of a virus in the present invention, a step of administering a pharmaceutical composition including a saikosaponin to a subject suffering from the infection is disclosed, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus, and non-enveloped viruses. The aim of the present invention is to develop a new drug to replace or assist the existing clinical drugs (IFN-α/ribavirin) so as to achieve the benefits of reducing side effects and economic burden.

[0007] In another method for preventing or treating an infection of a virus in the present invention, a step of administering an anti-viral agent in combination with a pharmaceutical composition including a saikosaponin to a subject suffering from the infection is disclosed, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus and non-enveloped viruses, and the anti-viral agent is at least one selected from a group consisting of Interferon alpha, pegylated Interferon-alpha, Ribavirin, Boceprevir and Telaprevir.

[0008] In addition, the present invention discloses a pharmaceutical composition including a main component and a polymer mixed with the main component, wherein the main component includes a saikosaponin. The pharmaceutical composition solves the poor solubility of a drug by nanoscaling the drug, which increases the bioavailability and antivirus effect.

[0009] The present invention further discloses a method for preparing a pharmaceutical composition, including pouring an organic solvent containing a saikosaponin into a solution including a polymer, distributing the saikosaponin throughout the polymer, and removing the organic solvent to obtain the pharmaceutical composition.

[0010] The inventors of the present invention verified that the saikosaponin has the effects of treating and preventing the HCV infection, and may prevent the measles virus, the respiratory syncytial virus, the vesicular stomatitis virus, the dengue virus, and the non-enveloped virus' invasion of the cells to serve as a broad-spectrum viral inhibitor. The present invention also discloses a successful dosage form of the saikosaponin at the nanoscale level that can be prepared in water to increase the bioavailability. In addition to being a drug for the prevention and

treatment of a viral infection, the saikosaponin and its nanoscaled dosage form can also be applied to masks, gloves, mosquito repellent, liquid soap, dry hand wash (a disinfecting fluid), and various cleaning supplies in the future, to achieve the effect of anti-virus protection, which would make epidemic prevention easier.

[0011] Other objects, advantages and efficacies of the present invention are described in detail below and taken from the preferred embodiments with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Fig. 1(A) is a High Performance Liquid Chromatography (HPLC) analysis result of the alcohol extract of *Bupleurum* (BK).

[0013] Fig. 1(B) is an HPLC analysis result of the saikosaponin SSa.

[0014] Fig. 1(C) is an HPLC analysis result of the saikosaponin SSb2.

[0015] Fig. 1(D) is an HPLC analysis result of the saikosaponin SSc.

[0016] Fig. 1(E) is an HPLC analysis result of the saikosaponin SSd.

[0017] Fig. 2(A) shows the effect of the alcohol extract of *Bupleurum* (BK) on inhibiting the HCV infection.

[0018] Fig. 2(B) shows the effect of the saikosaponin SSa on inhibiting the HCV infection.

[0019] Fig. 2(C) shows the effect of the saikosaponin SSb2 on inhibiting the HCV infection.

[0020] Fig. 2(D) shows the effect of the saikosaponin SSc on inhibiting the HCV infection.

[0021] Fig. 2(E) shows the effect of the saikosaponin SSd on inhibiting the HCV infection.

[0022] Fig. 3 shows the effects of the alcohol extract of *Bupleurum* (BK)

and the saikosaponin SSa, SSb2, SSc, and SSd on the time points of the HCV infection.

[0023] Fig. 4 is the result of entry assays for the alcohol extract of *Bupleurum* (BK) and the saikosaponin SSa, SSb2, SSc, and SSd.

[0024] Fig. 5 shows the result that the saikosaponin SSb2 inhibits the viruses from attaching to the cells.

[0025] Fig. 6 shows the result that the saikosaponin SSb2 inhibits the infections of different genotypes of HCV.

[0026] Fig. 7 shows the result that the saikosaponin SSb2 inhibits the viruses in the sera of the clinical patients from attaching to the cells.

[0027] Fig. 8(A) shows the effect of the saikosaponin SSb2 on inhibiting the measles virus (MV) infection.

[0028] Fig. 8(B) shows the effect of the saikosaponin SSb2 on inhibiting the respiratory syncytial virus (RSV) infection.

[0029] Fig. 8(C) shows the effect of the saikosaponin SSb2 on inhibiting the vesicular stomatitis virus (VSV) infection.

[0030] Fig. 8(D) shows the effect of the saikosaponin SSb2 on inhibiting the dengue virus (DENV) infection.

[0031] Fig. 8(E) shows the effect of the saikosaponin SSb2 on inhibiting the reovirus (RV) infection.

[0032] Fig. 9(A) shows the result of the crystal property of the pharmaceutical composition of the present invention determined by X-ray diffraction (XRD).

[0033] Fig. 9(B) shows the result of inter-molecular interactions between the two materials of the pharmaceutical composition of the present invention determined by Fourier transform infrared spectroscopy (FTIR).

[0034] Fig. 9(C) shows the determined solubility of the pharmaceutical

composition of the present invention.

[0035] Fig. 10(A) shows the effect of the nanoparticles of the present invention on inhibiting the HCV infection.

[0036] Fig. 10(B) shows the effect of the nanoparticles of the present invention on inhibiting the DENV infection.

[0037] Fig. 11(A) shows the effect of a combination of the alcohol extract of *Bupleurum* (BK) and various concentrations of interferon on inhibiting the HCV infection.

[0038] Fig. 11(B) shows the effect of a combination of the saikosaponin SSb2 and various concentrations of interferon on inhibiting the HCV infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0039] Further embodiments herein may be formed by supplementing an embodiment with one or more elements from any one or more of the other embodiments herein, and/or substituting one or more elements from one embodiment with one or more element from one or more of the other embodiments herein.

[0040] The following non-limiting examples are provided to describe particular embodiments. The embodiments throughout may be supplemented with one or more details from one or more examples below, and/or one or more elements from an embodiment may be substituted with one or more details from one or more of the examples below.

[0041] The present invention discloses a method for preventing or treating an infection of a virus, including a step of administering a pharmaceutical composition including a saikosaponin to a subject suffering from the infection, wherein the virus is one of the hepatitis C virus, the measles virus, the respiratory syncytial virus, the vesicular stomatitis virus,

the dengue virus, and non-enveloped viruses (including reovirus, enterovirus, norovirus, and adenovirus).

[0042] The saikosaponin used in the present invention is an alcohol extract of *Bupleurum kaoi*, which is extracted from *Bupleurum kaoi* (an endemic species in Taiwan) and includes the saikosaponins SSa, SSb2, SSc, and SSd. It should be realized that the phrase "saikosaponin" herein is not limited to the alcohol extract from *Bupleurum kaoi* and may be a product (including a synthesized product) obtained from other *Bupleurum* species using any solvent or any method. In another aspect, the saikosaponin in the present invention can refer to a collective composition of the alcohol extracts of *Bupleurum*, SSa, SSb2, SSc, SSd, and the combination thereof, for example, the saikosaponin could include SSb2 only, include SSa and SSb2, or include SSa, SSb2, SSc, and SSd, although the antivirus effects of the alcohol extract of *Bupleurum*, SSa, SSb2, SSc, and SSd are separately shown in the present invention.

[0043] To prepare a pharmaceutical composition using saikosaponin, the saikosaponin of the present invention can be mixed with a pharmaceutically acceptable carrier to obtain a formulation with more bioavailability. The pharmaceutically acceptable carrier above or the phrases "excipients" and "bioavailable carriers or excipients" include any appropriate compounds known to be used for preparing the dosage form, such as a solvent, a dispersing agent, a coating, an antibacterial or antifungal agent, and a preserving agent or delayed absorbent. Typically, such a carrier or excipient does not have therapeutic activity in and of itself. Each formulation prepared by combining the saikosaponin disclosed in the present invention and the pharmaceutically acceptable carriers or excipients will not cause undesired effects, allergies, or other inappropriate effects when administered to an animal or human. Accordingly, the saikosaponin

disclosed in the present invention in combination with the pharmaceutically acceptable carrier or excipient is adaptable for clinical usage and can achieve the treatment or prevention effect.

[0044] The carrier varies depending on the dosage form itself, and the composition for oral administration may use any orally acceptable formulation, which includes capsule, tablet, pill, emulsion, aqueous suspension, dispersing agent, and solvent. As to the carrier generally used in the oral formulation, taking the tablet as an example, the carrier may be a basic additive such as lactose, corn starch, lubricant, and magnesium stearate. The diluents used in the capsule include lactose and dried corn starch. To prepare the aqueous suspension or the emulsion formulation, the active ingredient is suspended or dissolved in an oil interface in combination with the emulsion or the suspension agent, and an appropriate amount of sweeteners, flavours, or pigments is added as needed.

[0045] In accordance with different uses, the pharmaceutical composition of the present invention can be manufactured as one of a drug, a disinfecting fluid, a pair of gloves, a mask, a dry hand wash, a liquid soap, or a mosquito repellent. The embodiments for preparing the pharmaceutical composition of the present invention are described in detail below.

[0046] Determination of the active ingredient of *Bupleurum*:

[0047] Please refer to Fig. 1(A) to Fig. 1(E), which show the HPLC analysis results of the alcohol extract of *Bupleurum*, the saikosaponins SSa, SSb2, SSc, and SSd. In the present invention, HPLC is used to determine the saikosaponins SSa, SSb2, SSc, and SSd indeed existed in the alcohol extract of *Bupleurum kaoi* (BK) and quantify their contents. The quantified results of Fig. 1(A) to Fig. 1(E) are shown below in Table 1.

Table 1

	Linear	equation*	r^2	Amount/8 mg

	range (μg/ml)			crude extract (μg/ml)[#]
SSa	19.53-312.4	y=1813.5x-18796	0.9963	192.46±2.84
SSb2	7.81-31.24	y=1880.3x-1629	1	13.52±0.87
SSc	9.27-37.08	y=2001.6x-4214	0.9988	20.74±1.17
SSd	78.1-624.8	y=904.73x-1182.2	0.9979	538.79±1.37

*x is the concentration of saikosaponin in μg/ml, y is peak area.

[#]Crude extract was injected 3 times. The contents of crude extract were expressed mean ± SEM.

[0048] Saikosaponin has the effect of inhibiting the HCV infection:

[0049] Figs. 2(A) to 2(E) show the effects of the alcohol extract of *Bupleurum* and saikosaponins SSa, SSb2, SSc, and SSd on inhibiting the HCV infection. The alcohol extract of *Bupleurum kaoi* (BK) and saikosaponins SSa, SSb2, SSc, and SSd were dissolved in dimethyl sulfoxide (DMSO) and prepared into various concentrations used to evaluate the antivirus activity. It was found that BK, SSa, SSb2, SSc, and SSd all have the effect of inhibiting the infection of cell-culture derived HCV genotype 2a and reveal dose dependency. According to the results above, the concentrations that were non-toxic to the cells and had the anti-virus activity, e.g. 50 μg/ml of BK, 8 μM of SSa, 50 μM of SSb2, 200 μM of SSc, and 2 μM SSd, were chosen for the experiments of the anti-virus mechanism. The dosages above have about 2 log (i.e. 100 times) the inhibition index for the virus protein. The quantified results of Fig. 2(A) to Fig. 2(E) are shown below in Table 2.

Table 2

Treatments	EC_{50}	CC_{50}	SI
BK	16.82±1.89 μM	215.4±10.7 μM	12.8
SSa	1.14±0.08 μM	29.62±1.75 μM	26
SSb2	16.13±2.41 μM	740.4±28.35 μM	45.9
SSc	86.12±6.84 μM	716.79±83.17 μM	8.32
SSd	2.8±0.17 μM	23.38±3.14 μM	8.35

[0050] Fig. 3 shows the effects of the alcohol extract of *Bupleurum*, and the saikosaponin SSa, SSb2, SSc, and SSd on the time points of the HCV infection. First, in order to investigate the complete infection cycle of the virus, the virus and drugs were added in three different ways: 1) pre-treatment: after the cells were treated with the drugs for 24 hours, the drugs were removed and the virus was added, and then the expression of the virus was detected by luminescence 3 days later, 2) co-addition: the virus and the drugs were added simultaneously and removed after 3 hours, and then the expression of the virus was detected by luminescence 3 days later, and 3) post-infection: the drugs were added after 3 hours from the virus infection, and then the expression of the virus was detected by luminescence 3 days later. In the co-addition experiment, it can be seen that the alcohol extract of *Bupleurum*, the saikosaponin SSa, SSb2, and SSd have good inhibition, which proves the drugs can interfere with the virus infection. In the pre-treatment experiment, all drugs were ineffective, and it is supposed *prima facie* that the drugs do not act to protect from the virus infection. In the results of the post-infection experiment, it can be seen that all drugs were ineffective, and it was proven that the drugs are not effective in the replication or translation stage of the virus. In addition, SSc had an

inhibitive effect only in the experiment where the drug was maintained throughout the virus infection process (data not shown). It can be seen from the above results that the antivirus mechanism of the alcohol extract of *Bupleurum*, the saikosaponin SSa, SSb2, and SSd inhibits the early entry stage of the HCV.

[0051] Fig. 4 is the result of entry assays for the alcohol extract of *Bupleurum* and the saikosaponin SSa, SSb2, SSc, and SSd. In the inactivation and attachment experiments, it can be seen that only SSb2 can neutralize the viral particles and inhibit the viruses from attaching to the cells, and in the fusion experiment, it can be seen that the alcohol extract of *Bupleurum*, SSa, and SSb2 can inhibit the viral fusion. The results above prove that the main mechanism by which the alcohol extract of *Bupleurum* and its active ingredients SSa and SSb2 against the virus is entry inhibition, and thus the alcohol extract of *Bupleurum* and its active ingredients SSa and SSb2 can function as entry inhibitors.

[0052] Fig. 5 shows the result that the saikosaponin SSb2 inhibits the virus from attaching to the cells. Cell-culture derived HCV (HCVcc) or soluble glycoprotein E2 (sE2) was added to Huh-7.5 cells under 4°C, and the cells were treated with SSb2 at the same time. After 3 hours of treatment, SSb2 and virus were washed with phosphate buffered saline (PBS), and then the anti-E2 antibody was used to target the virus or glycoprotein on the cell membrane. Finally, the level of virus was detected by enzyme-linked immunosorbent assay (ELISA). The result showed that SSb2 can avoid the viral entry by interfering with the binding of the HCVcc and sE2 to the cell membrane. In view of the above experiment, it can be seen that *Bupleurum kaoi* and its active ingredients (SSa, SSb2, SSc, and SSd) can serve as therapeutic drugs for chronic hepatitis C. In addition, the mechanism of *Bupleurum kaoi*, SSa, SSb2, SSc, and SSd inhibits the early entry of the

virus, these components also have preventive functions and can serve as drugs for liver transplantation (disinfectant drugs) to prevent the virus from invading new liver tissue, and this specific use overcomes the previous defect that IFN-α + ribavirin can only be used in treatment but not in prevention.

[0053] Fig. 6 shows the result that the saikosaponin SSb2 inhibits the infections of different genotypes of HCVs. As shown in Fig. 6, Huh-7.5 cells were infected with genotypes 2b, 3a, or 7a of HCV, respectively, and treated with SSb2 at the same time, and the results show that SSb2 can inhibit the infections of different genotypes of HCV.

[0054] Fig. 7 shows the result that the saikosaponin SSb2 inhibits the viruses from the sera of the clinical patients from attaching to the cells. The viruses from the sera of the clinical patients were added to Huh-7.5 cells under 4°C, and the SSb2 was added thereto at the same time. After 3 hours of treatment, the SSb2 and viruses were washed with PBS, and then the total RNA of the cells was extracted. Finally, the level of virus was quantified by quantitative polymerase chain reaction (qPCR). The result showed that SSb2 can inhibit the binding of the genotypes 1a, 1b, 2a, and 6 of HCV from the sera of the clinical patients to the cells. The quantified results are shown below in Table 3.

Table 3

	Genotype	Treatment	
		DMSO	SSb2
HCVser#1	1a	35103 IU/ml	<10 IU/ml
HCVser#2	1b	32887 IU/ml	<10 IU/ml
HCVser#3	2a	29130 IU/ml	<10 IU/ml
HCVser#4	6	35144 IU/ml	<10 IU/ml

[0055] By incorporating the experimental results in Fig. 6 and Fig. 7, the present invention proves that the saikosaponin SSb2 not only inhibits the genotype 2a of HCVcc, but also inhibits the infections of other genotypes 2b, 3a, and 7a of HCVcc as well as the attachment of the genotypes 1a, 1b, 2a, and 6 of HCV from the sera of the clinical patients to the cells, such that the clinical patients can resist the high variability of HCV. Based on the feature that SSb2 inhibits various genotypes of HCVs, it illustrates that saikosaponin can overcome the problem of poor therapeutic effect of IFN-α + ribavirin on the genotype 1 of HCV, and overcome the problems of two new drugs, boceprevir and telaprevir, which have poor therapeutic effect on other genotypes of HCVs except for the genotype 1 HCV and are not suitable for being administered alone (i.e. boceprevir and telaprevir only can be used in combination with IFN-α + ribavirin).

[0056] Saikosaponin has the effect of inhibiting the infection of various viruses:

[0057] Figs. 8(A) to 8(E) show the effect of SSb2 on inhibiting the infection of measles virus (MV), respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), dengue virus (DENV), and reovirus (RV). The quantified results are shown below in Table 4.

Table 4

	Envelope	Genome	CC_{50} (μM)	EC_{50} (μM)	SI
MV	+	(-)ssRNA	629.18±43.01	59.26±1.22	10.62
RSV	+	(-)ssRNA	985.18±27.98	105.35±1.69	9.35
VSV	+	(-)ssRNA	945.92±25.09	49.82±0.75	18.99
DENV	+	(+)ssRNA	945.92±25.09	30.96±62.94	30.55
RV	-	(+)dsRNA	721.26±62.94	139.03±3.51	5.19

[0058] According to Table 4, it can be seen that these viruses belong to different types, and the results show that SSb2 has inhibitory effects on the viruses regardless of DNA or RNA viruses, viruses with single or double stranded genomes, enveloped or non-enveloped viruses, and it is proven that SSb2 is a broad-spectrum antiviral drug. Based on the inhibitory effects of SSb2 on the non-enveloped virus, it can be seen that SSb2 also has inhibitory effects on other non-enveloped viruses such as enterovirus, norovirus, and adenovirus. The non-enveloped viruses referred to in the present invention, besides the reovirus (RV), enterovirus, norovirus, and adenovirus described above, further includes other non-enveloped viruses known in the art such as human papillomavirus, small RNA virus, and so on.

[0059] According to the characteristic that SSb2 inhibits infections from various viruses, the saikosaponin can also be applied to masks, gloves, mosquito repellent, liquid soap, dry hand wash (a disinfecting fluid), and various cleaning supplies in the future, to make epidemic prevention easier. For example, the saikosaponin can be added to mosquito repellent to inhibit the dengue fever epidemic that periodically spikes every summer in tropics and subtropics. Alternatively, based on the characteristic that SSb2 also has inhibitory effects on the non-enveloped viruses (such as enterovirus, norovirus, or adenovirus), the saikosaponin can be added to dry hand wash, to solve the problem that the alcoholic dry wash has no disinfecting effect on the non-enveloped viruses and to relieve the exploding crises of epidemic viruses in the world.

[0060] Because saikosaponin has poor solubility, the problem of low bioavailability may occur if it is made as a drug. In addition to combining the saikosaponin with the above-mentioned pharmaceutically acceptable carrier, in order to enhance the solubility of the saikosaponin, a

pharmaceutical composition is prepared using nanotechnology in the present invention. The saikosaponin, the main component of the pharmaceutical composition, was mixed with a polymer to create a nanoparticle, which is <50 nm in diameter. Because the pharmaceutical composition of the present invention can be refrigerated in liquid form, or be frozen in the form of dry powder, it can be made as a drug, various medical defenses (such as disinfecting fluid, gloves, mask, dry hand wash, liquid soap, and mosquito repellent) and commodities.

[0061] In addition, the present invention provides a method for manufacturing a pharmaceutical composition, including pouring an organic solvent containing the saikosaponin into a solution including a polymer, distributing the saikosaponin throughout the polymer, and removing the organic solvent to obtain the pharmaceutical composition. In the following embodiments of the present invention, SSb2 is used as an example to prepare SSb2 nanoparticles (SSb2-NP). It should be realized that the preparation method provided in the embodiments of the present invention is also suitable for preparing nanoparticles of other saikosaponin (SSa, SSc, and SSd) and nanoparticles of the alcohol extract of *Bupleurum*.

[0062] Preparation of SSb2 nanoparticles:

[0063] SSb2 was distributed and admixed to prepare SSb2 nanoparticles by the emulsion solvent diffusion method. This process can be performed using various polymers such as Pluronic® polymer, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), and others. According to the solubility of SSb2, PVP was used as an example of the present process. In this process, SSb2 was dissolved in an appropriate amount of ethanol to make 1 mg/ml solution, PVP was dissolved in an appropriate amount of pure water to prepare a 35% solution, and then the SSb2 solution was poured into the PVP solution. The distribution and admixing of the SSb2 and the PVP were

done using ultrasound, and the organic solvent (i.e. ethanol in this case) and part of the water were removed using a vacuum decompression concentrator, to create primary SSb2 nanoparticles. After filtration using qualitative filter paper to remove oversized pellets, the complete SSb2 nanoparticles in this example can be obtained. This formulation can be refrigerated in a liquid form, or frozen in powder form, and the latter can be re-suspended in pure water prior to being administered.

[0064] According to the preparation method in the embodiment of the present invention, SSb2 is dissolved and formulated in an ethanol solution, but the skilled person in the art could replace the ethanol solution with other suitable organic solvents.

[0065] The size of the nanoparticles was analyzed by photo correlation spectroscopy (PCS) as 16.57±3.88 nm, and the size of the nanoparticles was additionally verified by transmission electron microscopy (TEM) at about 10 nm, showing consistent results with that of PCS. The yield of the present process may achieve 81.45±6.14%. Fig. 9(A) shows the results of the crystallization property of the pharmaceutical composition of the present invention determined using X-ray diffraction (XRD). Based on the crystallization property determined by XRD, the individual groups of physical mixture (PM) at different ratios are compared, the nanoparticles in this example (SSb2-NP) lost the lattice signal of SSb2 at 3.7°, and it can be seen that the SSb2 lattice of the nanoparticles disappeared. Fig. 9(B) shows the results of inter-molecular interaction between the two materials of the pharmaceutical composition of the present invention determined using Fourier transform infrared spectroscopy (FTIR). The inter-molecular interaction between the two materials is determined by FTIR, a shift of the hydroxyl group (3,420 cm^{-1}) can be seen from the spectrum of the nanoparticles (SSb2-NP), and the shift was different from that of the

physical mixture (SSb2-PM), showing that the inter-molecular interactions indeed occurred in the nanoparticles in this case. The inter-molecular interaction between the two materials can explain why the SSb2 lattice property of the nanoparticles disappeared, which improves the solubility of SSb2. Fig. 9(C) shows the determined solubility of the pharmaceutical composition of the present invention. The solubility of the present nanoparticles was determined using dissolution assay. By comparing the un-formulated SSb2 with the SSb2 nanoparticles in this example, the dissolution assay shows that the nanoparticles in this example significantly enhanced the solubility of SSb2. Through the modification of the nanoparticles, SSb2 achieved 50% release in 20 minutes and may maintain a releasing trend during the subsequent test.

[0066] Antiviral activity of the nanoparticles of the saikosaponin:

[0067] Figs. 10(A) and 10(B) show the effects of the nanoparticles of the present invention on inhibiting the HCV and DENV infections, respectively. SSb2-NP has a characteristic of being active in water without another solubilizer (such as DMSO). The present experiment compared the SSb2-NP with the raw material drug in the solubilizer (SSb2-DMSO) and the raw material drug in water (SSb2-DW), and analyzed the antiviral effects themselves. The results show that SSb2-DW cannot exhibit any antiviral effect against either HCV (Fig. 10(A)) or DENV (Fig. 10(B)), and the antiviral effect of SSb2-NP almost equals that of SSb2-DMSO. The results reflect the fact that the nanoparticles of the saikosaponin, SSb2-NP, successfully prepared using only water in the formulation in the present invention exhibit antiviral effects and enhance the bioavailability and the antiviral use of the saikosaponin.

[0068] The effect of a combination of the pharmaceutical composition of the present invention and clinical drug (interferon, IFN) on inhibiting the

HCV infection in Huh-7.5 cells:

[0069] The pharmaceutical composition of the present invention can be used in combination with suitable clinical drug, to increase the validity of the therapy. Huh-7.5 cells were infected with HCV for 3 hours. After the infection is completed, the exceed viruses were washed and the culture medium containing 2% fetal bovine serum and the pharmaceutical composition of the present invention (BK: 10 μM, 17 μM, and 20 μM ; SSb2 : 10 μM, 16 μM, and 20 μM) in combination with interferon (0.5 unit, 1.0 unit, and 2.0 unit) were added for culturing another 72 hours. Then, the culture medium was collected and the inhibitory effects of the pharmaceutical composition of the present invention in combination with interferon on HCV were calculated by the expression of luminescence of the culture medium.

[0070] Fig. 11(A) shows the effect of a combination of the alcohol extract of *Bupleurum* (BK) and various concentrations of interferon on inhibiting the HCV infection, and Fig. 11(B) shows the effect of a combination of the saikosaponin SSb2 and various concentrations of interferon on inhibiting the HCV infection, wherein MOCK group is a negative control group without administering any drug, virus only group is a negative control group infected with HCV, DMSO group is a negative group administered dimethyl sulfoxide (DMSO). Compared with the virus only group, it can be seen that the pharmaceutical composition of the present invention, including the BK groups in Fig. 11(A) and SSb2 groups in Fig. 11(B), in combination with interferon can suppress the infectivity of HCV, showing that this combined administration indeed can inhibit HCV infection. In addition, under the same concentration of the pharmaceutical composition of the present invention, low dose interferon and high dose interferon exhibit similar inhibitory effects on HCV. Therefore, the combination of the

pharmaceutical composition of the present invention and interferon has partial synergistic effect. Based on this assay, it is proven that the pharmaceutical composition of the present invention can be combined with clinical drug to achieve a better inhibitory effect on HCV infection.

[0071] The specific drug (Interferon) used in the present invention is only exemplary embodiment which do not limit the scope of the present invention. The pharmaceutical composition of the present invention can be used in combination with other clinical drugs for treating or preventing a virus infection. For example, the skilled person in the art should realize that Interferon used in the above embodiments can also be Interferon alpha, pegylated Interferon-alpha, Ribavirin, Boceprevir, Telaprevir, or the combination thereof.

[0072] Embodiments:

[0073] 1. A method for performing one of preventing and treating an infection of a virus, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus, and non-enveloped viruses, the method comprising a step of administering a pharmaceutical composition including a saikosaponin to a subject suffering from the infection.

[0074] 2. A method according to Embodiment 1, wherein the saikosaponin is obtained from an alcohol extract of *Bupleurum*.

[0075] 3. A method according to Embodiment 1 or 2, wherein the saikosaponin comprises at least one selected from a group consisting of SSa, SSb, SSc, and SSd.

[0076] 4. A method according to any one of Embodiments 1-3, wherein the saikosaponin is derived from *Bupleurum kaoi*.

[0077] 5. A method according to any one of Embodiments 1-4, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

[0078] 6. A method according to any one of Embodiments 1-5, wherein the pharmaceutical composition is manufactured as one selected from a group consisting of a drug, a disinfecting fluid, a pair of gloves, a mask, a dry hand wash, a liquid soap, and a mosquito repellent.

[0079] 7. A method according to any one of Embodiments 1-6, wherein the non-enveloped viruses comprise one selected from a group consisting of a reovirus, an enterovirus, a norovirus, and an adenovirus.

[0080] 8. A method for performing one of preventing and treating an infection of a virus, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus, and non-enveloped viruses, the method comprising a step of administering an anti-viral agent in combination with a pharmaceutical composition including a saikosaponin to a subject suffering from the infection, and wherein the anti-viral agent is at least one selected from a group consisting of Interferon alpha, pegylated Interferon-alpha, Ribavirin, Boceprevir, and Telaprevir.

[0081] 9. A pharmaceutical composition, including:

a main component including a saikosaponin; and

a polymer mixed with the main component.

[0082] 10. A pharmaceutical composition according to Embodiment 9, wherein the pharmaceutical composition has a size smaller than

50 nm.

[0083] 11. A pharmaceutical composition according to Embodiment 9 or 10, wherein the saikosaponin is obtained from an alcohol extract of *Bupleurum*.

[0084] 12. A pharmaceutical composition according to any one of Embodiments 9-11, wherein the saikosaponin comprises at least one selected from a group consisting of SSa, SSb, SSc, and SSd.

[0085] 13. A pharmaceutical composition according to any one of Embodiments 9-12, wherein the saikosaponin is derived from *Bupleurum kaoi*.

[0086] 14. A pharmaceutical composition according to any one of Embodiments 9-13, further comprising a pharmaceutically acceptable carrier.

[0087] 15. A pharmaceutical composition according to any one of Embodiments 9-14, wherein the pharmaceutical composition is manufactured as one selected from a group consisting of a drug, a disinfecting fluid, a pair of gloves, a mask, a dry hand wash, a liquid soap, and a mosquito repellent.

[0088] 16. A pharmaceutical composition according to any one of Embodiments 9-15, wherein the polymer improves the solubility of the saikosaponin.

[0089] 17. A pharmaceutical composition according to any one of Embodiments 9-16, wherein the polymer is one selected from a group consisting of a Pluronic polymer, a polyvinyl alcohol (PVA), and a polyvinylpyrrolidone (PVP).

[0090] 18. A method for preparing a pharmaceutical composition, including steps of:

pouring an organic solvent containing a saikosaponin into a solution, wherein the solution includes a polymer;

distributing the saikosaponin throughout the polymer; and

removing the organic solvent to obtain the pharmaceutical composition.

[0091] 19. A method according to Embodiment 18, further including a step of dissolving the saikosaponin in the organic solvent, wherein the organic solvent is an alcohol.

[0092] 20. A method according to Embodiment 18 or 19, wherein the polymer improves the solubility of the saikosaponin.

[0093] 21. A method as according to any one of Embodiments 18-20, wherein the polymer is one selected from a group consisting of a Pluronic polymer, a polyvinyl alcohol (PVA), and a polyvinylpyrrolidone (PVP).

[0094] It is understood that this invention is not limited to the particular embodiments disclosed, but is intended to cover all modifications which are within the spirit and scope of the invention as defined by the appended claims, the above description, and/or shown in the attached drawings.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for performing one of preventing and treating an infection of a virus, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus, and non-enveloped viruses, the method comprising a step of administering a pharmaceutical composition including a saikosaponin to a subject suffering from the infection.
2. A method as claimed in Claim 1, wherein the saikosaponin is obtained from an alcohol extract of *Bupleurum*.
3. A method as claimed in Claim 1, wherein the saikosaponin comprises at least one selected from a group consisting of SSa, SSb, SSc, and SSd.
4. A method as claimed in Claim 1, wherein the saikosaponin is derived from *Bupleurum kaoi*.
5. A method as claimed in Claim 1, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.
6. A method as claimed in Claim 1, wherein the pharmaceutical composition is manufactured as one selected from a group consisting of a drug, a disinfecting fluid, a pair of gloves, a mask, a dry hand wash, a liquid soap, and a mosquito repellent.
7. A method as claimed in Claim 1, wherein the non-enveloped viruses comprise one selected from a group consisting of a reovirus, an enterovirus, a norovirus, and an adenovirus.
8. A method for performing one of preventing and treating an infection of a virus, wherein the virus is one selected from a group consisting of a hepatitis C virus, a measles virus, a respiratory syncytial virus, a vesicular stomatitis virus, a dengue virus, and non-enveloped viruses, the

method comprising a step of administering an anti-viral agent in combination with a pharmaceutical composition including a saikosaponin to a subject suffering from the infection, wherein the anti-viral agent is at least one selected from a group consisting of Interferon alpha, pegylated Interferon-alpha, Ribavirin, Boceprevir, and Telaprevir.

9. A pharmaceutical composition, comprising:

a main component including a saikosaponin; and

a polymer mixed with the main component.

10. A pharmaceutical composition as claimed in Claim 9, wherein the pharmaceutical composition has a size smaller than 50 nm.

11. A pharmaceutical composition as claimed in Claim 9, wherein the saikosaponin is obtained from an alcohol extract of *Bupleurum*.

12. A pharmaceutical composition as claimed in Claim 9, wherein the saikosaponin comprises at least one selected from a group consisting of SSa, SSb, SSc, and SSd.

13. A pharmaceutical composition as claimed in Claim 9, wherein the saikosaponin is derived from *Bupleurum kaoi*.

14. A pharmaceutical composition as claimed in Claim 9, further comprising a pharmaceutically acceptable carrier.

15. A pharmaceutical composition as claimed in Claim 9, wherein the pharmaceutical composition is manufactured as one selected from a group consisting of a drug, a disinfecting fluid, a pair of gloves, a mask, a dry hand wash, a liquid soap, and a mosquito repellent.

16. A pharmaceutical composition as claimed in Claim 9, wherein the polymer improves the solubility of the saikosaponin.

17. A pharmaceutical composition as claimed in Claim 9, wherein the polymer is one selected from a group consisting of a Pluronic polymer, a

polyvinyl alcohol (PVA), and a polyvinylpyrrolidone (PVP).

18. A method for preparing a pharmaceutical composition, comprising steps of:

pouring an organic solvent containing a saikosaponin into a solution, wherein the solution includes a polymer;

distributing the saikosaponin throughout the polymer; and

removing the organic solvent to obtain the pharmaceutical composition.

19. A method as claimed in Claim 18, further comprising a step of dissolving the saikosaponin in the organic solvent, wherein the organic solvent is an alcohol.

20. A method as claimed in Claim 18, wherein the polymer improves the solubility of the saikosaponin.

21. A method as claimed in Claim 18, wherein the polymer is one selected from a group consisting of a Pluronic polymer, a polyvinyl alcohol (PVA), and a polyvinylpyrrolidone (PVP).

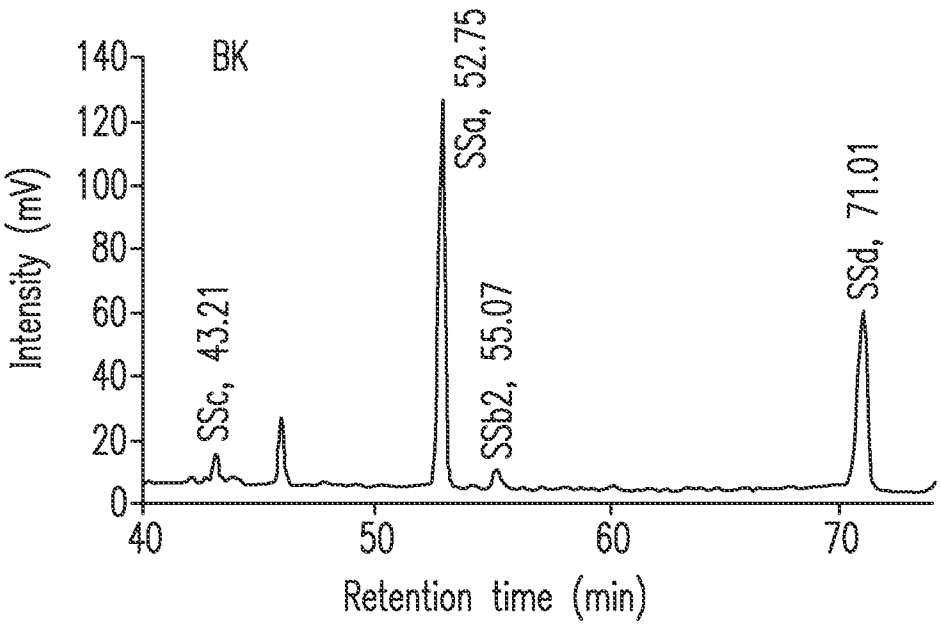
BK
SSa, 52.75
SSd, 71.01
SSc, 43.21
SSb2, 55.07
Intensity (mV)
Retention time (min)
140
120
100
80
60
40
20
0
40
50
60
70

Fig. 1(A)

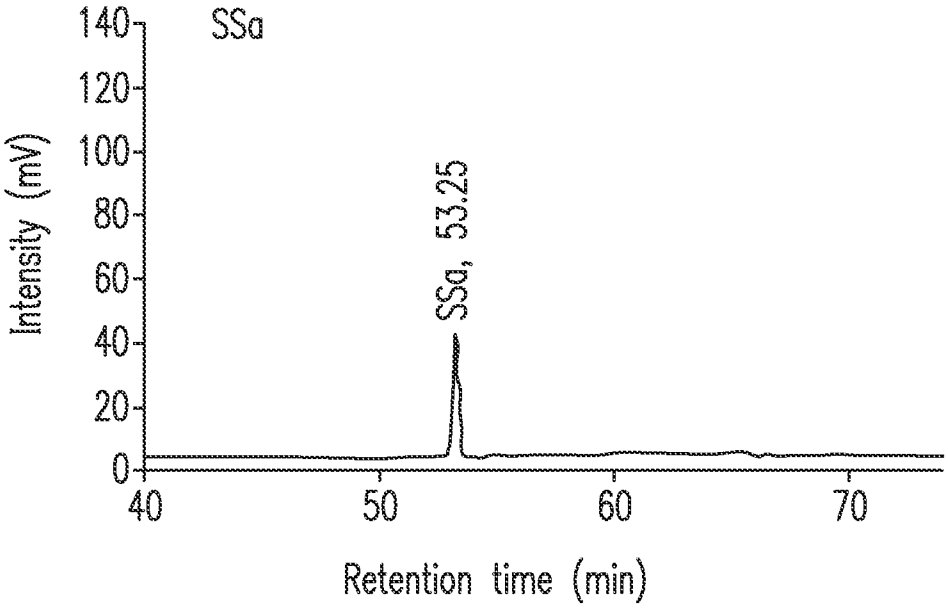
SSa
SSa, 53.25
Intensity (mV)
Retention time (min)
140
120
100
80
60
40
20
0
40
50
60
70

Fig. 1(B)

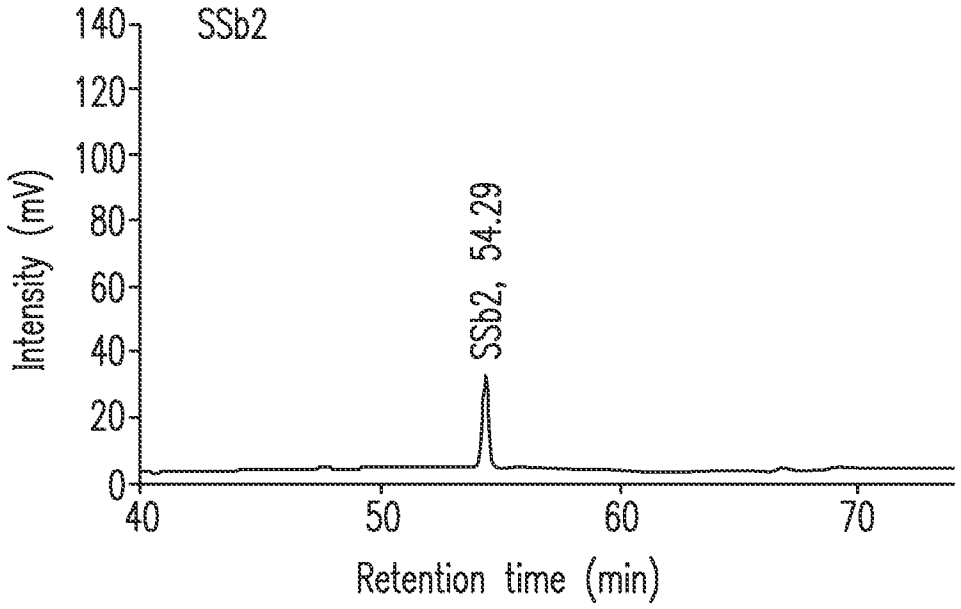
SSb2
140
120
100
80
60
40
20
0
Intensity (mV)
SSb2, 54.29
40
50
60
70
Retention time (min)

Fig. 1(C)

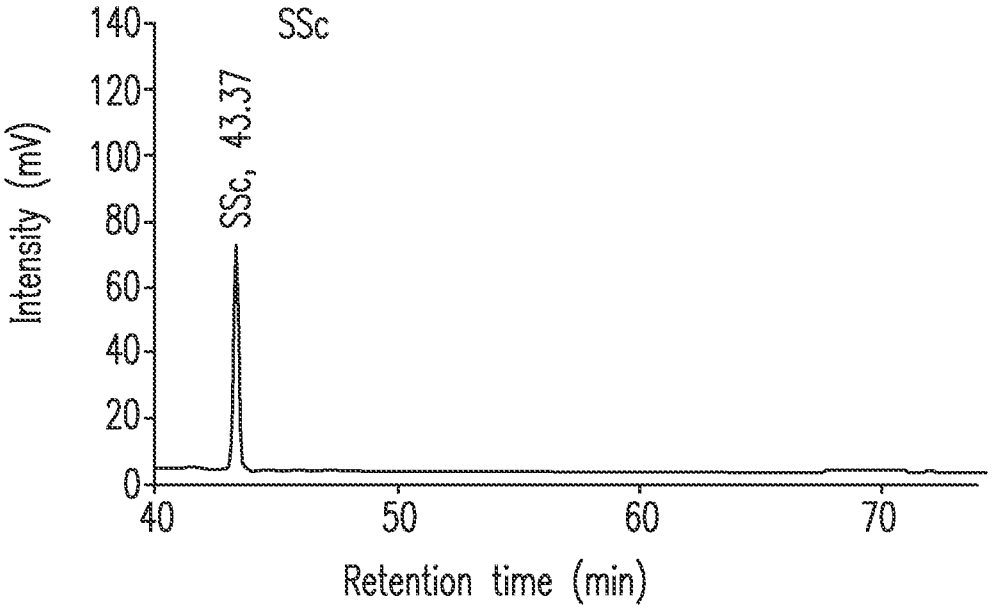
SSc
140
120
100
80
60
40
20
0
Intensity (mV)
SSc, 43.37
40
50
60
70
Retention time (min)

Fig. 1(D)

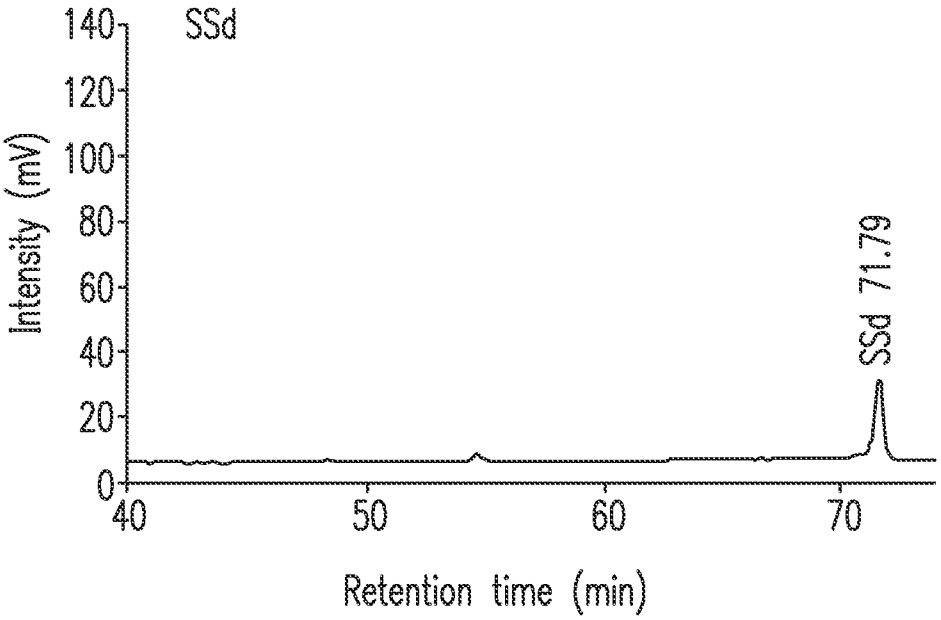
SSd
Intensity (mV)
140
120
100
80
60
40
20
0
40
50
60
70
Retention time (min)
SSd 71.79

Fig. 1(E)

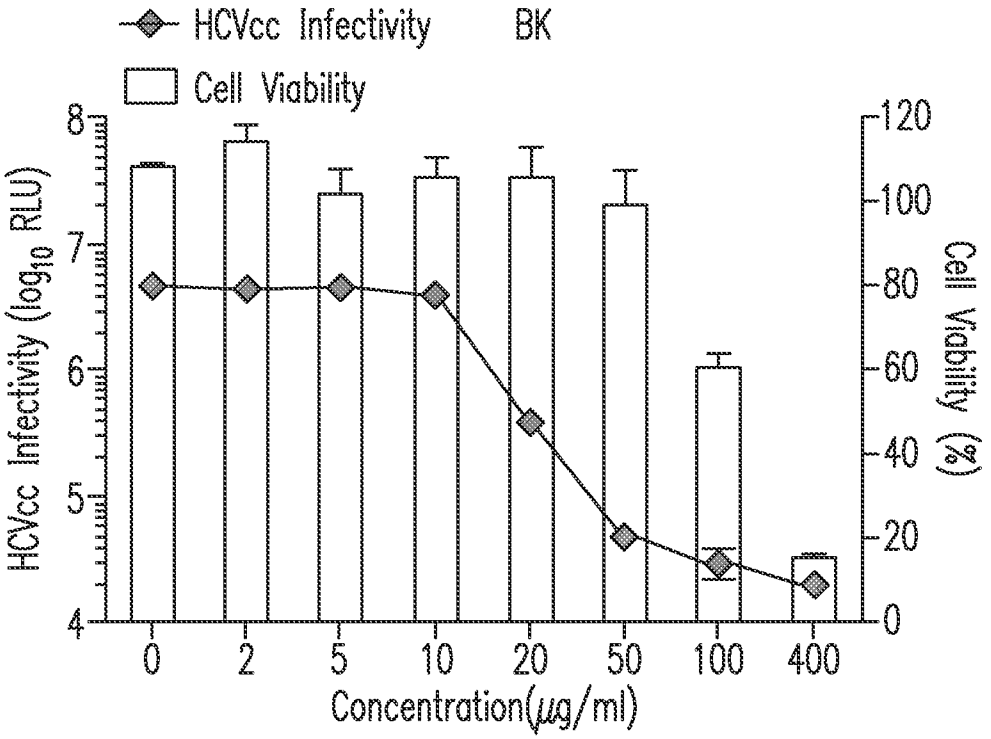
HCVcc Infectivity
Cell Viability
BK
HCVcc Infectivity (log10 RLU)
Cell Viability (%)
8
7
6
5
4
120
100
80
60
40
20
0
0
2
5
10
20
50
100
400
Concentration(μg/ml)

Fig. 2(A)

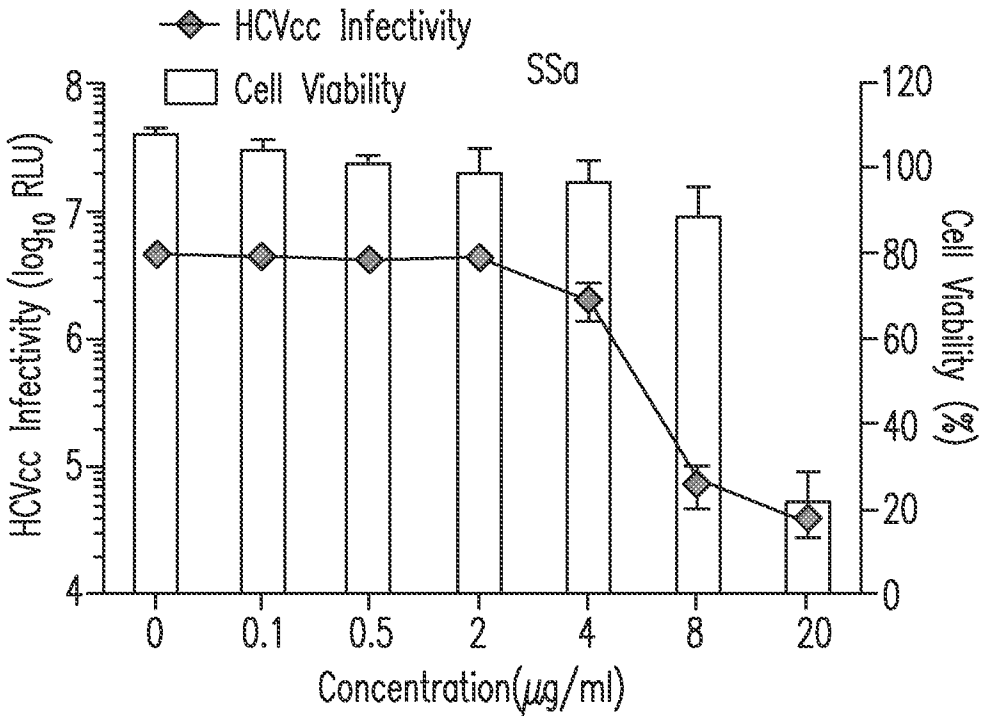
HCVcc Infectivity
Cell Viability
SSa
HCVcc Infectivity (log10 RLU)
Cell Viability (%)
8
7
6
5
4
120
100
80
60
40
20
0
0
0.1
0.5
2
4
8
20
Concentration(μg/ml)

Fig. 2(B)

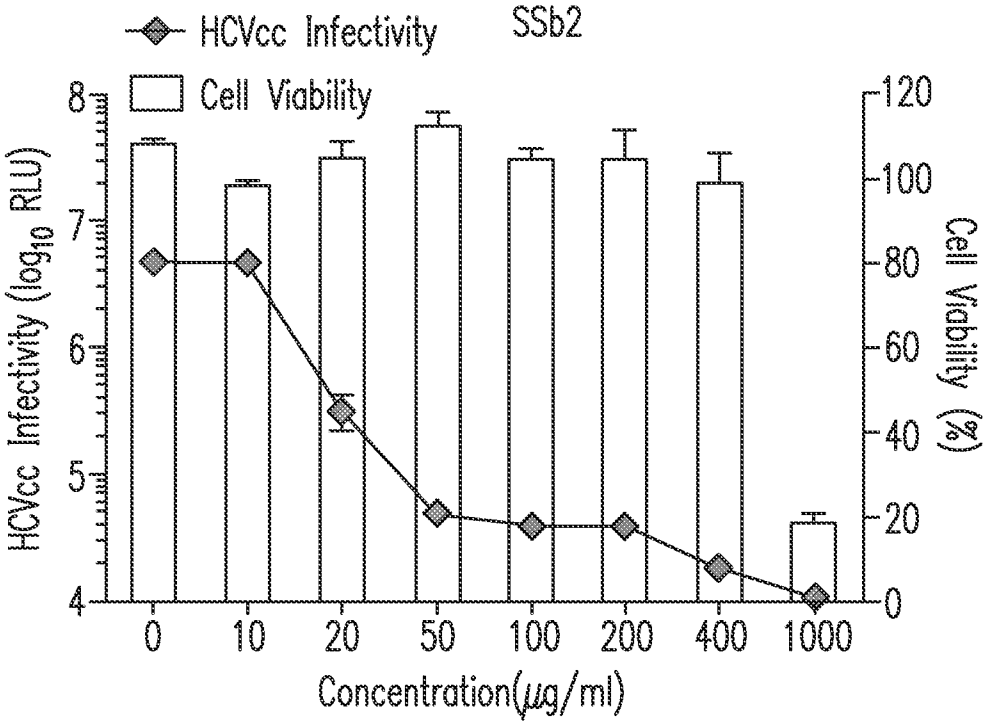
HCVcc Infectivity
SSb2
Cell Viability
HCVcc Infectivity (log10 RLU)
Cell Viability (%)
8
7
6
5
4
120
100
80
60
40
20
0
0
10
20
50
100
200
400
1000
Concentration(μg/ml)

Fig. 2(C)

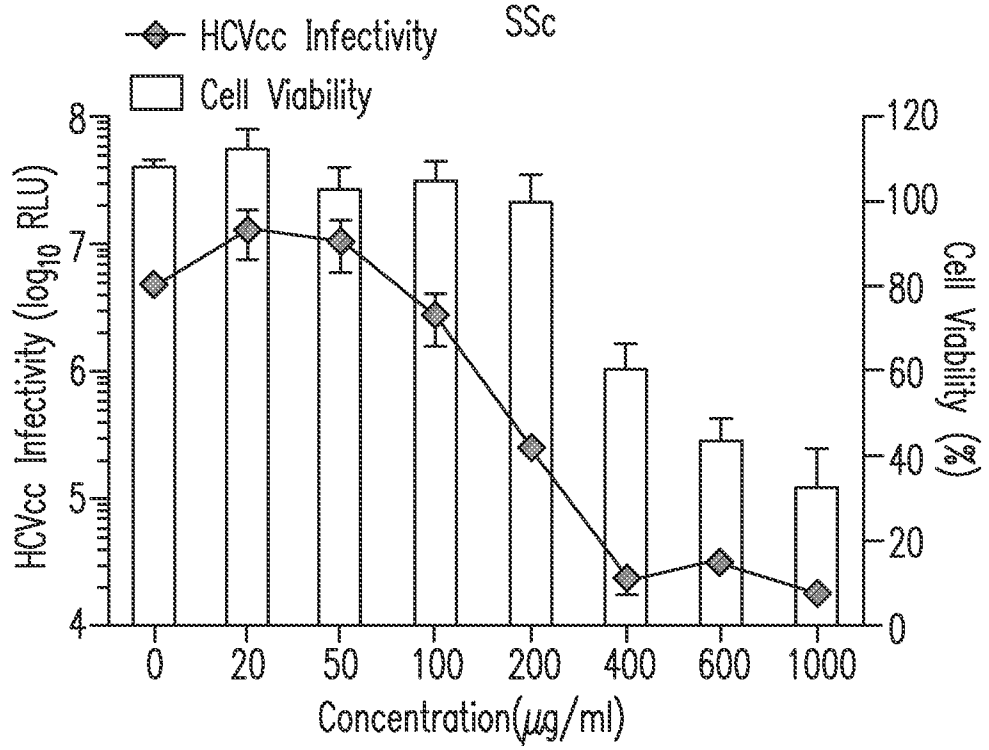
HCVcc Infectivity
SSc
Cell Viability
HCVcc Infectivity (log10 RLU)
Cell Viability (%)
8
7
6
5
4
120
100
80
60
40
20
0
0
20
50
100
200
400
600
1000
Concentration(μg/ml)

Fig. 2(D)

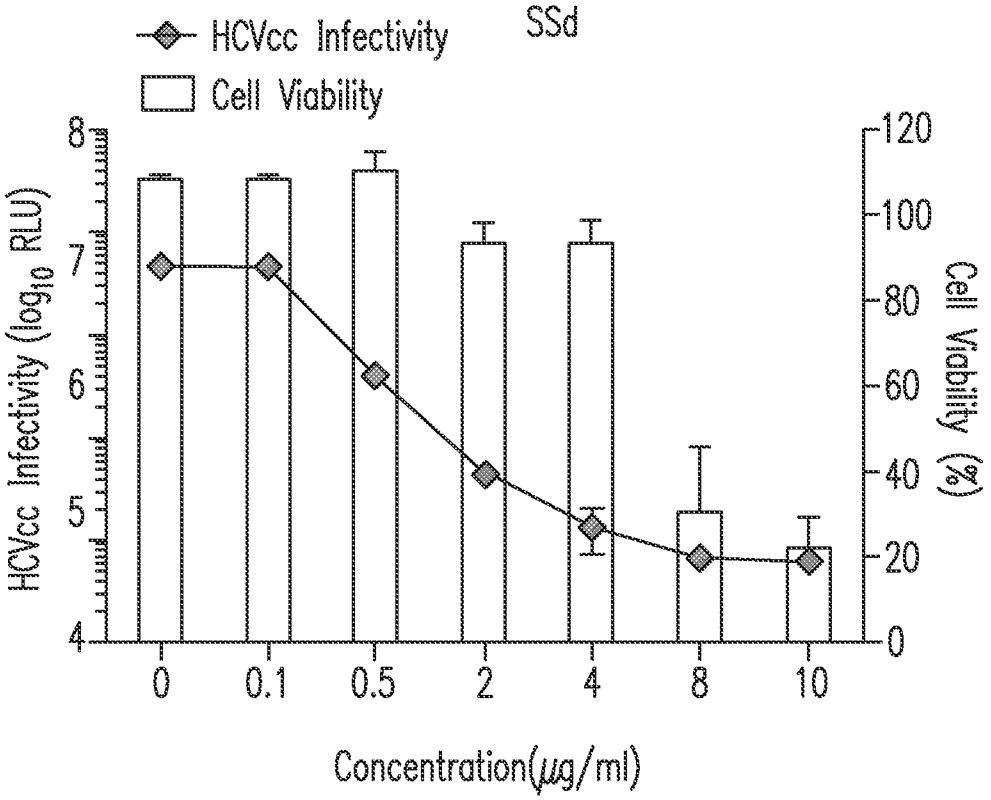
SSd
HCVcc Infectivity
Cell Viability
HCVcc Infectivity (log_{10} RLU)
Cell Viability (%)
8
7
6
5
4
120
100
80
60
40
20
0
0
0.1
0.5
2
4
8
10
Concentration(μg/ml)

Fig. 2(E)

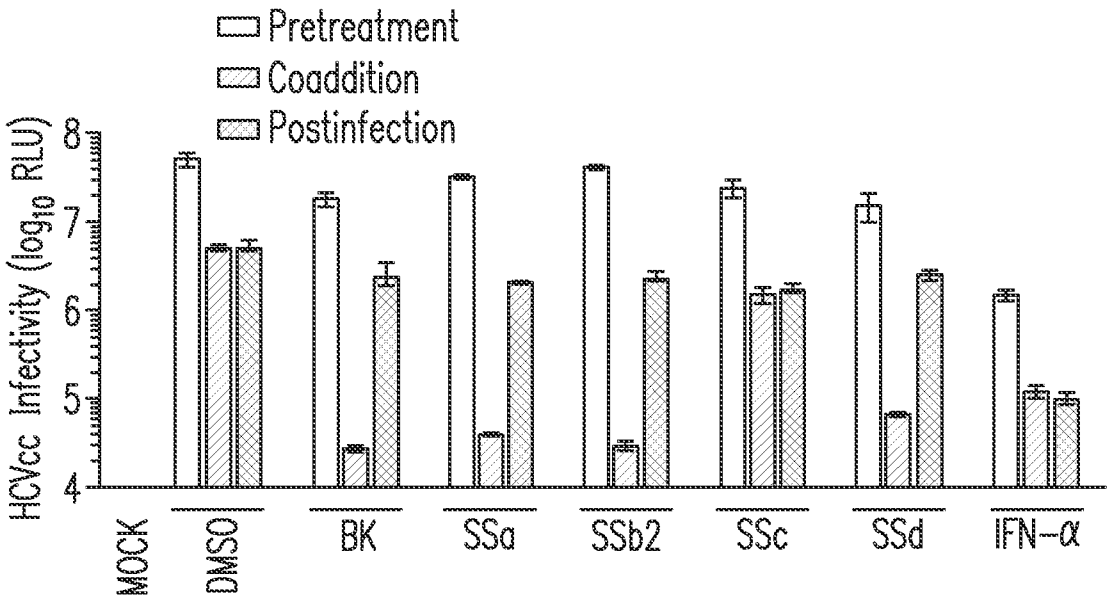
Pretreatment
Coaddition
Postinfection
HCVcc Infectivity (log_{10} RLU)
8
7
6
5
4
MOCK
DMSO
BK
SSa
SSb2
SSc
SSd
IFN-α

Fig. 3

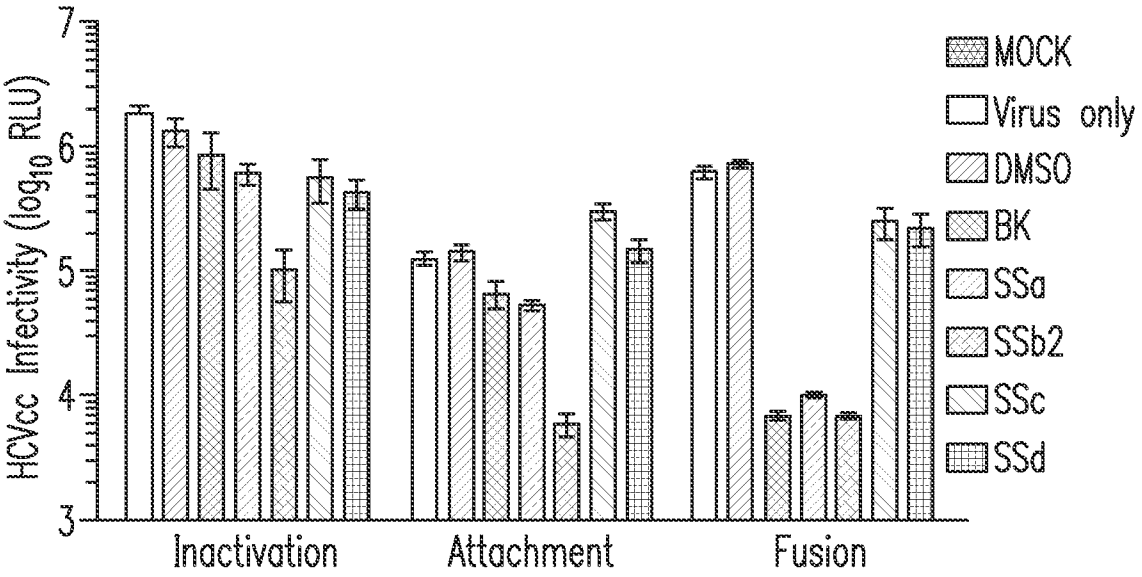
HCVcc Infectivity (log_{10} RLU)
7
6
5
4
3
MOCK
Virus only
DMSO
BK
SSa
SSb2
SSc
SSd
Inactivation
Attachment
Fusion

Fig. 4

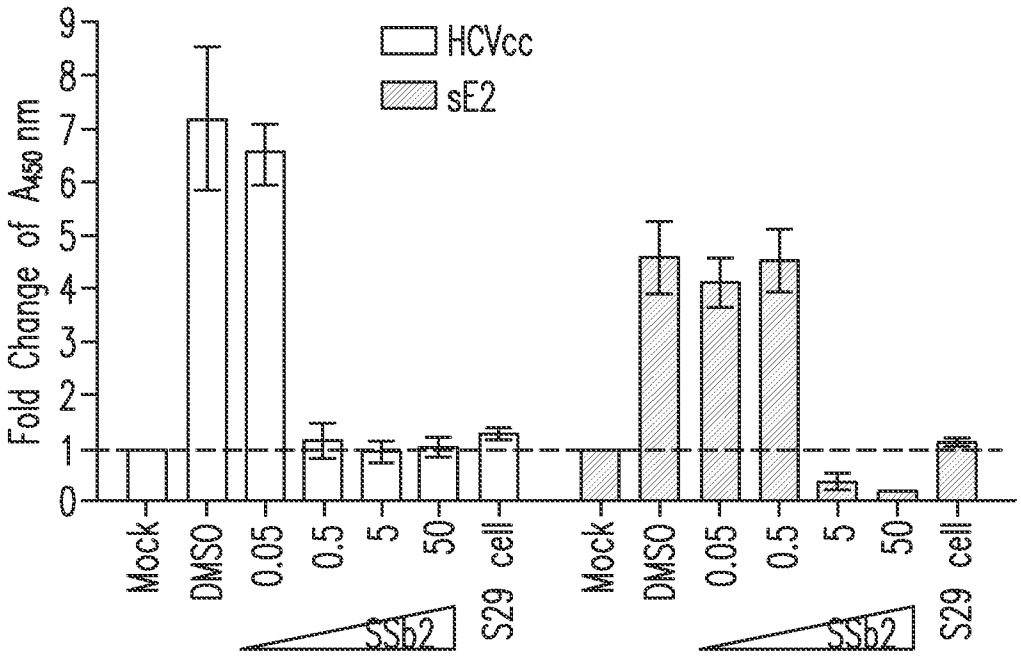
HCVcc
sE2
Fold Change of A450 nm
0
1
2
3
4
5
6
7
8
9
Mock
DMSO
0.05
0.5
5
50
S29 cell
SSb2
Mock
DMSO
0.05
0.5
5
50
S29 cell
SSb2

Fig. 5

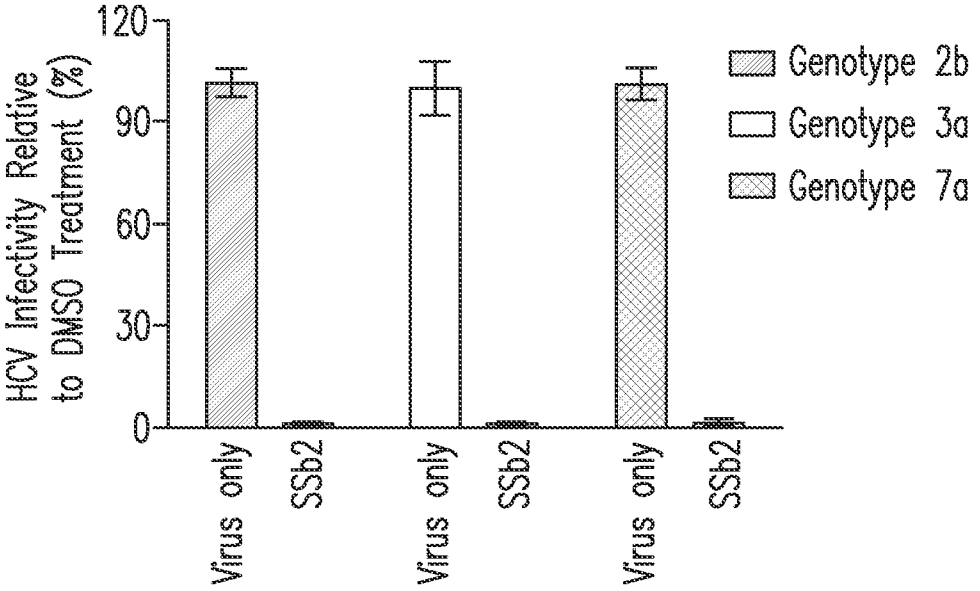
HCV Infectivity Relative to DMSO Treatment (%)
0
30
60
90
120
Genotype 2b
Genotype 3a
Genotype 7a
Virus only
SSb2
Virus only
SSb2
Virus only
SSb2

Fig. 6

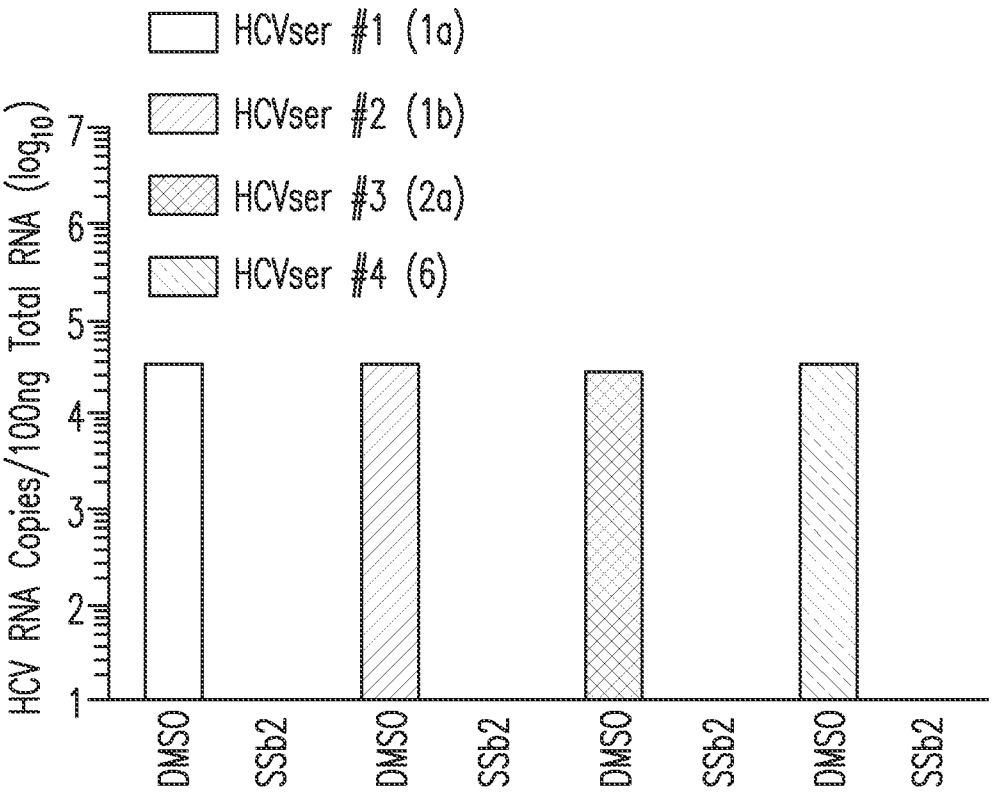
HCVser #1 (1a)
HCVser #2 (1b)
HCVser #3 (2a)
HCVser #4 (6)
HCV RNA Copies/100ng Total RNA (log10)
1
2
3
4
5
6
7
DMSO
SSb2
DMSO
SSb2
DMSO
SSb2
DMSO
SSb2

Fig. 7

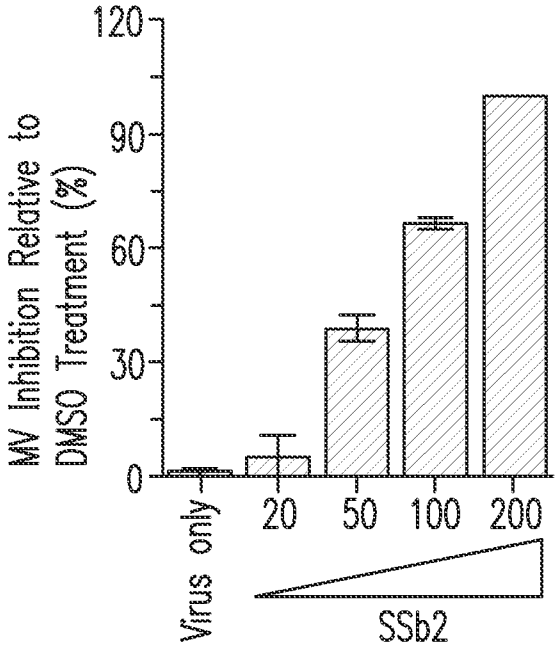
MV Inhibition Relative to DMSO Treatment (%)
120
90
60
30
0
Virus only
20
50
100
200
SSb2

Fig. 8(A)

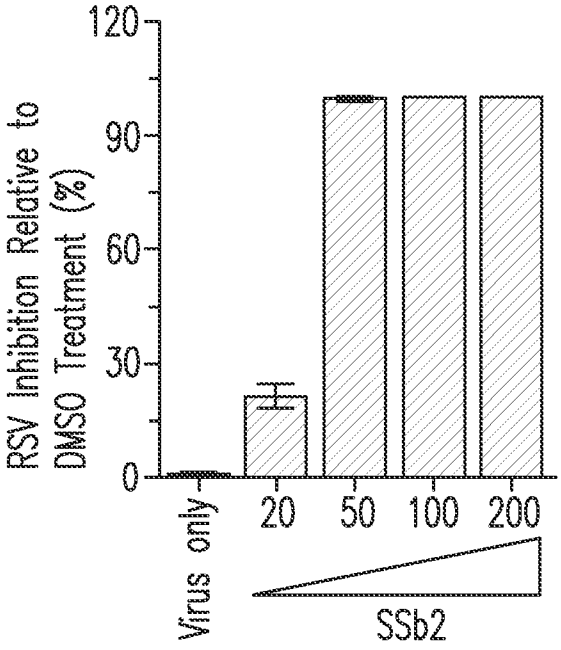
RSV Inhibition Relative to DMSO Treatment (%)
120
90
60
30
0
Virus only
20
50
100
200
SSb2

Fig. 8(B)

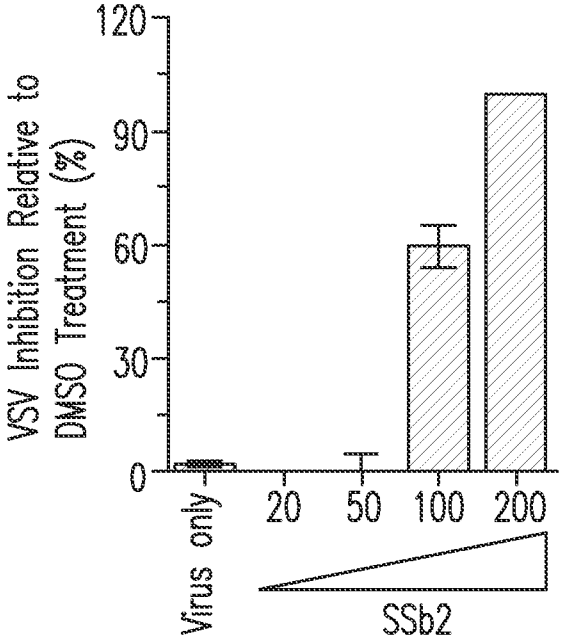
VSV Inhibition Relative to DMSO Treatment (%)
120
90
60
30
0
Virus only
20
50
100
200
SSb2

Fig. 8(C)

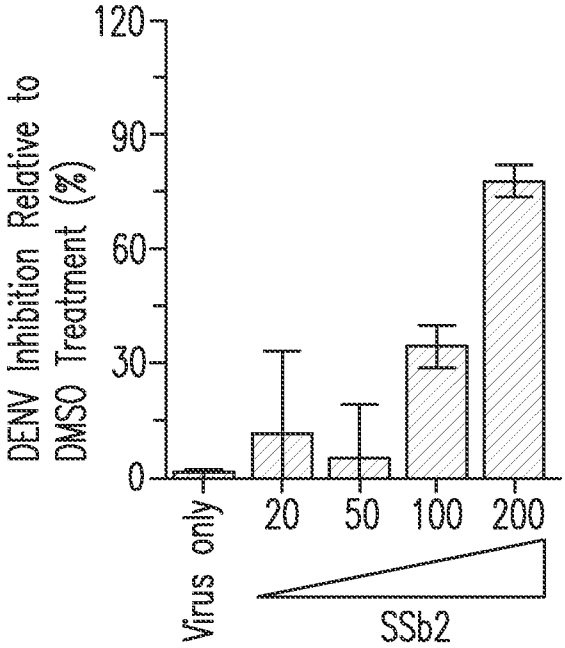
DENV Inhibition Relative to DMSO Treatment (%)
120
90
60
30
0
Virus only
20
50
100
200
SSb2

Fig. 8(D)

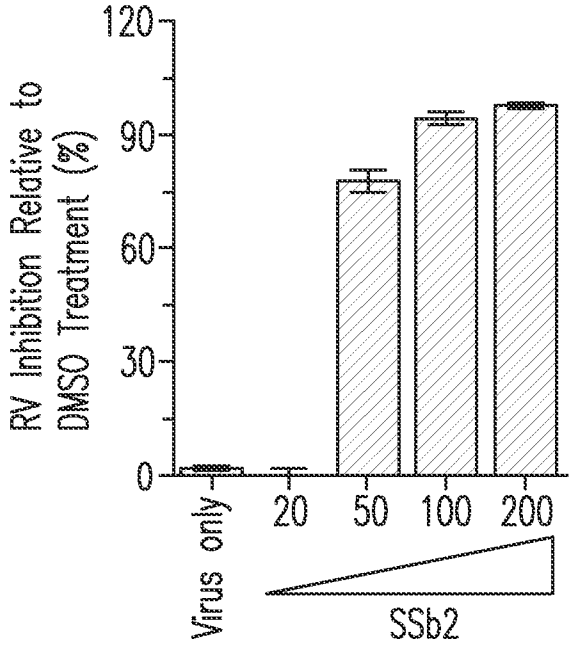
RV Inhibition Relative to DMSO Treatment (%)
120
90
60
30
0
Virus only
20
50
100
200
SSb2

Fig. 8(E)

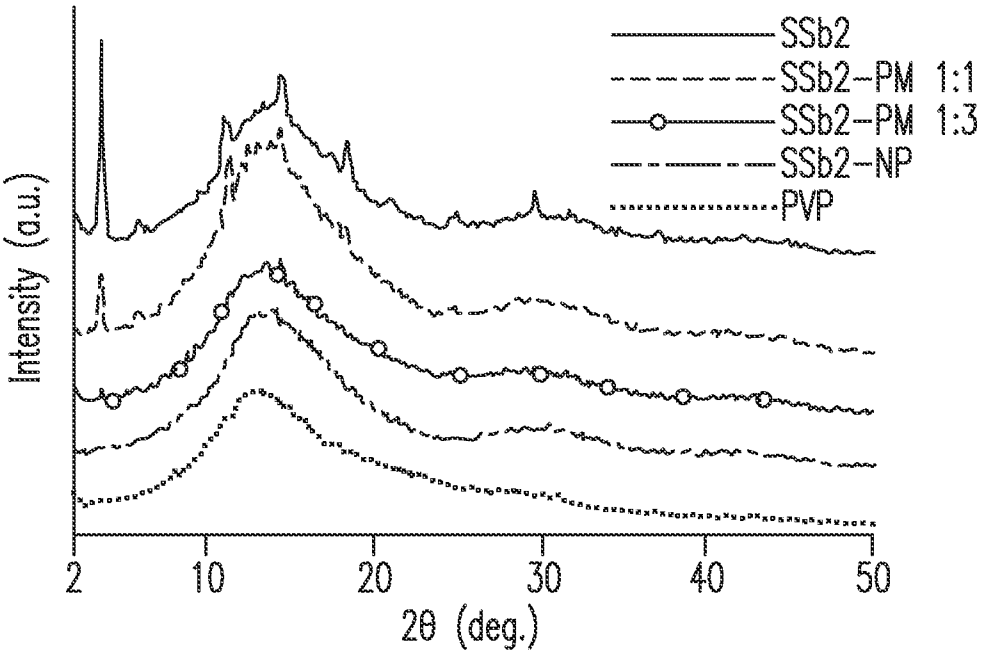
SSb2
SSb2-PM 1:1
SSb2-PM 1:3
SSb2-NP
PVP
Intensity (a.u.)
2
10
20
30
40
50
2θ (deg.)

Fig. 9(A)

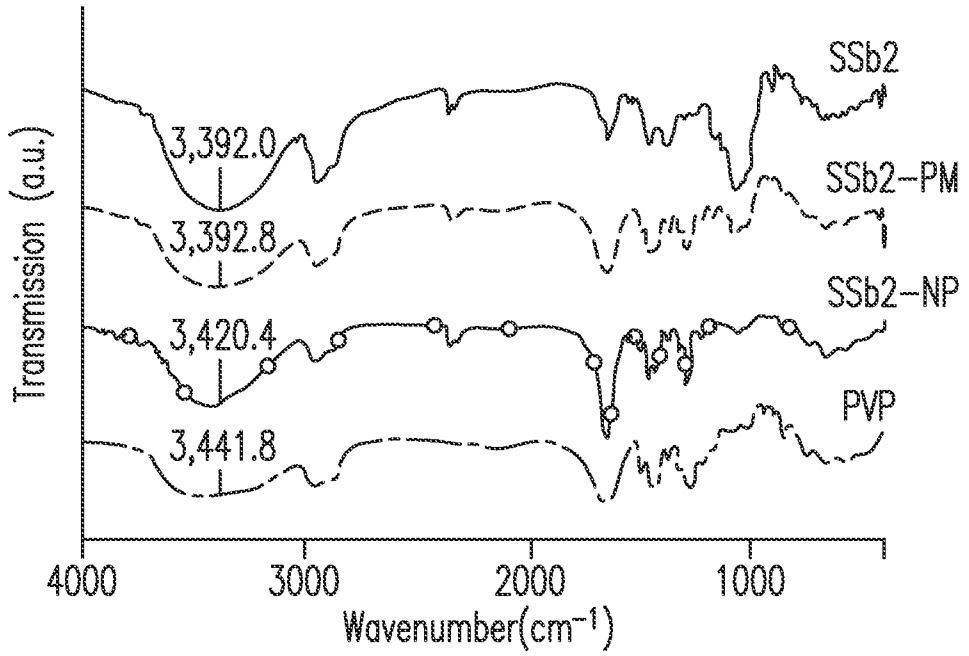
SSb2
SSb2-PM
SSb2-NP
PVP
3,392.0
3,392.8
3,420.4
3,441.8
Transmission (a.u.)
4000
3000
2000
1000
Wavenumber(cm⁻¹)

Fig. 9(B)

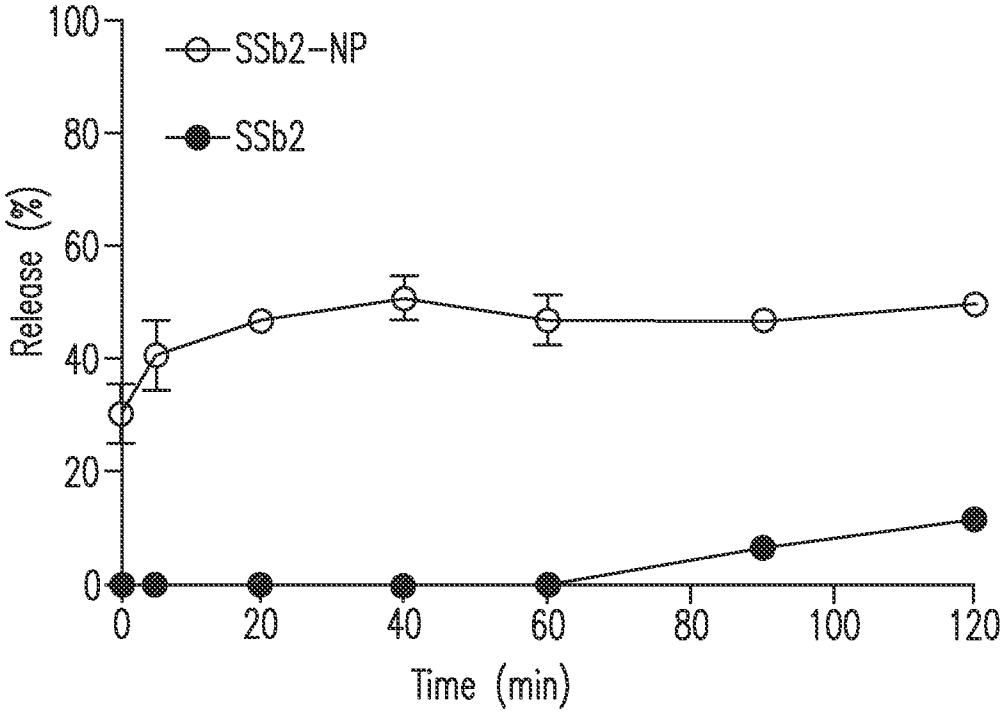
100
80
60
40
20
0
Release (%)
SSb2-NP
SSb2
0
20
40
60
80
100
120
Time (min)

Fig. 9(C)

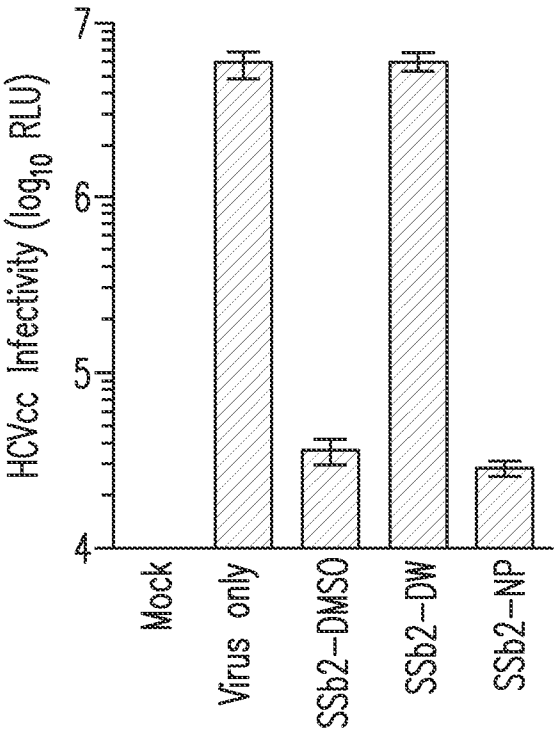

Fig. 10(A)

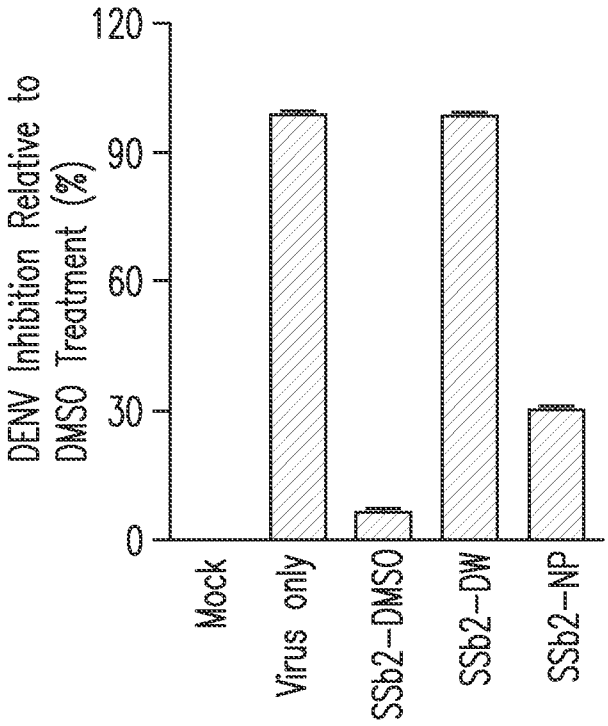

Fig. 10(B)

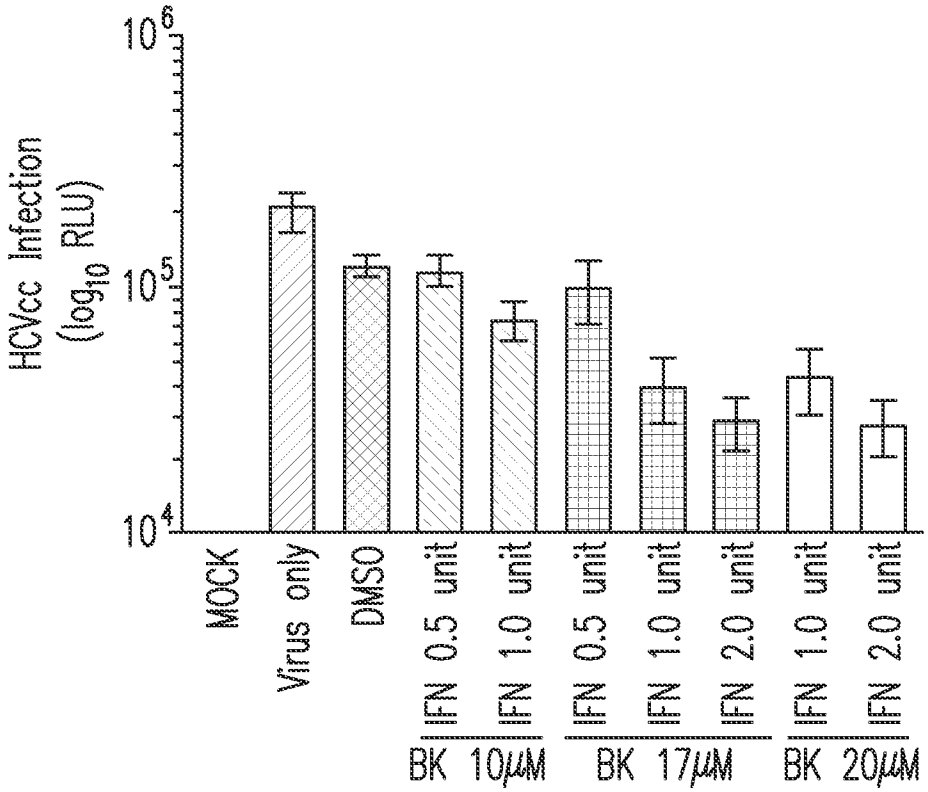

Fig. 11(A)

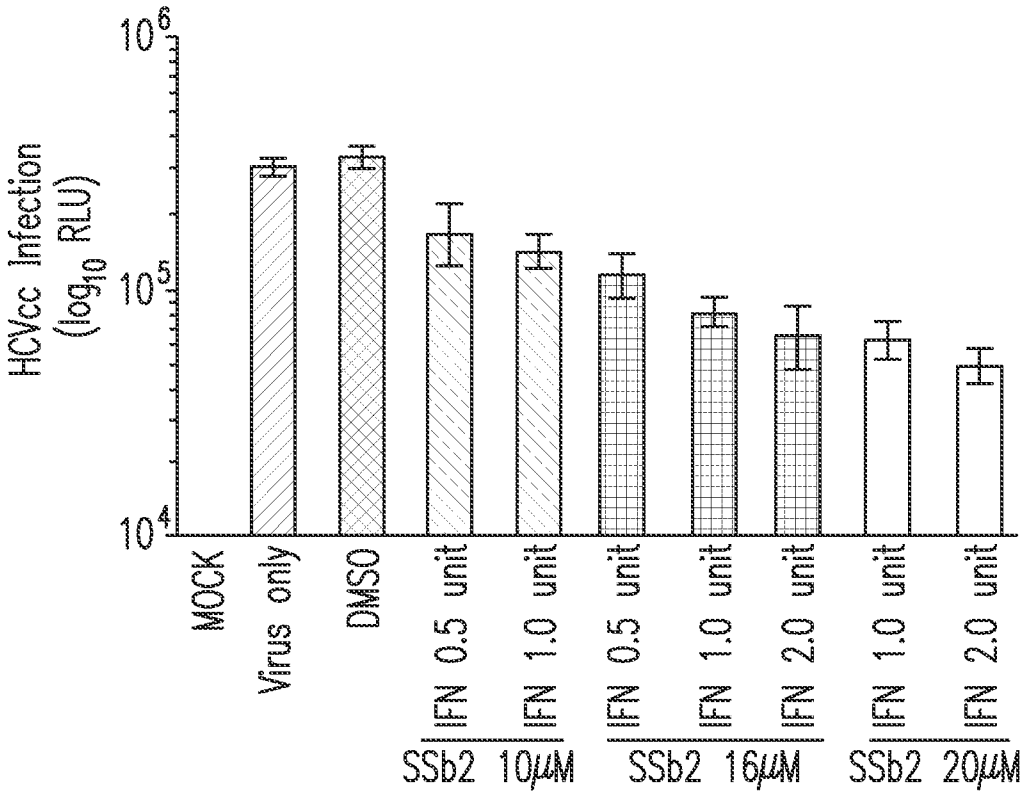

Fig. 11(B)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/040755

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61P 31/12 (2014.01)
CPC - A61K 36/00 (2014.09)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/194, 31/235; A61P 31/12, 31/14 (2014.01)
CPC - A61K 31/704, 36/00 (2014.09)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/33, 533

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2012/0165279 A1 (LEE et al) 28 June 2012 (28.06.2012) entire document	1, 3, 5 --- 2, 4, 6-8
X --- Y	US 7,105,186 B2 (ARNTZEN et al) 12 September 2006 (12.09.2006) entire document	9, 11, 12, 14 --- 10, 13, 15-17
X --- Y	US 2007/0059358 A1 (CHEN et al) 15 March 2007 (15.03.2007) entire document	18-21 --- 16, 17
Y	US 8,222,487 B2 (LIN et al) 17 July 2012 (17.07.2012) entire document	2, 4, 13
Y	US 2007/0185216 A1 (SNYDER et al) 09 August 2007 (09.08.2007) entire document	6, 7, 15
Y	US 2011/0268722 A1 (SIEGELIN et al) 03 November 2011 (03.11.2011) entire document	8
Y	WO 2005/007196 A2 (MACLACHLAN et al) 27 January 2005 (27.01.2005) entire document	10

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 September 2014	08 OCT 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774