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(54) Title: AN ANTIVIRAL PHARMACEUTICAL COMPOSITION

(57) Abstract: The present invention relates to a pharmaceutical composition for use in reducing dengue virus count and treating or preventing dengue virus infection, comprising a therapeutically or prophylactically effective amount of an aqueous extract or bioactive compound derived from *Scutellaria baicalensis*. Use of this pharmaceutical composition as an antiviral drug or prophylaxis for dengue virus infection is also disclosed in the present invention.

AN ANTIVIRAL PHARMACEUTICAL COMPOSITION

FIELD OF INVENTION

The present invention relates to an antiviral pharmaceutical composition. More particularly, the present invention provides an antiviral pharmaceutical composition derived from a natural medicinal plant, and use of this pharmaceutical composition for treating infection with any of the four distinct dengue virus serotypes.

BACKGROUND OF THE INVENTION

Dengue infection is a serious viral disease in the tropic and subtropic areas of the world. It accounts for at least 500,000 hospital admission annually and consumes massive hospital resources. This mosquito-borne disease is estimated to pose health threat to at least 2.5 billion people living in endemic regions of the tropic and subtropic regions. It is currently among one of the most rapidly spreading mosquito-borne diseases. Dengue virus (DENV) is an enveloped virus and a member of Flaviviridae family with four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Currently, there is no specific anti-dengue medication available and the treatment procedure is merely supportive. In addition, there is no approved dengue vaccine available. Therefore, there is an urgent need to find an effective antiviral for this disease.

Dengue can manifests as a self-limiting mild nonspecific fever to asymptomatic disease. However, the disease can also manifests as severe disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which can lead to death. The severity of the disease has been linked to a number of factors. Among the factors found important include the number virus in the blood during the viremic phase of the disease. It is therefore desired to find therapeutic remedy to reduce the virus count in

addition to inhibit virus replication in cells.

Among the different types of compounds and chemicals which have been studied for their antiviral properties include those from plants, plant extract in particular has been the natural form of remedy used in many communities as traditional medication in folk medicine. Natural nature-derived remedy is believed to have low side effects and the combination of the various plant constituents act to enhance potency of the effective compounds without exaggerating the potential cytotoxic effects.

Traditional Chinese medicinal herbs have been widely used for the treatment of various diseases. A number of these herbs have been traditionally used for the treatment of many ailments associated to viral infections. *Scutellaria baicalensis* is one of the most widely used medicinal plants, and is officially listed in the Chinese Pharmacopoeia. Extract of its roots have been used for the treatment of inflammation, cancer, reducing the total cholesterol level and decreasing blood pressure. The roots of this plant contain a variety of bioactive chemical constituents, including the different flavonoids such as baicalein, baicalin, scutellarein, apigenin, hispidulin, luteolin and wogonin.

A U.S. Patent No. US2002168426 has disclosed a composition comprising extracts of flos lonicerae, fructus forsythiae and radix scutellaria (root of the *Scutellaria baicalensis*) that is effective in inhibiting influenza virus, parainfluenza virus, herpes I virus and herpes II virus, but not viruses from the Flaviviridae family.

A PCT publication No. WO2005044281 also discloses a naturally occurring compound, baicalin, which is extracted from *Scutellaria baicalensis* Georgi, as a treatment for severe acute respiratory syndrome (SARS) virus infection, particularly in human. This invention also relates to a therapeutic method, using pharmaceutical compositions comprising baicalin compounds for the treatment, amelioration, management or prevention of SARS and other diseases associated with members of

the order Nidovirales of the Coronaviradae family, which are also different from that of the Flaviviridae family.

As revealed by the prior art, *Scutellaria baicalensis* may be a potential source to be used in the development of antiviral medication. However, none of the existing technologies has disclosed or suggested its use in the treatment of flavivirus-associated disease exemplified by dengue or in the development of anti-dengue drugs.

SUMMARY OF INVENTION

The primary object of the present invention is to provide a pharmaceutical composition containing extract of *Scutellaria baicalensis* for use in reducing virus count, the treatment, amelioration, management or prevention of dengue disease caused by any of the dengue virus serotypes: DENV-1, DENV-2, DENV-3 and DENV-4.

Another object of the present invention is to exploit the antiviral property of *Scutellaria baicalensis* and to develop an antiviral drug or pharmaceutical composition using this plant species as an immediate alternative and evidence-based product to combat dengue disease.

Yet another object of the present invention is to provide a method for reducing dengue virus count and treating dengue virus infection using a pharmaceutical composition containing therapeutic derivatives of *Scutellaria baicalensis.*

Still another object of the present invention is to diversify the medicinal uses of the *Scutellaria baicalensis* and provide another avenue for the commercialization of this useful therapeutic plant.

At least one of the preceding objects is met, in whole or in part, by the present invention, in which one of the embodiments of the present invention describes a pharmaceutical composition for use in reducing virus count and treating or preventing dengue virus infection, comprising a therapeutically or prophylactically effective amount of an aqueous extract or bioactive compound derived from *Scutellaria baicalensis*. Preferably, the pharmaceutical composition is having antiviral activity against dengue virus serotype DENV-1, DENV-2, DENV-3 and DENV-4.

Another embodiment of the present invention discloses the use of a pharmaceutical composition comprising a therapeutically or prophylactically effective amount of an aqueous extract or bioactive compound from *Scutellaria baicalensis* for reducing virus count and treating or preventing dengue virus infection, wherein the dengue virus serotype includes DENV-1, DENV-2, DENV-3 and DENV-4.

Still another embodiment of the present invention is a method for reducing virus count and treating or preventing dengue virus infection comprising the step of administering to a subject in need thereof a pharmaceutical composition having a therapeutically or prophylactically effective amount of an aqueous extract or bioactive compound derived from *Scutellaria baicalensis*.

In one of the preferred embodiments of the present invention, the subject in need is a human or a mammal.

According to another preferred embodiment of the present invention, the therapeutically effective amount is in a concentration range of 60 μg mL^{-1} to 900 μg mL^{-1}, whereas the prophylactically effective amount is in a concentration range of 270 μg mL^{-1} to 900 μg mL^{-1}.

Still another preferred embodiment of the present invention discloses that the aqueous extract is derived from root of *Scutellaria baicalensis*. Preferably, the extract is

containing the major bioactive compound from *Scutellaria baicalensis*, baicalein.

Yet another preferred embodiment of the present invention discloses that the dengue virus infection and virus count to be treated or prevented is related to the dengue virus serotypes of DENV-1, DENV-2, DENV-3 and DENV-4.

One skilled in the art will readily appreciate that the present invention is well adapted to perform the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The embodiments described herein are not intended as limitations on the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of facilitating an understanding of the invention, there is illustrated in the accompanying drawing the preferred embodiments from an inspection of which when considered in connection with the following description, the invention, its construction and operation and many of its advantages would be readily understood and appreciated.

Figure 1 shows the toxicity of *Scutellaria baicalensis* extract, labelled as AHPE-XA-09, against Vero cells, in which the data are presented as percentage of cell viability from a triplicate assay.

Figure 2 exhibits the prophylactic effects of *Scutellaria baicalensis* extract against DENVs, in which prophylactic activity of the plant extract on DENVs *in vitro* replication performed by the foci forming unit reduction assay (FFURA) is shown in (A); and the respective dengue virus count reflected by DENVs RNA reduction levels quantified by qRT-PCR for all 4 DENV serotypes are shown in (B).

Figure 3 exhibits the anti-adsorption activity of *Scutellaria baicalensis* extract against DENVs, in which the effect of plant extract on DENVs adsorption and attachment to the Vero cells obtained by FFURA is shown in (A); and the respective dengue virus count reflected by DENVs RNA production levels quantified by qRT-PCR for all 4 DENV serotypes are shown in (B).

Figure 4 exhibits the effects of *Scutellaria baicalensis* extract against DENVs intracellular replication, in which the antiviral activity of the plant extract on DENVs after adsorption to the Vero cells obtained by FFURA are shown in (A); and respective dengue virus count reflected by DENVs RNA production levels quantified by qRT-PCR for all 4 DENV serotypes are shown in (B).

Figure 5 exhibits the direct virucidal activity of *Scutellaria baicalensis* extract against DENVs, in which the direct virucidal effects of the plant extract on DENVs obtained by FFURA are shown in (A); and dengue virus count reflected by RNA production levels quantified by qRT-PCR for all 4 DENV serotypes are shown in (B).

Figure 6 shows the standard curve of the pure baicalein and LC/MS/MS chromatogram of the *Scutellaria baicalensis* extract, AHPE-XA-09, in which the pure baicalein standard linear regression curve was plotted from the area under the curve based on the different intensity height determined from the chromatogram of the different concentrations of pure baicalein. The intensity of baicalein in the AHPE-XA-09 was extraploted from the plot (indicated by a dot line) as shown in (A); whereas the LC/MS/MS chromatogram in (B) shows the intensity and retention time of baicalein in the AHPE-XA-09.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an antiviral pharmaceutical composition. More particularly, the present invention provides an antiviral pharmaceutical composition derived from a natural medicinal plant, and use of this pharmaceutical composition for treating infection with any of the four distinct dengue virus serotypes.

Hereinafter, the invention shall be described according to the preferred embodiments of the present invention and by referring to the accompanying description and drawings. However, it is to be understood that limiting the description to the preferred embodiments of the invention and to the drawings is merely to facilitate discussion of the present invention and it is envisioned that those skilled in the art may devise various modifications without departing from the scope of the appended claim.

The present invention discloses a pharmaceutical composition for reducing dengue virus count and use in treating or preventing dengue virus infection. As set forth in the preceding embodiment, this pharmaceutical composition comprises a therapeutically or prophylactically effective amount of an aqueous extract derived from *Scutellaria baicalensis*, which can contain bioactive compounds, their constituents or the combination thereof. Preferably, the aqueous extract is derived from root of *Scutellaria baicalensis*, which is also known as radix scutellaria. More preferably, the aqueous extract can be obtained from the water extract of the dried ground root of *Scutellaria baicalensis*. An example of preparing the aqueous extract of this pharmaceutical composition is further detailed in Example 1.

This extract can be stored in its aqueous form before being used or further processed into various types of consumable products. Apart from being formulated into an antiviral drug, this pharmaceutical composition can also be incorporated into health

supplements or health-promoting foods or beverages.

There are a number of bioactive chemical constituents and compounds which can be found in the root extract of *Scutellaria baicalensis*, including a few types of flavonoids. One of the bioactive flavonoids found from *Scutellaria baicalensis* that is having antiviral property is baicalein. Pure baicalein can also be extracted from the root of *Scutellaria baicalensis* in order to provide the pharmaceutical composition of the present invention. Identification and quantitation of the active compound, baicalein, can be performed by liquid-chromatography-mass spectrometry-mass spectrometry (LC/MS/MS).

Preferably, the pharmaceutical composition of *Scutellaria baicalensis* extract containing the major compound baicalein can be formulated as an antiviral drug or a prophylactic agent. According to yet another preferred embodiment of the present invention, the therapeutically effective amount of the composition is in a concentration range of 60 $\mu g\ mL^{-1}$ to 900 $\mu g\ mL^{-1}$, when it is used as antiviral drug that can act both intracellularly and extracellularly. More preferably, the concentration range applied is 60 $\mu g\ mL^{-1}$ to 375 $\mu g\ mL^{-1}$, as the composition at a concentration of less than 375 $\mu g\ mL^{-1}$ is generally non-cytotoxic. Whilst, when the composition is used as prophylactic agent, higher concentration shall be applied. Preferably, the prophylactically effective amount is in a concentration range of 270 $\mu g\ mL^{-1}$ to 900 $\mu g\ mL^{-1}$, more preferably, 270 $\mu g\ mL^{-1}$ to 375 $\mu g\ mL^{-1}$. A cytotoxicity test can be performed using selected cell line such as Vero cell, in order to determine the toxicity of the plant extract in the pharmaceutical composition of the present invention. An example of the cytotoxicity test is further detailed in Example 2.

Another further embodiment of the present invention discloses the use of a pharmaceutical composition comprising a therapeutically or prophylactically effective amount of an aqueous extract of *Scutellaria baicalensis*, which contains the major compound baicalein, for reducing virus count, treating or preventing dengue virus

infection. In accordance with another preferred embodiment of the present invention, the pharmaceutical composition is proven to have antiviral activity against the four dengue virus serotypes, DENV-1, DENV-2, DENV-3 and DENV-4.

In accordance with another preferred embodiment of the present invention, a method for reducing dengue virus count, treating or preventing dengue virus infection is also disclosed. This method comprises the step of administering to a subject in need thereof a pharmaceutical composition having a therapeutically or prophylactically effective amount of an aqueous extract of *Scutellaria baicalensis*, which contains the major compound baicalein.

According to one of the preferred embodiments of the present invention, the subject in need is a human or a mammal. Preferably, the pharmaceutical composition can be orally administered or administered by suppository route into the human or the mammal's body as a therapeutic or prophylactic drug to reduce virus count, treat or to prevent dengue virus infection.

A series of evaluation tests can be conducted in order to evaluate the effectiveness of the pharmaceutical composition. FFURA together with qRT-PCR can be used to evaluate the *in vitro* anti-dengue virus activity of the *Scutellaria baicalensis* water extract, as further detailed in Examples 3 to 8. The extract can be evaluated for its virus count reduction properties and potential antiviral activities, including its prophylactic effects, its activity against virus adsorption to the cells, its activity added after virus adsorption to the cells, and its activity directly to the viral suspension to evaluate its extracellular virucidal effect. Besides, identification and quantitation of the active compound, baicalein, in the *Scutellaria baicalensis* water extract can also be performed. Figure 6 shows the intensity, which reflects the concentration of baicalein in the extract.

The water extract of *Scutellaria baicalensis* exhibits a dose-dependent inhibition

effects against all 4 DENV serotypes replication in Vero cells. An example of the half maximal inhibition concentration (IC_{50}) values is presented in Table 2. It has been demonstrated that the plant extract shows antiviral activity against the different serotypes of DENV in all stages of *in vitro* replication but the most effective effects can be observed when the extract is evaluated during the time of virus adsorption.

As revealed by the experimental data, the pharmaceutical composition of the present invention, which contains the water extract of the roots of *Scutellaria baicalensis*, a Chinese medicinal herb, possesses anti-DENV activities. The reduction in virus count and anti-DENV activities can be notable in all stages, when cells are treated after virus adsorption, during the virus adsorption or even prior to the virus infection. The tested extract exerted significant direct virucidal effects on extracellular free DENVs particles which is a notable ability for anti-dengue candidate to neutralize free viruses. One of the possible mechanisms for the *Scutellaria baicalensis* extract extracellular and intracellular activities against DENVs may be attributed to its ability to bind or to inactivate important structural, non-structural protein(s), or both of DENVs. Such inhibitory mechanism has also been reported for several known plant-derived flavonoids such as pinostrobin against dengue virus NS2B/NS3 protease. Furthermore, activity of other plant-derived flavonoids such as wogonin and baicalein can also be demonstrated against cellular DNA and also cellular RNA beside their proved ability to inactivate the cellular RNA polymerases. Therefore, it may be a probable mechanism for the tested plant extract and its constituents to inhibit the dengue virus replication through interference with RNA polymerase of DENVs or bind to the viral RNA, or the combination thereof. The experimental data shown by qRT-PCR supports the findings from viral foci reduction but the inhibition of RNA production is more significant than inhibition of DENVs foci presentation suggesting a mechanism affecting the molecular pathways in dengue virus replication.

The present disclosure includes as contained in the appended claims, as well as that of the foregoing description. Although this invention has been described in its preferred

form with a degree of particularity, it is understood that the present disclosure of the preferred form has been made only by way of example and that numerous changes in the details of construction and the combination and arrangements of parts may be resorted to without departing from the scope of the invention.

EXAMPLE

Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention, which is limited only by the claims.

Example 1 Preparation of plant material, cells and virus

The plant of *S. baicalensis* was commercially obtained. The water soluble extract produced can be labelled as AHPE-XA-09. The water extract derived using the dried roots of *Scutellaria baicalensis* was prepared in sterile deionized water as a stock solution and stored at -20°C. The stock solution of the plant extract was sterilized by a syringe filter with 0.2 micron pore size (Millipore, MA, USA) right before the experiments. Eagle's Minimum Essential Medium (EMEM) (Gibco, NY, USA) was used as a diluent to prepare the different concentrations of the plant extract at the time of experiments. C6/36 mosquito cell line as a good host for dengue virus propagation, and Vero (African green monkey kidney) cell line for antiviral activity evaluation were used in this study. Both cells were maintained and propagated in EMEM (Gibco, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, NY, USA). Cultured C6/36 and Vero cells were incubated at 28°C and 37°C, respectively in the presence of 5% CO_2. At the time of virus inoculation and antiviral assay test, FBS concentration was reduced to 2%. There were 4 different clinical isolates of dengue virus representing 4 distinct serotypes of dengue virus (DENV-1, 2, 3 and 4) used in this study. These four serotypes were propagated in C6/36 cell line and harvested after CPE presentation on day seven post infection. After titration, viral stock was stored at

-70°C. The *Scutellaria baicalensis* extract was determined using mass spectrometry to contain at least 1% (1.03 µg/gm dried extract) baicalein.

Example 2 ***In vitro*** **cytotoxicity assay**

In order to determine the toxicity of the plant extract against Vero cells, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, the confluent Vero cells in 96-well microplate were treated by different concentrations of the plant extract in triplicates. The treated cells were incubated for four days which was same to the time period for antiviral assay tests at 37°C followed by the addition of 15 µl of MTT solution to each well. The microplate was incubated at 37°C for more than 4 hours. Then, 100 µl of the solublization/stopping solution was added to each well. The optical density (OD) of all wells including non-treated cells were read using 570 nm wavelength filter by plate reader (TECAN, Mannendorf, Switzerland). Cytotoxicity of the plant extract was calculated using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA). The toxicity of *Scutellaria baicalensis* extract against Vero cells are shown in Figure 1. As revealed by the data, the half maximal cytotoxic concentration (CC_{50}) value for the cold water extract of the *Scutellaria baicalensis* root was 912.6 µg mL^{-1}. Over 70% of the cells were viable after 4 days of treatment with the extract at concentration less than 375 µg mL^{-1}.

Example 3 **Prophylactic activity assay**

Effect of prophylactic treatment of cells with the plant extract prior to DENV infection was examined. A confluent monolayer of Vero cell line was treated with the different concentrations of the extract for 3 h before DENV inoculation. The treatment medium was aspirated after 5 h and cells were washed twice with sterile PBS and then infected with 200 FFU of each serotypes of DENV separately. The microplate was kept at 37°C for 1 h to allow virus adsorption. After virus adsorption, the infected cell monolayer was rinsed twice with sterile PBS and incubated in 2% FBS containing EMEM with 1.5% CMC and the different concentration of the compounds. The plates were incubated at 37°C for 4 days in the presence of 5% CO_2. Reduction in infectious

virus count was determined by counting the number of virus foci formed. Viral foci were stained and counted as described earlier. The prophylatic effects of *Scutellaria baicalensis* extract against DENVs are shown in Figure 2. It is effective at a concentration of approximately 260 μg mL^{-1} to 370 μg mL^{-1}.

Example 4 Quantitative real-time polymerase chain reaction (qRT-PCR)

In order to evaluate the effect of the plant extract on virus count and cellular replication, DENV RNA production was estimated by a quantitative RT-PCR method with different primer sets for 4 different DENV serotypes, as shown in Table 1. This experiment was done in order to confirm the foci forming assay results of the prophylactic activity studied as well as other antiviral studies.

Table 1

Virus Serotype	Primer Sequence
DENV-1	Forward: 5' CAA TAT GCT GAA ACG CGC GAG AAA 3' Reverse: 5' GCT CCA TTC TTC TTG AAT GA 3'
DENV-2	Forward: 5' CAA TAT GCT GAA ACG CGA GAG AAA 3' Reverse: 5' AAG ACA TTG ATG GCT TTT GA 3'
DENV-3	Forward: 5' CAA TAT GCT GAA ACG CGT GAG AAA 3' Reverse: 5' GAA GGT TCC CCA TCT AGC CA 3'
DENV-4	Forward: 5' GAA GTG AAA ACA TGT CTG TGG CCC A 3' Reverse: 5' TTC ACA GCA CAA TTA CCG CCA G 3'

As shown in Figure 2(B), 750 μg mL^{-1} of the plant extract decreased all DENVs RNA production by more than 70% compared to the untreated but dengue virus infected cells. These observations may be related to the plant extract uptake by the cells during pre-treatment and their probable activity against DENVs intracellular replication and/or could be related to the masking of the viral receptors due to binding of bioactive constituents and compounds such as different flavonoids within the plant extract. Therefore, it can be further improved including but not limited to optimizing

of exposure time and dose for effective DENVs pre-treatment.

Example 5 Anti-adsorption activity assay

Effect of the plant extract against DENVs adsorption and attachment to the Vero cells was determined by adding the different concentrations of the extract to the cells at the same time of 200 FFU DENV innoculation to the cells. The Cells were washed with PBS after 1 hour incubation at 37°C for virus adsorption and overlaid with 2% FBS containing EMEM with 1.5% CMC. The plates were kept at 37°C for 4 days with 5% CO_2. Viral foci were stained and counted as mentioned above. A qRT-PCR was also conducted using the protocol as set forth in Example 4 in order to confirm the foci forming assay results. Figure 3 shows the anti-adsorption activity of *Scutellaria baicalensis* extract against DENVs. The most effective antiviral activity was related to the anti-adsorption activity against DENVs with IC_{50} values ranging from 56.02 to 77.41 $\mu g\ mL^{-1}$ for different serotypes of DENV, as shown in Table 2 and Figure 3(A). The SI value for the plant extract in this study were ranging from 11.7 to 16.2 (DENV-1, SI= 13.1; DENV-2, SI= 16.2; DENV-3, SI= 11.7 and DENV-4 SI= 12.4). Production of DENVs viral RNA decreased ranging from 68 to 82% in the presence of 175 $\mu g\ mL^{-1}$ of the extract during the viral adsorption period, as revealed by Figure 3(B). This could be related to the direct virucidal activity of the plant extract which decrease the number of infectious DENVs particles during the adsorption time and/or binding to the DENVs cellular receptors. However, interfering with the virus attachment to the cells through the binding to cellular receptors and/or virus ligand could be another explanation for the anti-adsorption activity of the extract.

Example 6 Antiviral activity assay

In order to evaluate the effect of the plant extract against intracellular replication of DENVs, Vero cells were plated into 24 wells cell culture microplate. After attaining 80% confluency, virus inoculum consisting of 200 FFU of each DENV serotype was added to each well separately and viruses were allowed to absorb to the cells for 2 h at 37°C. Unabsorbed viruses were removed by rinsing cells with sterile PBS twice.

Different concentrations of the plant extract were mixed with 1.5% carboxymethylcellulose (CMC) containing cell-growth medium supplemented with 2% FBS and the plates were incubated at 37°C for 4 days. Then the DENV foci were visualized. Number of foci was expressed as foci forming unit (FFU). Reduction in infectious virus count and antiviral effects of plant extract was measured by calculating the percentage of foci reduction (% RF) compared against the controls maintained in parallel using the following formula; RF(%) = (C-T) × 100/C, where, C is the mean of the number of foci from triplicates without plant extract added, and T is the mean of the number of foci from triplicates of each treatment with the plant extract. A qRT-PCR was also conducted using the protocol as set forth in Example 4 in order to confirm the foci forming assay results. The reduction in virus count and antiviral effects of *Scutellaria baicalensis* against DENVs intracellular replication are shown in Figure 4. Intracellular anti-dengue activities of the plant extract were also significant based on the results in Table 2, Figure 4(A) and SI values (DENV-1, SI= 10.5; DENV-2, SI= 9.7; DENV-3, SI= 10.2 and DENV-4 SI= 9.6). These findings were supported by the results from q-RT-PCR, which showed a substantial decrease of viral RNA yield by more than 50% at 93 $\mu g\ mL^{-1}$ of the extract used, and a reduction of viral RNA by at least 50% and 750 $\mu g\ mL^{-1}$ of the extract used as compared to the non-treated cells, as shown in Figure 4(B).

Example 7 Extracellular virucidal activity

The potential direct virucidal effect of the extract on DENVs was evaluated by treating the viral suspension with 200 FFU of DENVs with increasing concentrations of the plant extract for 2 h at 37°C. The confluent Vero cells in 24 wells cell culture microplate were infected with treated viral suspensions. After 1 h adsorption at 37°C, cells were washed twice with PBS and overlaid with 1.5% CMC containing cell culture medium. The microplate was incubated for 3 days in a humidified 37°C incubator in the presence of 5% CO_2. Viral foci were visualized as set forth in the preceding description. A qRT-PCR was also conducted using the protocol as set forth in Example 4 in order to confirm the foci forming assay results and to determine the

number of virus. Results from direct virucidal activity of *Scutellaria baicalensis* were shown in Table 2. It was demonstrated that water extract of this plant exhibited a potent extracellular anti-DENVs activity with IC_{50} values ranging from 74.33 to 95.83 $\mu g\ mL^{-1}$ for different serotypes of DENV. Similarly, as illustrated in Figure 5(B), qRT-PCR analysis showed that 187 µg/mL of the extract decreased the DENVs RNA production ranging from 77% to 83% as compared to non-treated virus inoculum.

Example 8 Statistical analysis

In order to determine the CC_{50} and IC_{50} as the main parameters for this study, Graph Pad Prism for Windows, version 5 (Graph Pad Software Inc., San Diego, CA, 2005) was used. Selectivity Index value (SI) was determined as the ratio of CC_{50}/IC_{50} of the plant extract. Table 2 shows the IC_{50} values of the *Scutellaria baicalensis* extract against four different stages of DENVs replication cycle. Values were calculated by Graph Pad Prism Version 5 software (Graph Pad Software Inc., San Diego, CA.) through the results from FFURA.

Table 2

Antiviral Activity / Virus Serotype	Prophylactic Activity IC_{50} ($\mu g\ mL^{-1}$)	Anti-Adsorption Activity IC_{50} ($\mu g\ mL^{-1}$)	After Adsorption Activity IC_{50} ($\mu g\ mL^{-1}$)	Direct Virucidal Activity IC_{50} ($\mu g\ mL^{-1}$)
DENV-1	269.9	69.14	86.59	91.93
DENV-2	369.8	56.02	93.66	95.83
DENV-3	330.3	77.41	89.39	93.68
DENV-4	345.8	73.59	95.19	74.33

CLAIMS

1. A pharmaceutical composition for use in reducing dengue virus count, treating or preventing dengue virus infection, comprising a therapeutically or prophylactically effective amount of an aqueous extract derived from *Scutellaria baicalensis*.

2. A composition according to claim 1, wherein the therapeutically effective amount is in a concentration range of 60 $\mu g\ mL^{-1}$ to 900 $\mu g\ mL^{-1}$.

3. A composition according to claim 1, wherein the prophylactically effective amount is in a concentration range of 270 $\mu g\ mL^{-1}$ to 900 $\mu g\ mL^{-1}$.

4. A composition according to claim 1, wherein the aqueous extract is derived from the root of *Scutellaria baicalensis*.

5. A composition according to claim 1, wherein the aqueous extract contains bioactive compounds, constituents or a combination thereof.

6. A composition according to claim 5, wherein the bioactive constituents of the extract include baicalein.

7. A composition according to claim 1, wherein serotypes of the dengue virus includes DENV-1, DENV-2, DENV-3 and DENV-4.

8. Use of a pharmaceutical composition comprising a therapeutically or prophylactically effective amount of an aqueous extract derived from *Scutellaria baicalensis* for reducing dengue virus count, treating or preventing dengue virus infection.

9. Use of a pharmaceutical composition according to claim 8, wherein the therapeutically effective amount is in a concentration range of 60 μg mL^{-1} to 900 μg mL^{-1}.

10. Use of a pharmaceutical composition according to claim 8, wherein the prophylactically effective amount is in a concentration range of 270 μg mL^{-1} to 900 μg mL^{-1}.

11. Use of a pharmaceutical composition according to claim 8, wherein the aqueous extract is derived from the root of *Scutellaria baicalensis*.

12. Use of a pharmaceutical composition according to claim 8, wherein the aqueous extract contains bioactive compounds, constituents or a combination thereof.

13. Use of a pharmaceutical composition according to claim 12, wherein the bioactive constituents of the extract include baicalein.

14. Use of a pharmaceutical composition according to claim 8, wherein the dengue virus serotype includes DENV-1, DENV-2, DENV-3 and DENV-4.

15. Use of a pharmaceutical composition according to claim 8, wherein the pharmaceutical composition is orally or suppository administered into human or mammal's body as a therapeutic or prophylactic drug.

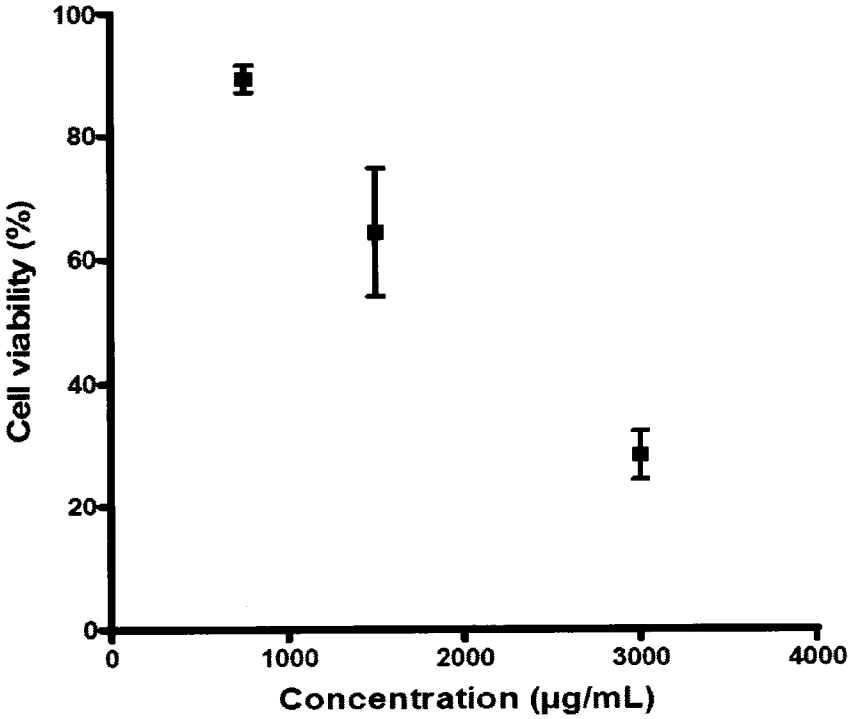


Figure 1

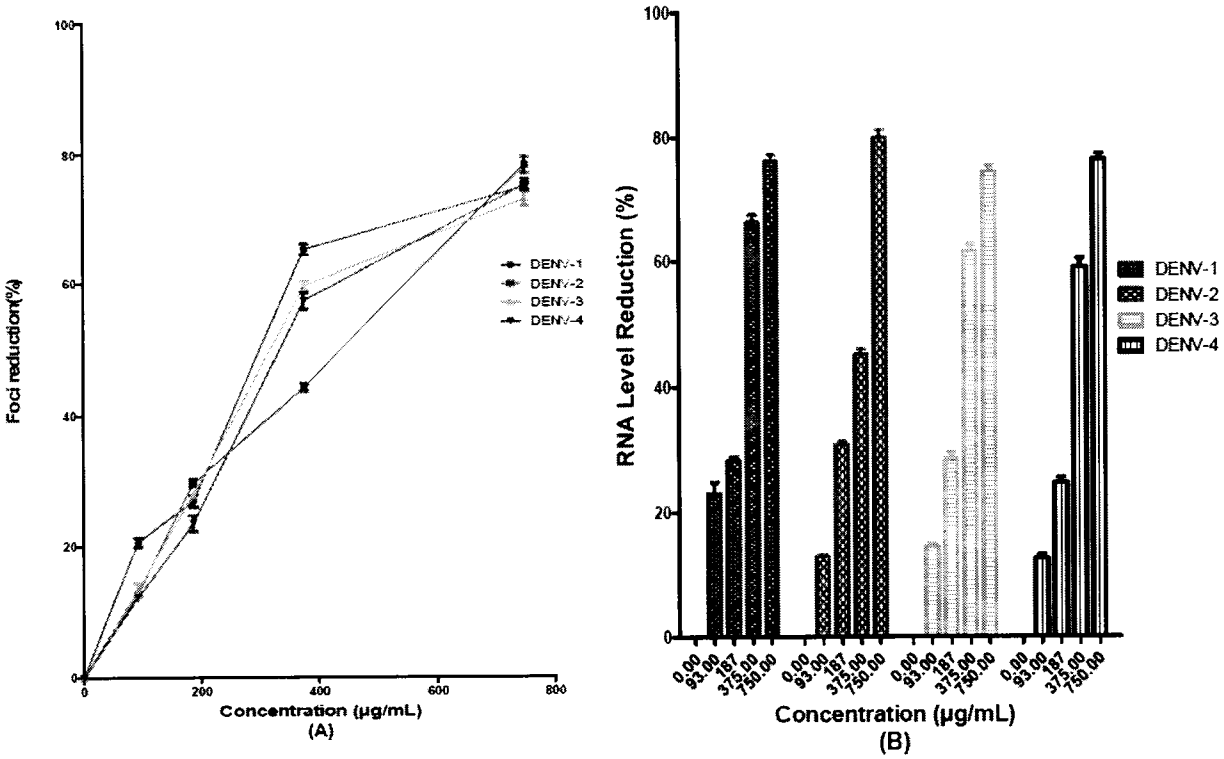


Figure 2

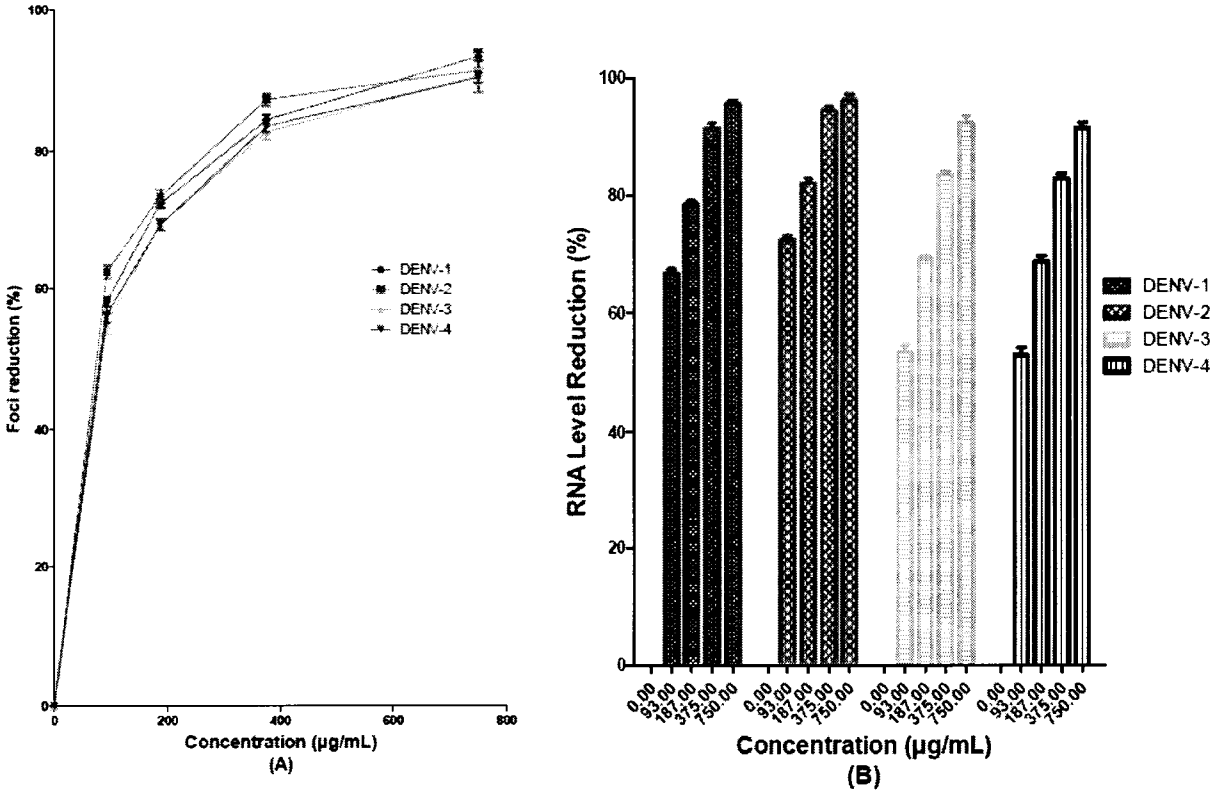


Figure 3

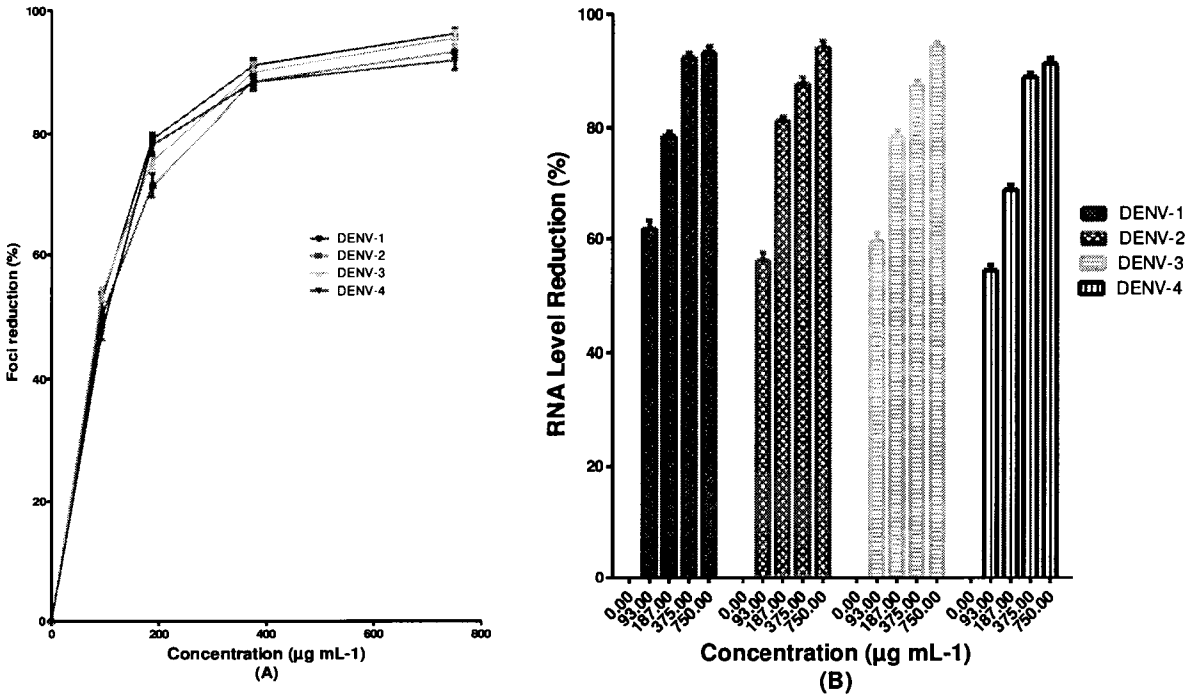


Figure 4

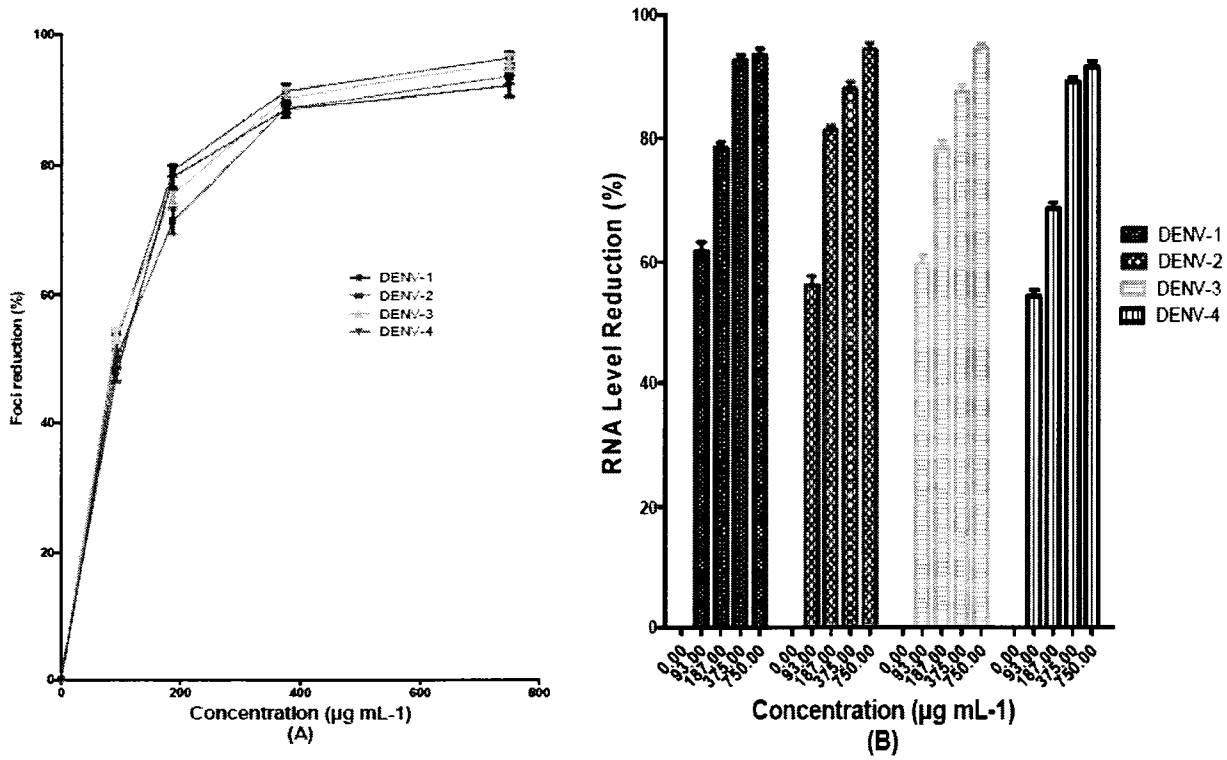


Figure 5

(A)

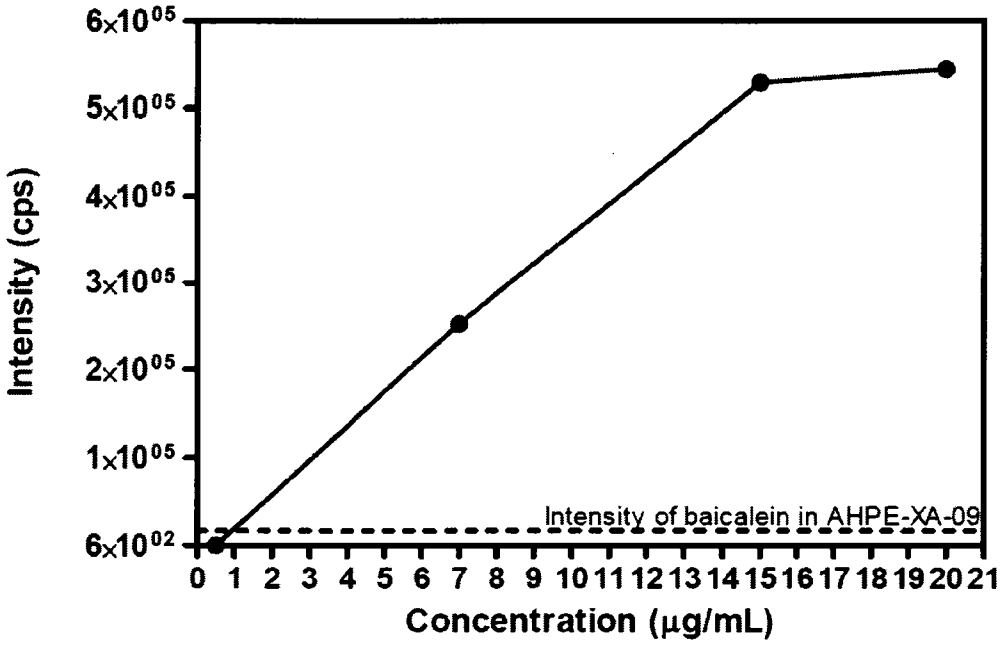
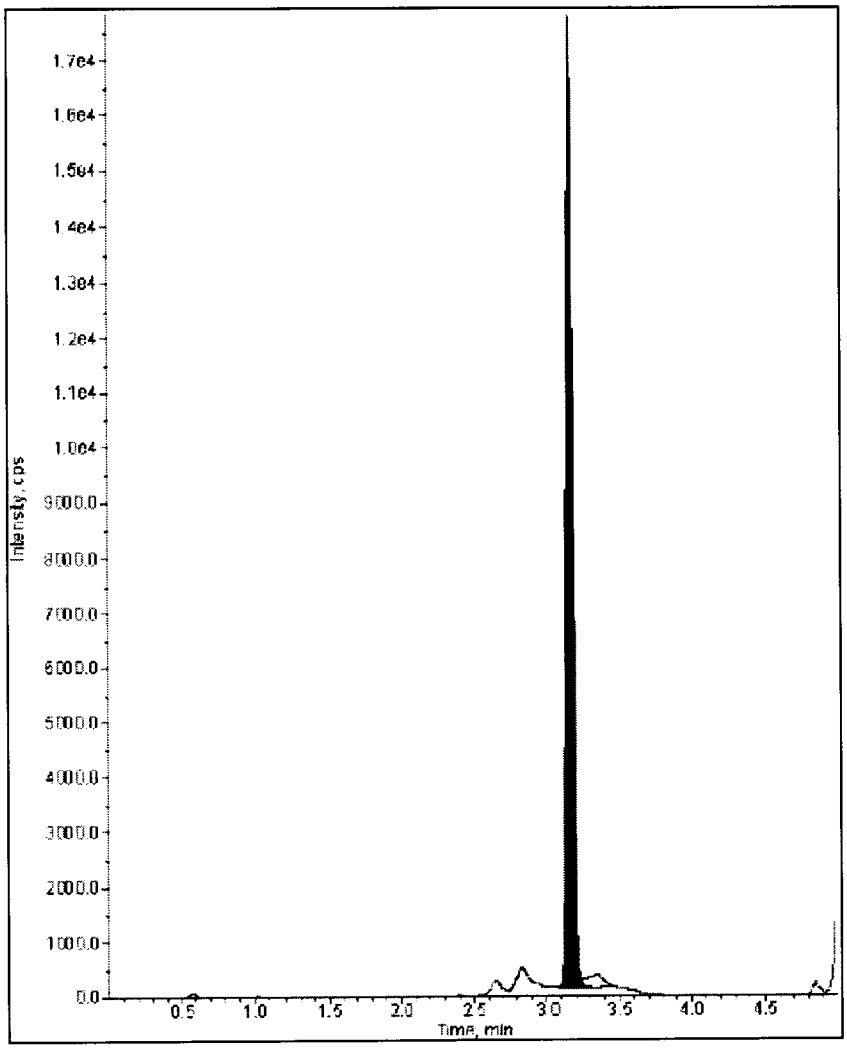


Figure 6(A)

(B)


1.7e4
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1.4e4
1.3e4
1.2e4
1.1e4
1.0e4
9000.0
8000.0
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
Intensity, cps
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time, min

Figure 6(B)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2014/000004

A. CLASSIFICATION OF SUBJECT MATTER

A61K 36/539 (2006.01) A61K 125/00 (2006.01) A61K 31/353 (2006.01) A61P 31/12 (2006.01)

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)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

WPI, Medline, EPODOC, XPTK. Keywords: Scutellaria baicalensis, baikal skullcap, Chinese Skullcap, bioactive, radix scutellaria, baicalein, Dengue, DENV-1, DENV-2, DENV-3, DENV-4, virus, breakbone fever and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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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
12 June 2014	12 June 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Ishanee Mookerjee AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262256167

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/MY2014/000004**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 2005/044281 A1 (THE UNIVERSITY OF HONG KONG) 19 May 2005	
X	abstract, page 4 lines 8-9, page 4 line 28, page 12 lines 7-10, page 14 line 19-page 15 line 5, page 24 lines 23-27	1, 4, 5
Y	abstract, page 4 lines 8-9, page 4 line 28, page 12 lines 7-10, page 14 line 19-page 15 line 5, page 24 lines 23-27	1-15
	WO 2009/097512 A1 (INNOVATIVE DRUG DISCOVERY INC.) 06 August 2009	
X	abstract, [0006], [0016], [0038], [0078]	1, 4, 5
Y	abstract, [0006], [0016], [0038], [0078]	1-15
	Zandi K. et al., Novel antiviral activity of baicalein against dengue virus, *BMC Complementary and Alternative Medicine* 2012, 12:214	
X	abstract, page 1 right-hand column, page 8 left-hand column - Conclusions	1-15
Y	abstract, page 1 right-hand column, page 8 left-hand column - Conclusions	1-15
	WO 2013/147584 A1 (UNIVERSITI MALAYA) 03 October 2013	
P,X	abstract, page 7, page 12 lines 14-17	1-15

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/MY2014/000004**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2005/044281 A1	19 May 2005	None	
WO 2009/097512 A1	06 August 2009	None	
WO 2013/147584 A1	03 October 2013	None	

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.