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Posted Date: 13 June 2023

doi: 10.20944/preprints202306.0906.v1

Keywords: Infectious Diseases; Arboviruses; Dengue Virus; Baicalin; Baicalein

Article

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Abstract: Dengue virus (DENV) is a member of the Flaviviridae family and is responsible for the most common mosquito-borne human disease. Currently, there are no available antiviral drugs to treat DENV infections. However, certain flavonoids, including baicalein and baicalin, have demonstrated significant anti-DENV effects in vitro. This study aimed to assess the bioavailability of baicalein and its metabolite, baicalin, in the blood serum of Albino Wistar rats using the LC/MS/MS method. The in vitro activity of sera obtained from rats administered baicalein was evaluated for its anti-DENV properties using quantitative RT-PCR and the Foci Forming Reduction Assay (FFRA). The results obtained through LC/MS/MS analysis indicated that the bioavailability of baicalin in rat blood serum reached 44.20 µg/ml one hour after oral administration of baicalein. Furthermore, the sera collected from rats given baicalein exhibited a significant 78.26% inhibition of DENV-2 replication when tested against Dengue-2 using both quantitative RT-PCR and FFRA. These findings suggest that baicalein and baicalin hold promise as potential therapeutic candidates for further investigation in the development of treatments against DENV infections.

Keywords: infectious diseases; arboviruses; dengue virus; baicalin; baicalein

1. Introduction

Dengue virus infection is endemic in several tropical and subtropical regions of the world where the mosquito vectors, *Aedes aegypti* and *Aedes albopictus* are found [3]. The disease is estimated to affect approximately 200 to 400 million cases annually in the world [1,2].

Dengue is caused by any of the four dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Dengue fever (DF) is usually a mild self-limiting febrile illness that may develop into more severe diseases such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [4,5]. To date, there is no approved specific antiviral therapy for dengue. Hence, an effective treatment against dengue is urgently needed.

Flavonoids are polyphenolic compounds with known potential biological activities including antioxidant, anti-inflammatory, anti-cancer, antimicrobial and antiviral activities [6]. It is also known that flavonoids are synthesized by plants in response to microbial infections [7]. In earlier studies, baicalein, a flavonoid and its main metabolite, baicalin were reported to exhibit *in vitro* anti-DENV replication activities [8,9]. Baicalein and baicalin are flavonoids mainly found in the roots of *Scutellaria baicalensis* a Chinese medicinal plant [8,10]. Previous pharmacokinetic studies of baicalein showed that the compound is metabolized in animal and human to baicalin [11,12].

 doi:10.20944/preprints202306.0906.v1

The antiviral properties of flavonoids relate to their chemical structure. However, to elucidate the anti-DENV activity of selected flavonoids *in vivo*, it is important to know the bioavailability and the type of metabolites that are present in serum after administration of these flavonoids. Therefore, it is essential to study the bioavailability and metabolism of both baicalein and baicalin.

This study is designed to evaluate the bioavailability of baicalein in Albino Wistar rat and evaluate sera of rats fed with baicalein on anti-DENV activity *in vitro*.

2. Results

Pharmacokinetic study of baicalein in rat

Baicalein was administered orally (500 mg/ml) and intraperitoneal injection (IP) to the Albino Wistar rats. Following thereafter baicalin the metabolite of baicalein, at concentration of 44.20 µg/ml was detected in rat blood serum at 1-hour oral consumption. Baicalin concentration in rat blood serum dropped to 27.70 µg/ml after 2 hours post-oral administration and maintained at the same concentration up to 4 hours. After 8-hours the baicalin concentration was detected 16.40 µg/ml in the rat blood serum. Intraperitoneal injection with baicalein the highest concentration of baicalin detected in IP blood serum was at 10.30 µg/ml at thirty minutes post injection. The concentration dropped to 3.10 µg/ml at one hour and two hours after injection and eventually not detectable in the rat blood serum at 4 hours onwards. (Figure 1).

Baicalein concentration in the rat serum was measured as well using LC/MS/MS. The results showed that the highest concentration of baicalein was detected at one hour after oral administration (24.80 µg/ml) and it was maintained at 18 µg/ml after two hours and four hours oral administration. Baicalein concentration dropped to 13.80 µg/ml eight hours after oral administration. Similar to baicalin, baicalein bioavailability was detected very low in rat serum when it was administrated intraperitoneally. The highest concentration of baicalein detected was equal to 8.37 µg/ml thirty min after IP injection. The concentration dropped to 3.21 µg/ml and maintained at the same range till 2 hours after injection and eventually became undetectable at 4-8 hours after IP injection.

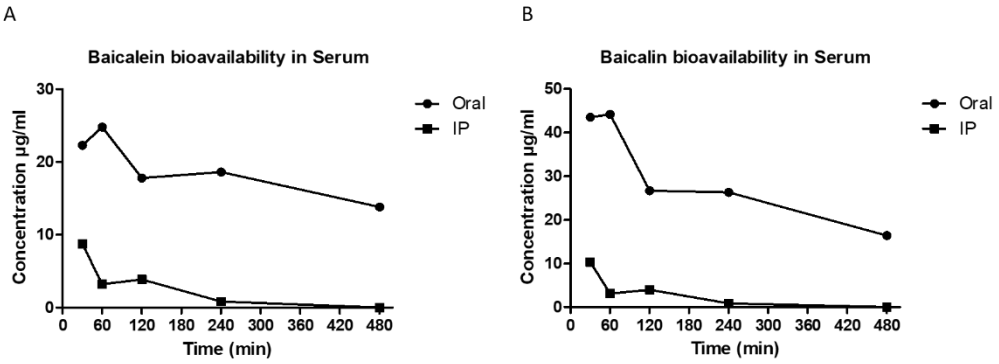

Figure 1. Serum concentration-time profile of baicalein and its main metabolite. After oral and intraperitoneal (IP) administration of baicalein at 500 mg/kg/rat, the serum concentration–time curve of baicalein (A), and its major active metabolite, baicalin (B) as well as are showed.

Cytotoxicity of sera of rats fed baicalein

The cytotoxicity of sera of rats fed with baicalein was determined by MTS assay as described above. The cytotoxicity results showed, there was no cytotoxicity from all sera of rats fed baicalein against Vero cells (Figure 2). In sera with the most concentrated more than 90% of cells were still viable, in comparison to the vehicle control (Sera of rats without compound).

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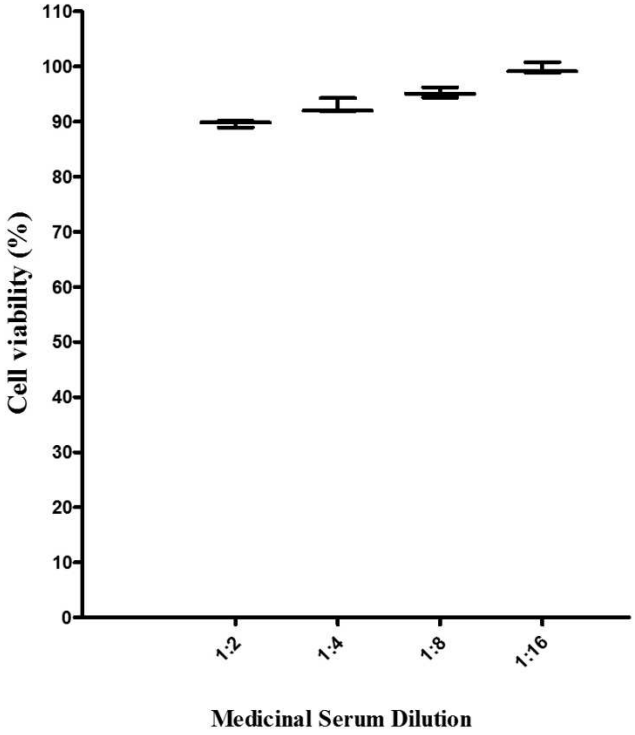

Figure 2. Cytotoxicity of sera of rats fed baicalein containing baicalein and baicalin against Vero cells using MTS assay. Different dilutions of sera were used to treat the Vero cells for 4 days. All experiments were conducted as three independent experiments in triplicates and the data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

In vitro antiviral activity of sera of rats fed baicalein

The effects of sera of rats fed with baicalein was evaluated against DENV-2 virus *in vitro* using the virus yield reduction assay and qT-PCR and the Focus Forming Unit Reduction Assay (FFURA). Results obtained suggested that at, 1:2 diluted serum prepared from the orally administered rat's blood sample inhibited 78.26% ± 2.04 of DENV-2 replication whereas at the same dilution prepared from the intraperitoneally administered rat's blood sample, 52.50 % ± 2.04 inhibition against DENV-2 replication was obtained. The same inhibition percentages were observed for other serum dilutions. The FFURA data showed the same reduction in number of dengue virus antigen compared to virus control group (Figure 3B). As the figure present, number of foci in sera of rat fed with baicalein significantly reduce compared to virus control. The same reduction can be observed in sera of rats injected intraperitoneally with baicalein, however the reduction is not significant.

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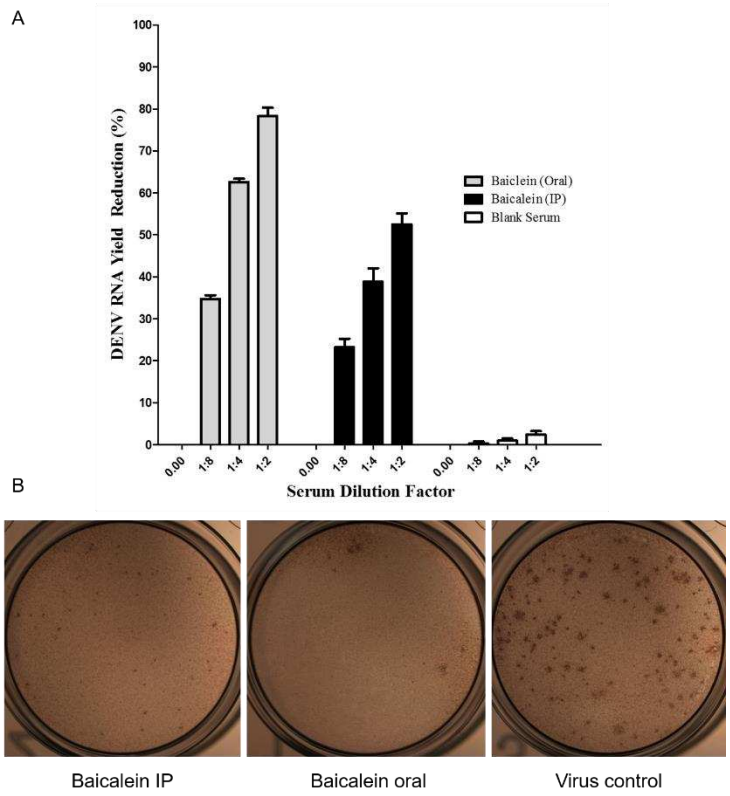

Figure 3. Anti-dengue effect of sera of rats fed baicalein containing baicalein and baicalin in Vero cells. (A) qRT-PCR was performed to evaluate the reduction of DENV yield production in vitro after 4 days post infection. It shows that baicalin from sera of rats fed baicalein (oral and IP) can inhibit around 78.26 and 52.5% of DENV-2 particles respectively. (B) Focus Forming assay performed to confirm qRT-PCR results of sera of rats fed baicalein against dengue 2 virus .

3. Discussion

Dengue continues to be a major mosquito-borne disease of significant worldwide health concern. To date, there is no specific drug to treat this infection and currently the most applied prevention measures lie in controlling the mosquito populations [15]. Earlier studies to discover antiviral against dengue showed that, both baicalein and its metabolite baicalin, process good anti-DENV inhibition properties *in vitro* [9,16]. Baicalein showed direct virucidal activity against DENV-2 with IC_{50}= 1.55 µg/mL besides its effects against dengue virus adsorption and intracellular replication with IC_{50} = 7.14 µg/mL and IC_{50}= 6.46 µg/mL respectively [16]. Baicalin on other hand, affected DENV-2 replication at 13.5 µg/ml concentration and showed anti-adsorption effect with IC_{50} = 18.07 µg/ml [9]. Baicalein, a flavonoid isolated mainly from the roots of *S.baicalensis*, when administrated to the animals and human is metabolized to baicalin as its main metabolite [17–19]. To determine if baicalein possess anti DENV replication property *in vivo*, baicalein was fed to adults' rats to evaluate the bioavailability and *in vitro* anti-DENV activity. The sera of rats fed with baicalein was tested for its anti DENV property ex-*vivo* since DENV could not productively infect rats.

The sera of rats fed baicalein containing baicalin and baicalein significantly inhibited DENV-2 replication in Vero cells in a dose-dependent manner in consistency with our previous findings [9,16].

We found out that the sera prepared from orally administered rats in the first hour after administration contain higher amount of baicalin and baicalein compared to the sera that were taken after 1 h post administration from rats that injected IP. This finding can confirm that the significant bioavailability of orally administered baicalein in Albino Wistar rat is 1 h post administration with significant *in vitro* anti-DENV activity as well. Since the amount of baicalein detected in serum was neglectable to exert that effect and as baicalin is the predominant metabolite of baicalein, it can be concluded that the baicalin in sera of rats fed baicalein plays an important role in observed anti-

 doi:10.20944/preprints202306.0906.v1

DENV-2 activity. We have reported *in vitro* anti-DENV-2 activity of baicalin in our previous study with various effects against different stages of DENV-2 replication cycle. Baicalin inhibited DENV-2 intracellular replication with IC50= 13.5 µg/mL besides its virucidal activity against DENV-2 extracellular particles with IC 50= 8.74 µg/mL and anti-adsorption effect with IC50 = 18.07 µg/ml. Nevertheless, obtained data in current study showed that sera of rats fed baicalein containing 21.1 µg/mL of baicalin exhibited significant reduction of dengue-2 virus with 78.26% ± 2.04.

Based on previous findings it has been shown that the conversion of baicalein to baicalin occurs during digestion by the removal of a glycoside moiety by ß-glucoronidase with 74% conversion [11,12,20]. ß-glucoronidase enzyme is found in lysosomes mainly available in the intestinal lumen. This enzyme is also produced by some certain intestinal commensal bacteria [12].

Results from the study suggested that oral administration of baicalein gives significant bioavailability of both baicalein and baicalin to the Albino Wistar rat blood serum compared to intraperitoneal injection. This finding suggests that baicalein can be absorbed through the digestive system in animal model efficiently. The of data this study showed that baicalein is also available as non-metabolized administered compound but with lower concentration compared to baicalin which is consistent with previous studies on pharmacokinetic of baicalein [10,12].

4. Materials and Methods

Materials

Baicalein and baicalin were purchased from Sigma Chemical Company (Sigma, St Louis,USA). The compound was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), prior to use. All other chemicals and solvents used in LC/MS/MS such as methanol and acetonitrile were of the HPLC grade purchased from Fisher Scientific Company (Fisher Scientific,USA).

Cells and virus

C6/36 mosquito cells and Vero cells (African green monkey kidney) purchased from American Type Culture Collection (ATCC) were cultured and maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA). Vero and C6/36 cells were incubated in humidified atmosphere at 37°C and 28°C and in the presence of 5% and 3% CO_2 respectively.

In this study dengue virus type-2 (DENV-2) New Guinea C strain (NGC) was used. The virus was kindly provided by The Virology laboratory of the Tropical Infectious Disease Research and Education Center (TIDREC), University of Malaya (Kuala Lumpur, Malaysia). DENV-2 was propagated in C6/36 cells and harvested after presentation of cytopathic effects (CPE) on the day seven post-infection (PI). Propagated viral stock was titrated on Vero cells by focus forming assay (FFA) as previously described [13] and stored at -80°C until needed.

Animal ethical issues

This study received approval from the Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya, Malaysia (No. MP/17/02/2012/KZ) and in full compliant of the National Academy of Science's Guide for the Care and Use of Laboratory Animals [21,22]

Animal experimental design

Fourteen mature male Albino Wistar rats (weighing approximately 220-250 g) were obtained from the Animal Experimental Unit (AEU) of the University of Malaya. Rats were housed 2 per cage and maintained in a dark room under controlled temperature of 24°C, respectively. Food and water were provided as prescribed by the AEU.

The half maximum lethal dose (LD_{50}) was not carried out for this study because the maximum non-lethal dose of baicalein has been previously reported [12] and in compliance to the to Ethical Guidelines, which recommended not to redo the same test that has been conducted and published.

 doi:10.20944/preprints202306.0906.v1

The rats were fasted for 12 h with free access to water before and throughout the experiment. Rats were randomly assigned to two experimental groups: group 1, normal control (n = 4) and group 2, experimental (n =10). Rats in the experimental group were further divided into two treatment groups with different administration routes, intraperitoneal (IP) (n=5) and oral administration (n=5).

Administration of baicalein

To investigate the bioavailability of baicalein at different time profiles, the compound was dissolved in 0.5% DMSO and administered by an intragastric probe and intraperitoneal injection for baicalein with doses of 500 mg/kg/d (n=5) and 100 mg/kg/d (n=5), respectively. Control rats in all groups were treated with 0.5% DMSO at a final concentration of 1 mL/kg per rat based on previous recommendation as well as different methods of administration. The rats were anesthetized with IP injection of ketamine\ xylazine and blood samples were collected by cardiac puncture separately at 30, 60, 120, 240- and 480-minutes post-dosing. Sera were obtained by centrifugation at 3000 x g for 20 min and stored at -80 C°.

Quantification of baicalein and its conjugated metabolite in serum

The concentration of baicalein and baicalin in serum samples were analyzed by liquid chromatography tandem mass spectrometric (LC/MS/MS) method. Sample extraction was carried out using the protein precipitation method**.** The frozen serum was thawed at room temperature (25±1º C), vortexed to ensure complete mixing of the content. To each 100µl serum sample, 50µl of internal standard (containing 500ng/mL of naringenin) was added followed by 300µl of acetonitrile. The mixture was vortexed for 20 sec and centrifuged for 5min at 16000x g. The supernatant was then transferred to 2 mL vials and injected into the LC/MS/MS system (AB SCIEX QTRAP 5500, Applied Biosystems, USA). The analytical column used was a Phenomenex, Gemini-NX C18 (150mm length X 2.1 mm I.D, particle size 5µm) and Phenomenex, Gemini-NX C18 guard column (4mm ID x 2.0mm length). Mobile phase used was 0.1% formic acid in water for pump A and the mixture of acetonitrile and methanol (1:1 v:v) for mobile phase B. The flow rate was set at 0.4 mL/min and a gradient elution was used at room temperature. The gradient program began with 5% B, then ramped up to 60% B at 1.00 min and held until 3.50 min. The gradient was then ramped again to 95% B at 4.00 min and held until 5 min. The gradient program was returned to 5% B at 5.01 min and this condition was held for a further 7.00 min. Sample injection volume was 10µl. An API 5500 triple quadrupole mass spectrometer fitted with ESI probe and operated in the negative ionization mode were used to perform mass spectral analyses. Analytes were then quantified by multiple reactions monitoring (MRM). For quantitative analysis, multiple reactions monitoring (MRM) was used and for baicalein, baicalin the precursor ion monitored were 445.05 and 269.01, respectively while fragment ions monitored were 175.20 and 241.2, respectively.

Cytotoxicity of sera of rats fed baicalein

The cytotoxicity of sera of rats fed baicalein was determined against Vero cells using the MTS assay. Briefly, Vero cells seeded at a concentration of 5×10^4 cells/well in 96-well cell culture microplate and kept at 37°C for 24 h in a humidified atmosphere with 5% CO_2.

When cell monolayer reached 90% confluency, two-fold dilutions of sera of rats fed baicalein were added to the cells in triplicates. After 4 days post treatment, MTS solution (Promega, Madison, WI, USA) was added to each well and the microplate was kept at 37°C for 4 h in a humidified atmosphere with 5% CO_2. Then the absorbance values of the wells were measured at 570 nm using a 96-well plate reader (TECAN, Mannendorf, Switzerland). Dose-response curve was plotted using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005). Results were represented as the means ± standard error of the mean (SEM) from triplicate assay from three independent experiments.

In Vitro Antiviral activity of sera of rats fed baicalein

 doi:10.20944/preprints202306.0906.v1

Antiviral activity of sera of rats fed with baicalein was evaluated by performing the virus yield reduction assay using DENV-2 specific quantitative RT-PCR and Foci Forming Reduction Unit Assay (FFRUA). Briefly, confluent monolayers of Vero cells were prepared in 24-wells cell culture plate and incubated with 100 µl of DENV-2 (NGC) suspension containing 100 FFU of DENV-2 and an equal volume of the diluted two-fold concentrations of sera of rats fed baicalein in FBS-free medium in the presence of blank serum (50%, 25%, 12.5% and 6.25%) which were sterile-filtered using 0.2 µm syringe filter (Sartorius Stedim Biotech, Germany) and added to the respective wells. Virus adsorption was performed with gentle rocking and incubated inside CO_2 Incubator for 1 h at 37ºC temperature. Subsequently, cells were washed twice with sterile PBS to remove unabsorbed viruses; then the cells were overlaid with 100 µl medium containing 2% FBS (Sigma-Aldrich, USA) with increasing two-fold dilutions of sera of rats fed baicalein. Treated cells were then incubated for 2 days at 37°C in 5% CO_2 atmosphere. After two days post-infection, the supernatants were collected and DENV-2 RNA were extracted using the Viral RNA extraction kit (Qiagen, Hilden, Germany DENV yield was determined using the q-RT-PCR.

DENV quantitative RT-PCR

The quantitative RT-PCR assay for DENV-2 was performed using SensiFAST™ SYBR® Hi- ROX One-Step Mix (Bio line, UK) in a total reaction volume of 20 µl which contained of ddH2O (6.4µl), 2× SensiFAST One-Step (10 µl), Reverse transcriptase (0.2 µl), RNase Inhibitor (0.4 µl), 0.5 µl of 400 nM of forward (DNF) and reverse (D2R) primers [14], and the extracted DENV-2 RNA (2 µl). All samples were performed in triplicates, amplification was performed using the DNA Engine Opticon system (MJ Research/Bio-Rad, Hercules, CA) with the following thermal cycling conditions: reverse transcription at 42°C for 10 min, initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 59°C for 15 sec and 72°C for 30 sec. Melting curve analysis was afterward performed at temperature from 60°C to 95°C to verify the assay specificity. For absolute quantities of viral RNA in the samples, standard curve was established with viral RNA extracted from DENV-2 virus inoculate of known infection virus titer.

Focus forming unit reduction assay (FFURA)

Antiviral activity of sera of rats fed with baicalein was determined and evaluated by measuring the reduction in the number of DENV infectious foci after treatment. Briefly, Vero cells were seeded in 24 wells plate and incubated overnight inside a CO2 incubator at 37ºC temperature. The following day the Vero cells monolayer were infected with DENV-2 and treated with sera of rats fed with baicalein prepared in 2% EMEM with 1% Carboxymethyl cellulose (CMC) for 4 days post-infection effect. Virus foci were visualized as previously described [8]. The number of DENV-2 foci was counted using a stereomicroscope and the virus titer was expressed as Foci Forming-Unit (FFU).

5. Conclusions

This The present study was designed to investigate antiviral activities of sera of rats fed baicalein against DENV due to administration of baicalein in Wistar rats. The results showed significant *in vitro* anti-dengue activity properties for sera of rats fed baicalein. As conclusion, oral administration of baicalein gives higher bioavailability of baicalin compared to the intraperitoneal administration. Based on baicalin effects against different stages of DENV replication, further studies may require revealing the specific antiviral target(s) of the compound. Furthermore, based on the acceptable bioavailability of baicalein and its metabolite baicalin and significant anti-DENV activity of sera of rats fed baicalein, further *in vivo* study it required using suitable animal models for DENV. It also furthers studies are required to investigate the molecular and intracellular pathways, especially identification of target viral gene(s) and cellular element(s) that could play a vital role in facilitating anti-DENV function of baicalein and baicalin as potential candidates for dengue infection treatment.

Author Contributions: This work was approved by all the co-authors. E.M, P.H, K.Z and SAB, made a significant contribution to the concept study design. Data and logistics for sample acquisition were completed by D.E and

 doi:10.20944/preprints202306.0906.v1

Z.C. R.P. E.M and P.H contributed to data analysis and interpretation. E.M P.H and D.E contributed to writing the draft of the article and critically revising it. SAB, K.Z contributed with grant resources. SAB and K.Z took part in project investigation and supervision.

Funding: We acknowledge the funding from the Ministry of Education, Malaysia for niche area research under the Higher Institution Centre of Excellence (HICoE) program (Project MO002-2019).

Conflicts of Interest: The authors declare no conflicts of interest. The work described in this manuscript is original. It has not been published and is not considered for publication elsewhere, partially, or completely. All authors read and approved the final work

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