Punica granatum Leaf Ethanolic Extract and Ellagic Acid as Inhibitors of Zika Virus Infection

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Abstract

Zika virus (ZIKV), an arthropod-borne flavivirus, is an emerging healthcare threat worldwide. ZIKV is responsible for severe neurological effects, such as paralytic Guillain-Barrè syndrome, in adults, and also congenital malformations, especially microcephaly. No specific antiviral drugs and vaccines are currently available, and treatments are palliative but medicinal plants show great potential as natural sources of anti-ZIKV phytochemicals. This study deals with the investigation of the composition, cytotoxicity and anti-ZIKV activity of *Punica granatum* leaf ethanolic extract, fractions and phytoconstituents. *Punica granatum* leaves were collected from different areas in Italy and Greece in different seasons. Crude extracts were analyzed and fractionated, and the pure compounds were isolated. The phytochemical and biomolecular fingerprint of the pomegranate leaves was determined. The antiviral activities of the leaf extract, fractions and compounds were investigated against the MR766 and HPF2013 ZIKV strains *in vitro*. Both the extract and its fractions were found to be active against ZIKV infection. Of the compounds isolated, ellagic acid showed particular anti-ZIKV activities, with EC_{50} values of 30.86 μM for MR766 and 46.23 μM for HPF2013. The mechanism of action was investigated using specific antiviral assays and it was demonstrated that ellagic acid was primarily active as it prevented ZIKV infection and was able to significantly reduce ZIKV progeny production. Our data demonstrate the anti-ZIKV activity of pomegranate leaf extract and ellagic acid for the first time. These findings identify ellagic acid as a possible anti-ZIKV candidate compound that can be used for preventive and therapeutic interventions.

Keywords

Zika virus, *Punica granatum,* Lythraceae, leaf ethanolic extract, phytochemical and biomolecular fingerprint, antiviral, ellagic acid

Abbreviations

Aut, autumn samples; BHK21, Baby hamster kidney cell; BLAST, basic local alignment search tool; BSTFA, *N,O*-Bis(trimethylsilyl) trifluoroacetamide; CC_{50}, 50%-cytotoxic concentrations; C.I., Confidence intervals; DAPI, 4′,6-diamidino-2-phenylindole; EC_{90}, effective concentration-90; ESI, Electrospray ionization; FBS, foetal bovine serum; FDA, Food and Drug Administration; HBeAg, Hepatitis B e Antigen; HBV, Hepatitis B; HIV-1, Human immunodeficiency virus type 1; HSV-2, Herpes simplex virus type 2; HPV, Human Papilloma virus I_s, linear retention indices; *ITS*, Internal transcribed spacer; MEM, Minimum Essential Medium; MOI, Multiplicity of infection; MTS, 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt;

PC, Principal component; PDA, Photo diode array; PFU, Plaque-forming unit; PG, *Punica granatum*; PGL8, *Punica granatum* leaf extract 8; Prep, preparative; *psbA-trnH*, chloroplast photosystem II protein D1; RP, Reverse phase; RSD, Relative standard deviation; SPE, Solid phase extraction; SI, Selectivity index; SIM, Selected Ion Monitoring; SRM, Selected Reaction Monitoring; Sum, summer samples; VACV, Vaccinia virus; WHO, World Health Organization; ZIKV, Zikavirus

Introduction

Zika virus (ZIKV) is a mosquito-borne virus that belongs to the Flaviviridae family. It is primarily transmitted by the bite of an infected mosquito from the *Aedes* genus, mainly *Aedes aegypti*, in tropical and subtropical regions [1]. Outbreaks of ZIKV disease have been recorded in Africa, the Americas, Asia and the Pacific and it is considered a global emerging healthcare threat. Since *Aedes albopictus* has the capability to be a vector for ZIKV, other countries in temperate regions, such as the Mediterranean basin, are potentially at risk [2]. ZIKV is usually responsible for asymptomatic or mild self-limiting dengue-like diseases, which are characterized by fever, rash, conjunctivitis, arthralgia and malaise. During the recent outbreak in Brazil, it has been associated with severe neurological effects, such as Guillain-Barré syndrome and meningoencephalitis, in adults, and congenital malformations, especially microcephaly, in infants born to infected mothers [3]. Despite the severity of ZIKV complications, there are currently no FDA-approved vaccines. No specific antiviral drugs are currently available and treatments are palliative and mainly directed towards the relief of symptoms [1]. For these reasons, new effective preventive and therapeutic strategies against ZIKV infection are urgently needed. Harnessing the potential of medicinal plants as natural sources of anti-ZIKV phytochemicals, such as polyphenols and alkaloids [4], is a complementary and alternative strategy. *Punica granatum* L. (Lythraceae family), commonly known as pomegranate, is a domesticated tree that is widely grown as an evergreen in tropical regions, and as a deciduous tree in temperate areas. It is an ancient plant that is well known in folk medicine and is becoming increasingly popular as a functional food and nutraceutical source due to its high polyphenol content, not only in the edible part, but also in other parts of the fruit and plant, including the peel, bark, leaves and flowers [5]. Pomegranate is a rich source of a wide variety of compounds with beneficial physiological activities, in particular antioxidative, anti-inflammatory and anti-cancerous properties [6]. Nearly every part of the plant has been tested for antimicrobial activity, and roles in the suppression of enteric infections, food preservation, wound healing, as well as gut and oral health have been demonstrated [7]. Most antiviral studies have been performed on the fruit's peel and juice, and it has been found that extracts exerted inhibitory activity against herpes simplex virus type 2 (HSV-2), human immunodeficiency virus type 1 (HIV-1) and the influenza virus [8-10]. Little information has been reported on pomegranate leaf extracts, compared to other edible and non-edible parts of the plant, although some recent studies have indicated that they may be an important source of specialized bioactive metabolites and they possess a broad range of biological properties, such as *in-vitro* antioxidant, anti-inflammatory, anti-cholinesterase and anti-proliferative activities [5,11,12]. This study explores the cytotoxicity and anti-ZIKV activity of pomegranate leaf ethanolic extracts, as well as of the corresponding fractions and phytoconstituents after a phytochemical and

biomolecular characterization of the leaves, which were collected from various sites, after different vegetative periods and in different years.

Results and discussion

Preliminary tests were carried out on a reference pomegranate leaf ethanolic extract (PGL8) to investigate its anti-ZIKV activity in a specific virus plaque reduction assay against the African lineage strain, 1947 Uganda MR766. A range of extract concentrations were added before and during the infection, as well as after the removal of the virus inoculum. As reported in Figure 1S, Supporting Information, the extract exerts remarkable antiviral activity, generating dose-response curves. Under these conditions, the extract reduced the number of viral plaques with an EC_{50} value of 11.4 µg/mL (Table 1). To exclude the possibility that antiviral activity was due to cytotoxicity, cells were treated with the serially diluted extract and added to the cell culture medium for 72 hours at 37°C, and the cellular viability was then determined by MTS assay. The CC_{50} values were above 100 µg/mL, indicating that the antiviral activity observed was not due to cytotoxicity (Table 1, Figure 2S, Supporting Information). Since the extract was resuspended in a DMSO/H_2O solution (50%/50%), a control sample with equal volumes of DMSO/H_2O was included in all cell-culture experiments in order to rule out the possibility of the solvent having a cytotoxic effect. The selectivity index (SI), which measures the preferential antiviral activity of a drug in relation to its cytotoxicity, was 10.84. Two unrelated DNA viruses, HSV-2 and VACV, were assessed in order evaluate the antiviral specificity of PGL8. As reported in Table 1, the extract exerted relevant inhibitory activity against HSV-2, with an SI of 47.08. These data confirmed the anti-HSV-2 effect that had been observed in extracts derived from pomegranate fruit, including rind and juice [8]. By contrast, we did not observe any inhibition of VACV infectivity.

The characterization of the pomegranate leaf ethanolic extract (sample PGL8) was carried out by HPLC-PDA-MS/MS and GC-MS after derivatization with bis(trimethylsilyl)trifluoroacetamide to obtain trimethylsilyl derivatives, and three different chemical classes of specialized metabolites were revealed: phenolics, flavonoids and triterpenes. A list of the identified and putatively identified compounds is reported in Table 2, while the HPLC-PDA and GC-MS profile are found in Figures 3S and 4S, Supporting Information, respectively. In accordance with the current literature, flavones and flavonols are the most representative specialized metabolites in the extract, and often exist as glycosides of luteolin, apigenin and quercetin. Ellagic acid is the most abundant compound, while hydrolyzable tannins, such as punicalins and punicalagins, which are markers of the other parts of the pomegranate plant, were not detected. In addition, the presence of a pseudomolecular ion at *m/z* 455, in negative mode, with a fragment at *m/z* 407 $(M\text{-}HCHO\text{-}H_2O\text{-}H)^-$, and of a pseudomolecular ion at

134 *m/z* 457, in negative ionization mode, in the LC-MS profiles indicates the present of triterpenoid
135 molecules, but with evident coelution. A GC-MS analytical platform was therefore used and enabled
136 oleanolic, betulinic and ursolic acids to be identified after their derivatization in the extract.
137 To ensure consistent quality and reproducible activity in the pomegranate leaf extracts, genotypic and
138 phenotypic stability were evaluated by comparing the phytochemical and biomolecular patterns of
139 leaves that belonged to plants of different origins (see table 1S, Supporting Information), that were
140 harvested in different vegetative periods (summer and autumn) and in different years (2017 and
141 2018).
142 HPLC-PDA-MS/MS and GC-MS profiles were qualitatively consistent and all markers were detected
143 in all of the samples. Quantitation results, reported in Table 2S, Supporting Information, showed
144 differences in the abundances of some compounds in the leaf extracts. The repeatability results
145 showed that RSD% never exceeded 5%, while intermediate precision in the different extracts showed
146 RSD% of below 15%. The accuracy of the data was determined by comparing, when available, the
147 UV and MS quantification results, and the RSD% never exceeded 20%. Principal Component
148 Analysis was then applied to highlight similarities and differences between the samples. Figure 1
149 reports the score and loading plots of the first principal component (PC1) against the second principal
150 component (PC2), showing homogenous sample distribution in the score plot (no cluster of samples
151 are formed) and a good explained variance (39.14% for PC1 and 19.72% for PC2). No clear
152 discrimination between the samples was observed, although the autumn samples show slightly higher
153 contents of ellagic acid and its hexoside (both positively correlated with PC2, as can be seen in the
154 loading plot). In general, the few differences in the phenolics and triterpenes can be ascribed to
155 phenotypical variability and environmental factors.
156 To further confirm the quality and reproducibility of the pomegranate leaf extracts, a genotypic
157 fingerprint of the collected leaves was obtained using a DNA barcoding approach [13]. The nuclear
158 internal transcribed spacer region (*ITS*) and the chloroplast photosystem II protein D1 (*psbA-trnH*)
159 genes were amplified and sequenced for each site from which pomegranate leaf samples were
160 harvested. The sequences were deposited in the GenBank (Table 3S, Supporting Information) and
161 compared to those present in the database (fifty-nine *P. granatum ITS* sequences originating from
162 India, Iran and China and twenty-seven *P. granatum psbA-trnH* sequences from Iran, Tunisia, China
163 and Italy (Apulia, Latium, Sardinia, Padua and Trieste)).
164 Figures 5S and 6S, Supporting Information report no variation in the *ITS* and *psbA-trnH* nucleotide
165 composition for the eleven sites, suggesting that these biomolecular markers are stable. A consensus
166 sequence for each DNA region was obtained from the alignment of all the samples. A BLAST
167 alignment of the consensus, with all the data present in the database, showed a percentage of identity

among the *P. granatum* sequences that ranged from 100% to 96.89% for *ITS,* and from 100% to 97.64% for *psbA-trnH,* confirming intra-species stability and higher inter-species variability. Interestingly, the comparison with *psbA-trnH* from Italian regions (accession numbers: HG765008, HG765007, HG765006, HG765005), showed 100% similarity, supporting the stability of the species in Italy.

Based on the data obtained from the phytochemical characterization, representative summer and autumn pool extracts were created.

A bio-guided fractionation procedure was performed to attribute the antiviral activity to a specific fraction and/or single components of the pomegranate leaf extracts. The summer and autumn extracts were submitted to SPE fractionation, resulting in two fractions: a phenolic fraction (yield 73%) eluted with methanol/water 85:15 (PGAut85, PGSum85), and a triterpenoid fraction (yield 5%) eluted with methanol/water 95:05 (PGAut95, PGSum95). Table 2S, Supporting Information reports the quali-quantitative composition of the four samples. The phenolic fraction was characterized by ellagic acid, rutin, apigenin, quercetin and luteolin glycosides. In the PGAut85 fraction, ellagic acid was the most abundant compound, while, in the PGSum85 one, luteolin 4'-*O*-glucoside was the main compound, although a good amount of ellagic acid was detected. The triterpenoid fraction was characterized by oleanolic, betulinic and ursolic acids.

As reported in Table 1 and Figure 7S, Supporting Information, both the PGAut85 and PGSum85 fractions were active against ZIKV at increasing doses with EC_{50} values of 10.40 and 16.20 μg/mL, respectively. However, no PG95 fractions exerted antiviral activity. No statistical differences were observed in the EC_{50} values of the summer and autumn fractions, confirming the demonstrated similar chemical compositions of the pomegranate leaves. The main components of the PG85 and PG95 fractions were therefore tested. A preliminary screening was performed to test the activity of the phenolic and triterpenic compounds at three doses (33, 11, 3.7 μg/mL) against the MR766 strain by treating cells before, during and after infection. Table 3 demonstrates that ellagic acid was active against ZIKV infection in a dose-dependent manner. No inhibitory effect was observed at any dose for the other compounds. These data, obtained on cell cultures, did not confirm the predictive inhibitory activity of luteolin, apigenin and rutin as inhibitors of the ZIKV NS2B-NS3 protease, as identified by molecular docking [14,15]. Furthermore, the ability of quercetin 3-*O*-glucoside to inhibit ZIKV *in vitro*, as reported in the literature, was not reproduced [16]. According to our data, ellagic acid showed the highest antiviral activity against ZIKV, and was therefore isolated from the pomegranate extract, by Prep-LC, and selected for further study. The isolated ellagic acid was characterized by ^{1}H NMR and its spectrum compared with that of the commercial standard (Figure

8S, Supporting Information). The purity of the compound was determined by HPLC-PDA and calculated to be >97%.

To confirm the inhibition of ZIKV infectivity that the isolated ellagic acid demonstrated in the preliminary standard plaque reduction assay, a wider range of concentrations was tested against the MR766 strain in order to determine the EC_{50} values. High inhibitory activity was observed with an EC_{50} value of 30.86 μM (Table 4, Figure 2A). It is worth noting that the compound was also active against the Asian lineage strain, 2013 French Polynesia HPF2013, with an EC_{50} of 46.23 μM, indicating the broad spectrum of its action against different ZIKV strains. These data were confirmed using the commercially available standard, endowed with comparable EC_{50} values (Table 4 and Figure 2B). To corroborate the inhibition of ZIKV infectivity by ellagic acid, immunofluorescence experiments that incubated fixed cells with a flavivirus group antigen antibody, were performed in the same conditions as described previously for both MR766 and HPF2013. As reported in Figure 2C and 2D, the analysis by confocal laser scanning microscope, revealed a strong red signal from ZIKV protein E in the cytosol of untreated cells. A dose-dependent signal was observed in treated cells; the highest tested dose (109.2 μM) completely inhibited the infectivity of both strains, while the number of infected cells was considerably reduced at 36.4 μM.

Recently, the antiviral activity of ellagic acid, which had been isolated from other plants, has been demonstrated *in vitro* against different RNA viruses, such as the influenza virus, Ebolavirus, Hepatitis C virus, and HIV-1 [17-20]. Furthermore, ellagic acid has revealed potential activity against HBV infection due to its hepatoprotective properties and ability to effectively block HBeAg secretion in cells [21]. By contrast, ellagic acid partially inhibited HSV-2 infection [8]. Herein, we have demonstrated, for the first time, the antiviral activity of pomegranate-derived ellagic acid against ZIKV, a member of the Flaviviridae family. Previously, other polyphenols, such as delphinidin and epigallocatechin gallate, have been shown to have anti-flaviviral effects [22]. Our data have demonstrated that the isolated ellagic acid inhibited, *in vitro,* the infection of two lineages, the African one, which is responsible for more acute infection, and Asian ZIKV, which is associated with neurological impairments [23]. Interestingly, the compound also exerted adulticidal activity against *Aedes aegypti* mosquito, the main vector of the virus [24].

As ellagic acid was identified as an inhibitor compound of ZIKV infectivity, further studies were performed to elucidate its mechanism of action. Firstly, we tested the ability of isolated ellagic acid to reduce ZIKV progeny production *in vitro* by performing a virus yield reduction assay. The experimental procedure for this assay is similar to the one described for the viral plaque reduction assay, but the viral titers of the samples were evaluated after infection. As reported in Figure 3, 109.2

and 36.4 μM concentrations significantly reduced the production of infectious viruses 100- and 10-fold, respectively.

A virucidal assay was performed to investigate the possible direct virus-inactivating activity of the isolated compound on both MR766 and HPF2013. To this aim, 10^5 pfu of the ZIKV strains and the compound, at the dose corresponding to the EC_{90} values, were mixed and incubated for 2 hours at either 4 or 37°C. As reported in Figure 9S, Supporting Information, no inhibition by the isolated ellagic acid was observed under any experimental conditions either for MR766 or HPF2013, thus excluding the possibility that the direct inactivation of extracellular virus particles may be a mode of antiviral action. The time-of-addition assay allowed us to investigate the stage of the virus replication cycle at which the compound acts by targeting the cellular surface or intracellular processes. To this aim, the compound was added to the cells at different times of infection only before, during, or after infection. In all of the experiments, DMSO-treated infected cells were used as controls, and plaque-formation inhibition was evaluated. Figure 3C shows that the isolated compound exerted inhibitory activity against the MR766 strain in a dose-response manner, when added 2 hours before infection, with an EC_{50} value of 74.48 μM. By contrast, inhibition was absent in the during-treatment assay, whereas weak inhibition was observed at the higher doses tested in the post-treatment assay. These data were confirmed using the HPF2013 (panel D) strain with inhibitory activity being observed in the pre-treatment assay with a value of 93.01 μM. The ability to inhibit viral infection during the pre-treatment assay was also observed when cells were treated with the commercial compound (data not shown). These data suggest that ellagic acid primarily reduces cell susceptibility to virus infection by tethering to the cell surface.

Our results demonstrate that ellagic acid did not affect the ZIKV infection by directly inactivating the virus particles. The time-of-addition experiments indicated that ellagic acid, added before viral exposure, suppressed viral replication, which suggests that, mechanistically, the compound interferes with the cell surface, likely masking ZIKV receptors, including Axl9, on target cells, prior to viral/cell membrane fusion. Similar ellagic-acid activity was observed against HIV-1. However, in this case, it was also shown to be able to specifically block viral integrase activity [18]. Furthermore, ellagic acid has been observed to have a HPV-preventive effect in clinical trials; women treated with the ellagic acid complex were less likely to be diagnosed with an abnormal Pap smear at 6 months [25].

The absence of activity when the compound was added, with the virus, to the cells indicates that it did not impair the early intracellular steps of viral replication or viral targets. This hypothesis was confirmed by performing a binding assay, and it was shown that a high concentration of isolated compound did not inhibit the binding of either MR766 or HPF2013 to the host-cell surface (Figure 10S, Supporting Information).

Further studies are required to clarify whether the anti-ZIKV activity of ellagic acid may also occur indirectly via an alteration in the innate response of the infected target cells. In recent years, ellagic acid has gained attention due to its antioxidant, anticancer, antiallergic and anti-inflammatory activities. Its antioxidant properties have been associated with hepatoprotective activity, the attenuation of liver injury during Hepatitis B infection and with therapeutic effects on the survival of influenza-challenged mice, in combination with an antiviral drug and an immunomodulator [26,27]. Our current data suggest that ellagic acid may be a promising candidate for the development of a novel anti-ZIKV compound. Further structural modifications might be needed to improve its selectivity index.

In summary, we have demonstrated, for the first time, that pomegranate leaf extract and its fractions possess anti-ZIKV activity. The lack of a protective vaccine and specific treatment against ZIKV has prompted us to develop safe and effective anti-ZIKV compounds that are also able to prevent infection by impairing the chain of congential transmission. The pomegranate leaf ethanolic extract is characterized by hydrolyzable tannins, flavonoids and triterpenes, its phytochemical pattern is stable and does not depend on geographical conditions or season. Furthermore, no differences were found in the *ITS* and *psbA-trnH* sequences extracted from leaves collected in different sites. Moreover, leaf collection is sustainable as it does not cause damage to the plant during spring pruning or in the fall. Ellagic acid was identified, from among the isolated constituents, as an interesting antiviral compound for its inhibitory activity, ability to prevent infection and reduce the transmission of extracellular free virus at high titers. Further work must still be done to elucidate the cellular targets involved in this antiviral action and to assess ellagic acid's clinical potential as a preventive and/or therapeutic compound.

Materials and methods

Plant materials

Punica granatum leaves were collected from different sites in Sardinia, and occasionally other Italian regions, and in Greece from June to October 2017 and 2018 (Table 1S, Supporting Information). Sample 8 was from the botanical garden of the University of Cagliari, Italy. All individuals sampled from other sites were collected randomly. Voucher specimens (Table 1S) were deposited at the Cagliari's botanical garden and at the Department of Drug Science and Technology of the University of Torino. The fresh plant materials were dried at 40°C to constant weight.

Chemicals

LC-MS grade acetonitrile, HPLC-grade methanol, pyridine, *N,O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA), formic acid (>98% purity), ellagic acid, rutin and apigenin were purchased from Merck. De-ionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore)). Luteolin, apigenin 7-*O*-glucoside, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 4'-*O*-glucoside, betulinic acid, oleanolic acid and ursolic acid were obtained from Extrasynthese.

Ethanolic extract preparation

Two extracts were prepared from each sample. 0.500 g of dried and ground powder was extracted using an ultrasonic bath (Soltec, Sonica S3 EP 2400) operating at 40 KHz with 10 mL of ethanol, three times for 10 min each. The supernatants were combined and centrifuged at 5000 rpm for 10 min, poured into a glass balloon and evaporated in a rotary evaporator under vacuum at a temperature below 50°C. In order to reduce chlorophyll interference, 30 mg of crude extract were resuspended in 1 mL of methanol/water (20:80, v/v), loaded onto a Bond Elut Jr 500mg SPE-C18 cartridge (Agilent Technologies), eluted with 8 mL of methanol/water (95:5, v/v) and evaporated in a rotary evaporator. Pool samples, obtained by mixing the leaves that were harvested in summer (PGSum) and autumn (PGAut), were also created and extracted in the same way.

HPLC-PDA-MS/MS analysis

For each extract, a 10 mg/mL stock solution in methanol was prepared, subsequently diluted with acetonitrile/water (95:5, v/v) and filtered through a 13 mm diameter, 0.22 µm PTFE syringe hydrophilic filter before HPLC-PDA-MS/MS analyses. Each extract (5 µL) was analyzed using a Shimadzu Nexera X2 system equipped with a photodiode array detector SPD-M20A that was connected, in series, to a Shimadzu LCMS-8040 triple quadrupole system outfitted with an electrospray ionization (ESI) source (Shimadzu). The chromatographic conditions were: column: Ascentis Express RP-Amide (10 cm × 2.1 mm, 2.7 µm, Supelco); mobile phases: A water/formic acid (999:1, v/v) and B acetonitrile/formic acid (999:1, v/v); flow rate: 0.4 mL/min; column temperature: 30 °C; gradient: 5% B for 5 min, 5–25% B in 35 min, 25–100% B in 10 min, 100% B for 1 min. UV spectra were acquired over the 220-450 nm wavelength range. The mass spectrometer operation conditions and identification criteria were as reported by Marengo *et al.* (2017) [33]. Quantitation was performed using the external standard calibration method via UV (at the λ max for each compound) and Selected Reaction Monitoring (SRM) acquisition in ESI^+ (collision energy: 35.0 V for ESI^+, dwell time: 20). The results are expressed as mg of compound per g of dried leaves (mg/g). When the commercial standard was not available, quantification was based on the UV calibration curves of compounds belonging to the same chemical class. The calibration ranges, λ max, SRM

transitions and analytical performance of the method are reported in Table 5S. Analyses were performed in triplicate. All data were processed using LabSolution software (Shimadzu).

GC-MS analysis

GC analysis were carried out on a Shimadzu 2010 GC unit that was coupled to a Shimadzu QP2010 Mass spectrometer and that made use of a MPS-2 multipurpose sampler (Gerstel). The derivatization of the extracts was performed with bis(trimethylsilyl)trifluoroacetamide to obtain trimethylsilyl derivatives, as reported by Rubiolo *et al.* 2013 [34]. GC-MS analyses were carried out on a MEGA-1 column (100% methylpolysiloxane, 15 m × 0.18 mm d_c, 0.18 µm d_f) from MEGA S.r.l. (Milan, Italy). Analytical conditions: injector temperature: 300°C, transfer line temperature: 320°C, carrier gas: He (0.8 ml/min), split ratio 1:10. Temperature program: 50°C(2min)//5°C/min//300°C. MS conditions: source temperature: 200°C, ionization mode: electron impact (70 eV), scan rate: 0.2 u/s, mass range: 100–650 *m/z*. Compounds were identified via comparisons of mass spectra and linear retention indices (I_s), calculated versus a C9-C25 hydrocarbon mixture, with those reported in the literature. The identity of the triterpenoids was confirmed by the co-injection of commercially available standards. The quantitation of the triterpenoids was performed in SIM-acquisition mode using the external standard calibration method, according to Rubiolo *et al.* 2013 [34] (Table 6S). Data were processed using Shimadzu GCMS Solution software (Shimadzu)

DNA extraction, PCR amplification and sequencing

The DNA extraction, PCR amplification and sequencing of the *ITS* and *psbA-trnH* regions were performed according to Marengo *et al.* (2019) [13] without modifications. Table 4S, Supporting Information reports the list of primers used in PCR and sequencing.

SPE-C18 cartridge and preparative LC (Prep-LC) fractionation

Crude PGSum and PGAut ethanolic extracts were fractionated using a SPE-C18 cartridge: 30 mg of each crude extract were resuspended in 1 mL of methanol/water (20:80, v/v), loaded onto the Bond Elut Jr 500mg SPE-C18 cartridge, first eluted with 5 mL of methanol/water 85:15, v/v (PG85) and subsequently with 5 mL of methanol/water 95:05, v/v (PG95). Both fractions were evaporated to dryness. Fraction PGAut85, at a concentration of 40 mg/mL, was injected into a Shimadzu LC-10AT system to isolate the ellagic acid. Chromatographic conditions: column: Ascentis Express RP-Amide (15cm × 10mm, 5 µm, Supelco) mobile phases: see HPLC-PDA-MS/MS analysis; flow rate: 1 mL/min; column temperature: 30°C; gradient program: 10% B for 1 min, 10–30% B in 60 min, 30–51% B in 9 min, 51–100% B in 1 min, 100% B for 4 min; injection volume: 100 µL. Ellagic acid was collected via multiple injections, the organic solvent was evaporated with a rotary evaporator and the

sample was subsequently freeze-dried. The purity of the isolated ellagic acid was confirmed via a HPLC-PDA-MS/MS analysis at a concentration of 1mg/ml in methanol, and was calculated as a percentage peak area at 254 nm. Isolated and commercial ellagic acid were also characterized by ^{1}H NMR. Spectra were collected in deuterated DMSO using a JEOL ECZR600 (600 MHz) nuclear magnetic resonance (NMR) spectrometer.

Cell cultures

African green monkey fibroblastoid kidney cells (Vero cells, ATCC CCL-81) were grown as monolayers in Eagle's Minimum Essential Medium (MEM) (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic solution (Penicillin-Streptomycin, Sigma-Aldrich) in a humidified 5% CO_2 atmosphere at 37° C. The antiviral assays, against ZIKV and HSV-2, were performed on Vero cells. BHK-21 cells (ATCC CCL-10) were grown in DMEM 10% FBS, and used for antiviral assays against the Vaccinia virus (VACV). The embryonic human kidney cells (293T) (ATCC CRL-3216) were grown as monolayer in DMEM 10% FBS supplemented with 1% Glutamax-I (Invitrogen).

Viruses

Two ZIKV strains were used to investigate the antiviral potential of pomegranate: the 1947 Uganda MR766, and the 2013 French Polynesia HPF2013, representing the African and the Asian lineages respectively. The viruses were produced via the transfection of 293T cells with two plasmids (pCDNA6.2 Zika MR766 Intron3115 HDVr MEG 070916 5 and pCDNA6.2 Zika HPF2013 3864,9388Intron HDVr MEG091316 2) kindly provided by Prof. F. Di Cunto and Prof. M. J. Evans, and were propagated and titred in Vero cells, as described in Francese *et al.*, 2019 [23]. The HSV-2 strain (ATCC VR-540) was propagated, collected and titrated, via plaque assay, on Vero cells [28]. The Vaccinia virus (VACV, ATCC VR-1354) strain was propagated, collected and titrated, via plaque assay, on BHK-21 cells.

Viability assay

Cell viability was assessed using the MTS assay, as described in Donalisio *et al.*, 2016 [29]. PGL8, PGAUT85 and PGEST85 were re-suspended in a DMSO/H_2O solution (50%/50%), at 10 mg/mL concentration; PGAUT95 and PGEST95 were re-suspended in a DMSO/H_2O solution (87.5%/12.5%) at 2.5 mg/mL concentration; ellagic acid was re-suspended in DMSO at 10 mg/mL concentration. The effects of the extract, fractions and ellagic acid on Vero-cell viability were evaluated at 24 and 72 hours. 50%-cytotoxic concentrations (CC_{50}) and 95% confidence intervals (C.I.) were determined using Prism 5 software (Graph-Pad Software).

Inhibition assays

The anti-ZIKV activity of the extract, fractions and ellagic acid was determined using a viral plaque reduction assay on Vero cells as described in Francese *et al.*, 2019 [23]. For the HSV-2 and VACV plaque reduction assays, the cells were infected with virus at MOI 0.001 and 0.006 PFU/cell, respectively, the cells were fixed and the plaques were counted at 24 (HSV-2) and 72 hours (VACV) post infection. PGL8, PGAUT85 and PGEST85 were re-suspended in a DMSO/H_2O solution (50%/50%) at 10 mg/mL concentration; PGAUT95 and PGEST95 were re-suspended in a DMSO/H_2O solution (87.5%/12.5%) at 2.5 mg/mL concentration. Betulinic acid, oleanolic acid, ursolic acid and apigenin were re-suspended in a DMSO/H_2O solution (70%/30%) at 7 mg/mL concentration; apigenin 7-O-glucoside, luteolin, luteolin 4-O-glucoside, luteolin 7-O-glucoside, quercetin 3-O-glucoside, rutin and ellagic acid were re-suspended in DMSO at 10 mg/mL concentration. The concentrations that reduced viral infectivity by 50% (half maximal effective concentration, EC_{50}) and concentrations that reduced viral infectivity by 90% (effective concentration-90, EC_{90}) were calculated using the software Prism. The results are reported for three independent experiments. The selectivity index (SI) was calculated as the ratio CC_{50} / EC_{50}.

Virus inactivation assay

Ellagic acid preparations were investigated for their ability to directly inactivate ZIKV particles at 4°C and 37 °C [23]. Ellagic acid was re-suspended in DMSO at 10 mg/mL concentration.

Time-of-addition assays

Serial dilutions of ellagic acid were either added to Vero cells before infection for 2 hours at 37 °C, during infection with MR766 (MOI 0.005), or after the infection [28]. Ellagic acid was re-suspended in DMSO at 10 mg/mL concentration.

Immunofluorescence assay

Vero cells that were seeded on coverslips were treated with serial doses of plant-isolated ellagic acid for 2 hours prior to infection, for two hours during infection with MR766 (MOI 1) and for 30 hours after the absorption process, at 37 °C. The experiment was performed as described in Francese *et al.*, (2019) [23], with the exception of the nucleic staining, which was performed using 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) 0.5 µg/mL for 10 minutes at room temperature. Ellagic acid was re-suspended in DMSO at 10 mg/mL concentration.

Virus yield reduction assay

The experiment was conducted as described in Francese *et al.* (2019) [23]. Ellagic acid was re-suspended in DMSO at 10 mg/mL concentration.

Binding assay

The experiment was conducted as described in Francese *et al.* (2019) [23]. Ellagic acid was re-suspended in DMSO at 10 mg/mL concentration.

Statistical analysis

Antiviral data were analyzed using the Student's t-test and F-test on GraphPad Prism version 5.00 software, as appropriate. The Student's t-test was used to compare viral titers in virus inactivation assays. Significance was reported for p-value <0.05. Principal Component Analysis was carried out using Statistica 10 (StatSoft. Inc.) software. Sequence quality and alignment were performed as reported by Marengo *et al.* (2019) [13].

Supporting Information

HPLC-PDA-MS/MS analysis conditions, GC-MS analysis conditions, SPE-C18 cartridge and preparative LC (Prep-LC) fractionation conditions, Anti-ZIKV activity of a reference pomegranate leaf ethanolic extract PGL8 (Fig. 1S), Cell viability assays (Fig. 2S), LC chromatograms of the pomegranate leaf extract PGL8 (Fig. 3S), GC-MS chromatograms of the pomegranate leaf extract PGL8 (Fig. 4S), Comparison of *ITS* sequences between the eleven *Punica granatum* samples (Fig. 5S), Comparison of *psbA-trnH* sequences between the eleven *Punica granatum* samples (Fig. 6S), Anti- MR766 activity of PGSum85, PGSum95, PGAut85 and PGAut95 (Fig. 7S), ^{1}H 600 MHz NMR of ellagic acid isolated with LC-Prep and of the commercial standard reference (Fig. 8S), Inactivation of MR766 particles by isolated ellagic acid (Fig. 9S), Effect of isolated ellagic acid on virus binding to cells (Fig. 10S), Location, coordinates, and code of pomegranate leaf samples (Table 1S), Concentration of phenolic compounds and triterpenes in the different pomegranate leaf extracts and in the fractions (Table 2S), List of the sequences obtained from *P. granatum* samples deposited in GenBank (table 3S), List of primers used in PCR and sequencing (Table 4S), Quantification method, linearity range, R^2 and calibration curve of the main compounds by UV and SRM methods (Table 5S) and Target ion, qualifier ions, linearity range, R^2 and calibration curve of triterpenoids quantified by GC-MS (Table 6S) are available as Supporting Information.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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555 **Figure legend**

556 **Figure 1.** Score plot (A) and loading plot (B) of the principal component analysis relative to the
557 quantity of the main markers of the pomegranate's leaf extracts.

558 **Figure 2.** Panels A and B. Plaque reduction assays infecting cells with MR766 (panel A) and
559 HPF2013 (panel B) in the presence of isolated and commercial ellagic acid. Vero cells were treated
560 with ellagic acid prior to infection, during the infection period, and after the infection for 72 hours.
561 Results are reported as percentage of infection in comparison to untreated controls (Y-axis); the
562 concentrations tested are reported on X-axis. Error bars represent the standard error of the mean for
563 three independent experiments. Panel C and D. Representative MR766 foci (panel C) and HPF2013
564 foci (panel D) in Vero cells by immunofluorescence assay. Vero cells were treated with ellagic acid
565 before, during, and after the infection. The ZIKV protein E is visualized in red, nuclei in blue. NI, not
566 infected; UT, untreated. Scale bar, 20 μm. Ellagic acid was resuspended in a DMSO solution.

567 **Figure 3.** Panel A and B. Virus yield reduction assays. Effects of isolated ellagic acid on multiple
568 cycles of MR766 (panel A) and HPF2013 (panel B) replication. Viral titres are expressed as PFU/mL.
569 Error bars represent the standard error of the mean for three independent experiments ($p<0.05$). Panel
570 C and D. Time of addition assays with isolated ellagic acid. Vero cells were treated with compound
571 prior to infection (pre-treatment), during the infection period (during infection) or after infection
572 (post-treatment) with MR766 (panel C) and HPF 2013 (panel D). Data are reported as percentage of
573 infection in comparison to untreated control. Error bars represent the standard error of the means for
574 three independent experiments. Ellagic acid was resuspended in a DMSO solution.

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576 **Table 1.** Anti-ZIKV-MR766 activity of the pomegranate leaf ethanolic extract (PGL8)

Virus	Sample	EC_{50}[a] (µg/mL) (95% CI[b])	EC_{90}[c] (µg/mL) (95% CI)	CC_{50}[d] (µg/mL) (95% CI)	CC_{90}[e] (µg/mL) (95% CI)	SI[f]
MR766	PGL8	11.40 (7.84-16.57)	75.32 (25.7-113.3)	123.6 (104.0-146.7)	443 (313.3-559.2)	10.84
HSV-2	PGL8	3.29 (1.64-6.56)	120.2 (18.5-224.1)	154.9 (112.3-213.7)	1633 (860-2023.3)	47.08
Vaccinia virus	PGL8	n.a.[g]	n.a	-	-	n.a.
	Fractions	**EC_{50} (µg/mL) (95% CI)**	**EC_{90} (µg/mL) (95% CI)**	**CC_{50} (µg/mL) (95% CI)**	**CC_{90} (µg/mL) (95% CI)**	**SI**
MR766	PGSum85	16.20 (12.4-21.2)	95.27 (45.5-129.5)	76.1 (48.4-120)	143 (74.5-184.4)	4.69
	PGAut85	10.4 (7.08-15.2)	43.96 (17.3-111.7)	73.4 (51.6-104)	156 (88.3-216.2)	7.05
	PGSum95	n.a.	n.a.	14.2 (12.3-16.4)	32.2 (22.3-46.6)	-
	PGAut95	n.a.	n.a.	17.5 (11.9-25.8)	37.2 (18-76.6)	-

577 [a]EC_{50}: half maximal effective concentration; [b]CI: confidence interval; [c]EC_{90}: 90% effective concentration; [d]CC_{50}: half
578 maximal cytotoxic concentration; [e]CC_{90}: 90% cytotoxic concentration; [f]SI: selectivity index; [g]n.a.: not assessable.

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Table 2. List of identified and putatively identified compounds in leaf extract. Each compound is referred through its relative retention time, UV maxima λ absorption, molecular formula, pseudomolecular ions (ESI^+ and ESI^-), ion fragments generated by Product Ion Scan mode (PIS) and identified or tentatively identified compound names. The identification confidence value and the literature reference that indicates the presence of the compounds in pomegranate are also reported.

N°	RT	λmax (nm)	Molecular formula	$[M+H]^+$	$[M-H]^-$	Supp. MW	MS^{2+} *m/z*	MS^{2-} *m/z*	Compound name	Identif. conf.[b]	Ref.
1[a]	1.649	272	$C_7H_6O_5$	/	169	170		125 110	Gallic acid	1	[30]
2	7.373	314	$C_{15}H_{18}O_8$	/	325	326		163 145	Coumaric acid hexoside	2	[31]
3	11.657	273 357	$C_{13}H_8O_8$	/	291	292		247	Brevifolin – carboxyl acid	2	[31]
4	14.364	268		801 151	799	800	151	301	Ellagic derivative	2	[30]
5	19.032	270 350		611	935 655 609	610	151	301 137	Ellagitannin	3	
6	19.420	251 360	$C_{20}H_{16}O_{13}$	465	463	464	/	301	Ellagic acid glucoside	2	[30]
7	24.091	274 365	$C_{41}H_{28}O_{27}$	953	951	952	/	933 301	Galloyl-HHDP-DHHDP-hexoside (Granatin B)	2	[30]
8[a]	24.366	253 347	$C_{21}H_{20}O_{11}$	449	447	448	287	285	Luteolin 7-*O*-glucoside	1	[31]
9[a]	25.994	252 366	$C_{14}H_6O_8$		301	302	/	284 229 185	Ellagic acid	1	[30]
9a[a]	25.994		$C_{27}H_{30}O_{16}$	611	609	610			Rutin	1	[30]
10[a]	27.426	255 353	$C_{21}H_{20}O_{12}$	465	463	464	303 229 153	301 255 151	Quercetin 3-*O*-glucoside	1	[30]
11[a]	28.885	266 336	$C_{21}H_{20}O_{10}$	433	431	432	271	269	Apigenin 7-*O*-glucoside	1	[31]

12	30.971	268 332		433	431	432	271	269 195 151 117	Apigenin glycoside	3	
13 [a]	31.719	267 337	$C_{21}H_{20}O_{11}$	449	447	448	287	285 257	Luteolin 4'-*O*-glucoside	1	[30]
14	33.223	268 340		449	447	448	287 153	285 151	Luteolin glycoside 1	3	
15	37.253	268 340		419	417	418	287 153	285 257 175 151	Luteolin glycoside 2	3	
16 [a]	42.958	252 347	$C_{15}H_{10}O_6$	287	285	286	153 135 117	171 151 133 115	Luteolin	1	[30]
17 [a]	45.538	267 336	$C_{15}H_{10}O_5$	271	269	270	163 153 119	151 117	Apigenin	1	[31]
*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Oleanolic acid	1	[31]
*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Betulinic acid	1	[31]
*	50.201	-	$C_{30}H_{48}O_3$	457	455	456	333 239 191 189	407	Ursolic acid	1	[31]

a Compounds identified by comparing with reference standards.

b An Identification Confidence according to the request of the Chemical Analysis Working Group (CAWG, 2007) [32] is indicated: Level 1:Identified compound (A minimum of two independent orthogonal data (such as retention time and mass spectrum) compared directly to an authentic reference standard; Level 2:Putatively annotated compound (Compound identified by analysis of spectral data and similarity to bibliographic data); Level 3: Putatively characterised class compound; Level 4: unknown compound.

Table 3. Anti-ZIKV-MR766 activity of phenolic and triterpenic compounds. For each concentration tested, the percentage of infection in comparison to control is reported as mean value ± SD. The molarities of the compounds is reported in square brackets, referred to 3.7 µg/mL, 11 µg/mL and 33 µg/mL concentrations, respectively.

Compounds	3.7 µg/mL	11 µg/mL	33 µg/mL
Apigenin	99.0 ± 7.1 [13.7 µM]	n.a.[a] [40.7 µM]	n.a. [122.1 µM]
Apigenin 7-O-glucoside	103.6 ± 8.9 [8.5 µM]	101.2 ± 3.7 [25.4 µM]	102.5 ± 2.8 [76.2 µM]
Betulinic acid	99.3 ± 1.1 [8.1 µM]	101.5 ± 6.4 [24.1 µM]	95.5 ± 10.7 [72.3 µM]
Luteolin	102.9 ± 4.1 [13 µM]	n.a. [38.5 µM]	n.a. [115.5 µM]
Luteolin 4-O-glucoside	103.0 ± 1.4 [8.3 µM]	96.0 ± 5.7 [24.5 µM]	102.8 ± 8.8 [73.6 µM]
Luteolin 7-O-glucoside	105.0 ± 5.7 [8.3 µM]	102.0 ± 11.3 [24.5 µM]	100.0 ± 3.3 [73.6 µM]
Oleanolic acid	99.5 ± 9.2 [8.1 µM]	103.8 ± 3.2 [24.1 µM]	104.3 ± 6.7 [72.3 µM]
Quercetin 3-O-glucoside	105.5 ± 6.4 [8.0 µM]	103.1 ± 9.8 [23.8 µM]	106.5 ± 4.6 [71.3 µM]
Rutin	103.3 ± 9.5 [6.1 µM]	95.4 ± 20.6 [18.0 µM]	107 ± 2.4 [54.1 µM]
Ursolic acid	100.0 ± 11.9 [8.1 µM]	79.7 ± 2.3 [24.1 µM]	n.a. [72.3 µM]
Ellagic acid	83.8 ± 11.5 [12.2 µM]	43.3 ± 1.3 [36.4 µM]	0.0 ± 0.8 [109.2 µM]

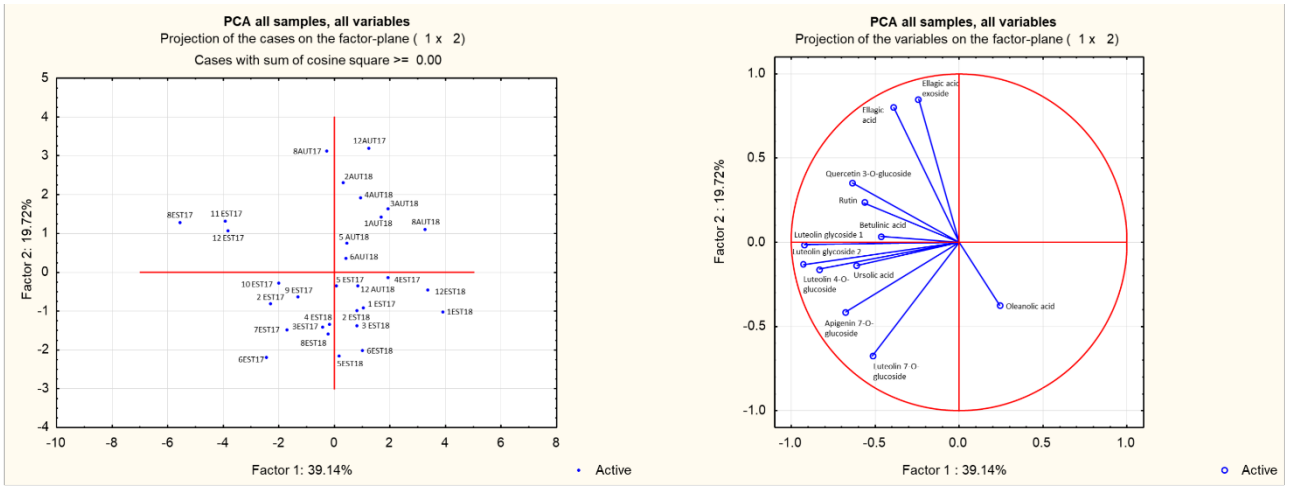
[a]n.a., not assessable

Betulinic acid, oleanolic acid, ursolic acid, apigenin were resuspended in a DMSO/H_2O solution (70%/30%); apigenin 7-O-glucoside, luteolin, luteolin 4-O-glucoside, luteolin 7-O-glucoside, quercetin 3-O-glucoside, rutin and ellagic acid were were resuspended in a DMSO solution.

Table 4. Anti-ZIKV activity of ellagic acid

	Virus	EC_{50}[a] (μM) (95% CI[b])	EC_{90}[c] (μM) (95% CI[b])	CC_{50}[d] (μM)	SI[e]
Commercial ellagic acid	MR766	36.22 (28.91-45.37)	93.05 (53.17-162.8)	496.5	13.7
	HPF2013	20.99 (16.48-26.74)	53.23 (31.11-91.09)	496.5	23.7
Isolated ellagic acid	MR766	30.86 (26.02-36.6)	42.64 (33.98-53.51)	446.85	14.5
	HPF2013	46.23 (37.88-56.41)	141.2 (85.90-232.0)	446.85	9.7

[a]EC_{50}: half maximal effective concentration; [b]CI: confidence interval; [c]EC_{90}: 90% effective concentration; [d]CC_{50}: half maximal cytotoxic concentration; [e]SI: selectivity index. Ellagic acid was resuspended in a DMSO solution.



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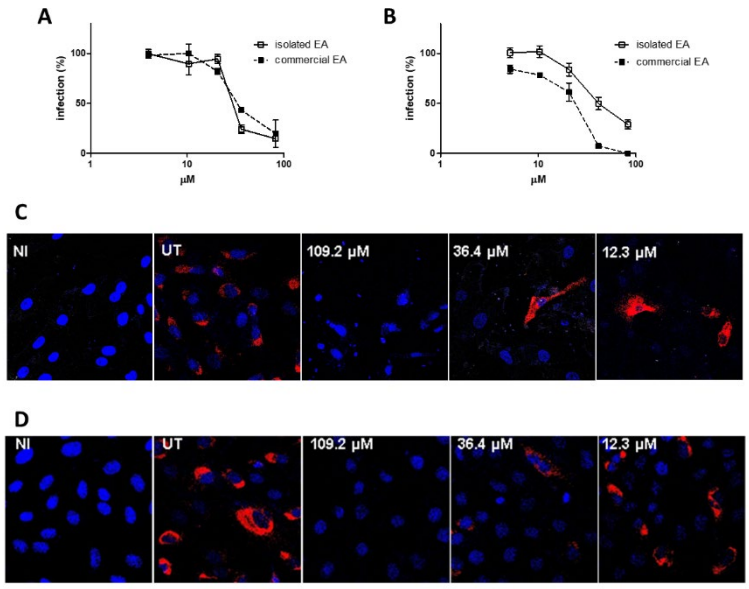

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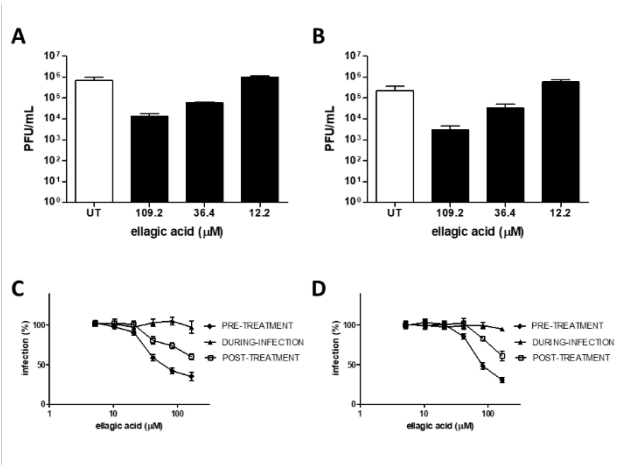

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