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

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

**ABSTRACT**
Zika virus, an arthropod-borne flavivirus, is an emerging healthcare threat worldwide. Zika virus 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-Zika phytochemicals. This study deals with the investigation of the composition, cytotoxicity, and anti-Zika activity of *Punica granatum* leaf ethanolic extract, fractions, and phytoconstituents. *P. 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 Zika virus strains in vitro. Both the extract and its fractions were found to be active against Zika virus infection. Of the compounds isolated, ellagic acid showed particular anti-Zika activities, with EC50 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 Zika virus infection and was able to significantly reduce Zika virus progeny production. Our data demonstrate the anti-Zika activity of pomegranate leaf extract and ellagic acid for the first time. These findings identify ellagic acid as a possible anti-Zika candidate compound that can be used for preventive and therapeutic interventions.

* These authors contributed equally to this work.
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 antioxidant, 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 HSV-2, HIV-1, and the influenza virus [8–10]. Little information has been reported on pomegranate leaf extracts, compared to other edible and nonedible 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, anticholinesterase, and antiproliferative 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 Fig. 15, 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 EC50 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 h at 37°C, and the cellular viability was then determined by MTS assay. The CC50 values were above 100 μg/ml, indicating that the antiviral activity observed was not due to cytotoxicity (►Table 1, Fig. 25, Supporting Information). Since the extract was resu-
pended in a DMSO/H₂O solution (50%/50%), a control sample with equal volumes of DMSO/H₂O was included in all cell-culture experiments in order to rule out the possibility of the solvent having a cytotoxic effect. The 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 to 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 the 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 3 different chemical classes of specialized metabolites were revealed: phenolics, flavonoids, and triterpenoids. A list of the identified and putatively identified compounds in the extract and often exist as glycosides of luteolin, apigenin, and quercetin. Ellagic acid is the most abundant compound, while hydroxytyrosol 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-HCHO-H₂O-H⁻), and of a pseudomolecular ion at m/z 457, in negative ionization mode, in the LC-MS profiles indicates the presence of triterpenoid molecules, but with evident coelution. A GC-MS analytical platform was therefore used and enabled oleanolic, betulinic, and ursolic acids to be identified after their derivatization in the extract.

To ensure consistent quality and reproducibility activity in the pomegranate leaf extracts, genotypic and phenotypic stability were evaluated by comparing the phytochemical and biomolecular patterns of leaves that belonged to plants of different origins (see Table 1S, Supporting Information) that were harvested in different vegetative periods (summer and autumn) and in different years (2017 and 2018).

HPLC-PDA-MS/MS and GC-MS profiles were qualitatively consistent, and all markers were detected in all of the samples. Quantitation results, reported in Table 2S, Supporting Information, showed differences in the abundances of some compounds in the leaf extracts. The repeatability results showed that RSD% never exceeded 5%, while intermediate precision in the different extracts showed RSD% of below 15%. The accuracy of the data was determined by comparing, when available, the UV and MS quantification results, and the RSD% never exceeded 20%. Principal component analysis was then applied to highlight similarities and differences between the samples. Fig. 1 reports the score and loading plots of the PC1 against the PC2, showing homogeneous sample distribution in the score plot (no cluster of samples is formed) and a good explained variance (39.14% for PC1 and 19.72% for PC2). No clear discrimination between the samples was observed, although the Aut show slightly higher contents of ellagic acid and its hexoside (both positively correlated with PC2, 19.72% for PC2). No clear discrimination between the samples was observed, although the Aut show slightly higher contents of ellagic acid and its hexoside (both positively correlated with PC2, as can be seen in the loading plot). In general, the few differences in the phenolics and triterpenes can be ascribed to phenotypical variability and environmental factors.

To further confirm the quality and reproducibility of the pomegranate leaf extracts, a genotypic fingerprint of the collected leaves was obtained using a DNA barcoding approach [16]. The nuclear ITS region and the psbA-trnH genes were amplified and sequenced for each site from which pomegranate leaf samples were harvested. The sequences were deposited in the GenBank (Table 3S, Supporting Information) and compared to those present in the database (59 P. granatum ITS sequences originating from India, Iran, and China and 27 P. granatum psbA-trnH sequences from Iran, Tunisia, China, and Italy [Apulia, Latium, Sardinia, Padua and Trieste]).

Figs. 5S and 6S, Supporting Information, report no variation in the ITS and psbA-trnH nucleotide composition for the 11 sites, suggesting that these biomolecular markers are stable. A consensus sequence for each DNA region was obtained from the align-
Table 2. List of identified and putatively identified compounds in leaf extract. Each compound is referred through its relative retention time, UV maxima $\lambda$ 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.

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<th>$[M – H]^–$ MS$^2+$ m/z</th>
<th>$[M – H]^–$ MS$^3+$ m/z</th>
<th>MS$^2–$ m/z</th>
<th>Compound name</th>
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<td>800 151</td>
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<td>271 269 270 163 153 119</td>
<td>151 117</td>
<td>Apigenin</td>
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<td>C$<em>{30}$H$</em>{48}$O$_3$</td>
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* Continued
For Table 2 Continued

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<th>RT (min)</th>
<th>Amax (nm)</th>
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<th>[M – H]*</th>
<th>Supp. MW</th>
<th>MS* m/z</th>
<th>MS* m/z</th>
<th>Compound name</th>
<th>Identif. conf.</th>
<th>Ref.</th>
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<td>189</td>
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</tr>
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</table>

* Compounds identified by comparing with reference standards; \(^\text{b}\) An identification confidence according to the request of the Chemical Analysis Working Group (CAWG, 2007) \(^{[15]}\) is indicated: Level 1: Identified compound (A minimum of 2 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 characterized class compound; Level 4: unknown compound.

**Fig. 1** Score plot (a) and loading plot (b) of the principal component analysis relative to the quantity of the main markers of the pomegranate’s leaf extracts.
Table 3 Anti-ZIKV 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.

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

Betulinic acid, oleanolic acid, apigenin were resuspended in a DMSO/H₂O 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 re-suspended in a DMSO solution. n. a.: not assessable.

Table 4 Anti-ZIKV activity of ellagic acid.

<table>
<thead>
<tr>
<th>Virus</th>
<th>EC₅₀ a (µM) (95% CI b)</th>
<th>EC₉₀ c (µM) (95% CI b)</th>
<th>CC₅₀ d (µM)</th>
<th>SI e</th>
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</thead>
<tbody>
<tr>
<td>Commercial</td>
<td></td>
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<tr>
<td>ellagic acid</td>
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<td></td>
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</tr>
<tr>
<td>MR766</td>
<td>36.22 (28.91–45.37)</td>
<td>93.05 (53.17–162.8)</td>
<td>496.5</td>
<td>13.7</td>
</tr>
<tr>
<td>HPF2013</td>
<td>20.99 (16.48–26.74)</td>
<td>53.23 (31.11–91.09)</td>
<td>496.5</td>
<td>23.7</td>
</tr>
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<tr>
<td>ellagic acid</td>
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<tr>
<td>MR766</td>
<td>30.86 (26.02–36.6)</td>
<td>42.64 (33.98–53.51)</td>
<td>446.85</td>
<td>14.5</td>
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<tr>
<td>HPF2013</td>
<td>46.23 (37.88–56.41)</td>
<td>141.2 (85.90–232.0)</td>
<td>446.85</td>
<td>9.7</td>
</tr>
</tbody>
</table>

* EC₅₀: half maximal effective concentration; b CI: confidence interval; c EC₉₀: 90% effective concentration; d CC₅₀: half maximal cytotoxic concentration; e SI: selectivity index. Ellagic acid was resuspended in a DMSO solution.

As reported in Table 1 and Fig. 7S, Supporting Information, both the PAGaut85 and PGSum85 fractions were active against ZIKV at increasing doses with EC₅₀ values of 10.40 and 16.20 µg/mL, respectively. However, no PG95 fractions exerted antiviral activity. No statistical differences were observed in the EC₅₀ 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 3 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 inhibitory activity of luteolin, apigenin, and rutin as inhibitors of the ZIKV NS2B-NS3 protease, as identified by molecular docking [17, 18]. Furthermore, the ability of quercetin 3-O-glucoside to inhibit ZIKV in vitro, as reported in the literature, was not reproduced [19]. 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 1H NMR, and its spectrum compared with that of the commercial standard (Fig. 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 EC₅₀ values. High inhibitory activity was observed with an EC₅₀ value of 30.86 µM (Table 4, Fig. 2a). It is worth noting that the compound was also active against the Asian lineage strain, 2013 French Polynesia HPF2013, with an EC₅₀ 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 EC₅₀ values (Table 4 and Fig. 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 Figs. 2c and d, 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, Ebola virus, hepatitis C virus, and HIV-1 [20–23]. Furthermore, ellagic acid has revealed potential activity against HBV infection due to its hepatoprotective properties and ability to effectively block HBeAg secretion in cells [24]. 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 antiflaviviral effects [25]. Our data have demonstrated that the isolated ellagic acid inhibited, in vitro, the infection of 2 lineages, the African one, which is responsible for more acute infection, and Asian ZIKV, which is associated with neurological impairments [26]. Interestingly, the compound also exerted adulticidal activity against *Aedes aegypti* mosquito, the main vector of the virus [27].

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 ▶ Fig. 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, 10⁵ pfu of the ZIKV strains and the compound, at the dose corresponding to the EC₉₀ values, were mixed and incubated for 2 h at either 4 or 37°C. As reported in Fig. 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 di-

▶ Fig. 2. Panels a and b. Plaque reduction assays infecting cells with MR766 (panel a) and HPF2013 (panel b) in the presence of isolated and commercial ellagic acid. Vero cells were treated with ellagic acid prior to infection, during the infection period, and after the infection for 72 h. Results are reported as percentage of infection in comparison to untreated controls (Y-axis); the concentrations tested are reported on X-axis. Error bars represent the standard error of the mean for 3 independent experiments. Panel c and d. Representative MR766 foci (panel c) and HPF2013 foci (panel d) in Vero cells by immunofluorescence assay. Vero cells were treated with ellagic acid before, during, and after the infection. The ZIKV protein E is visualized in red, nuclei in blue. NI: not infected; UT: untreated. Scale bar, 20 µm. Ellagic acid was resuspended in a DMSO solution.
rect 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. ▶ Fig. 3 c shows that the isolated compound exerted inhibitory activity against the MR766 strain in a dose-response manner, when added 2 h before infection, with an EC50 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 [21]. 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 [28].

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 (Fig. 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, anti-allergic, 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 [29, 30].

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 congenital 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, and its 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

*P. 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, Supporting Information) were deposited at the Cagliari’s botanical garden and at the Department of Drug Science and Technology of the University of Turin. The fresh plant materials were dried at 40 °C to constant weight.

Chemicals

LC-MS grade acetonitrile, HPLC-grade methanol, pyridine, 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, 3 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 500 mg 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 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. [31]. Quantitation was performed using the external standard calibration method via UV (at the λ max for each compound) and 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, Supporting Information. 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 Rubiol et al. [32]. GC-MS analyses were carried out on a MEGA-1 column (100% methylpolysiloxane, 15 m × 0.18 mm, 0.18 µm df) 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 (2 min)/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 *I*ₙ 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 Rubiol et al. [32] (Table 6S, Supporting Information). Data were processed using Shimadzu GCMS Solution software (Shimadzu).
DNA extraction, PCR amplification and sequencing
The DNA extraction, PCR amplification, and sequencing of the rITS and psbA-trnH regions were performed according to Marengo et al. [16] without modifications. Table 4S, Supporting Information, reports the list of primers used in PCR and sequencing.

SPE-C18 cartridge and Prep-LC fractionation
Crude PGSum and PAGut 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 500 mg 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 PAGut85, 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 (15 cm × 10 mm, 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 1 mg/ml in methanol and was calculated as a percentage peak area at 254 nm. Isolated and commercial ellagic acid were also characterized by 1H 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 MEM (Sigma-Aldrich) with 10% heat-inactivated FBS (Sigma-Aldrich) and 1% antibiotic solution (penicillin-streptomycin, Sigma-Aldrich) in a humidified 5% CO2 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 VACV. The embryonic human kidney cells (293 T) (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 MR766 and were propagated, collected and titrated during infection with MR766 (MOI 1), and for 30 h after the absorption process, at 37 °C. The experiment was performed as described in Francese et al. [26], with the exception of the nucleic staining, which was performed using DAPI (Sigma-Aldrich) 0.5 µg/ml for 10 min at room temperature. Ellagic acid was resuspended in DMSO at 10 mg/mL concentration.

Viability assay
Cell viability was assessed using the MTS assay, as described in Donalisio et al. [34]. PGL8, PAGut85, and PGSum85 were resuspended in a DMSO/H2O solution (50%/50%), at 10 mg/mL concentration; PAGut95 and PGSum95 were resuspended in a DMSO/H2O solution (87.5%/12.5%) at 2.5 mg/mL concentration; ellagic acid was resuspended in DMSO at 10 mg/mL concentration. The effects of the extract, fractions, and ellagic acid on Vero cells viability were evaluated at 24 and 72 h. CC50 and 95% CI 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. [26]. 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 h (VACV) post infection. PGL8, PAGut85, and PGSum85 were resuspended in a DMSO/H2O solution (50%/50%) at 10 mg/mL concentration; PAGut95 and PGSum95 were resuspended in a DMSO/H2O solution (87.5%/12.5%) at 2.5 mg/mL concentration. Betulinic acid, oleandric acid, ursolic acid, and apigenin were resuspended in a DMSO/H2O 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 resuspended in DMSO at 10 mg/mL concentration. The concentrations that reduced viral infectivity by 50% (half maximal effective concentration, EC50) and concentrations that reduced viral infectivity by 90% (effective concentration-90, EC90) were calculated using the software Prism. The results are reported for 3 independent experiments. The selectivity index (SI) was calculated as the ratio CC50/EC50.

Virus inactivation assay
Ellagic acid preparations were investigated for their ability to directly inactivate ZIKV particles at 4 °C and 37 °C [26]. Ellagic acid was resuspended 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 h at 37 °C, during infection with MR766 (MOI 0.005), or after the infection [33]. Ellagic acid was resuspended 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 h prior to infection, for 2 h during infection with MR766 (MOI 1), and for 30 h after the absorption process, at 37 °C. The experiment was performed as described in Francese et al. [26], with the exception of the nucleic staining, which was performed using DAPI (Sigma-Aldrich) 0.5 µg/ml for 10 min at room temperature. Ellagic acid was resuspended in DMSO at 10 mg/mL concentration.
Virus yield reduction assay

The experiment was conducted as described in Francese et al. [26]. Ellagic acid was resuspended in DMSO at 10 mg/mL concentration.

Binding assay

The experiment was conducted as described in Francese et al. [26]. Ellagic acid was resuspended 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. [16].

Supporting Information

Anti-ZIKV activity of a reference pomegranate leaf ethanolic extract PGL8 (Fig. 15); cell viability assays (Fig. 25); LC chromatograms of the pomegranate leaf extract PGL8 (Fig. 35); GC-MS chromatograms of the pomegranate leaf extract PGL8 (Fig. 45); comparison of ITS sequences between the 11 P. granatum samples (Fig. 55); comparison of psbA-trnH sequences between the 11 P. granatum samples (Fig. 65); anti-MR766 activity of PGSum85, PGSum95, PGAut85, and PGAut95 (Fig. 75); 'H 600 MHz NMR of ellagic acid isolated with LC-Prep and of the commercial standard reference (Fig. 85); inactivation of MR766 particles by isolated ellagic acid (Fig. 95); effect of isolated ellagic acid on virus binding to cells (Fig. 105); location, coordinates, and code of pomegranate leaf samples (Table 15); concentration of phenolic compounds and triterpenes in the different pomegranate leaf extracts and in the fractions (Table 25); list of the sequences obtained from P. granatum samples deposited in GenBank (Table 35); list of primers used in PCR and sequencing (Table 45); quantification method, linearity range, R², and calibration curve of the main compounds by UV and SRM methods (Table 55); and target ion, qualifier ions, linearity range, R², and calibration curve of triterpenoids quantified by GC-MS (Table 65) are available as Supporting Information.

Contributors’ Statement

Conception and design: C. Sanna; C. Bertea; C. Cagliero; B. Sgorbini; D. Lembo; M. Donalisio; P. Rubiolo. Data collection: C. Sanna; S. Acquardo; A. Civa; A. Marengo; R. Francese; M. Rittà. Analysis and interpretation: S. Acquardo; A. Civa; A. Marengo; R. Francese; M. Rittà; C. Cagliero; C. Sanna; C. Bertea; B. Sgorbini; D. Lembo; M. Donalisio; P. Rubiolo. Statistical analysis: S. Acquardo; A. Civa; A. Marengo; R. Francese; M. Rittà; C. Cagliero; C. Bertea; B. Sgorbini; D. Lembo; M. Donalisio; P. Rubiolo. Drafting of the manuscript: S. Acquardo; A. Civa; A. Marengo; R. Francese; M. Rittà. Critical revision of the manuscript: C. Sanna; C. Bertea; C. Cagliero; B. Sgorbini; D. Lembo; M. Donalisio; P. Rubiolo.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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