1 **Inhibitory Effects of Pycnogenol® on Hepatitis C Virus Replication**

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7 **List of abbreviations**

8 CC₅₀, 50% cytotoxic concentration; CI, combination index; DAA, direct acting

9 antivirals; EC₅₀, 50% effective concentration; HCV, hepatitis C virus; IC₅₀, 50%

10 inhibitory concentration; IRES, Internal Ribosome Entry Site; NS, nonstructural

11 protein; NF- κ B, nuclear factor- κ B; PC, procyanidin; PEG-IFN- α -2a,

12 pegylated interferon- α -2a; PEG-IFN- α -2b, pegylated interferon- α -2b; RBV,

13 ribavirin; PYC, Pycnogenol[®]; qRT-PCR, quantitative real-time reverse transcription

14 polymerase chain reaction; ROS, reactive oxygen species; SI, selectivity index

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1 **Abstract**

2 Chronic hepatitis C virus (HCV) infection increases the risk of liver cirrhosis and
3 hepatocellular carcinoma. In the last decade, the current standard HCV treatment,
4 pegylated interferon and ribavirin, have limited efficacy and significant side effects.
5 Novel direct acting antivirals show promise, but escape mutants are expected, along
6 with potential side effects. Pycnogenol[®], a French maritime pine extract, has been
7 reported to have antioxidant and antiviral effects. Here, we evaluated the effect of
8 Pycnogenol[®] on HCV replication.
9 Wild-type and protease inhibitor (VX-950; telaprevir)-resistant HCV replicon cells were
10 treated with Pycnogenol[®], Pycnogenol[®] and interferon-alpha, and ribavirin and
11 telaprevir. Pycnogenol[®] effects on replication were also evaluated in HCV-infected
12 chimeric mice.
13 Pycnogenol[®] treatment showed antiviral effects without cytotoxicity at doses up to 50
14 µg/mL. Pycnogenol[®] in combination with interferon-alpha or ribavirin showed
15 synergistic effects. Moreover, Pycnogenol[®] inhibited HCV replication in
16 telaprevir-resistant replicon cells; telaprevir and Pycnogenol[®] acted additively to reduce
17 HCV RNA levels in wild-type HCV replicon cells without significantly increasing
18 cytotoxicity. Pycnogenol[®] antiviral activity was higher than its components procyanidin

1 and taxifolin. Further, treatment of infected chimeric mice with Pycnogenol®
2 suppressed HCV replication and showed a synergistic effect with interferon-alpha. In
3 addition, Pycnogenol® treatment resulted in dose-dependent reduction of reactive
4 oxygen species in HCV replicon cell lines.
5 Pycnogenol® is a natural product that may be used to improve the efficacy of the current
6 standard antiviral agents and even to eliminate resistant HCV mutants.

7 **Keywords:** Natural product, Resistant, ROS, antioxidant, replication

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1 **1. INTRODUCTION**

2 Approximately 130–170 million people are chronically infected, leading to 54,000
3 deaths and 955,000 disability-adjusted life-years associated with acute HCV infection
4 (Mohd Hanafiah et al., 2013). Chronic hepatitis C can lead to a large spectrum of
5 diseases, including steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (Perz and
6 Alter, 2006). To date, no protective vaccine is available for HCV infection; over the last
7 decade, therapy has consisted of a 24–48-week course of peginterferon-alpha-2a
8 (PEG-IFN-alpha-2a) or peginterferon-alpha-2b (PEG-IFN-alpha-2b) in combination
9 with the guanosine analogue, ribavirin (RBV). The therapy leads to sustained virologic
10 response (SVR) in 42–52%, 65–85%, and 76–82% of individuals infected with HCV
11 genotype 1; 4, 5, or 6; and 2 or 3, respectively (Antaki et al., 2010; Hoofnagle and
12 Seeff, 2006). The recently approved non-structural protein (NS) 3/4A protease
13 inhibitors (PIs) boceprevir (approved by the FDA on May 13, 2011) and telaprevir
14 (approved by the FDA on May 23, 2011), used in combination with PEG-IFN-alpha and
15 RBV for HCV genotype 1 infections, have increased cure rates to approximately 70%
16 (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011). However, these
17 triple-therapy regimens may result in unfavourable side effects and emergence of
18 drug-resistant HCV (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011),

1 which may reduce virus susceptibility and applicability of current HCV triple therapies
2 (Ozeki et al., 2011). Recently, two more effective compounds have been approved for
3 HCV treatment: the protease inhibitor simeprevir (approved by the FDA in November,
4 2013) and the nucleotide polymerase inhibitor sofosbuvir (approved by the FDA on
5 December 6, 2013). Among patients infected with HCV, less than 10% are treated and
6 cured, and the major challenge in controlling HCV infections is the identification of
7 those already infected, most of whom are situated in the poorest regions of the world
8 (Thomas, 2013), and to find the most effective, tolerable and affordable direct acting
9 antivirals (DAA) combination that can cure people in the shortest period (Poveda et al.,
10 2014). In the NEUTRINO phase III trial of treatment-naïve patients, 12 weeks of triple
11 combination therapy with sofosbuvir (400 mg) once daily resulted in SVR rates of 89%
12 in patients with HCV genotype 1 (92% for subtype 1a and 82% for subtype 1b), and
13 96% in patients with genotype 4 (Lawitz et al., 2013). Moreover, in the FISSION trial
14 of HCV-2/3 treatment-naïve patients receiving sofosbuvir/RBV for 12 weeks, 95% of
15 patients with genotype 2 and 56% of patients with genotype 3 achieved an SVR (Lawitz
16 et al., 2013). In addition, most DAA agents are characterized by a low genetic barrier to
17 the development of resistance, except sofosbuvir, which showed a very high resistance
18 barrier. This is the reason most current DAA-based therapies under evaluation must be

1 co-administered with either PEG-IFN-alpha and ribavirin or different compounds
2 belonging to different DAA classes (Poveda et al., 2014).

3 Pycnogenol® (PYC; trademark of Horphag Research, Geneva, Switzerland) is a
4 French maritime pine extract produced from the outer bark of *Pinus pinaster* ssp.
5 *atlantica*, and is generally considered safe for human use (American Botanical Council,
6 2010). The main PYC constituents are procyanidins (68.4%), taxifolin (21.87%), ferulic
7 acid (3.70%), catechin (2.53%), and caffeic acid (3.51%) (Lee et al., 2010). PYC has
8 been reported to have antioxidative and anti-inflammatory effects, and to reduce
9 cardiovascular risk factors associated with type 2 diabetes (Maimoona et al., 2011;
10 Zibadi et al., 2008). A recent report suggests that PYC can inhibit encephalomyocarditis
11 virus replication in the mouse heart by suppressing expression of proinflammatory
12 cytokines, and genes related to cardiac remodelling and mast cells (Matsumori et al.,
13 2007). PYC has also been reported to inhibit binding of human immunodeficiency virus
14 type-1 to host cells, and to cause other significant changes, including increased
15 expression of manganese superoxide dismutase (Feng et al., 2008).

16 HCV gene expression elevates reactive oxygen species (ROS) levels via
17 calcium signalling. In addition, HCV Core, NS3, and NS5A proteins have all been
18 shown to induce oxidative stress (Choi et al., 2004). The reported link between HCV

1 and oxidative stress makes this pathway a promising anti-HCV therapeutic strategy. To
2 date, however, the effect of PYC on HCV infection has not been investigated. This
3 study evaluated the inhibitory effects of Pycnogenol® on HCV replication *in vitro* and *in*
4 *vivo*.

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2 **2. Materials and methods**

3 ***2.1. Cell culture and reagents***

4 Genotype 1b HCV subgenomic replicon cell lines, R6FLR-N (R6, genotype 1b, strain
5 N) (Watanabe et al., 2006), FLR3-1 (genotype 1b, Con-1) (Sakamoto et al., 2005) and
6 Rep JFH Luc3-13 genotype 2a (Takano et al., 2011), strain JFH-1 (Wakita et al., 2005)
7 (Supplementary Fig. 1) were cultured at 37°C (5% CO₂) in Dulbecco's modified Eagle
8 medium-GlutaMAX-I (DMEM-GlutaMAX-I; Invitrogen, Carlsbad, CA, USA)
9 containing 10% foetal bovine serum and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA,
10 USA) (Sakamoto et al., 2005; Watanabe et al., 2006). The JFH-1/K4 cell line, which
11 comprises HuH-7 cells persistently infected with the HCV JFH-1 strain, was maintained
12 in DMEM with 10% FCS (Takano et al., 2011).

13 PYC was supplied by Horphag Research Co. Pegylated IFN-alpha-2a was obtained
14 from Chugai Pharmaceutical Co. Japan.

15 ***2.2. HCV replicon cell reporter assay***

16 Cells were seeded into 96-well plates (5×10^3 per well). After incubation for 24 hours at
17 37°C (5% CO₂), the medium was removed and replaced with growth medium
18 containing serial dilutions of PYC, IFN-alpha, RBV, telaprevir or simeprevir (Janssen

1 Pharma Co., Tokyo, Japan). After 72 hours, luciferase activity was measured using the
2 Bright-Glo luciferase assay kit (Promega, Madison, WI). Measurements were made in
3 triplicate using an AccuFLEX Lumi 400 luminometer (Aloka, Tokyo, Japan), and the
4 results expressed as the average percentage of the control.

5 ***2.3. Generation of telaprevir-resistant replicon cell lines and analysis***

6 Telaprevir-resistant R6FLR-N subgenomic replicon cell lines were established as
7 described previously (Katsume et al., 2013). Briefly, wild-type R6FLR-N replicon cells
8 were seeded in 10-cm dishes in the presence of 0.5 mg/mL G418 and treated with
9 telaprevir. The cells were incubated for 51 days with no-compound control or telaprevir
10 (1.8 μ M and 2.7 μ M serially diluted in media). Fresh media and telaprevir were added
11 every 3 days. Most cells incubated with 2.7 μ M telaprevir died; however, after 3 weeks
12 small colonies started to appear and were expanded for 4 weeks. Deep sequencing was
13 performed as described previously (Katsume et al., 2013) and revealed a mutation
14 profile in NS3 (V36A, T54V and A156T) and NS5A (Q181H, P223S and S417P) which
15 confer resistance to telaprevir. Resistant replicon cells were seeded at 5×10^3 /well. After
16 incubation for 24 hours at 37°C (5% CO₂), culture medium was removed and replaced
17 with growth medium containing serial dilutions of PYC or telaprevir alone or in
18 combination. After 72 hours, luciferase activity was determined using the Bright-Glo

1 luciferase assay kit (Promega, Madison, WI, USA). Measurements were made in
2 duplicate using a GloMax-Multi detection system (Promega, Madison, WI, USA).
3 Cytotoxicity was measured using WST-8 cell counting kit (Dojindo, Kumamoto, Japan).
4 Western blot analysis was performed, as described previously (Nishimura et al., 2009).
5 Briefly, HCV replicon cells (2×10^5) were grown in a 60-mm cell culture dish. After 24
6 h, cells were treated with PYC for 72 h. Cells were collected and lysed with
7 radioimmunoprecipitation buffer (1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 150
8 mmol NaCl, 0.5 mmol ethylenediaminetetraacetic acid, 1 mmol dithiothreitol, and 10
9 mmol Tris, pH 7.4). Total protein (30 μ g) was electrophoresed on a 12% sodium
10 dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride
11 membrane (Immobilon-P; Millipore, Billerica, MA, USA). HCV NS3 and NS5B
12 proteins were detected using rabbit NS3 (R212) polyclonal antibody or anti-NS5B
13 (5B14) monoclonal antibody. Beta-actin was detected using an actin monoclonal
14 antibody (Sigma, St. Louis, MO, USA).

15 ***2.4. Quantitative real-time polymerase chain reaction***

16 Quantification of HCV RNA was performed using real-time reverse transcription
17 polymerase chain reaction (qRT-PCR) based on TaqMan chemistry using the forward
18 primer R6-130-S17 (nucleotides 130–146), 5'-CGGGAGAGCCATAGTGG-3'; the

reverse primer R6-290-R19 (nucleotides 290–272),
5'-AGTACCACAAGGCCTTTTCG-3'; and the Taq-Man probe R6-148-S21FT
(nucleotides 148–168), 5'-FAM-CTGCGGAACCGGTGAGTACAC-TAMRA3', as
described previously (Takeuchi et al., 1999). HCV RNA was extracted from
PYC-treated, persistently-infected JFH-1/K4 HCV cells, using the ISOGEN RNA
extraction kit (Nippon Gene, Japan).

2.5. In vivo effects of PYC

We produced chimeric mice by transplanting human primary hepatocytes into severe
combined immunodeficient mice carrying a urokinase plasminogen activator transgene
controlled by the albumin promoter (Mercer et al., 2001; Tateno et al., 2004). All
animals received humane care according to National Institute of Health criteria outlined
in the Guide for Care and Use of Laboratory Animals. The hepatocytes were infected
with HCV-G9 (genotype 1a) (Inoue et al., 2007). HCV 1a RNA levels reached 2.9–18.0
 $\times 10^6$ copies/mL in mice sera after 1–2 months of infection. PYC (40 mg/kg) was
administered intraperitoneally once daily. PEG-IFN (30 μ g/kg) was administered
subcutaneously at 0, 3, 7, and 10 days either alone or in combination with PYC. Each
treated group contained at least 3 chimeric mice. HCV RNA was purified from 2 μ L
chimeric mouse serum using SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan).

1 HCV RNA levels were quantified using qRT-PCR as reported previously (Takeuchi et
2 al., 1999).

3 **2.6. ROS assay**

4 Formation of ROS in the HuH-7 cell-based HCV-replicon-harboring cell line
5 (R6FLR-N), and in R6FLR-N cured of HCV by interferon treatment (Blight et al.,
6 2002) was measured using the OxiSelect ROS assay kit (Cell Biolabs, San Diego, CA,
7 USA) according to the manufacturer's instructions. Duplicate samples at 1×10^7
8 cells/mL from each culture were then incubated with dichlorodihydrofluorescein
9 DiOxyQ (DCFH-DiOxyQ). Under these conditions, ROS species rapidly oxidize DCFH
10 into the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Fluorescence
11 intensity, which is proportional to the total ROS levels in the sample, was measured
12 with a fluorescence spectrophotometer reader at 480-nm excitation and 530-nm
13 emission.

14 **2.7. Statistical analysis**

15 Data are presented as means $\pm$ standard error of triplicate experiments. Data were
16 analysed using Kruskal-Wallis test and Mann-Whitney U tests. A p -value <0.05 was
17 considered statistically significant. To evaluate the synergistic or antagonistic effects of
18 combinations of PYC and IFN-alpha or PYC and telaprevir, we calculated the

1 combination index (CI) using CalcuSyn (Biosoft, Ferguson, MO, USA), where $CI < 1$
2 indicates synergism, $CI = 1$ indicates an additive effect, and $CI > 1$ indicates
3 antagonism. The weighted average CI was calculated using the formula: $CI = [CI_{50} +$
4 $2CI_{75} + 3CI_{90} + 4CI_{95}]/10$, where CI_{50} , CI_{75} , CI_{90} , and CI_{95} are the CI values at 50%,
5 75%, 90% and 95% inhibition, respectively (Bassit et al., 2008; Chou and Talalay,
6 1984).

7

3. RESULTS

3.1. *PYC inhibits HCV replication and protein synthesis in vitro*

We assessed the effect of PYC on HCV in R6FLR-N and FLR3-1 cell lines after 72 hours (**Fig. 1**). The data are expressed as relative values using the relative light unit count for the 0 µg/mL treatment sample as 100% (**Fig. 1A**). The results showed that PYC inhibited luciferase activity in R6FLR-N cells (50% inhibitory concentration $[IC_{50}] = 5.78 \pm 3.75$ µg/mL, 50% effective concentration $[EC_{50}] = 4.33$ µg/mL (2.2–8.5) in a dose-dependent manner. To rule out the possibility that the antiviral activity was caused by cytotoxic effects, cell proliferation was analysed using the WST-8 assay; no significant differences in cell viability (50% cytotoxic concentration $[CC_{50}] > 60$ µg/mL PYC; Selectivity index $[SI] > 14.1$) (**Fig. 1B**). These results indicate that PYC suppresses HCV (genotype 1b) replication.

Consistent with results showing the inhibitory effects of PYC on HCV replication, we observed that HCV NS3 protein levels decreased significantly in PYC and IFN-alpha-treated HCV replicon cell lines (**Fig. 1C**). HCV NS3 and NS5B proteins levels were progressively suppressed in HCV replicon cell lines at various PYC concentrations (0, 5, 10, and 20 µg/mL) (**Fig. 1D & Fig. 1E**). These results suggest that HCV protein synthesis was inhibited by PYC in a concentration-dependent manner.

3.2. Combinations of IFN-alpha, RBV, and PYC inhibit HCV replication

R6FLR-N cells were treated with IFN-alpha and RBV alone or in combination with several concentrations of PYC and incubated for 48 h (**Fig. 2A**). HCV replication was suppressed by approximately 20% following treatment with 5 µg/mL RBV, and by approximately 40% following treatment with 1 IU/mL IFN-alpha. Treatment with both RBV and IFN-alpha led to approximately 50% suppression. PYC showed a dose-dependent additive effect when administered in combination with RBV and IFN-alpha (**Fig. 2A**). Treatment with both PYC (5 µg/mL) and IFN-alpha (1 IU/mL) showed a synergistic effect ($CI = 0.253$) in suppressing HCV replication without cytotoxicity (**Fig. 2A & 2B**).

3.3. Effect of PYC on HCV JFH-1 replication

JFH Luc3-13-N cells were inoculated with IFN-alpha (5 IU/mL) or several concentrations of PYC (5–50 µg/mL) and incubated for 72 h (**Fig. 2C**). HCV (genotype 2a) replication was suppressed by approximately 50% following treatment with 40 µg/mL PYC (**Fig. 2C**) without significant cytotoxicity (**Fig. 2D**).

3.4. Anti-HCV activity of PYC in the HCV JFH-1 infection system

PYC, IFN-alpha, and RBV treatments were also evaluated in JFH-1/K4 HCV (genotype 2a) infected cells (**Fig. 2E**). HCV RNA levels decreased in the presence of

PYC (10 or 20 µg/mL) to levels comparable to treatment with 1 IU/mL IFN-alpha in cell culture supernatant after 72 h. Treatment with a combination of IFN-alpha (1 IU/mL), RBV (5 µg/mL), and PYC (10 or 20 µg/mL) had a greater effect than IFN-alpha or PYC alone (**Fig. 2E**).

3.5. PYC inhibits HCV replication in telaprevir-resistant replicon cells and inhibits additively with telaprevir

We next examined the efficacy of PYC in DAA-resistant HCV. To select telaprevir-resistant replicons, cells with genotype 1b HCV replicons were treated for 14 passages with 1.8 µM and 2.7 µM telaprevir, concentrations 4–6 times the reported IC₅₀ (Katsume et al., 2013). These telaprevir-resistant replicon cells showed some cross-resistance to another protease inhibitor, simeprevir (**Supplementary Fig. 2**). We investigated whether incubation of the wild-type HCV and telaprevir-resistant replicon with PYC alone or with telaprevir would inhibit HCV replication. The susceptibility of the replicon to PYC was measured after treating the cells with increasing concentrations of PYC and telaprevir for 72 hours (**Fig. 3**). **Fig. 3A** shows that PYC reduced luciferase activity in a dose dependant manner in a wild-type HCV replicon and 2 telaprevir-resistant replicon cell lines. In addition, PYC had an additive effect with telaprevir (CI = 1.05) (**Fig. 3B**). Further, inhibition was greater in telaprevir (1.8 µM)

1 than telaprevir (2.7 μ M) and combined PYC (10 μ g/mL) and telaprevir (1.8 μ M and 2.7
2 μ M) treatment reduced luciferase levels to those reached by PYC alone at 10 μ g/mL.
3 Moreover, the resistant mutants remain as sensitive to IFN- α as the wild-type
4 replicon (**Fig. 3A**). After a 72-hour incubation with PYC and telaprevir, no significant
5 cytotoxicity, as evaluated in the WST-8 based cell viability assay, was observed in the
6 replicon cells (**Fig. 3C**).

7 **3.6. PYC suppresses HCV replication more effectively than procyanidin or taxifolin**

8 Because procyanidin and taxifolin are the main constituents of PYC (Lee et al.,
9 2010), we examined their ability to suppress HCV replication (**Supplementary Fig. 3**).
10 Procyanidin could not inhibit HCV replication in R6FLR-N cells at concentrations
11 between 15–60 μ g/mL (**Supplementary Fig. 3A**). Cytotoxicity was not observed even
12 at this high dose (data not shown). In JFH-1/K4 HCV-infected cell lines, procyanidin
13 suppressed supernatant HCV RNA levels after 72 hours and worked synergistically with
14 IFN- α (**Supplementary Fig. 3B**). Moreover, we also examined taxifolin efficacy,
15 but did not observe any effect on HCV replication (**Supplementary Fig. 3C**) or HCV
16 infection in JFH-1/K4 cells (data not shown).

17 **3.7. PYC inhibits HCV replication in chimeric mice**

18 To evaluate the *in vivo* effects of PYC on HCV, we used chimeric mice with a

1 humanized liver infected with HCV G9 (genotype 1a). In the untreated control group
2 (n=3 mice), no decrease in HCV genome RNA levels was observed. In the group treated
3 with PYC (40 mg·kg⁻¹·day⁻¹) (n=3 mice), serum HCV RNA levels decreased rapidly,
4 and within 9 days the effect was greater than with PEG-IFN treatment (30 µg/kg)(n=3
5 mice) (**Fig. 4A**). Treatment with both PYC (40 µg/kg) and PEG-IFN (30 µg/kg)
6 significantly reduced HCV RNA levels after 14 days compared to either PEG-IFN or
7 PYC monotherapy (Kruskall-Wallis test, $p = 0.0008$). To exclude the possibility that
8 this decline was caused by indirect effects on human hepatocyte viability, we measured
9 human albumin levels in chimeric mice and found no significant variation during
10 treatment ($p = 0.728$) (**Fig. 4B**), indicating that PYC has an antiviral effect and acts
11 synergistically with PEG-IFN in chimeric mice with humanized livers infected with
12 HCV.

13 ***3.8. PYC suppresses intracellular ROS production in R6FLR-N cells***

14 A ROS assay was used to assess the ability of PYC to act as a free radical scavenger.
15 Fluorescence intensity was measured for each sample. Total ROS production was
16 significantly decreased by PYC in the HCV replicon cell line in a dose-dependent
17 manner (**Fig. 5**). Treatment with PYC at 40 µg/mL reduced ROS to levels comparable
18 to cells cured of the HCV replicon by IFN treatment (Blight et al., 2002), suggesting

1 that PYC may scavenge ROS in HCV replicon cell lines.

3 **4. DISCUSSION**

4 Oxidative stress has been identified as a key mechanism of HCV-induced
5 pathogenesis (de Mochel et al., 2010; Ke and Chen, 2012; Quarato et al., 2013; Tardif et
6 al., 2005). Moreover, several studies have reported a correlation between oxidative
7 stress and IFN treatment response, and have observed that oxidative stress was reduced
8 to normal levels after viral eradication (Levent et al., 2006; Serejo et al., 2003). These
9 data provide a firm theoretical basis for investigation of antioxidants as therapeutics.
10 PYC is a mixture of various chemical groups and exhibits radical-scavenging
11 antioxidant, anti-inflammatory, and antiviral activities (Maimoona et al., 2011). In
12 addition, PYC protects biomolecules such as proteins against oxidative damage (Voss et
13 al., 2006). To our knowledge, this is the first report to demonstrate a direct antiviral
14 effect of PYC against HCV. Our results show that PYC inhibits HCV replication in
15 HCV replicon cell lines and JFH-1 without cytotoxicity. Moreover, this result is in line
16 with a recent report, based on data obtained from 5,723 subjects that showed side effect
17 incidence rates of 2.4% and 0.19% in patients and healthy subjects, respectively
18 (American Botanical Council, 2010). The study also found PYC to be nontoxic at doses

1 of 20–100 mg per day for extended periods (months) and 100–300 mg for shorter
2 periods (American Botanical Council, 2010).

3 Treatments of replicon and JFH-1 cell lines using combinations of PYC with
4 RBV, IFN, and telaprevir showed that co-administration of these compounds increased
5 HCV antiviral activity. In addition, we found that PYC suppressed HCV replication in
6 telaprevir-resistant replicon cells and may improve the response to protease inhibitors.

7 In this report, we found that procyanidins, oligomeric compounds formed from catechin
8 and epicatechin, but not taxifolin, inhibited HCV replication at doses between 15 and 60
9 µg/mL and had a synergistic effect with IFN treatment without cytotoxicity. Moreover,
10 procyanidin B1 extracted from *Cinnamomum cassia* cortex suppresses hepatitis C virus
11 replication (Li et al., 2010). Other studies have also shown that epicatechin,
12 catechin-derived compounds, and caffeic acid phenethyl ester inhibit HCV replication
13 and attenuate the inflammation induced by the virus (Khachatoorian et al., 2012; Lin et
14 al., 2013; Shen et al., 2013). However, PYC showed greater antiviral effects than
15 procyanidin and taxifolin. PYC efficacy was much stronger than procyanidin or
16 taxifolin; therefore, a combination of components or unknown factor(s) in PYC may
17 contribute to inhibition of viral replication.

18 Constitutive activation of NF-kappa B and STAT-3 by HCV is implicated in

1 acute and chronic liver disease (Gong et al., 2001; Waris et al., 2003; Waris et al.,
2 2005). Consistent with these data, a previous study showed that PYC inhibits NF-kappa
3 B and activator protein-1, and abolishes the degradation of I-kappa B alpha (Cho et al.,
4 2000). Moreover, a recent study showed that PYC also inhibits expression and secretion
5 of tumour necrosis factor-alpha and interleukin 6, reducing calcium uptake and
6 suppressing NF-kappa B activation (Choi and Yan, 2009). We observed PYC free
7 radical scavenging activity against ROS in HCV replicon cell lines. These data support
8 our finding that PYC exerts its antioxidant effects directly by scavenging of ROS and
9 indirectly by enhancing cellular antioxidant enzymes (Packer et al., 1999).

10 **5. Conclusion**

11 Our study shows that the natural product PYC inhibits HCV replication both *in vitro*
12 and *in vivo*. Our results indicate that *in vitro* combinations of PYC/IFN-alpha/RBV and
13 PYC/telaprevir lead to a much stronger antiviral response than with either agent alone
14 and that PYC suppresses replication in telaprevir-resistant replicon cells. Future clinical
15 trials are necessary to assess which patients, for example, naïves, non-responders, or
16 those with severe liver disease, could benefit from co-administration of PYC with
17 PEG-IFN-alpha, RBV, or DAAs. Addition of PYC may be a viable strategy to improve
18 the efficacy of HCV therapies using the recently licenced antiviral molecules.

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Figure legends

Fig. 1. Effect of PYC on HCV RNA replication in HCV replicon cell lines

(A) Dose-dependent inhibition of HCV by PYC in the R6 replicon cell line. (B) Cytotoxicity in the R6 HCV-replicon cell line treated with PYC. The ratio (%) of viability compared to the no-treatment control value is indicated. (C-D) HCV protein expression level decreased with PYC treatment in the HCV R6 and FLR3-1 replicon cell lines. (C, D) Dose-dependent effects of PYC on HCV protein synthesis (C NS3, D NS5B) in R6 and FLR3-1 replicon cell lines. Error bars = standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The ratio of luciferase was calculated as compared to non-treatment values.

Fig. 2. Effect of PYC and IFN-alpha on HCV replication

(A) Effect of PYC (5 $\mu\text{g/mL}$), IFN-alpha (1 IU/mL), and RBV (5 $\mu\text{g/mL}$) on antiviral activity in the R6 HCV replicon cell line. (B) Cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and RBV. (C) Effect of IFN-alpha (5 IU/mL), and PYC (5–50 $\mu\text{g/mL}$) on JFH-1 replicon cells (RepJFH Luc3-13-N) (replication; left, viability; right). (D) Cytotoxicity in the RepJFH Luc3-13-N cell treated with PYC. (E) Synergistic effect of PYC, IFN-alpha, and RBV in the culture supernatant of HCV

infection system in JFH-1/K4 cell line.

Error bars = standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Effect of PYC on HCV replication in telaprevir-resistant replicon cells

(A) Dose-dependent inhibition of HCV by PYC in wild type (WT) and telaprevir-resistant replicon cell lines (V1.8, V2.7). (B) Effect of PYC and telaprevir in the R6 replicon cell line. (C) PYC cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and telaprevir. Ratio of viability (%) to cells without PYC is indicated.

Error bars = standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001

Fig. 4. *In vivo* effects of PYC on HCV replication

(A) Median change from baseline serum relative HCV RNA levels (HCV RNA amount at the beginning; average 4.8×10^7 copies/mL) in chimeric mice treated with PYC (40 mg/kg/day, intraperitoneally) or PEG-IFN (30 µg/kg, subcutaneously) alone, in combination with PEG-IFN, and untreated controls (in each group, n=3). (B) Serum human albumin levels in chimeric mice during treatment.

Fig. 5. Effect of PYC on ROS production in HCV replicon cells

- 1 HCV replicon and cured cell lines were incubated for 72 hours with various
- 2 concentrations of PYC and without PYC treatment. Data are shown as mean $\pm$ standard
- 3 error of mean. ROS levels in HCV-negative cured cells are also shown. *P < 0.05;
- 4 **P < 0.01

Figure(s)

Fig.1 Ezzikouri et al.

A R6

B R6

C RepJFH Luc3-13-N

D RepJFH Luc3-13-N

E JFH-1/K4

Fig.2 Ezzikouri et al.

A

B

C

Fig.3 Ezzikouri et al.

A **B** **Fig.4 Ezzikouri et al.**

Fig.5 Ezzikouri et al.