Organ- and species-specific biological activity of rosmarinic acid

Iswandana, R.; Pham, B. T.; van Haaften, W. T.; Luangmonkong, T.; Oosterhuis, D.; Mutsaers, H. A. M.; Olinga, P.

Published in:
Toxicology in Vitro

DOI:
10.1016/j.tiv.2016.01.009

Publication date:
2016

Toxicology in Vitro 32 (2016) 261–268

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Organ- and species-specific biological activity of rosmarinic acid

R. Iswandana [a,b], B.T. Pham [a,c], W.T. van Haaften [a], T. Luangmonkong [a,d], D. Oosterhuis [a], H.A.M. Mutsaers [a,1], P. Olinga [a,*,1]

[a] Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, University of Groningen, The Netherlands
[b] Faculty of Pharmacy, Universitas Indonesia, Indonesia
[c] Department of Pharmaceutics, Hanoi University of Pharmacy, Vietnam
[d] Department of Pharmacology, Faculty of Pharmacy, Mahidol University, Thailand

ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 11 January 2016
Accepted 18 January 2016
Available online 19 January 2016

Keywords:
Antifibrotic
Anti-inflammatory
Precision-cut intestinal slices
Precision-cut liver slices
Rosmarinic acid
Toxicity

ABSTRACT

Rosmarinic acid (RA), a compound found in several plant species, has beneficial properties, including anti-inflammatory and antibacterial effects. We investigated the toxicity, anti-inflammatory, and antifibrotic effects of RA using precision-cut liver slices (PCLS) and precision-cut intestinal slices (PCIS) prepared from human, mouse, and rat tissue. PCLS and PCIS were cultured up to 48 h in the absence or presence of RA. Gene expression of the inflammatory markers: IL-6, IL-8/CXCL1/KC, and IL-1β, as well as the fibrosis markers: pro-collagen 1a1, heat shock protein 47, α-smooth muscle actin, fibronectin (Fn2) and plasminogen activator inhibitor-1 (PAI-1) were evaluated by qPCR. RA was only toxic in murine PCIS. RA failed to mitigate the inflammatory response in most models, while it clearly reduced IL-6 and CXCL1/KC gene expression in murine PCIS at non-toxic concentrations. With regard to fibrosis, RA decreased the gene levels of Fn2 and PAI-1 in murine PCLS, and Fn2 in murine PCIS. Yet, no effect was observed on the gene expression of fibrosis markers in human and rat PCIS. In conclusion, we observed clear organ- and species-specific effects of RA. RA had little influence on inflammation. However, our study further establishes RA as a potential candidate for the treatment of liver fibrosis.

1. Introduction

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (See Fig. 1). It is commonly found in plants of the Boraginaceae family (forget-me-not) and the subfamily Nepetoideae of the mint family Lamiaceae. It is also found in some fern and hornwort species (Petersen and Simmonds, 2003). RA has a gamut of beneficial biological activities, such as anti-inflammatory (Rocha et al., 2015), antioxidant, antiapoptotic, and antifibrotic effects (Domitrović et al., 2013; Li et al., 2010).

Rocha et al., 2015 demonstrated that RA might be useful in the pharmacological modulation of injuries associated with inflammation (Rocha et al., 2015). The anti-inflammatory properties of RA are thought to be based on the inhibition of lipoxygenase and cyclooxygenases, interference with the complement cascade (Petersen and Simmonds, 2003; Mirzoeva and Calder, 1996; Krol et al., 1996) and down-regulation of inflammatory cytokines (Sanbongi, 2003). Because chronic inflammation is an important trigger for fibrogenesis, RA might mitigate fibrosis by dampening the inflammatory response during chronic diseases. Liver fibrosis, especially the end stage cirrhosis, is a major cause of mortality worldwide (Poynard et al., 2010). Similarly, intestinal fibrosis is found in most patients with inflammatory bowel disease (IBD), which affects at least 2.2 million Europeans (Poynard et al., 2010; Loftus, 2004). Currently, the only available treatment for liver and intestinal fibrosis is surgery, therefore there is an urgent need for alternative and effective treatment modalities.

Previously, Westra et al. (2014a) showed that RA decreased the expression of the fibrosis markers collagen 1α1 (Col1α1), heat shock protein 47 (Hsp47), and α-smooth muscle actin (αSma) in both human and rat precision-cut liver slices (Westra et al., 2014a). In addition, RA also showed therapeutic activity against acute liver toxicity in vivo (Li et al., 2010). RA ameliorated hepatic oxidative/nitrosative stress, suppressed inflammation, and inhibited activation of hepatic stellate cells (HSCs) and apoptosis in CCl_4-injured livers. The hepatoprotective activity of RA was accompanied by induction of the Nrf2/HO-1 pathway (Domitrović et al., 2013). Moreover, it has also been shown that RA inhibits COX-2 activation in colon cancer HT-29 cells (Scheckel et al., 2008; Hossan et al., 2014). These results suggest that RA may be a promising anti-inflammatory and antifibrotic compound in both liver and intestinal fibrosis. Yet, the discovery of effective antifibrotics is hampered by the absence of good translational models, variability in the observed efficacy of drug candidates in rodent models due to species- and strain-dependent

* Corresponding author at: GUIDE (Groningen University Institute for Drug Exploration), Department of Pharmaceutical Technology and Biopharmacy, Antonius Deusinglaan 1, 9713 AV, Groningen, – The Netherlands.
E-mail addresses: raditya.iswandana@rug.nl (R. Iswandana), b.t.pham@rug.nl (B.T. Pham), w.t.van.haaften@umcg.nl (W.T. van Haaften), t.luangmonkong@rug.nl (T. Luangmonkong), d.oosterhuis@rug.nl (D. Oosterhuis), h.a.m.mutsaers@rug.nl (H.A.M. Mutsaers), p.olinga@rug.nl (P. Olinga).
[1] These authors shared senior authorship.

http://dx.doi.org/10.1016/j.tiv.2016.01.009
0887-2333/

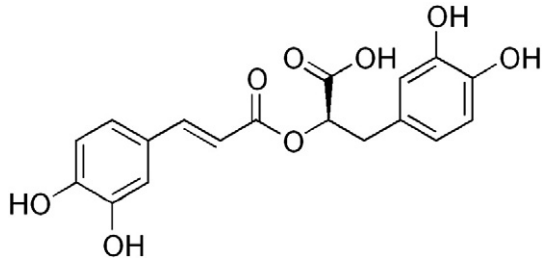

Fig. 1. Rosmarinic acid structure.

responses and the inability to replicate the multicellular pathophysiology of fibrosis in vitro (Westra et al., 2012; Torok et al., 2015; Mutsaers et al., 2015). To tackle these issues, the objective of this study was to investigate organ- and species-specific biological activity of RA with a focus on the putative anti-inflammatory and antifibrotic effects using precision-cut liver slices (PCLS) and precision-cut intestinal slices (PCIS) prepared from human, murine, and rat tissue.

This ex vivo/in vitro model is ideal to study multicellular processes, e.g. fibrosis, because the slices contain all the different cells in their original environment allowing for cell–cell and cell-matrix interactions (Baumgart and Carding, 2007).

2. Materials and methods

2.1. Chemical

All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise. Stock solutions of RA were prepared in milli-Q and stored at −20 °C. During experiments, stocks were diluted in culture medium with a final solvent concentration of ≤1%.

2.2. Animals

Tissue was obtained from male Wistar rats (Harlan Laboratories B.V., Horst, The Netherlands) and C57BL/6 mice (De Centrale Dienst Proefdieren, University Medical Center Groningen, Groningen, The Netherlands). Animals were housed under controlled conditions with a twelve-hour light/dark cycle and free access to water and food (Harlan chow no. 2018, Horst, The Netherlands). Organs were harvested via a terminal procedure performed under isoflurane/O_2 anesthesia (Nicholas Piramal, London, UK). All experiments were approved by the Animal Ethical Committee of the University of Groningen.

2.3. Preparation of murine precision-cut liver slices

Murine liver slices (PCLS) were prepared according to the protocol by de Graaf et al., (2010) In short, liver cores were obtained using a 5-mm biopsy-punch. Subsequently, slices were made using a Krumdieck tissue slicer (Alabama Research and Development, USA), filled with ice-cold Krebs–Henseleit buffer (KHB) supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH), saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4. PCLS with a wet weight of approximately 3 mg, have an estimated thickness of 300–400 μm. To prevent rapid loss of viability after slicing, PCLS were directly transferred to ice-cold University of Wisconsin organ preservation solution (UW-solution).

2.4. Preparation of intestinal slices

Healthy human jejunum tissue was obtained from pylorus preserving pancreaticoduodenectomies. Use of human tissue was approved by the Medical Ethical Committee of the University Medical Center Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining the need of written consent for 'further use' of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG.

Rat jejunum (about 25 cm distal from the stomach and 15 cm in length) or mouse jejunum (about 15 cm distal from the stomach and 10 cm in length) were excised and preserved in ice-cold KHB until use.

Table 1
Fibrotic and inflammatory primers and probes gene expression.

Species	Primer	Forward sequence	Reverse sequence	Probe sequence
Human	GAPDH	ACCAGGGCTGCTTTTAACTCT	GGTGCCATGGAATTTGCC	TGCCATCAATGACCCCTTCA
	Col1α1	CAATCACCTGCGTACAGAACGCC	CGGCAGGGCTCGGGTTTC	CAGGTACCATGACCGAGACGTG
	Hsp47	GCCCACCGTGGTGCCGCA	GCCAGGGCCGCCTCCAGGAG	CTCCCTCCTGCTTCTCAGCG
	αSma	AGGGGGTGATGGTGGGAA	ATGATGCCATGTTCTATCGG	GGGTGACGAAGCACAGAGCA
	Fn2	AGGCTTGAACCAACCTACGGATGA	GCCTAAGCACTGGCACAACAGTTT	ATGCCGTTGGAGATGAGTGGGAA
	PAI-1	CACGAGTCTTTCAGACCAAG	AGGCAAATGTCTTCTCTTCC	–
	IL-6	Hs00985639_m1		
	IL-8	Hs00174103_m1		
	IL-1β	Hs01555410_m1		
Mouse	GAPDH	ACAGTCCATGCCATCACTGC	GATCCACGACGGACACATTG	–
	Col1α1	TGACTGGAAGAGCGGAGAGT	ATCCATCGGTCATGCTCTCT	–
	Hsp47	AGGTCACCAAGGATGTGGAG	CAGCTTCTCCTTCTCGTCGT	–
	αSma	ACTACTGCCGAGCGTGAGAT	CCAATGAAAGATGGCTGGAA	–
	Fn2	CGGAGAGAGTGCCCCTACTA	CGATATTGGTGAATCGCAGA	–
	PAI-1	GCCAGATTTATCATCAATGACTGGG	GGAGAGGTGCACATCTTTCTCAAAG	–
	IL-6	Mm00446190_m1		
	CXCl1/KC	Mm04207460_m1		
	IL-1β	Mm00434228_m1		
Rat	GAPDH	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA	CTTGCCCACAGCCTTGGCAGC
	Col1α1	CCCACCGGCCCTACTG	GACCAGCTTCACCCTTAGCA	CCTCCTGGCTTCCCTG
	Hsp47	AGACGAGTTGTAGAGTCCAAGAGT	ACCCATGTGTCTCAGGAACCT	CTTCCCGCCATGCCAC
	αSma	AGCTCTGGTGTGTGACAATGG	GGAGCATCATCACCAGCAAAG	CCGCCTTACAGAGCC
	Fn2	TCTTCTGATGTCACCGCCAACTCA	TGATAGAATTCCTTGAGGGCGGCA	–
	PAI-1	AACCCAGGCCGACTTCA	CATGCGGGCTGAGACTAGAAT	–
	IL-6	CCGGAGAGGAGACTTCACAG	ACAGTGCATCATCGCTGTTC	–
	IL-8	GGCAGGGATTCACTTCAAGA	GCCATCGGTGCAATCTATCT	–
	IL-1β	AGGCAGTGTCACTCATTGTG	GGAGAGCTTTCAGCTCACAT	–

2.5. Slicing of precision-cut intestinal slices

Preparation of intestinal slices (PCIS) was carried out according to the protocol of de Graaf et al., (2010) In short, tissue was cleansed by flushing KHB through the lumen and subsequently divided into 2 cm segments. Afterwards, intestinal cores were prepared using 3% (*w*/*v*) agarose (Sigma-Aldrich, Steinheim, Germany) in 0.9% NaCl at 37 °C and embedded in an agarose core-embedding unit. Next, PCIS were prepared using a Krumdieck tissue slicer. Similar to PCLS, PCIS had a wet weight of approximately 3 mg, and an estimated thickness of 300–400 μm. Following slicing, PCIS were directly transferred to KHB to prevent loss of viability.

2.6. Incubation of slices

After slicing, PCLS and PCIS were cultured in 12-well plates or 24-well plates (murine PCIS) in Williams' Medium E + GlutaMAX (Gibco, New York, USA) supplemented with 14 mM Glucose (Merck, Darmstadt, Germany) and 50 μg/ml gentamycin (Gibco). PCIS medium also contained 2.5 μg/ml fungizone (amphotericin B; Invitrogen, Paisly, Scotland). Slices were incubated for 24 h (rat PCIS) or 48 h at 37 °C in an 80% O_2/5% CO_2 atmosphere. The culture plates were horizontally shaken at 90 rpm (amplitude 2 cm). For experiments, PCLS and PCIS were incubated with RA (100 μM–500 μM) for 24–48 h.

2.7. Viability

Viability of the slices was assessed by measuring the adenosine triphosphate (ATP) content using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany), as previously described (de Graaf et al., 2010). Determined ATP values (pmol) were normalized to the total amount of protein (μg) estimated by the Lowry method (BIO-rad RC DC Protein Assay, Bio Rad, Veenendaal, The Netherlands). Values displayed are relative values compared to the related controls.

2.8. Gene expression

After incubation, PCLS and PCIS were snap-frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA of three to six pooled snap-frozen slices was isolated using the Qiagen RNAeasy mini kit (Qiagen, Venlo, The Netherlands), and the amount of isolated RNA was measured with the BioTek Synergy HT (BioTek Instruments, Vermont, USA). Afterwards, RNA (1 μg) was reverse transcribed using the reverse transcriptase kit (Promega, Leiden, The Netherlands). The RT-PCR reaction was performed in the Eppendorf mastercycler using the following gradient: 25 °C for 10 min, 45 °C for 60 min and 95 °C for 5 min.

Subsequently, gene expression was studied via RT-qPCR using the SYBR green method or TaqMan gene expression assays (Applied Biosystems, Bleiswijk, The Netherlands). Samples were analyzed using a 7900 HT Fast Real-Time RT-PCR (Applied Biosystems) with 45 cycles of 10 min 95 °C, 15 s at 95 °C, and 25 s at 60 °C following by a

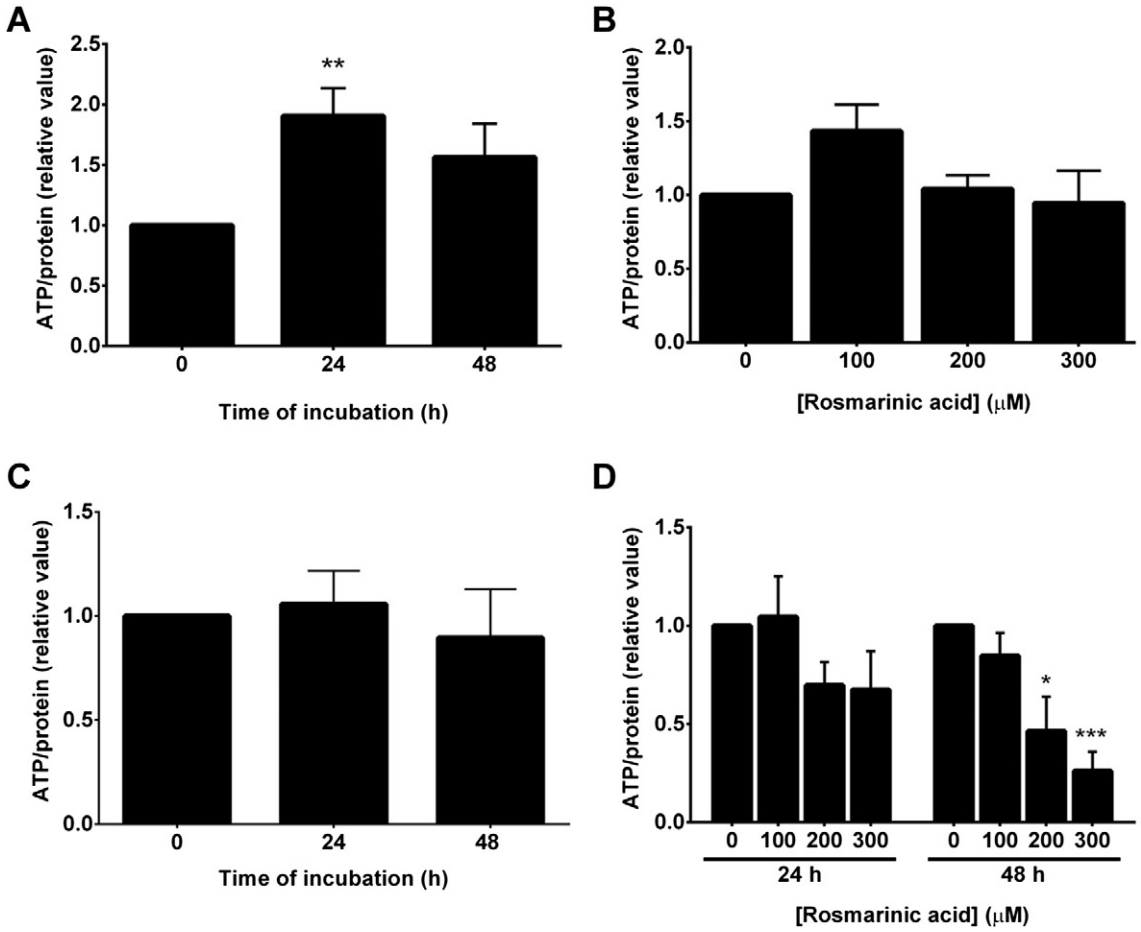

Fig. 2. The effect of incubation and rosmarinic acid on murine PCLS (n = 4) and PCIS 48 h (n = 4): (A) PCLS viability (relative value; incubation); (B) PCLS viability (relative value; rosmarinic acid); (C) PCIS viability (relative value; incubation); (D) PCIS viability (relative value; rosmarinic acid). Data are expressed as mean +/− SEM. Student's t-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

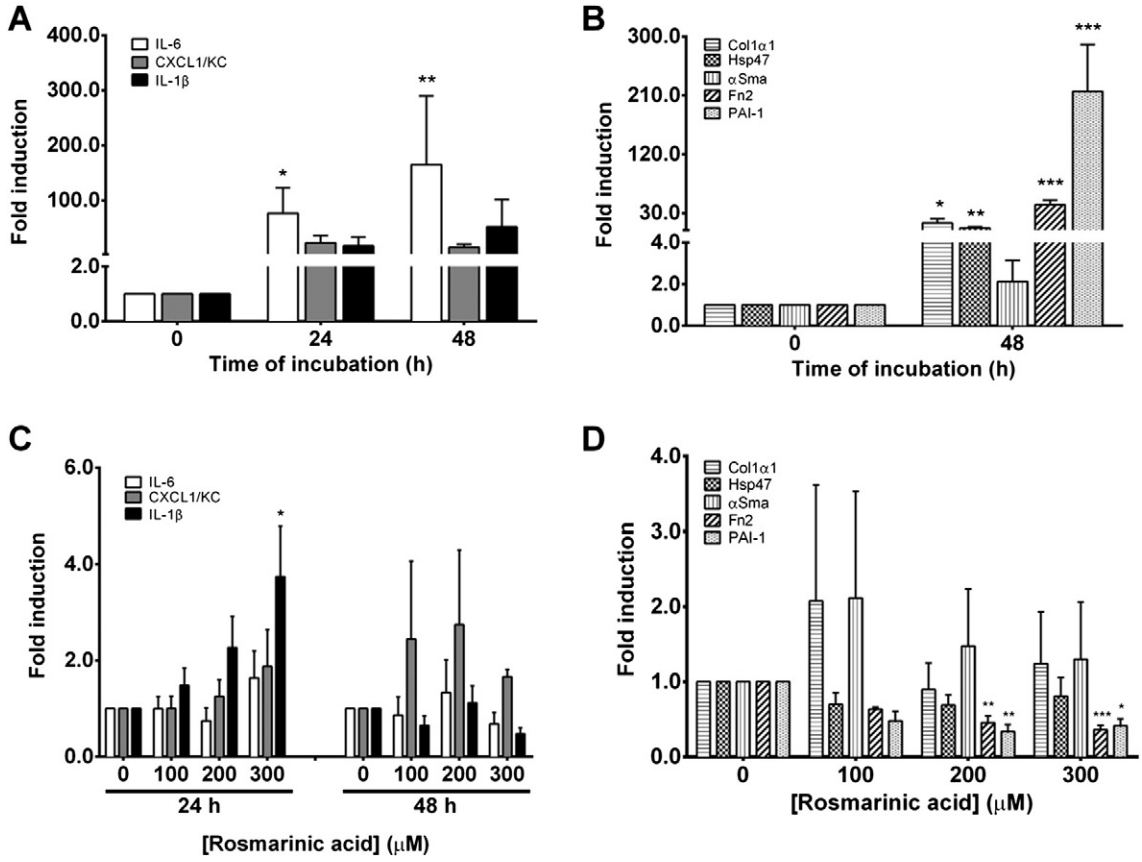

Fig. 3. The effect of incubation and rosmarinic acid on murine PCLS 48 h (n = 4): (A) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (incubation); (B) Fibrosis markers *Col1α1*, *Hsp47*, *αSma*, *Fn2*, and *PAl-1* expressions (incubation); (C) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (rosmarinic acid); (D) Fibrosis markers *COl1α1*, *Hsp47*, *αSma*, *Fn2* and *PAl-1* expressions (rosmarinic acid). Data are expressed as mean +/− SEM. ANOVA; *p < 0.05, **p < 0.01; ***p < 0.001 vs. control.

dissociation stage (SYBR green) or with 40 cycles of 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C (TaqMan). GAPDH was used as housekeeping gene, and relative expression was calculated as fold change ($2^{-\Delta\Delta Ct}$). Used primers and probes are listed in Table 1.

2.9. Statistics

Statistics were performed using GraphPad Prism 6.0 via one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or an unpaired, two-tailed Student's t-test as appropriate. A minimum of four different intestines/liver was used for each experiment, using 3–6 slices from each intestine/liver per condition. The results are expressed as mean ± standard error of the mean (SEM). Statistical differences in ATP levels were determined using the values relative to the control values. Regarding the RT-qPCR results, statistics were performed using the ΔΔCt values, while the data is presented as fold change ($2^{-\Delta\Delta Ct}$). Differences between groups were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Antifibrotic effect of RA in murine PCLS

Our lab previously demonstrated that RA mitigated fibrogenesis in human and rat PCLS. To elucidate potential species differences, we investigated whether the same effect could be observed in murine PCLS (n = 4). First we characterized the viability, by ATP content, and both the inflammatory and fibrotic response in the slices during culture. As shown in Fig. 2A (n = 4), ATP content of murine PCLS significantly increased after 24 h of culture, as compared to the 0 h control. Furthermore, ATP levels remained elevated, indicating that the slices were viable for 48 h. In addition, we observed an increase in inflammatory markers. Gene expression of IL-6 was significantly up-regulated 77 fold after 24 h and IL-6 levels were further elevated at 48 h (165 fold; Fig. 3A, n = 4). Concurrently, qPCR revealed a marked increase in multiple fibrosis markers after 48 h. Gene expression of Col1α1, Hsp47, Fn2, and PAI-1 were significantly elevated 15, 7, 43, and 216 fold, respectively. These results indicate the presence of both an inflammatory and fibrotic response in murine PCLS during culture. Fig. 2B (n = 4) demonstrates that RA (100–300 μM) does not exert toxicity in PCLS, yet we observed a concentration-dependent induction of IL-1β after 24 h (Fig. 3C, n = 4). Conversely, RA effectively mitigated fibrogenesis in PCLS as shown by a clear reduction in the expression of both Fn2 and PAI-1 at all the tested concentrations (Fig. 3D, n = 4). Thus, despite the observed induction of IL-1β, RA shows great potential as antifibrotic compound in murine PCLS, in concordance with our previous findings in human and rat PCLS (Westra et al., 2014a, 2014b).

3.2. Antifibrotic effect of RA in human, murine, and rat PCIS

Next, we investigated whether RA had a similar positive effects in intestinal slices prepared from tissue obtained from man, mouse, and rat.

3.2.1. Human PCIS

Fig. 4A demonstrates that the viability of human PCIS (n = 9) remained constant during culture. In addition, we observed a significant up-regulation of IL-6, IL-8, and IL-1β gene expression (Fig. 5A, n = 5) as well as elevated PAI-1 levels during culture (Fig. 5B, n = 5). The latter is in line with the observed onset of fibrosis in human PCIS reported previously (Pham et al., 2015). These results indicate the presence of both

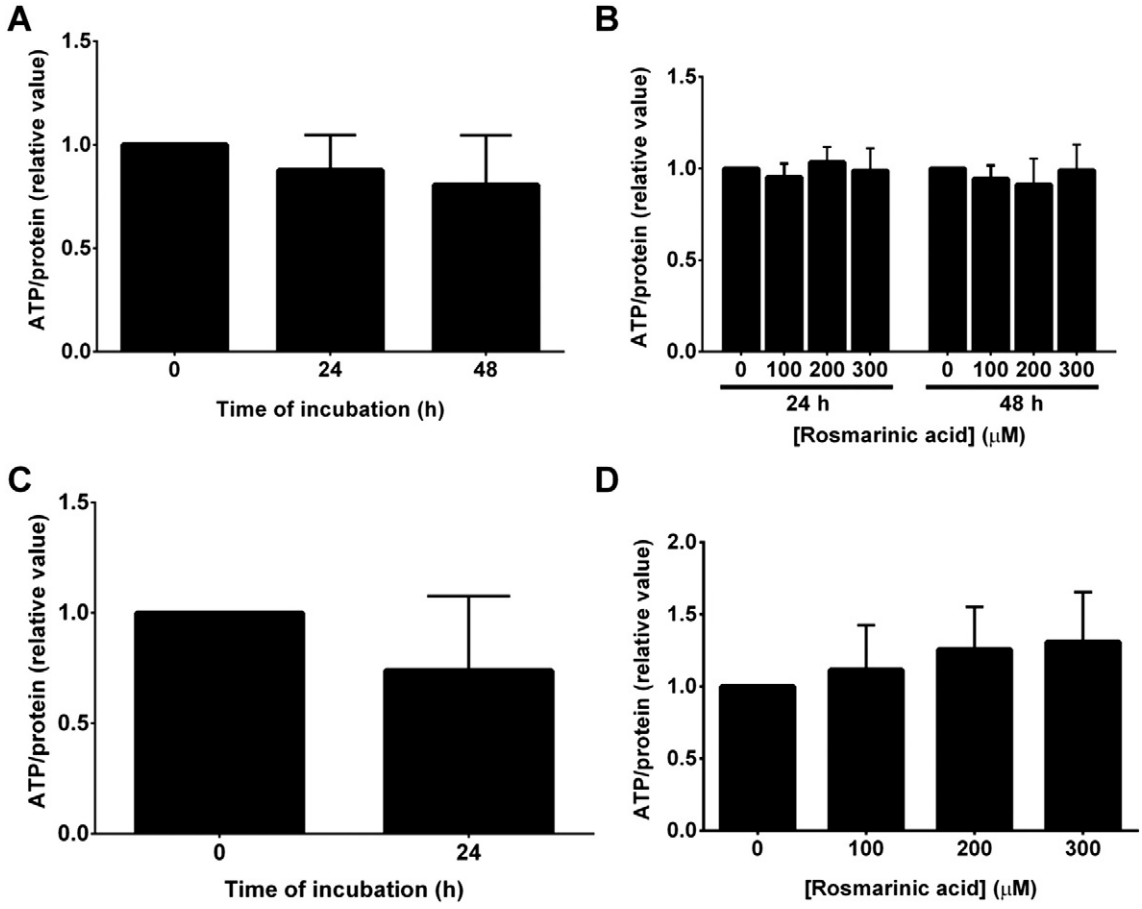

Fig. 4. The effect of incubation and rosmarinic acid on human PCIS 48 h (n = 9) and rat PCIS 24 h (n = 6): (A) Human PCIS viability (relative value; incubation); (B) Human PCIS viability (relative value; rosmarinic acid); (C) Rat PCIS viability (relative value; incubation); (D) Rat PCIS viability (relative value; rosmarinic acid). Data are expressed as mean +/− SEM. Student's t-test.

an inflammatory and fibrotic response in human PCIS during culture. As shown in Fig. 4B, RA (100–300 μM) had no impact on the viability of human PCIS (n = 9) as illustrated by stable ATP levels. In contrast to liver slices, RA had no significant influence on the expression of the investigated inflammatory and fibrotic markers in human PCIS. Out of interest, PAI-1 level was elevated during culture with RA (Figs. 5C and 5D, 3F, n = 5).

3.3. Murine PCIS

Similar to human PCIS, murine PCIS (n = 4) remain viable during culture for 48 h (Fig. 2C). In addition, gene expression of IL-6 and CXCL1/KC, the murine IL-8 homolog (Bozic et al., 1994), was up-regulated 2483 and 1721 fold respectively after 48 h incubation (Fig. 6A, n = 4). Moreover, PAI-1 levels increased more than 40 fold during culture (Fig. 6B, n = 4). Of interest, when murine PCIS were treated with RA, there was a slight reduction of viability after 24 h, yet viability remained at an adequate level to study the effects of RA. However, there was a significant reduction in viability after exposure to 200–300 μM RA for 48 h (Fig. 2D, n = 4). Fig. 4C (n = 4) shows that there was a significant reduction in IL-6 expression when PCIS were treated with 300 μM RA for 24 h. Furthermore, the expression of IL-6 and CXCL1/KC also showed a significant reduction following exposure to 100 μM of RA for 48 h. In addition, we observed a concentration-dependent reduction of the studied fibrosis markers with significant effect on Fn2 expression during 24 h, while RA had no effect after 48 h (Fig. 4D, n = 4).

3.3.1. Rat PCIS

Rat PCIS can only be cultured for 24 h (Fig. 4C, n = 4), still during this time, gene levels of IL-6, IL-8 and PAI-1 were significantly up-regulated (Fig. 7A,B, n = 4). Furthermore, in contrast to the results obtained with murine PCIS, RA did not affect the viability of rat PCIS, nor did it affect the inflammatory and fibrotic response (Figs. 4D, 7C, 7D, n = 4). Taken together, it is clear that RA elicits species-specific effects in the intestine.

4. Discussion

RA is an ester of caffeic acid found in a variety of plants, including the forget-me-not family. A multitude of beneficial properties has been contributed to RA, such as anti-inflammatory and antibacterial effects. Moreover, there is evidence indicating that RA might mitigate fibrosis (Domitrović et al., 2013; Li et al., 2010; Westra et al., 2014a, 2014b; Scheckel et al., 2008; Hossan et al., 2014), a detrimental pathophysiological process associated with various chronic diseases. In this study, we further evaluated to biological effects of RA.

4.1. Organ toxicity of RA

Our results demonstrated that RA concentration-dependent decreased the viability of murine PCIS, whereas both rat and human PCIS were unaffected. This discrepancy might be caused by species differences in the metabolism of RA. Several studies have previously shown

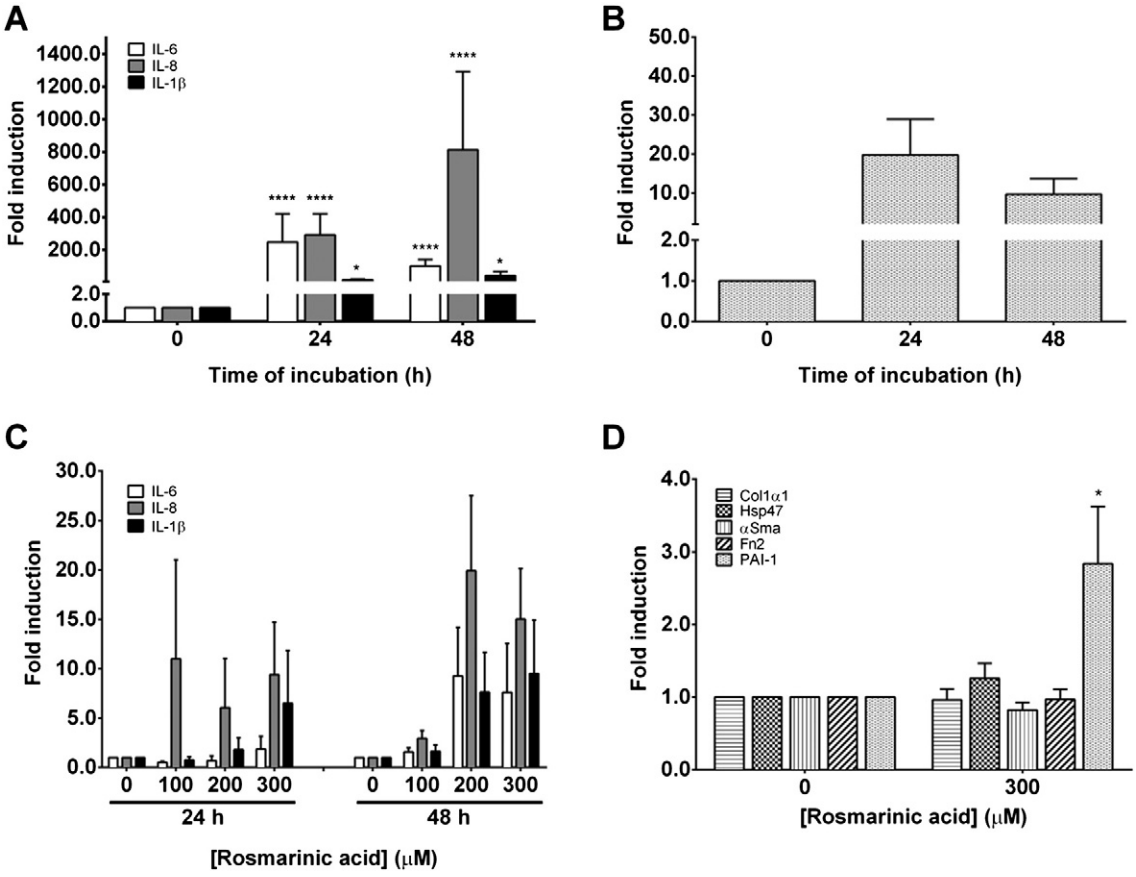

Fig. 5. The effect of incubation and rosmarinic acid on human PCIS (n = 5): (A) Inflammatory markers *IL-6*, *IL-8* and *IL-1β* expressions (incubation); (B) Fibrosis marker *PAI-1* expressions (incubation); (C) Inflammatory markers *IL-6*, *IL-8*, and *IL-1β* expressions (rosmarinic acid); (D) Fibrosis markers *Colα1*, *Hsp47*, *αSma*, *Fn2*, and *PAI-1* expressions (rosmarinic acid). Data are expressed as mean +/− SEM. ANOVA; *p < 0.05, ****p < 0.0001 vs. control.

that there were variances in absorption, metabolism, degradation and urinary excretion of RA between rats and humans, with rats excreting more of the glucuronide conjugate and humans the sulfate conjugate (Baba et al., 2004; Baba et al., 2005; Nakazawa and Ohsawa, 1999; Nakazawa and Ohsawa, 1998). Yet, RA metabolism in mice requires further investigation. Of note, several studies have shown that RA induces cell death in a variety of human colorectal carcinoma cell lines (Cheng et al., 2011; Xavier et al., 2009) as well as cells derived from mouse adenomas (Karmokar et al., 2012). These findings suggest that, under certain circumstances, RA might indeed be toxic for intestinal cells. Conversely, our results, and previous work from our group, showed that RA is not toxic for liver slices prepared from murine, rat and human tissue (Westra et al., 2014a, 2014b). Furthermore, a multitude of studies demonstrated that RA protects neural cell against apoptosis (Lee et al., 2008). Thus, RA appears to be generally non-toxic.

4.2. Anti-inflammatory effect of RA

To our knowledge this is the first study that addresses the effect of RA on the inflammation in human, rat and murine intestine. Our results with intestinal murine PCIS showed that RA only has anti-inflammatory effects in the murine intestine. As illustrated, RA potently reduced IL-6 and CXCL1/KC expression. Previously, Wang et al., showed that IL-6 is a potent proinflammatory cytokine which plays an important role in the pathogenesis of inflammatory bowel disease (IBD) (Wang et al., 2003). Our PCIS studies indicate that RA will not be a potential treatment of IBD.

4.3. Antifibrotic effect of RA

Our results demonstrated that RA can hamper fibrogenesis in murine PCLS, similar to previous observations from our lab using human and rat PCLS (Westra et al., 2014a, 2014b). In contrast, RA did not affect the fibrotic response in PCIS of these species. A possible explanation for the observed discrepancy could be the mechanisms underlying fibrogenesis in both organs. In the liver, fibrosis is mainly caused by activated resident cells, whereas infiltrating immune cells are key players in the fibrotic response in the intestine (Rieder et al., 2007; Friedman, 2008). Activated hepatic stellate cells (HSCs) have numerous interactions with the immune system by means of antigen presentation, secretion of chemokines and via expression of adhesion molecules (Friedman, 2008), and they produce the majority of the ECM components associated with liver fibrosis (Wynn and Barron, 2010). On the other hand, intestinal fibrosis is mainly caused by damaging processes that elicit infiltration of immune cells, which will ultimately result in destruction of the mucosal and submucosal layers via oxidant activity (Rieder et al., 2007). Thus, RA might directly affect profibrotic resident cells (i.e. HSCs) in PCLS, thereby reducing the fibrotic response, whereas RA fails to target the effector cells in PCIS. Further research is needed to elucidate whether RA can mitigate intestinal fibrosis in a co-culture model using PCIS and activated macrophages.

Taken together, our results clearly demonstrate that RA has potential as a therapeutic agent for the treatment of liver fibrosis. In addition, RA appears to elicit anti-inflammatory and antifibrotic effects in murine PCIS. Conversely, these beneficial effects were not observed in human and rat PCIS. Thus, the advantageous effects of RA are clearly organ- and species-specific.

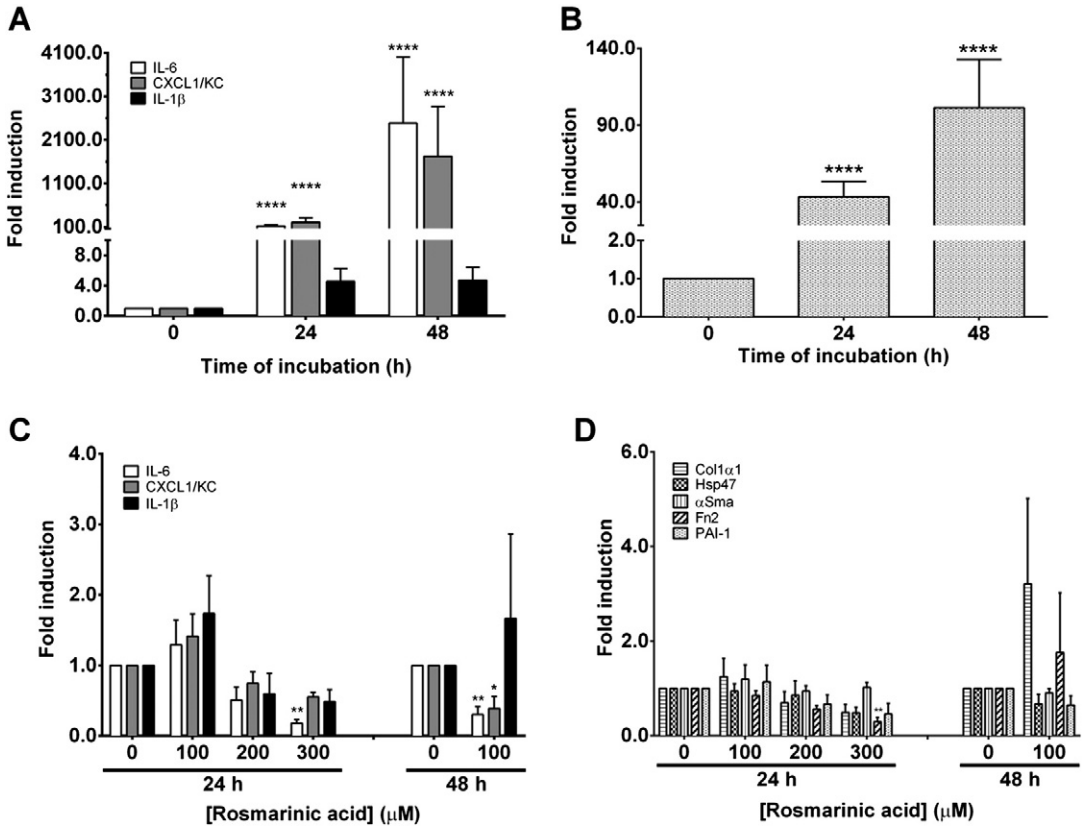

Fig. 6. The effect of incubation and rosmarinic acid on murine PCIS (n = 4): (A) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (incubation); (B) Fibrosis markers PAI-1 expressions (rosmarinic acid); (C) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (rosmarinic acid); (D) Fibrosis markers *Col1α1*, *Hsp47*, *αSma*, *Fn2*, and *PAI-1* expressions (rosmarinic acid). Data are expressed as mean +/− SEM. ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. control.

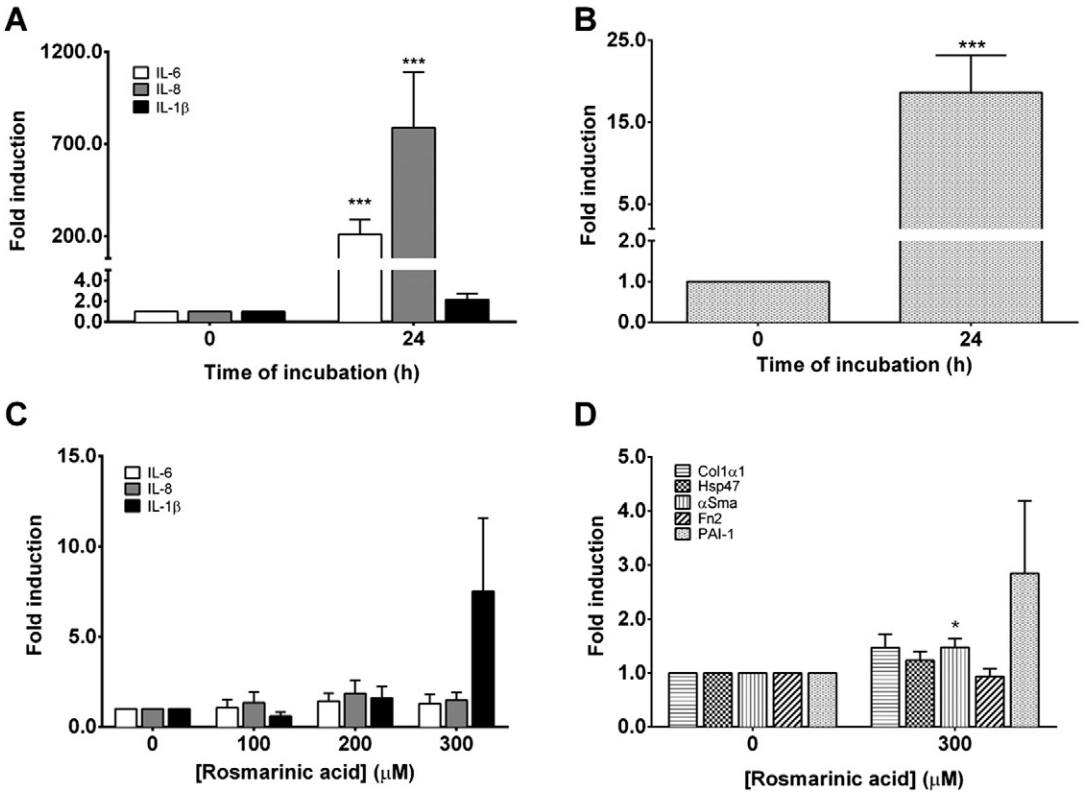

Fig. 7. The effect of incubation and rosmarinic acid on rat PCIS (n = 4): (A) Inflammatory markers *IL-6*, *IL-8*, and *IL-1β* expressions (incubation); (B) Fibrosis markers *PAI-1* expression (incubation); (D) Viability (relative value) (rosmarinic acid); (E) Inflammatory markers *IL-6*, *IL-8*, and *IL-1β* expressions (rosmarinic acid); (F) Fibrosis markers *Col1α1*, *Hsp47*, *αSma*, *Fn2*, and *PAI-1* expressions (incubation). Data are expressed as mean +/− SEM. ANOVA; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

We gratefully acknowledge the funding from *De Nederlandse organisatie voor gezondheidsonderzoek en zorginnovatie* (ZonMW)-The Netherlands number: 114021010 supporting this work. The authors thank Dr. Koert P. de Jong (Hepatobiliary Surgery and Liver Transplantation, University Medical Center Groningen) for the arrangements for obtaining human intestinal tissue.

References

Baba, S., Osakabe, N., Natsume, M., Terao, J., 2004. Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. Life Sci. 75, 165–178. http://dx.doi.org/10.1016/j.lfs.2003.11.028.

Baba, S., et al., 2005. Absorption, metabolism, degradation and urinary excretion of rosmarinic acid after intake of *Perilla frutescens* extract in humans. Eur. J. Nutr. 44, 1–9. http://dx.doi.org/10.1007/s00394-004-0482-2.

Baumgart, D.C., Carding, S.R., 2007. Inflammatory bowel disease: cause and immunobiology. Lancet 369, 1627–1640. http://dx.doi.org/10.1016/S0140-6736(07)60750-8.

Bozic, C.R., et al., 1994. The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization. J. Biol. Chem. 269, 29355–29358.

Cheng, A.-C., et al., 2011. Rosmanol potently induces apoptosis through both the mitochondrial apoptotic pathway and death receptor pathway in human colon adenocarcinoma COLO 205 cells. Food Chem. Toxicol. 49, 485–493. http://dx.doi.org/10.1016/j.fct.2010.11.030.

de Graaf, I.a.M., et al., 2010. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. Nat. Protoc. 5, 1540–1551. http://dx.doi.org/10.1038/nprot.2010.111.

Domitrović, R., et al., 2013. Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice. Food Chem. Toxicol. 51, 370–378. http://dx.doi.org/10.1016/j.fct.2012.10.021.

Friedman, S.L., 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology 134, 1655–1669. http://dx.doi.org/10.1053/j.gastro.2008.03.003.

Hossan, S., Rahman, S., Bashar, A.B.M.A., Jahan, R., 2014. Rosmarinic Acid : A Review of Its Anticancer Action 3 pp. 57–70.

Karmokar, A., et al., 2012. Dietary intake of rosmarinic acid by Apc Min mice, a model of colorectal carcinogenesis: levels of parent agent in the target tissue and effect on adenoma development. Mol. Nutr. Food Res. 56, 775–783. http://dx.doi.org/10.1002/mnfr.201100617.

Krol, W., et al., 1996. Inhibition of neutrophils' chemiluminescence by ethanol extract of propolis (EEP) and its phenolic components. J. Ethnopharmacol. 55, 19–25. http://dx.doi.org/10.1016/S0378-8741(96)01466-3.

Lee, H.J., et al., 2008. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology 250, 109–115. http://dx.doi.org/10.1016/j.tox.2008.06.010.

Li, G.-S., et al., 2010. In vitro and in vivo antifibrotic effects of rosmarinic acid on experimental liver fibrosis. Phytomedicine 17, 282–288. http://dx.doi.org/10.1016/j.phymed.2009.05.002.

Loftus, E.V., 2004. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. Gastroenterology 126, 1504–1517. http://dx.doi.org/10.1053/j.gastro.2004.01.063.

Mirzoeva, O.K., Calder, P.C., 1996. The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukot. Essent. Fat. Acids 55, 441–449. http://dx.doi.org/10.1016/S0952-3278(96)90129-5.

Mutsaers, H.A.M., Stribos, E.G.D., Glorieux, G., Vanholder, R., Olinga, P., 2015. Chronic kidney disease and fibrosis: the role of uremic retention solutes. Front. Med. 2, 1–7. http://dx.doi.org/10.3389/fmed.2015.00060.

Nakazawa, T., Ohsawa, K., 1998. Metabolism of rosmarinic acid in rats. J. Nat. Prod. 61, 993–996. http://dx.doi.org/10.1021/np980072s.

Nakazawa, T., Ohsawa, K., 1999. Metabolites of orally administere *Perilla frutescens* extract in rats and humans. Biol. Pharm. Bull. 23, 122–127.

Petersen, M., Simmonds, M.S.J., 2003. Rosmarinic acid. Phytochemistry 62, 121–125.

Pham, B.T., et al., 2015. Precision-cut rat, mouse, and human intestinal slices as novel models for the early-onset of intestinal fibrosis. Physiol. Rep. 3, e12323. http://dx.doi.org/10.14814/phy2.12323.

Poynard, T., et al., 2010. Prevalence of liver fibrosis and risk factors in a general population using non-invasive biomarkers (FibroTest). BMC Gastroenterol. 10, 40. http://dx.doi.org/10.1186/1471-230X-10-40.

Rieder, F., Brenmoehl, J., Leeb, S., Scholmerich, J., Rogler, G., 2007. Wound healing and fibrosis in intestinal disease. Gut 56, 130–139. http://dx.doi.org/10.1136/gut.2006.090456.

Rocha, J., et al., 2015. Anti-inflammatory effect of rosmarinic acid and an extract of *Rosmarinus officinalis* in rat models of local and systemic inflammation. Basic Clin. Pharmacol. Toxicol. 116, 398–413. http://dx.doi.org/10.1111/bcpt.12335.

Sanbongi, C., 2003. Rosmarinic acid inhibits lung injury induced by diesel exhaust particles. Free Radic. Biol. Med. 34, 1060–1069. http://dx.doi.org/10.1016/S0891-5849(03)00040-6.

Scheckel, K.a., Degner, S.C., Romagnolo, D.F., 2008. Rosmarinic acid antagonizes activator protein-1-dependent activation of cyclooxygenase-2 expression in human cancer. J. Nutr. 2098–2107 http://dx.doi.org/10.3945/jn.108.090431.

Torok, N.J., Dranoff, J.A., Schuppan, D., Friedman, S.L., 2015. Strategies and endpoints of antifibrotic drug trials: summary and recommendations from the AASLD Emerging Trends Conference, Chicago, June 2014. Hepatology 62, 627–634. http://dx.doi.org/10.1002/hep.27720.

Wang, L., et al., 2003. IL-6 induces NF-B activation in the intestinal epithelia. J. Immunol. 171, 3194–3201. http://dx.doi.org/10.4049/jimmunol.171.6.3194.

Westra, I.M., Pham, B.T., Groothuis, G.M.M., Olinga, P., 2012. Evaluation of fibrosis in precision-cut tissue slices. Xenobiotica.

Westra, I.M., Oosterhuis, D., Groothuis, G.M.M., Olinga, P., 2014a. Precision-cut liver slices as a model for the early onset of liver fibrosis to test antifibrotic drugs. Toxicol. Appl. Pharmacol. 274, 328–338. http://dx.doi.org/10.1016/j.taap.2013.11.017.

Westra, I.M., Hadi, M., Oosterhuis, D., de Jong, K.P., Groothuis, G.M.M., Olinga, P., 2014b. Precision-cut liver slices an ex vivo model for the early onset and end-stage of liver fibrosis (134 pp.) .

Wynn, T., Barron, L., 2010. Macrophages: master regulators of inflammation and fibrosis. Semin. Liver Dis. 30, 245–257. http://dx.doi.org/10.1055/s-0030-1255354.

Xavier, C.P.R., Lima, C.F., Fernandes-Ferreira, M., Pereira-Wilson, C., 2009. *Salvia fruticosa*, *Salvia officinalis*, and rosmarinic acid induce apoptosis and inhibit proliferation of human colorectal cell lines: the role in MAPK/ERK pathway. Nutr. Cancer 61, 564–571. http://dx.doi.org/10.1080/01635580802710733.