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## Anticancer Characteristics of *Fomitopsis pinicola* Extract in a Xenograft Mouse Model—a Preliminary Study

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### ABSTRACT

**Introduction:** Medicinal mushrooms have been used for the treatment of diseases and general promotion of health for many centuries. Recent pharmacological research into medicinal mushrooms has identified various therapeutic properties, with applications in modern medicine.

**Aim:** To evaluate the anti-cancer activities of *Fomitopsis pinicola* (*F. pinicola*) alcoholic extract in an in vivo setting.

**Methods:** The anti-tumour effect of the *F. pinicola* extract was tested in a xenograft immune-compromised Rag-1 mouse model. This was followed by RT-PCR and metabolomics analyses.

**Results:** There were no observable differences in tumor growth between treated and non-treated groups. The bioactive components were not detected in the mouse plasma or the tumor site.

**Conclusions:** The extract was poorly absorbed; this is likely due to the timing of treatment, dosage levels and modifications made to the extract where the alcohol-based solvent was replaced with water. This, in combination with fractionation studies which identified most anti-cancer compounds to be hydrophobic, largely explained the lack of anti-cancer activities in vivo.

### ARTICLE HISTORY

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prostate cancer;  
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## Introduction

Mushrooms have been used in oriental medicine for the general promotion of health and longevity for centuries (1). With a steady growth in the interest in medicinal mushrooms, investigations into their pharmaceutical potential are on the rise, with an increased number of published books and articles, and the addition of new scientific journals focusing on medicinal mushrooms (1,2). This is also reflected in industry, with the market value of medicinal mushrooms and their derivative supplements increasing from US\$6 billion worldwide in 1999 to US\$35.08 billion in 2015 and is expected to reach above US\$59.48 billion by 2021 (3).

Although medicinal mushrooms have been used by various cultures for centuries, they have only relatively recently been investigated for their application in modern medicine. Medicinal mushrooms have been used as an adjuvant to cancer therapy by alleviating the side-effects of chemo- and radiation therapy such

as nausea, anemia, and a lowered immune system (4). Furthermore, numerous clinical trials have been conducted to assess the potential anticancer properties of commercially prepared medicinal mushrooms (4).

We have previously tested a whiskey extract from the fungus *F. pinicola* on various human cancer cell lines and have found positive growth inhibitory effects. In addition, we identified several biologically active pathways associated with the anticancer activities of the extract in prostate cancer (PCa) cell lines (5). Since PCa is one of the most commonly diagnosed cancers in Australian and New Zealand men (6,7), and the fifth most common cancer worldwide (7), we have continued to focus on PCa in this study.

PCa originates from the prostate gland, a male-specific organ associated with the reproductive system. The growth and survival of prostate cells is highly dependent on stimulation by androgens (8), steroid hormones that play a specific role in the differentiation,

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development, and maintenance of male sexual characteristics via activation of androgen receptors (8). Androgens regulate the prostate epithelium by stimulating cell proliferation (9). Cancer occurs when the cell cycle becomes deregulated, and the rate of cell division exceeds the rate of cell death. Tumor progression and metastasis involve mutations in key genes involved in cell cycle control and proliferation, such as *TP53* and *RB* (*retinoblastoma*) (10). Over 95% of PCa are adenocarcinomas, which originate from prostatic epithelial cells (11). Immortalized human PCa cell lines are commonly used as models of PCa. The PC3 cell line is a PCa adenocarcinoma, and consists of metastatic and androgen-independent cells derived from prostate metastasis in bone (12), and is known to form tumors in a mouse xenograft model (13,14).

The aims of this study were to investigate the *in vivo* anticancer activities of a *F. pinicola* extract and to compare the results with previously identified anticancer activities *in vitro*. Further *in vivo* studies were designed to investigate the pharmacokinetic properties of the *F. pinicola* extract in mice. Using fractionation, the nature of the *F. pinicola* extract was also evaluated.

## Material and Methods

### Animal Ethics Statement

All animal studies were conducted under the approval of the University of Auckland Animal Ethics Committee (Ref No. 001190). All mice used in this study were housed at 20 °C in a 12 h light/dark cycle with food and water available *ad libitum*.

### Preparation and Modification of Extract

The *F. pinicola* whiskey extract tested in this study was provided by NZFocus Ltd (Auckland, New Zealand). The commercial extract had been prepared by submerging dried *F. pinicola* bracket fungi from the Himalayan region in whiskey for six months. We reduced the volume of *F. pinicola* whiskey extract by freeze-drying, resulting in a dry white powder. The powder was resuspended using 5% dextrose.

### PC3 Tumor Xenograft Study

Age-matched male-specific pathogen-free Rag1<sup>-/-</sup> mice (Jackson Laboratory, Bar Harbor, ME) weighing 18–22 g were subcutaneously inoculated with  $5 \times 10^6$  PC3 cells suspended in PBS (100 µl/mouse) on the right flank. Once tumors reached 200 mm<sup>3</sup>, animals were randomized to receive control vehicle (5%

dextrose) or 500 or 1,000 mg/kg *F. pinicola* extract ( $n = 10$  per group) daily for 15 days by oral gavage. Tumor measurements were recorded by electronic calipers. All quantified data are expressed as mean  $\pm$  standard error of the mean (SEM). P-values were calculated using one-way ANOVA and data were considered significant when  $P$ -value  $< 0.05$ . At the completion of treatment, tumor and liver tissues were collected for RT-PCR analysis.

### Metabolomics Evaluation

Age-matched male-specific pathogen-free CD-1 mice (Charles River Laboratories, Wilmington, MA) weighing 18–23 g were treated with 1,000 mg/kg *F. pinicola* extract by oral gavage and were culled 15 min, 30 min, 1 h, 4 h, and 24 h after dosing ( $n = 3$  per time point). Blood and liver were collected immediately after culling, with blood being centrifuged at 2,000g for 15 min in EDTA tubes (Becton Dickinson, Franklin Lakes, NJ) for the extraction of plasma. Fecal samples were also collected prior to culling.

### Q-Exactive Mass Spectrometry

Plasma and fecal samples were mixed with ice cold methanol then centrifuged at 12,000g for 10 min at 4 °C to remove protein precipitates. The process was repeated three times. Fifty micro liter of supernatant was taken and added to 50 µl of ASTM (American Society for Testing and Materials) Type 1 (ULTRAPURE) water and mixed. Five micro liter of each sample was injected into a Q-Exactive Hybrid Quadrupole-Orbitrap MS with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Waltham, MA) for analysis. A Syncronis C18 100  $\times$  2.1 mm, 1.7 µm column (Thermo Fisher Scientific) was used with a mobile phase gradient of ASTM type 1 water (0.1% formic acid) and acetonitrile (0.1% formic acid) at a flow rate of 0.4 mL/min. Deuterated internal standards (tryptophan D5, cortosterone D8, progesterone D9) purchased from Sigma-Aldrich (Auckland, New Zealand) and prepared in methanol were used to correct for analyte response. Mass spectrometry data were analyzed using the MaxQuant software. Alignment and principal component analysis were completed using the SIEVE software.

### RT-PCR

RNA was extracted from tumor, liver, and plasma using RNeasy Plus Mini Kits (QIAGEN, Venlo, the Netherlands) following the manufacturer's protocol.

One microgram of total RNA was reverse transcribed into cDNA using a Quantitect Reverse Transcription Kit (QIAGEN) following the manufacturer's protocol. The primers (Thermo Fisher Scientific) used for RT-PCR analysis included *RAD51* (Hs00947967\_m1, 58 bp), *Rad51* (Mm00487905\_m1, 58 bp), *BRCA1* (Hs01556193\_m1, 59 bp), *Brca1* (Mm00515386\_m1, 90 bp), *IRF1* (Hs00971960\_m1, 60 bp), *Irf1* (Mm01288580\_m1, 66 bp) and house-keeping genes *GAPDH* (Hs02758991\_g1, 93 bp), *Gapdh* (Mm99999915\_g1, 109 bp), and *18S* (4310893E, 187 bp). Primers targeting human genes were used for the analysis of tumor tissue and primers targeting mouse genes were used for liver tissue. RT-PCR reactions were performed with TaqMan<sup>TM</sup> Fast Advanced Master Mix (Thermo Fisher Scientific). The thermocycling steps were programmed with QuantStudio 6 (Thermo Fisher Scientific) as follows: a pre-cycling stage at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. The fluorescence signal was measured at the end of each annealing step. Microsoft Excel with the  $2^{-\Delta\Delta Ct}$  method was used to determine the mRNA expression levels of genes of interest relative to the house-keeping genes (15). One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)). *P*-values <0.05 were considered significant.

### Fractionation of Extract

Fractionation was used to separate *F. pinicola* extract into aqueous and organic fractions. The ratio used for separation was 1:2:1 water: alcohol: chloroform solution. As the original extract already contained 50% ethanol and 50% water, the proportion used was 1:1:1:1 (water: methanol: ethanol: chloroform).

### Tissue Culture

PCa cell line PC3 was obtained from Dr Ronnie Cohen (Perth) and was authenticated by analysis of short tandem repeats (STR) by DNA Diagnostic Ltd (Auckland, New Zealand). PC3 cells were cultured in Minimum Eagle Media (Sigma-Aldrich) supplemented with 10% Fetal Calf Serum (Moregate Biotech, Hamilton, New Zealand) and 1% Penicillin/Streptomycin/Glutamine (Sigma-Aldrich).

PC3 cells were seeded at a cell density of 2,000 cells/well in 96-well plates (Corning, New York, NY) and incubated overnight at 37 °C with 5% CO<sub>2</sub> before being treated with the aqueous/organic fractions of *F.*

*pinicola* extract. The cells were incubated under the same conditions for four ×24 h doubling cycles. Growth inhibition was measured using the sulforhodamine B-(SRB)-based assay (16) with IC<sub>50</sub> values determined by four parameter logistic fit using the SigmaPlot 11.0 (Systat Software Inc.).

## Results

### Antitumor Efficacy

To determine if *F. pinicola* extract alters PC3 tumor growth, Rag1<sup>-/-</sup> mice with PC3 tumors were treated with 500 or 1,000 mg/kg *F. pinicola* extract. Figure 1 shows the changes in the average tumor size over time in the three xenograft mouse groups. The control group showed gradual increase in average tumor weight until day 12, where reduction in average tumor size was observed from day 12 to day 14. This coincide with reduced number of mice in the control group, as the mice with the largest tumors had to be culled thus leading to reduction in average tumor size of the surviving mice. All three groups showed gradual increase in tumor size over time. However, there were no significant differences between the changes in tumor size among the three groups.

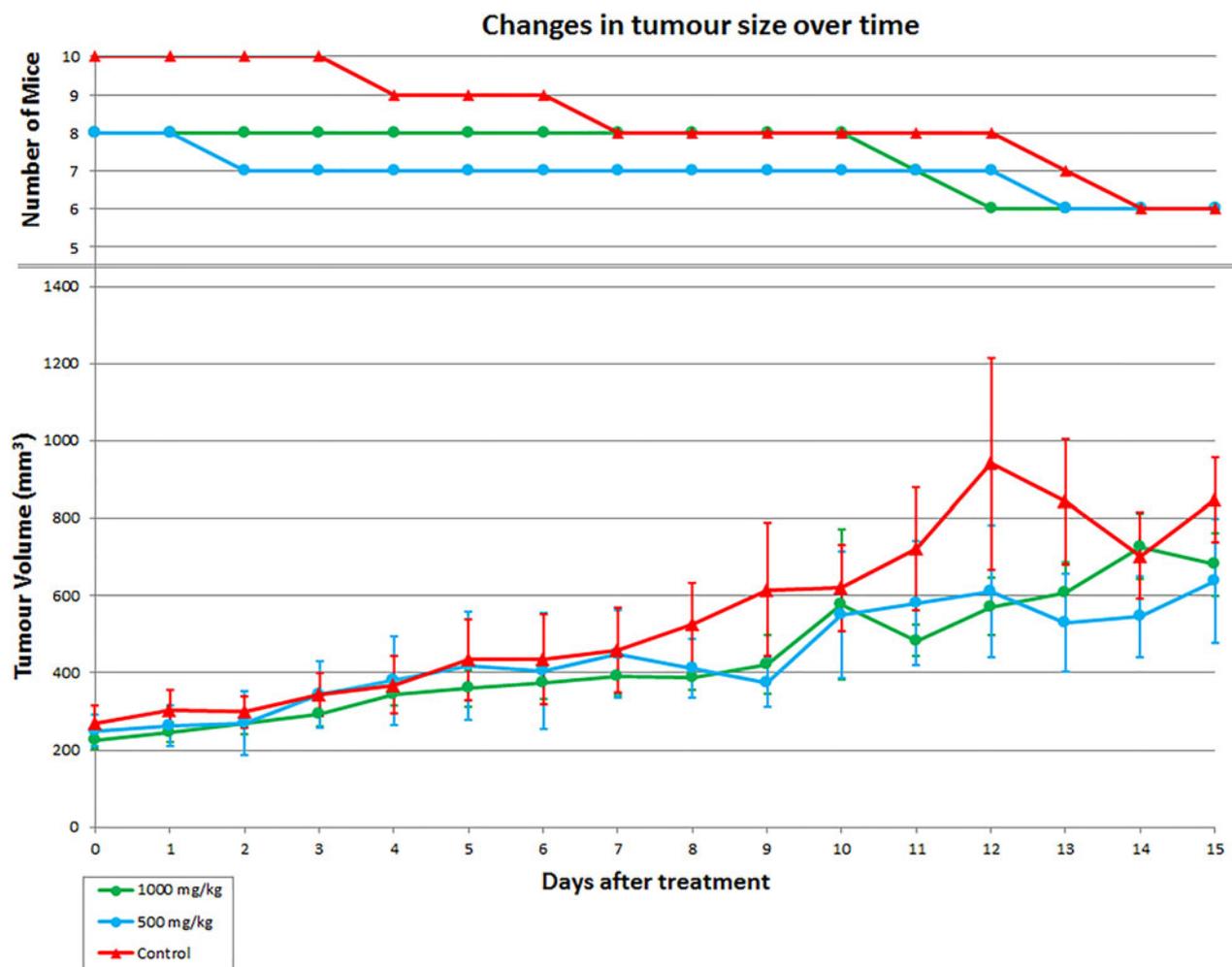
### Xenograft Mice Tumor and Liver Gene Expression Analysis

The expression of human *BRCA1*, *IRF1*, and *RAD51* genes in the PC3 tumors of Rag1<sup>-/-</sup> mice treated with *F. pinicola* extract were evaluated by RT-PCR, since the expression of these genes was altered by *F. pinicola* extract in prostate cancer cell lines in vitro (5). There were no significant changes in gene expression levels between treated and untreated groups for all three genes tested (Figure 2a).

We also used RT-PCR to evaluate the expression of the orthologous mouse genes (*Brca1*, *Irf1*, and *Rad51*) in the liver of the Rag1<sup>-/-</sup> mice treated with *F. pinicola* extract. All three genes showed significant changes in gene expression levels after treatment (*P* < 0.05) (Figure 2b). For *Brca1* and *Rad51*, the expression levels in the 1,000 mg/kg treatment group were significantly greater than those in the 500 mg/kg treatment group (*P* < 0.01).

### CD1 Mice Liver and Plasma Gene Expression Analysis

CD1 mice were treated with 1,000 mg/kg *F. pinicola* extract with plasma and liver samples collected at



**Figure 1.** The number of surviving mice and changes in tumor size over time in Rag1<sup>-/-</sup> mice with PC3 tumors treated daily with Bracket fungal extract at 500 or 1,000 mg/kg or control vehicle. Values represent the mean  $\pm$  SEM. Number of mice = 6–10. For 1,000 mg/kg and 500 mg/kg groups, the number of mice started at 8 due to both having two mice with failed tumor implantation. The number of mice reduced over time due to tumor ulceration (resulting in ethically required euthanasia).

multiple time points after dosing. Changes in gene expression over time were determined by RT-PCR. Figure 3a shows the changes in gene expression levels in the plasma of CD1 mice. In the plasma, while gene expression ranged between 1.37 and 0.79, no significant changes relative to baseline for any of the three genes were observed.

*Rad51* and *Brcal* showed significant increases in gene expression in the liver in the first 4 h after dosing ( $P < 0.05$ ) (Figure 3b). Liver *Irf1* showed a significant 1.5-fold increase in expression 30 min after dosing ( $P < 0.05$ ), and returned to predosing level 1 h after dosing. All three genes showed similar liver expression levels as untreated controls 24 h after dosing.

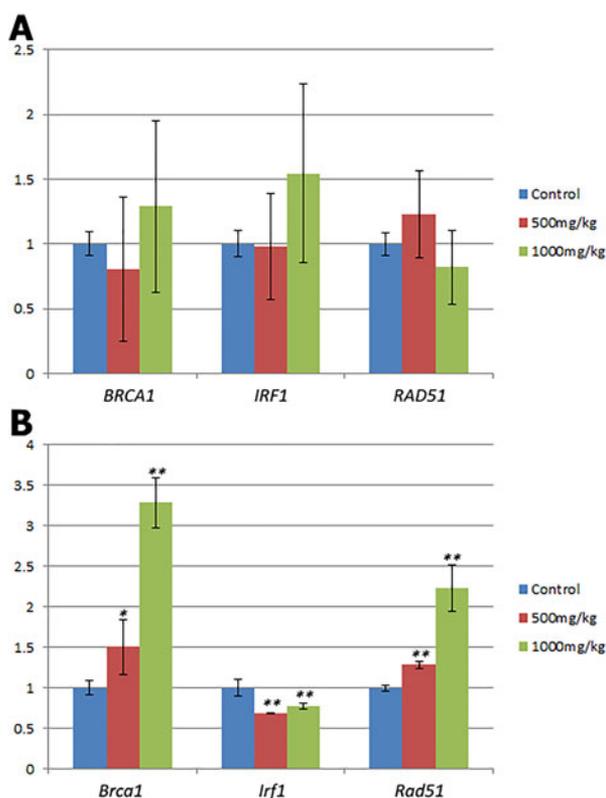
### Q-Exactive Analysis

Alignment and component extraction of mouse fecal and plasma samples are shown in Figure 4. For the

plasma samples, there is a lack of clustering between treated and control groups, which suggest no consistent differences in the metabolomics profiles between treated and control groups. For the mouse fecal samples, both positive and negative ions show clustering separate from controls, which suggest differences in the metabolomics profiles between the treated groups across all time points compared to controls. A scan of product ions revealed that multiple unknown positive and negative ions were observed in fecal samples of mice treated with *F. pinicola* compared to untreated control mice (Table 1). No unique peaks were found in treated mouse plasma samples compared to controls (data not shown).

### Extract Analysis

Fractionation was performed on *F. pinicola* whiskey extract. The extract was separated into an organic



**Figure 2.** Changes in gene expression levels in Rag1<sup>-/-</sup> mice treated with *F. pinicola* extract. **A.** Changes in human gene expression levels in the PC3 tumors. **B.** Changes in gene expression levels of mouse genes in the liver tissues. Bars represent the mean and standard error ( $n=3$ ). Statistically significant differences vs. controls are shown at \* $P < 0.05$ , \*\* $P < 0.01$ .

fraction (56.4% of total weight) and an aqueous fraction (43.6% of total weight).

The antiproliferative activity of the aqueous and organic fractions of *F. pinicola* whiskey extract were tested on the PC3 cell line (Figure 5). Stronger growth inhibitory activities were observed with the organic fraction compared to the aqueous fraction (Organic  $IC_{50} = 1.85 \mu\text{l} \pm 0.97 \mu\text{l}$  vs. Aqueous  $IC_{50} = 27.7 \mu\text{l} \pm 7.2 \mu\text{l}$ ).

## Discussion

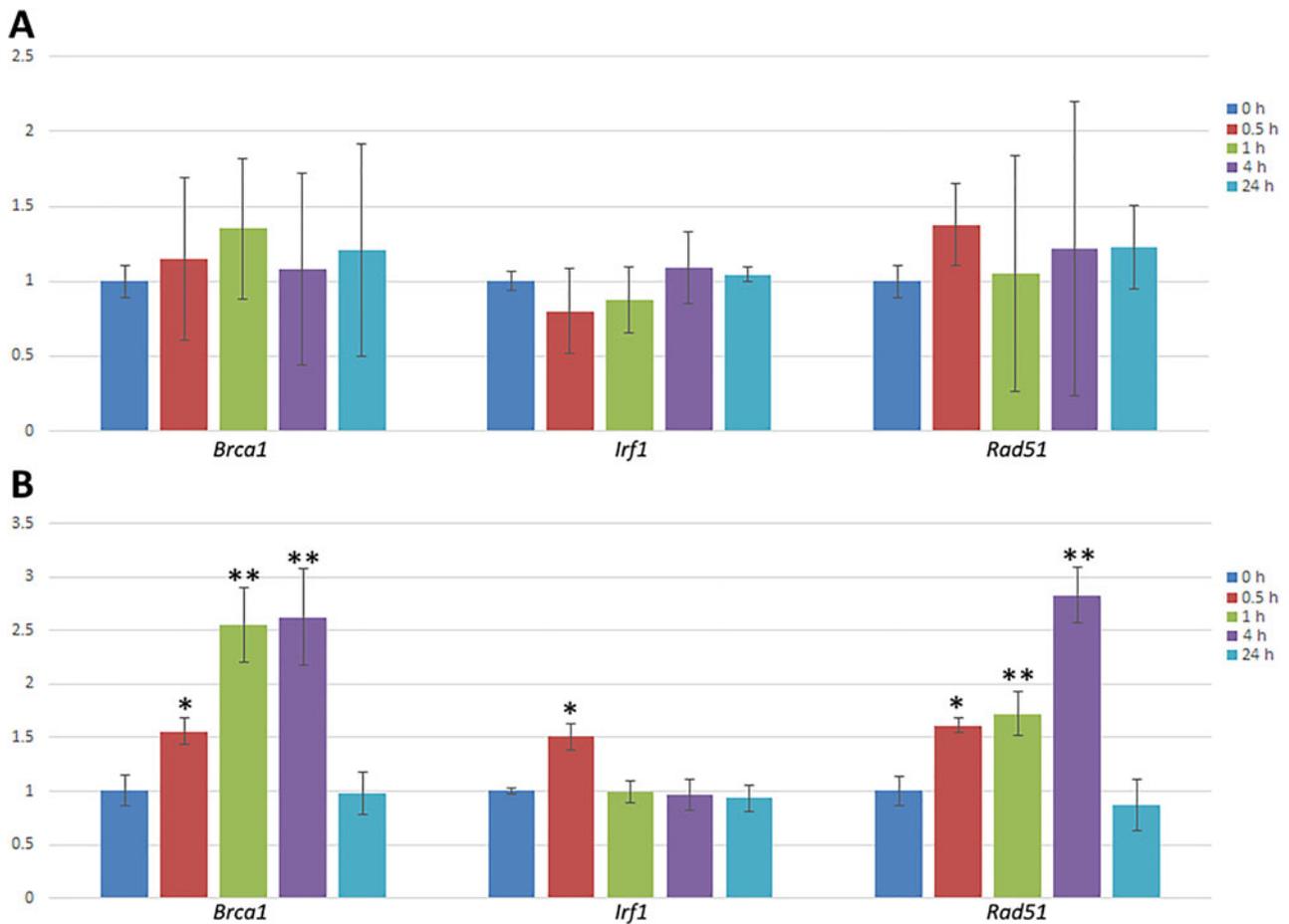
Following our identification of anticancer activities and associated biologically active pathways of *F. pinicola* extract in cancer cell lines (5), we wanted to ascertain if the same anticancer activities could be observed in an in vivo setting using a xenograft model. Despite observing antiproliferative activity in PC3 cells in vitro, the extract did not alter PC3 tumor growth at well-tolerated doses.

The tumor and liver tissues were harvested upon termination of the xenograft mice. The expression of

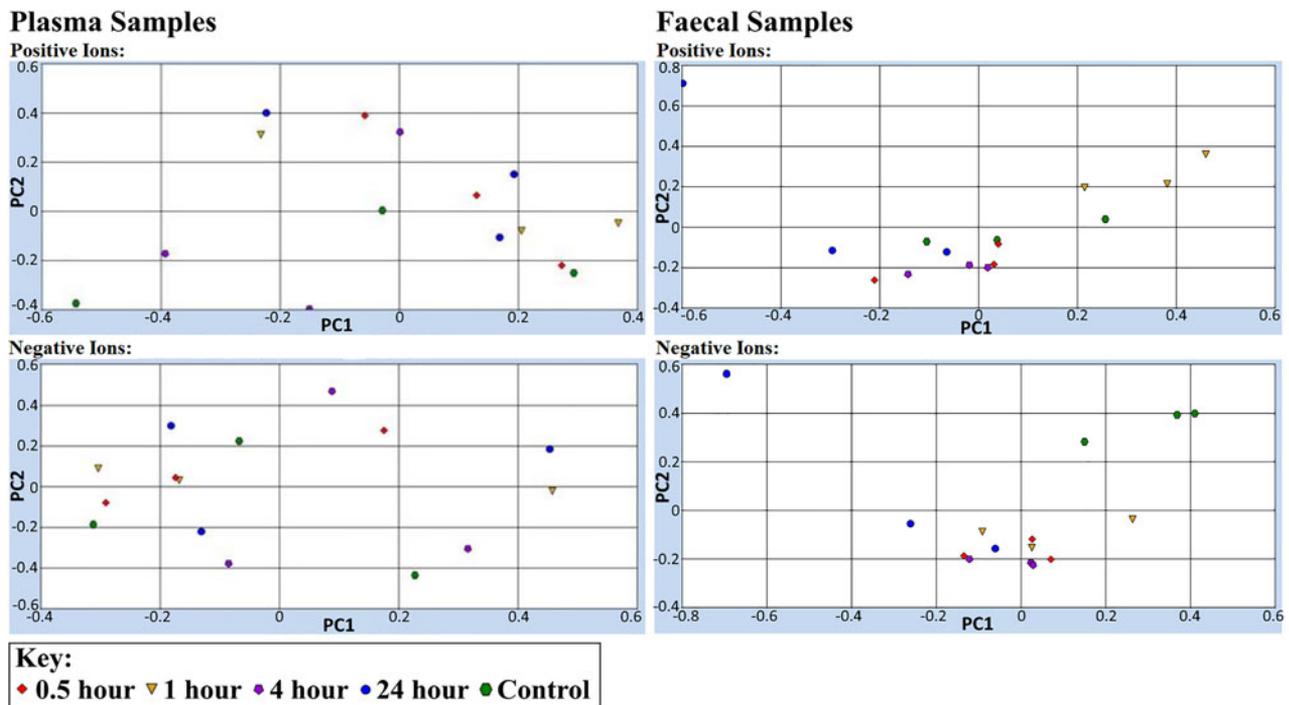
three genes, previously found to be differentially expressed in the earlier in vitro assays in response to *F. pinicola* extract (5) were measured using RT-PCR. We found significant changes in gene expression levels in the liver in the same direction as was observed by Affymetrix analysis in PCa cell lines (5), but these changes were not detected in the tumor tissues. It is not clear why the extract altered gene expression in the liver but not in the tumor, although the minimal effect on tumor gene expression correlates with the lack of efficacy against tumor growth. One possibility is that the compound is metabolized or excreted through the bile into the feces during first pass metabolism and therefore the active component does not reach the tumor. This is supported by the mass spectrometry analysis of the fecal samples, where, unlike the plasma samples, the treated fecal samples cluster together and unique product ions were detected, indicating the likely presence of extract components or their metabolites. If the extract is indeed rapidly hepatically eliminated, the active component would still reach the liver where it could alter gene metabolism prior to being metabolized or excreted.

We also observed that changes in gene expression levels in the healthy mouse liver were similar to that of the immune-compromised mouse liver in response to treatment with *F. pinicola* extract. However, similar to the situation in tumors, there were minimal changes in gene expression observed in plasma, further suggesting that the active components of the extract are restricted from reaching the systemic circulation through elimination during first pass through the liver. The changes in gene expression in the liver returned to baseline level within 24 h after treatment (Figure 3b), suggesting that the active components were not retained for long periods within the liver, presumably due to its elimination there.

The original *F. pinicola* extract was an alcoholic extract containing ~50% ethanol. Due to mice being extremely sensitive to ethanol, the alcoholic content was removed and the extract was resuspended in water as a 5% dextrose solution. The removal of ethanol increased the dosage the mice could tolerate without inducing alcohol toxicity. However, fractionation, in combination with tissue culture studies, showed the majority of the cell proliferative inhibitory activity to be in the hydrophobic (organic) fraction. The use of a hydrophilic-based solvent may have reduced the solubility of the active component in the GI tract of the mice, limiting absorption and promoting excretion in the feces. Dissolving the extract in an organic-based solvent, such as corn or palm oil, could potentially



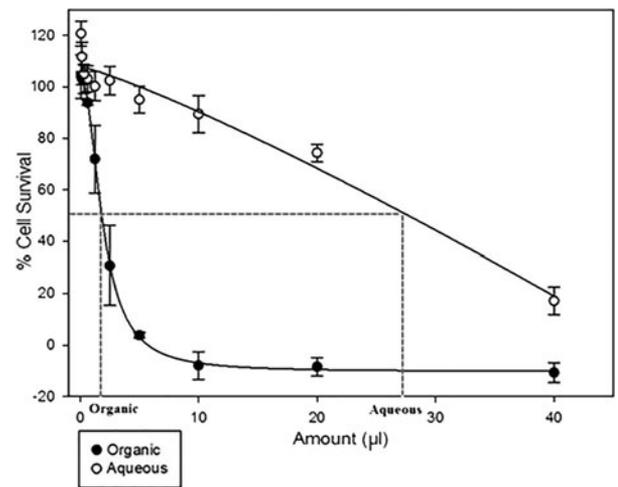
**Figure 3.** Changes in mouse gene expression levels in CD1 mice treated with *F. pinicola* extract over time. **A.** Changes in gene expression levels in the plasma. **B.** Changes in gene expression levels in the liver tissues. Bars represent the mean and standard error ( $n = 3$ ). Statistically significant differences vs. baseline are shown at  $*P < 0.05$ ,  $**P < 0.01$ .



**Figure 4.** Principal component analysis plot of mouse fecal and plasma samples.

**Table 1.** Top positive and negative ion intensities in fecal samples of mice treated with *F. pinicola* extract. Criteria: *p*-value < 0.0001 in 2 of the 4 time points measured.

Comp ID	Ions	Comp MW	m/z	Time	30 min			1 h			4 h			24 h			Control		
					1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
236	+	194.057055	195.0643	1.41	420903	254624	384911	452567	468475	0	277958	267284	338538	52405625	761579	642005	0	0	0
612	+	301.204241	302.2115	4.92	4244004	709078	403514	1473154	36125	0	27573955	2011448	5239506	7503101	19414791	6195028	0	0	0
701	+	325.284289	326.2916	18.35	76192	0	0	229359	0	0	95473	140857	123130	313805115	2356607	2293609	0	0	0
788	+	344.245959	345.2532	0.69	0	220619	190915	708859	0	0	512833	182830	266765	19540898	879553	1052688	0	0	0
1017	+	422.344836	423.3521	18.65	104632418	6378336	61807	61105	59672	73994	57383530	26568193	43543236	758106	80788414	55744600	0	0	0
1185	+	488.260974	489.2682	2.35	257542	7421	0	3348560	0	0	7266840	75542	3635733	37783596	7306762	3440643	0	0	0
1193	+	491.34627	492.3535	14.21	0	0	555219	0	0	23473929	6498274	5396326	1155768	85172064	97485113	38407302	0	0	0
1209	+	496.196887	497.2042	0.64	701801	13210669	2820004	54221591	39620225	0	11388213	4515558	15432000	36565342	10797316	20627349	0	0	0
1362	+	618.32561	619.3329	2.35	799310	59372	0	11566285	0	0	23496649	298343	11999968	131513965	21324879	11548210	0	0	0
1404	+	653.452624	654.4599	16.02	0	0	909486	29239	77115	25977495	9996033	6805382	2538309	46958314	85729215	24694846	0	0	0
1464	+	815.558825	816.5661	18.50	0	0	42115	0	0	13270852	8034838	3532050	771239	21741822	62083737	16524654	0	0	0
1484	+	884.24602	885.2533	7.90	17836090	31445409	8789710	4960636	2098228	0	19655006	13794804	12323028	3027381	26226702	21580866	0	0	0
203	+	209.103696	208.0964	7.64	576234	502820	0	3446462	0	0	65315704	1211589	21700875	21090665	54749827	5303870	0	0	0



**Figure 5.** Antiproliferative activity of *F. pinicola* extract organic fraction and aqueous fraction in PC3 cells using SRB staining method. Symbols represent the mean and standard error of the mean (*n* = 4 technical replicates).

enhance absorption and increase bioavailability of the active compounds without inducing toxicity (17).

The maximum dose tested was 1,000 mg/kg with no observable toxicity. A previous study by Wu et al. (18) used dosages of 0.5% *F. pinicola* ethanol extract in the diet of up to 5,000 mg/kg in mice with no observable toxicity. They did not detect any inhibition of tumor growth at a dosage of 1,250 mg/kg but found a statistically significant inhibition of tumor growth at higher dosages (>2,500 mg/kg). This suggests that the dosages we used may have been too low to have any observable anti-cancer effects. In addition, we could potentially have treated the mice with much higher doses without the risk of toxicity. Furthermore, other similar animal studies began treating mice with *F. pinicola* extract prior to xenografting human tumor cells, and subsequently found tumor growth inhibition (18–20). This prophylactic approach, rather than treating after tumors are well established, perhaps better represents the medicinal mushroom’s role as a nutraceutical for cancer prevention rather than a drug for the treatment of cancer.

### Conclusion

Treatment with doses of the *F. pinicola* extract up to 1,000 mg/kg had no statistically significant effect on PC3 PCa tumor growth in mice, despite showing strong growth inhibitory effects in an in vitro analysis with the same cell line.

Using a combination of molecular analysis, we have found that the lack of tumor growth inhibition is likely due to the low bioavailability of active ingredients at tumor site. Thus, future in vivo studies using

this extract will require improvements in the delivery mechanisms to ensure sufficient active components reach the tumor site for an observable response. Overall, we believe *F. pinicola* is still a potentially valuable source of anticancer (especially PCa) bio-active compounds, however, better understanding of the active components in the mushroom will be required moving forward.

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## Author Contributions

*Overall design of study:* Chi H.J. Kao, David R. Greenwood, Lynnette R. Ferguson, and Karen S. Bishop

*Tissue Culture Study & RT-PCR:* Chi H.J. Kao and Pamela M. Murray

*Animal Study:* Stephen M.F. Jamieson and Chi H.J. Kao

*Q-Exactive:* Margaret E. Coe and Chi H.J. Kao

*Fractionation:* David R. Greenwood and Chi H.J. Kao

## Disclosure Statement

The authors report no conflict of interest.

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