Polysaccharide peptide (PSP) extract from Coriolus versicolor decreased Tmax of tamoxifen and maintained biochemical serum parameters, with no change in the metabolism of tamoxifen in the rat

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Abstract

Background: Natural products such as mushrooms are increasingly used as adjunct therapies in cancer to stimulate the immune system. However, little is known about their interaction with chemotherapy drugs. This study investigated whether a commercial polysaccharide peptide (PSP) extract of the mushroom *Coriolus (C.) versicolor* interacts with tamoxifen, a common chemotherapy drug used in breast cancer.

Methods: The pharmacokinetic and biochemical parameters of tamoxifen in female Sprague-Dawley rats' serum were determined after orally administering tamoxifen (20 mg/kg) at a single dose and repeated dosing with and without *C. versicolor* (340 mg/kg).

Results: Although the area-under-curve and maximum concentration showed no significant differences in both the single dose and repeated dosing, time to reach maximum concentration (T_{max}) increased by 228% and 93%, respectively, in the rats treated with *C. versicolor* ($p < 0.05$). The repeated dosing of *C. versicolor*, when co-administered with repeated dosing of tamoxifen, maintained 19 out of 23 biochemical serum parameters in rats compared to control ($p < 0.05$). Using hepatic female rat microsomes, an *in vitro* study showed that the extract (10 − 100 µg/mL) did not significantly alter the rate of tamoxifen depletion which is known to be mediated by cytochromes P450 (CYP450).

Conclusions: These results indicate that *C. versicolor* may delay the intestinal absorption of tamoxifen and maintain biochemical serum parameters, without any change in tamoxifen's metabolism. If clinical trials confirm this result, the timing of tamoxifen and *C. versicolor* administration would need to be considered to avoid potential drug interactions resulting from a delay in achieving the systemic level of the anti-cancer medication.

Introduction

In recent times, cancer sufferers have turned to natural products as an adjunct support to providing relief from chemotherapy and its deleterious effects on the immune system, and to enhance recovery (1); (2); (3); (4). *Coriolus (C.) versicolor* ((L.) Quél. (1886)) or 'Yunzhi' in Chinese, is an edible, colourful mushroom with prized energising and healing effects, and has been used in folk medicine, especially traditional Chinese medicine, to treat herpes, liver disorders including hepatitis, chronic fatigue syndrome, upper respiratory, urinary and digestive tract infections, strengthen physique and increase energy (5). Most of the immune-stimulating properties of *C. versicolor* have been attributed to complex sugars in the form of polysaccharide peptide (PSP) (6) and polysaccharide krestin (PSK). Scientists have suggested that chemicals in *C. versicolor* may be able to fight cancer and boost the immune system, reduce the toxic effects and pain of chemotherapy and radiation therapy; increase the effectiveness of chemotherapy; prolong life and raise the quality of life of cancer patients (7); (5); (8); (9). In Japan, *C. versicolor* is approved as an immunotherapeutic agent for cancer. Thus, the popularity of combinational therapy makes it important to understand potential interactions between natural products and prescription drugs as interactions can undermine the chemotherapy regimen and/or illicit toxic responses to the body (10); (4); (11); (12).

The nonsteroidal, antineoplastic agent tamoxifen (13) is a first line drug in the prevention and treatment of oestrogen receptor-positive breast cancer which make up around 60-65% of breast cancers (Johnson et al., 2004). However, its side effects include hot flushes, an increased risk of blood clots in the legs and the lungs, and a two- to three-fold increased risk of endometrial cancer in postmenopausal women (14, 15). Although clinical evidence suggests that *C. versicolor* is beneficial as an adjunct treatment in breast cancer (15) (16), there is little information on whether or how it interacts with tamoxifen. Thus, the aim of this study was to examine the pharmacokinetic, biochemical interaction and depletion of tamoxifen with a commercial *C. versicolor* PSP extract using a female Sprague-Dawley rat model.

Materials And Methods

Materials and reagents

Tamoxifen citrate salt, internal standard verapamil hydrochloride, potassium dihydrogen phosphate and *ortho*-phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isoflurane was purchased from Cenvet (Kings Park, WA, Australia) while gavage needles (18G) were purchased from Livingstone, NSW, Australia. An 18G needle was used in these studies

because it is thinner than 16G and can easily pass through the oesophagus without causing any damage to mucosal membrane and distress to the animal. The high-performance liquid chromatography (HPLC) grade acetonitrile, methanol and hexane were from Analytical Science (Sydney, NSW, Australia). Irradiated rat pellet diet was purchased from Speciality Feeds (Glen Forrest, WA, Australia). The commercial *C. versicolor* extract (ONCO-Z®) was supplied by PuraPharm International (H. K.) Ltd. Central, Hong Kong. The PSP extract is made from 100% wild *C. versicolor* and contains absorbable peptidoglycan (APG). The standardised extract (340 mg) is equivalent to 2.83 g of dried *C. versicolor* and comes from a USP-accredited biological procedure for standardisation. The extract was verified by the U.S. Pharmacopoeia (17) in August 2009 and comprises of at least one peptide linked glucan, with glucose molecules as a monosugar connected by a 1→3 linkage. The crude extract (molecule weight of 0.5-40 kDa) comprises of an average 4.7% peptide/protein composition, 55% neutral sugar and 4.8% uronic acid. The purified extract has a molecular weight of 0.3 kDa to 5 kDa (average 2.6 kDa) and is highly water soluble. The amino acid sequence of the protein/peptide moiety was determined to be Asp-Cys-Pro-Pro-Cys-Glu (18). To maintain verification status, the manufacture of the extract must continue to take place under the same conditions. A voucher specimen was deposited at NICM Health Research Institute, Western Sydney University, Australia (Batch no.: A1301475). A partial structure of the PSP polysaccharide component is proposed in a previous study (6).

Dosing preparations

The PSP extract was prepared in distilled water at a concentration of 170 mg/mL and administrated according to the animal weight (2 mL/kg). The concentration was chosen to reflect a high dose and the company's amount of extract in one capsule (340 mg) and was under the considered lethal dose of more than 5000 mg/kg (6).

Tamoxifen was prepared at a concentration of 15.2 mg/mL dissolved in water and administrated by oral gavage according to the animal weight (2 mL/kg). As tamoxifen is given orally to patients, oral gavage allowed the drug to be delivered directly to the stomach and this minimises error associated with free feeding. The tamoxifen dose was chosen to keep the plasma concentrations above the detection limit (19).

Animals

Adult female Sprague-Dawley rats (over 12 weeks of age; 215 – 340 g) were bred in house in the School of Medicine Animal Housing Facility, Western Sydney University. Rats were chosen as the metabolic disposition of tamoxifen in rat resembles that in human (13). This rat species and gender has been used in other tamoxifen drug interaction studies (20) (19). Throughout the experiment, three rats were weight-matched and randomly housed in the facility in Green Line individually ventilated cages (IVC) (Techniplast, USA), in a temperature-controlled room (22 ± 3 °C), 50-60% relative humidity, under a 12 h light-dark cycle. The animals were acclimatised for at least one week with a normal diet and water ad libitum. During the study, rat observations included posture, coat hair, activity, movement, breathing, alertness, appetite changes/vomiting, dehydration, eyes, nose, faeces and abnormal bleeding. This was monitored daily using an animal health monitoring sheet. Initial and final weights were recorded. The rats were anaesthesised using a high dose of isoflurane (and/or ketamine and xylazine as an option) to bring them into a surgical plane of anaesthesia and bled completely by cardiac puncture. Isoflurane anaesthesia has shown no significant effects on plasma metabolites (21).

Pharmacokinetic interaction study of tamoxifen

The pharmacokinetic interaction study consisted of two parts: a single oral dose of tamoxifen and repeated dosing of tamoxifen (to reflect chronic dosing and achieve a steady state), with *C. versicolor* fed orally for seven days in both scenarios. The inclusion of five rats in each group of the pharmacokinetic and pharmacodynamics studies is an adequate number to be able to obtain statistically significant results to show that the effects are real and reproducible and are not by chance. Thirty rats were randomly divided into six groups (one control and five experimental groups) consisting of five rats in each: the control group treated with water; *C. versicolor* extract control (*C. versicolor* fed orally once a day at 340 mg/kg for seven days, with sampling on day 8); tamoxifen control single dose (dose of 30.4 mg/kg tamoxifen citrate equivalent to 20 mg/kg tamoxifen base); *C. versicolor* extract (once a day for seven days) and single dose of tamoxifen was administered orally on day 8 and serial blood sampling done on the same day; tamoxifen (control repeated dosing for 13 days at 20 mg/kg per day; sampling day 13) and tamoxifen

administered for 13 days and *C. versicolor* extract (340 mg/kg) administered daily from day 6-12 (to reflect daily usage of the natural product and to examine its protective effect on chronic tamoxifen administration), with blood sampling done on day 13. Tamoxifen was administered in the morning, whilst *C. versicolor* extract was administered in the afternoon to provide a time gap between the doses.

Only three rats were sampled, in the morning, at any time which allowed sampling times to be achieved. The rats were fasted for at least 12 h prior to blood sampling to attain whether the extract continued to have any effect on the drug. During blood sampling, each rat was anaesthetised by inhalation with isoflurane which allowed quicker and cleaner access for blood sampling and rapid recovery. Blood samples (up to 0.3 mL) were collected from the lateral saphenous vein at 0, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12 and 24 h. Multiple small samples are unlikely to cause hypovolaemia to the animal (22). The blood was centrifuged after sampling, and the serum samples were stored at -80 ºC until analysis.

All the animal experiments were carried out in accordance with the strict guidelines of the Animal Research Regulation 2005 (NSW) and the Australian code of practice for the care and use of animals for scientific purposes. Approval was endorsed by the Animal Ethics Committee of Western Sydney University (Animal Research and Teaching Proposal (ARTP) Approval Number: A9873) using the principles of replacement, reduction and refinement. The reporting of the results followed the Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guideline (23).

Liquid chromatography conditions

Chromatographic separations were performed a Shimadzu LC-20AT delivery unit with DGU-20A degassing solvent delivery unit, SIL-20A auto injector, CTO-20A column oven and SPD-A detector (Kyoto, Japan). Chromatographic separation was achieved by a Synergy MAX-RP-80A (4μ, 150 × 4.6 mm) (Phenomenex, Torrance, CA, USA) attached to a 1 mm Optic-guard C-18 pre-column (Optimize Technologies, Alpha Resources, Thornleigh, Australia) in ambient temperature. The isocratic mobile phase (using a modified method as described previously (24); (25) comprised of 50 mM potassium phosphate buffer (pH 2.15) and acetonitrile (55:45, v/v) using verapamil hydrochloride as the internal standard. The mobile phase was delivered at a flow rate of 1 mL/min. The eluent was monitored at 280 nm ultra-violet detection.

Preparation of stock and working solutions of tamoxifen

A stock solution of tamoxifen citrate (2 mM: equivalent to 0.371 mg/mL of tamoxifen) was prepared in methanol and was further diluted with methanol to give a series of working solutions of 0.12, 0.23, 0.46, 0.93, 1.86, 3.71, and 7.42 μg/mL. A stock solution of verapamil (50 μg/mL) was prepared in methanol. Both stock solutions were stored at -20 °C and working solutions were freshly prepared from the prepared stock solution when required. Standard curves were prepared by adding known concentrations of tamoxifen and verapamil to drug-free rat plasma.

Preparation of standard and quality control samples of tamoxifen

For the method validation, low, middle and high concentrations of tamoxifen samples (0.023, 0.186, and 0.742 μg/mL) were prepared by spiking 10 μL working solutions (0.23, 1.86, and 7.42 μg/mL) of tamoxifen into 100 μL of blank rat serum and stored at -20 °C. Serum calibration standards (0.012 – 0.742 μg/mL) were freshly prepared for each analysis by spiking 10 μL of working solutions of tamoxifen into 100 μL of blank pooled rat serum which was pre-thawed at room temperature.

Sample preparation

Briefly, 100 μL of serum samples were spiked with 5 μL of verapamil (final concentration: 2.5 μg/mL) after the deproteinisation with 100 μL of acetonitrile. The samples were extracted twice with n-hexane (900 μL) and the organic layer was removed and dried at 30 °C for 30 min under vacuum in a Speed Vac concentrator (Thermo Scientific, USA). The dried samples were reconstituted with the mobile phase (90 μL), and 30 μL was injected into HPLC system.

Pharmacokinetic analysis of tamoxifen

The pharmacokinetic parameters of tamoxifen were determined by a non-compartmental analysis using PKSolver (26). Maximum serum concentrations (C_{max}) and time to achieve maximum concentration (T_{max}) for both single and repeated-dose experiments were determined by visual inspection of the serum concentration vs time curve. The elimination constant rate (k_{el}) was estimated by semi-log linear regression of the terminal slope, and elimination half-life ($t_{1/2}$) was estimated by ln2 / k_{el}. Area under the curve (AUC) and area under the first moment curves (AUMC) from 0 to last observed concentration (AUC_{0-t} and $AUMC_{0-t}$, respectively) were determined by the linear trapezoidal method.

Serum biochemical parameters of single and repeated doses of tamoxifen

For the 24-hour time point of the rat's serum, twenty-three biochemical serum parameters, incorporating hepatic (e.g. alkaline phosphatase (ALP), alanine aminotransferase (ALT), total protein, albumin, globulins, cholesterol, total bilirubin), renal (e.g. albumin, creatinine, urea, calcium, phosphate) and cardiac (e.g. cholesterol, triglycerides, bile acids) diagnostics, were examined for all six *in vivo* studies. The samples were analysed by an external veterinary diagnostic laboratory (Veterinary Science Diagnostic Service (VSDS), University of Queensland, Australia) who were blinded to the actual experimental/control groups.

Measurement of V_{max}, K_m and CL_{int} for depletion of tamoxifen, alone and in combination with C. versicolor.

The depletion of tamoxifen alone and in the presence of *C. versicolor* was performed using female rat liver microsomes (Sigma-Aldrich, Australia) *in vitro* (25). Briefly, 5 µM of tamoxifen was preincubated with female rat microsomes with or without the presence of the *C. versicolor* extract (10, 50 and 100 µg/mL) in 0.5 mL of 0.1 M phosphate buffer (pH 7.4) containing a nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (1 mM NADP, 0.8 U glucose-6-phosphate dehydrogenase and 3 mM glucose 6 phosphate) and 3 mM magnesium chloride ($MgCl_2$,) in an open air shaking water bath at 37 °C for about 3 minutes. The organic solvent acetonitrile (MeCN) in the final incubation was 0.25%. After the preincubation, the enzymatic reaction was then initiated by adding a 0.5 mg/mL of female rat hepatic microsomes. During the incubation, 100 µL aliquots were removed at time (t) = 0, 20 and 30 minutes. Each extracted aliquot was mixed with 200 µL of ice-cold MeCN to deactivate the enzymatic reaction. The resultant mixture was vortexed and centrifuged at 14,000 × *g* for 10 minutes, and the supernatant (30 µL) was directly injected to the HPLC system for analysis as described in the previous section. The *in-vitro* intrinsic clearance (Cl_{int}) was estimated by the substrate depletion method using *in-vitro* $t_{1/2}$ approach. Briefly, using the AUC of tamoxifen at t = 0 as 100% of substrate, the AUC of the other time points were converted to a percentage of the substrate remaining, plotted as natural log of remaining drug vs. incubation time and the slope of the regression line, represented as depletion rate of constant (-k), was used for estimation of the *in vitro* $t_{1/2}$ by the following equation: *in vitro* $t_{1/2}$ = -0.693 / k. Subsequently, *in vitro* Cl_{int} was calculated by following formula: (0.693 / *in vitro* $t_{1/2}$) × (µL incubation volume/mg microsomal protein). All incubation conditions were within the linear range of the rate of reaction and greater than 10 % of substrate was depleted compared to the initial substrate amount (t = 0 min). Using substrate depletion constant rates (27), estimated K_m value (substrate concentration at half the maximum velocity (V_{max})) of tamoxifen was 18 µM indicating tamoxifen concentration used in this study was acceptable.

Statistical analysis

The data was expressed as the means ± standard error of mean (SEM) of the separate experiments using Windows Excel. The pharmacokinetic data was compared using unpaired t- test with two-tailed p value. One of the authors listed who completed the pharmacokinetic data was blinded to the actual experimental groups. The initial and final weights of the rats in each group were compared using paired t- test with two-tailed p value. The biochemical parameters were compared by one-way analysis of variance (ANOVA), followed by Tukey's method for multiple comparisons using the program GraphPad Prism (San Diego, USA). The author who performed the biochemical statistics was not blinded to the groups. P values less than 0.05, 0.01 and 0.001 were considered statistically significant. All pharmacokinetic parameters are expressed as mean ± standard deviation, except for the elimination $t_{1/2}$ (harmonic mean ± pseudo-standard deviation).

Results

HPLC method validation for the determination of tamoxifen in rat serum

The retention times for both tamoxifen and verapamil were approximately at 7.9 and 2.2 minutes, respectively. The total run time for each sample was 16 minutes. The concentrations of pharmacokinetic samples were determined by non-weighted least square linear regression of the serum calibration curve prepared daily and ranged from 0.0116 - 0.742 µg/mL ($r^2 \geq 0.998$), where peak area ratios of tamoxifen to verapamil was used. The calibration curve was y=4.042x-0.015 (based on the average of three calibration curves). Based on the calibration curves (28), a lower limit of quantification was estimated at 0.0232 µg/mL where the precision and accuracy were within accepted criteria (<15% of coefficient of variation (CV) and within 20% of nominal concentration, respectively). A test for lack of fit revealed no departure from linearity for the calibration curve (F = 0.186; df = 5, 14; P = 0.186), indicating that the linear model was valid. For quality control (QC) samples (0.0232, 0.0927 and 0.742 µg/mL), the intra- (n=3) and inter-day (n=3 days) precision, expressed as CV, ranged from 2.81 to 4.69% and 8.53 to 14.10%, respectively. Intra-day and inter-day accuracy of QC samples were within 95.08 to 115.43% and 84.00 to 117.55%, respectively.

Serum concentration-time profile of tamoxifen (single dose) and with rats fed C. versicolor extract

The tamoxifen single dose serum concentration-time profile is shown in Fig. 1. It is observed that tamoxifen (single dose) plus *C. versicolor* extract showed a different serum-concentration profile compared to tamoxifen alone. The curve for tamoxifen plus extract exhibits a slower rate to its peak concentration in the first 10 hours compared to the steeper tamoxifen only curve. The error bars indicate greater variability in the time points for the single dose which may indicate the extract's control on tamoxifen's absorption.

Effect of C. versicolor extract on single dose pharmacokinetics of tamoxifen

The pharmacokinetic parameters for the single dose study of tamoxifen are shown in Table 1. The half-life ($t_{1/2}$) for tamoxifen was around 17.7 hours which is comparable to literature values (19), (20). There was a significant difference between the time to reach maximum concentration (T_{max}) values between the groups, with *C. versicolor* extract increasing the value by 3 times. Although there was no significant difference between the two regimens for maximum concentration (C_{max}) and area under the serum concentration-time curve from 0 h to 24 hours (AUC_{0-24hr}) (p>0.05), *C. versicolor* extract pretreatment showed a tendency to reduce both the C_{max} and AUC_{0-24hr} of tamoxifen. *C. versicolor* extract pretreatment also reduced the rate and extent of absorption of tamoxifen as seen from the decreased slope of the absorption phase, thus affecting the C_{max} and AUC_{0-24hr}. Limitations in sampling time points precluded further calculations of the parameters using the PKSolver software.

Table 1 Non-compartmental pharmacokinetic parameters of single oral tamoxifen dose with/without extract in rat serum.

Parameter	Tamoxifen only	Tamoxifen + extract
AUC_{0-24hr} (µg/mL*h)	2.41 ± 0.82	1.55 ± 0.48
T_{max} (h)	4.15 ± 1.15	13.6 ± 2.71*
C_{max} (µg/mL)	0.17 ± 0.05	0.11 ± 0.03
$t_{1/2}$ (h)	17.7 ± 3.42	NC

Single oral dose of tamoxifen (20 mg/kg on day 8) with or without of *C. versicolor* extract (340 mg/kg orally for seven days) in the serum of female Sprague-Dawley rats. The data was expressed as mean ± SEM (n = 5) and the PK parameters AUC_{0-24hr} (area under the serum concentration-time curve from 0 h to 24 hours), C_{max} (maximum concentration), T_{max} (time to reach maximum concentration) and $t_{1/2}$ (terminal half-life) were calculated with non-compartmental analysis. There was significant difference (*p<0.05) of T_{max} (tamoxifen plus extract) compared to tamoxifen only. NC = Not calculated due to limited sampling time points.

Repeated dose serum concentration-time profile of tamoxifen and with rats fed C. versicolor extract

In humans, the steady state for tamoxifen is achieved in about 4-6 weeks (29). In rats, it has been shown that steady state is reached in three days (13). Thus, 13 days depicted chronic administration of tamoxifen in this study. The tamoxifen repeated-dose serum concentration-time profile with and without *C. versicolor* extract is shown in Fig. 2.

Effect of C. versicolor extract on repeated-dose pharmacokinetics of tamoxifen

The tamoxifen repeated-dose pharmacokinetic parameters are shown in Table 2. As for the single dose experiments, the results showed a significant difference ($p<0.05$) between the T_{max} values, with T_{max} almost doubling with the administration of *C. versicolor* extract. There was no significant difference between the tamoxifen only and tamoxifen plus *C. versicolor* extract for the other measured parameters. There was no significant difference between the T_{max} values of the single and multiple dose experiments for tamoxifen only and for tamoxifen and *C. versicolor* extract ($p>0.05$).

Table 2 Non-compartmental pharmacokinetic parameters of repeated tamoxifen oral dose with/without extract in the rat serum.

Parameter	Tamoxifen only	Tamoxifen + extract
AUC_{0-24hr} (µg/mL*h)	6.40 ± 1.51	7.63 ± 0.95
T_{max} (h)	6 ± 2.17	11.6 ± 0.4*
C_{max} (µg/mL)	0.40 ± 0.08	0.48 ± 0.068
$t_{½}$ (h)	26 ± 13.7	NC

Repeated oral dose of tamoxifen (20 mg/kg for 13 days) with or without *C. versicolor* extract (340 mg/kg orally on days 6-12) in the serum of female Sprague-Dawley rats. The data was expressed as mean ± SEM (n = 5) and the PK parameters AUC_{0-24hr} (area under the serum concentration-time curve from 0 h to 24 hours), C_{max} (maximum concentration), T_{max} (time to reach maximum concentration) and $t_{½}$ (terminal half-life) were calculated with non-compartmental analysis. There was significant difference (*$p<0.05$) of T_{max} (tamoxifen plus extract) compared to tamoxifen only. NC = Not calculated due to limited sampling time points.

Serum biochemical parameters of single and repeated doses of tamoxifen

The results of the biochemical serum parameters for control, single and repeated dose tamoxifen-treated rats in the presence or absence of *C. versicolor* extract are shown in Table 3. Twenty-three parameters were examined. There were no significant differences *C. versicolor* extract, *C. versicolor* extract plus single dose tamoxifen and single tamoxifen dose groups compared to control ($p>0.05$) for all the parameters tested. However, significant differences were seen for the repeated tamoxifen dose and repeated tamoxifen plus *C. versicolor* extract groups compared to control in some of the parameters.

There was no significant difference between the control and treatment groups for sodium (Na), magnesium (Mg), phosphate (PO_4), total bilirubin (TBIL), triglycerides (TRIG), urea (UREA), creatinine (CREAT), glucose (GLUC), non-esterified fatty acids (NEFA) and globulin (GLOB).

For potassium (K) and chlorine (Cl), there was a significant increase between control and repeated dose tamoxifen levels ($p<0.01$ and 0.001, respectively). Repeated dose tamoxifen + extract reduced these values to control levels.

For carbon dioxide (CO_2), there was a significant decrease between control and repeated dose tamoxifen ($p<0.05$). Repeated dose tamoxifen plus extract increased this to control levels.

For calcium (Ca), there was a significant decrease between control and repeated dose tamoxifen levels ($p<0.05$). Although there was no significant difference, repeated dose tamoxifen plus extract increased this slightly to control levels.

Tamoxifen markedly increased serum activity of both alanine aminotransferase (ALT) ($p<0.05$) and aspartate aminotransferase (AST) ($p<0.001$) compared to control. Repeated dose tamoxifen + extract reduced AST and ALT.

Alkaline phosphatase (ALP) results showed that repeated dose tamoxifen was not significantly different to control ($p<0.05$). However, there was a significant increase between control and repeated dose tamoxifen plus extract ($p<0.001$). Although there

was an increase in gamma-glutamyl transpeptidase (GGT) with tamoxifen (and a concomitant decrease with tamoxifen + extract), GGT values were not significantly different between the groups.

For TP and ALB, there was a significant decrease between control and repeated dose tamoxifen levels ($p < 0.05$ and $p < 0.001$, respectively). However, there was no significant difference between repeated dose tamoxifen plus extract and repeated dose tamoxifen.

For creatine kinase (CK), there was a significant increase between control and repeated dose tamoxifen ($p < 0.05$), with repeated dose tamoxifen plus extract decreasing values to control levels.

Although there was a tendency to increase bile acids (BA) with repeated tamoxifen, there was no significant differences compared to control. Repeated dose tamoxifen plus extract tended to reduce BA compared to control.

Weights of the rats

All rats lost weight during the experimental period, ranging from 1.58 g for single dose tamoxifen plus extract to 19.83 g for repeated dose of tamoxifen. The difference in the initial and final rat weights for the control ($p = 0.05$) was non-significant. Although the repeated-dose tamoxifen difference in weights ($p = 0.076$) was non-significant, repeated dose tamoxifen plus extract ($p = 0.22$) and single dose tamoxifen plus extract ($p = 0.311$) showed a trend for weight protection with the *C. versicolor* extract.

Single dose tamoxifen ($p = 0.032$) and the extract alone ($p = 0.027$) showed significant changes to weight.

Table 3 Female Sprague Dawley rat biochemical serum parameters

Parameter	Control	Extract only	Tamoxifen + extract (single)	Tamoxifen (single)	Tamoxifen + extract (repeated dose)	Tamoxifen (repeated dose)
Na (mmol/L)	142.74±0.55	144.70±0.32	143.84±0.96	145.84±2.42	145.52±1.82	148.34±3.78
K (mmol/L)	5.30±0.35	5.50±0.14	5.50±0.12	5.68±0.29	5.78±0.64	7.66±0.61**
Mg (mmol/L)	1.04±0.07	1.01±0.03	1.08±0.04	1.00±0.02	1.07±0.08	1.11±0.07
Ca (mmol/L)	2.65±0.07	2.67±0.04	2.66±0.02	2.60±0.04	2.39±0.09	2.25±0.17*
PO$_4$ (mmol/L)	2.45±0.16	2.22±0.14	2.54±0.12	2.22±0.04	2.84±0.21	2.86±0.22
Cl (mmol/L)	101.80±0.55	103.48±0.31	102.40±0.65	104.64±1.69	107.74±0.91	112.78±3.04***
CO$_2$ (mmol/L)	23.94±0.71	23.32±1.50	20.46±0.66	25.22±1.16	21.14±0.61	18.48±1.65*
AST (U/L)	121.96±20.27	163.92±25.86	158.62±18.85	137.34±19.12	219.36±57.26	401.18±50.34***
ALT (U/L)	49.42±6.46	44.82±4.26	45.54±5.34	42.76±3.13	76.00±20.42	100.14±12.63*
ALP (U/L)	112.00±9.91	129.60±13.58	122.40±3.23	163.60±36.55	253.00±21.00***	150.80±22.55
GGT (U/L)	0.19±0.14[a]	0.06±0.01[a]	0.05±0.00	0.23±0.18[b]	2.08±1.51[a]	5.65±3.31[a]
TP (g/L)	63.42±1.88	66.46±2.11	65.72±0.38	66.14±2.15	52.08±2.00**	52.88±2.99*
ALB (g/L)	35.96±1.03	37.16±1.16	37.40±0.28	37.84±1.14	29.04±0.90***	28.96±1.33***
TBIL (µmol/L)	1.84±0.47	2.10±0.25	2.30±0.29	2.68±0.10[a]	1.60±0.23	1.10±0.40[c]
CHOL (mmol/L)	1.58±0.15	2.20±0.20	1.68±0.12	1.48±0.19	0.80±0.06*	1.04±0.20
TRIG (mmol/L)	1.46±0.13	1.26±0.09	1.82±0.30	2.12±0.24	0.90±0.21	0.90±0.19
BA (µmol/L)	25.50±6.68	34.74±4.88	22.30±8.69	38.98±8.19	31.32±5.58	69.96±30.57
UREA (mmol/L)	5.44±0.32	4.55±1.14	5.16±0.20	5.34±0.36	5.72±0.35	5.26±0.27
CREAT (µmol/L)	44.78±1.01	45.58±0.85	45.98±1.64	42.34±0.83	47.06±1.64	49.86±2.04
GLUC (mmol/L)	11.60±0.65	10.86±0.63	10.50±1.00	9.44±0.32	13.00±1.89	12.38±1.64
NEFA (mEq/L)	0.56±0.06	0.47±0.04	0.87±0.07	0.78±0.07	0.48±0.07	0.99±0.29
GLOB (g/L)	27.46±0.90	29.30±1.10	28.32±0.21	28.26±1.03	23.04±1.26	23.92±1.74
CK (U/L)	243.22±84.37	492.86±128.98	927.02±298.71	420.04±111.36	1378.24±867.53	3255.10±1358.96*

Tamoxifen (20 mg/kg); *C. versicolor* extract (340 mg/kg). Key: Na = Sodium; K = Potassium; Mg = Magnesium; Ca = Calcium; PO$_4$ = Phosphate; Cl = Chloride; CO$_2$ = Carbon dioxide; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; ALP = Alkaline phosphatase; GGT = Gamma-glutamyl transpeptidase; TP = Total protein; ALB = Albumin; TBIL = Total bilirubin; CHOL =

Cholesterol; TRIG = Triglycerides; BA = Bile acids; UREA = Urea; CREAT = Creatinine; GLUC = Glucose; NEFA = Non-esterified fatty acids: GLOB = Globulin; CK = Creatine kinase. The data was expressed as mean ± SEM; n= 5 unless otherwise stated: a(n=4) b(n=3); c(n=2) due to haemolysis in blood. Note: GGT<0.1 added as 0.05 in the table for statistical purposes. *P <0.05; **P < 0.01; ***P < 0.001 compared to control rat (no *C. versicolor* or tamoxifen).

Metabolism of tamoxifen in vitro

In this study, all microsomal incubation conditions were within the linear range of the reaction rate, and greater than 10% of the substrate was depleted compared to the initial substrate amount (t = 0 min) (25). The overall *in vitro* Cl_{int} was estimated using the substrate depletion method (30) rather than the product formation method (31) as it is known that tamoxifen undergoes multiple CYP mediated metabolism. Tamoxifen was stable with microsomal incubation that lacked the NADPH regenerating system indicating that the depletion of tamoxifen in female rat hepatic microsomes was NADPH dependent. Compared with the *in vitro* Cl_{int} of the control (12 ± 4.2 µL/min/mg microsomal proteins), there were no significant differences observed for the *in vitro* Cl_{int} of tamoxifen when co-incubated with different concentrations of the *C. versicolor* extract (10, 50 and 100 µg/mL) (Table 4). Consequently, it is unlikely that the extracts (between 10 – 100 µg/mL) will significantly inhibit the metabolism (likely mediated via CYP) of tamoxifen.

The disappearance of tamoxifen was also examined in male rat microsomes and it was found that the clearance rate of tamoxifen was about three-fold faster than the female rat microsome. As in the female microsomes, *C. versicolor* extract did not appear to alter the depletion of tamoxifen in the male rat microsome (data not shown).

Table 4 *In vitro* Cl_{int} of tamoxifen (5 µM) with or without *C. versicolor* extract.

	Control (n = 3)	+ Extract (10 µg/mL) (n = 3)	+ Extract (50 µg/mL) (n = 3)	+ Extract (100 µg/mL) (n = 2)
-k	-0.0060 ± 0.0021	-0.0037 ± 0.0022	-0.007 ± 0.0025	-0.0045 ± 0.0005
In vitro $t_{1/2}$ (min)	146.3 ± 46.8	375.4 ± 175.6	123.2 ± 34.2	155.9 ± 17.3
In vitro Cl_{int} (µL/min/mg)	12 ± 4.2	7 ± 4	14 ± 5.0	9 ± 1.0

Using female rat microsomes. All data is expressed as mean ± SEM of n = 3 (10, 50 µg/mL *C. versicolor* extract), except for 100 µg/mL (n =2) compared to the control. P < 0.05 was deemed to be statistically significant by using ANOVA (Dunnett's Multiple Comparison Test).

Discussion

Tamoxifen is a lipophilic molecule that is well absorbed from the gastrointestinal tract and provides dissolution rate-limited absorption (32). *C. versicolor* extract compositions are quite varied depending on strains (with PSP prepared from the cultured mycelium of the Cov-1 strain) and preparation. From what is presented in the literature, the PSP moiety is a highly branched β-glucan−protein complex, high in aspartic and glutamic acids. PSP contains α-1, 4 and β- 1, 3 glucosidic linkages and presents D-glucose as the main monosaccharide, with rhamnose and arabinose also found in PSP. The polysaccharide moiety is stable to enzymatic proteolysis (9, 33, 34).

In this study, the results showed that the time to achieve peak serum concentration of tamoxifen in the *C. versicolor* extract-treated group was longer than the control group, without affecting tamoxifen's bioavailability. One suggested reason behind this is the relatively high viscosity of the β-glucans which may be delaying tamoxifen's absorption (35) thus, increasing T_{max}, with no change in C_{max} and AUC. To create significant viscosity, β-glucans have to be soluble, with molecular weights ranging from 26.8 to 3000 kDa (36). However, the molecular weight of the crude PSP extract used in this study is around 0.5-40 kDa, with the purified extract averaging 2.6 kDa (18).

It is well known that interactions occur between natural products and oral drugs. The partly digested *C. versicolor* extract in the gut is possibly slowing gastric emptying time, lengthening the passage time of tamoxifen in the small intestine, interfering with the mixing of the drug and digestive enzymes, and altering the diffusion and interaction of nutrients/tamoxifen with the mucosal surface, and hence its absorption rate. This suggests that tamoxifen is absorbed more gradually due to the delayed emptying of the extract (even after 12-hour fasting of the rats for both experiments before sampling), thus hindering acute therapeutic activity (36, 37). However, this may not be clinically relevant as most anticancer drugs are administered chronically to achieve continued pharmacodynamic effects.

Jordan (2008) noted that the key to tamoxifen's success was a sustained duration of action. In rats, tamoxifen acts as a tumouristatic agent – the drug is effective for long as the drug is present to suppress tumour growth. Although T_{max} was higher for the tamoxifen + *C. versicolor* extract single and repeated dose groups, and as tamoxifen was still present in the system, this may not affect tamoxifen's anti-tumour activity (38). Although delayed absorption may cause a delay in therapeutic activity, concomitant delivery of the extract with tamoxifen may be beneficial to sustain bioactivity and avoid acute toxicity or other side effects, given that cancer drugs are administered on a long-term basis and can irritate the gastrointestinal tract (37, 39). In another pharmacokinetic study in rats, PSP decreased the cyclophosphamide (a widely used anti-cancer agent) clearance and increased the plasma half-life of the anticancer drug (40). This pharmacokinetic interaction may also be beneficial to the concomitant use of PSP and cyclophosphamide as a combined therapy in patients, such that the doses of cyclophosphamide maybe adjusted to prevent anticancer drug-induced side effects.

Analysing the biochemical serum parameters allows for an initial insight for any toxicity effects of the drug/natural product. In this study, *C. versicolor* extract combined with tamoxifen (repeated dose) had comparable values to the control, except for ALP, TP, ALB, and CHOL. Chronic tamoxifen administration has been shown to induce low levels of DNA adducts in the kidney and liver (41). These changes due to prolonged tamoxifen treatment may indicate reduced kidney function and the extract may be exerting a protective effect on kidney function. For Ca, there was a significant decrease between control and repeated dose tamoxifen levels ($p < 0.05$). This agrees with a previous study which may reflect a decrease in bone minerals release and supports the protective effect of tamoxifen against bone resorption-related diseases (42). PSP extract did not interfere with this mechanism.

The results indicated some signs of hepatotoxicity in the female rats treated with multiple doses of tamoxifen compared to control, by markedly increased serum activity of both ALT ($p < 0.05$) and AST ($p < 0.001$). This confirms a previous study and implicates oxidative stress to DNA and protein and lipid peroxidation in the pathogenesis of tamoxifen-induced liver injury. *C. versicolor* extract plus repeated dose tamoxifen reduced AST and ALT (42). In contrast, CREAT was not significantly raised by tamoxifen in the current study. Previous studies showed marked decreases in serum ALB and TP in female rats treated with tamoxifen, suggesting decreased liver synthesis of proteins (43). In serum, it is known that more than 98% of tamoxifen is bound to albumin (13). Similarly, the present study exhibited marked decreases in serum levels of TP and ALB in female rats treated with tamoxifen ($p < 0.05$ and $p < 0.001$, respectively). However, there was no significant difference between repeated doses of tamoxifen + *C. versicolor* extract and repeated doses of tamoxifen for TP and ALB.

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins and alkaloids through dephosphorylation. ALP results showed repeated dose tamoxifen was not significantly different to control which agreed with a previous study (42). However, there was a significant increase between control and repeated dose tamoxifen plus *C. versicolor* extract ($p < 0.001$). A study showed that *C. versicolor* stimulated ALP activity in osteoblast cells (44). The source of elevated ALP levels can be deduced by obtaining serum levels of GGT. The concomitant increases of ALP with GGT may indicate hepatobiliary disease (45). Although there was an increase in GGT with tamoxifen (and a concomitant decrease with *C. versicolor* extract), GGT values were not significantly different between the groups.

High levels of CK are indicative of skeletal muscle or metabolic changes (46). Repeated-dose tamoxifen plus *C. versicolor* extract decreased this to control levels.

In Hemieda's study, tamoxifen lowered serum contents of total lipid, total CHOL and TRIG in the female rats. Although the results in this study showed this tendency, the results were not significant. However, repeated dose tamoxifen plus *C. versicolor* extract

significantly decreased CHOL (p<0.05) compared to control. Approximately 40% of total cholesterol is removed by conversion to bile acids in the liver (47). Although not significant, tamoxifen showed a tendency to increase the production of BA in the serum in this study.

In a study examining acute and subchronic oral toxicity of *C. versicolor* standardised water extract, there was no significant difference between *C. versicolor* water extract-treated rats compared to control rats for TP, ALB, TBIL, ALP, ALT, AST, UREA, K, Na, Cl, CREAT and uric acid (6). The administration of the water extract to female rats resulted in a statistically significant decrease in GLOB after 28 days treatment. In agreement, there was no significant difference between *C. versicolor* extract-treated rats and control rats for all twenty-three parameters in the current study, thus supporting the extract's safety profile and its transit through the gastrointestinal tract.

β-Glucans such as PSP and PSK are known to be orally bioavailable and contribute to the bioactivity of the extract (33); (48). Pinocytosis is considered responsible for such absorption. Molecular weights of no more than 3 kDa are advantageous to ensure passage through the intestinal wall. This was verified for the purified company extract as determined by the Caco-2 cell monolayer Transwell method (18). Similarly, a study found that PSK achieved its peak concentration at 0.5 − 1 h after administration and nearly completely disappeared after 72 h, with a $t_{1/2}$ = 5.9 h in rats and appeared in the blood in its original form or in a partly degraded form in the body (49). This study showed that the extract was still in the rat after 12 hours fasting. In this study, the highest concentration of PSK was observed in the intestinal tract (stomach and small intestine). PSK, or its derivative, was absorbed and transferred in comparatively high concentration to the liver and bone marrow.

Studies with PSP have shown immunomodulation and protection against the adverse and toxic effects of chemotherapeutics which has led to improved survival rates (8). The extract of PSP used in this study has activated lymphocytes *in vitro* (18). A Phase I trial in breast cancer patients showed that up to 9 g of *C. versicolor* was well tolerated and increased lymphocyte counts, natural killer cell functional activity, cytotoxic T lymphocytes (CD8^{+} T cells) and cluster of differentiation (CD19^{+}) B-lymphocyte antigen cells. This may explain *C. versicolor*'s immunostimulating properties on the body (50).

In addition to *C. versicolor*'s bioavailability, its immunostimulant properties may be due to enhanced colonisation by beneficial microbes in the human gut which would provide health benefits (known as prebiotics). In a previous study, PSP extract was shown to alter human gut microbiota and pH (33). Therefore, the time that *C. versicolor* remains in the gut before its transit may be modifying the intestinal bacteria and modulating the immune system in the rats.

In the rat, tamoxifen is metabolised via N-demethylation to N-desmethyltamoxifen by CYP3A2 (an ortholog of CYP3A4 in humans) which contributes approximately 92% of tamoxifen metabolism (51). N-desmethyltamoxifen has greater infinity to the oestrogen receptor than tamoxifen itself. In a previous study, polysaccharide peptides from *C. versicolor* competitively inhibited model cytochrome P450 enzyme probe substrates metabolism in human liver microsomes. However, the relatively high inhibitor constant (K_i) values for CYP1A2 and CYP3A4 suggested a low potential for PSP to cause herb−drug interaction related to these CYP isoforms (52). However, this study used substrates *in vitro* did not examine tamoxifen *in vivo*. Another study in humans showed that PSP was safe and was not expected to be associated with significant herb-drug interactions through CYP3A4 (53). Our results confirmed that the *C. versicolor* extract did not interfere with the depletion of tamoxifen *in vitro*. In contrast to other rat studies, tamoxifen has shown enhanced bioavailability with other natural products such as quercetin (20), silybinin (54), curcumin (55), baicalin (56) and naringin (19) potentially due to the inhibition of CYP3A4.

In this study, adverse effects were minimal: with tamoxifen, one rat had lost 50 g of weight during extended treatment (with the blood more dense and harder to collect) and one rat died at the 10 hr sampling point for single dose tamoxifen plus extract. The limitations of the study included not sampling past the 24-hr point and not measuring the metabolites. However, C_{max} and AUC were able to be calculated and showed no significant differences between the treatment groups. Another limitation is that some of the biochemical parameters were unreadable due to haemolysis. The weights between the rat groups were not identical.

Conclusion

This study has shown that *C. versicolor* interacted with tamoxifen as indicted by the increased time to reach the maximum concentration (T_{max}) of tamoxifen without any concomitant changes to C_{max} and AUC in both single and repeated dose tamoxifen experiments. The extract is delaying gastric emptying and intestinal transit, which in turn, allows for more time for tamoxifen's dissolution and absorption (consequently slower absorption), thus causing an increase in T_{max}. It appears that more than 12 hours of fasting is required to eliminate the extract from the gastrointestinal tract of the rats. The results confirmed that the extract (using increasing doses) would not inhibit tamoxifen's metabolism *in vitro*. This is vital to tamoxifen's chemotherapeutic properties as the metabolite is more active. *C. versicolor* exhibited a protective effect when tamoxifen was chronically administered as seen by the stable biochemical parameters compared to control. This could be advantageous in reducing the side effects of the chemotherapy over a long treatment cycle. If clinical trials confirm the results in the rat model, the tamoxifen dose may not need to be adjusted, but rather the time of administration may need to be considered if used with *C. versicolor* extract supplements, especially in the initial stages of chemotherapy. A systematic review and meta-analysis has shown overall survival benefits in breast, gastric and colorectal cancer chemotherapy when combined with *C. versicolor* (57).

Thus, it is important to consider the timing of chemotherapy agents and natural products so that the actions of the chemotherapy drug are not compromised. In addition, we need to utilise the immunostimulating properties of *C. versicolor* to combat the deleterious side effects of long-term cancer therapy.

Abbreviations

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; APG, absorbable peptidoglycan; AST, aspartate aminotransferase; AUC, area under the curve; BA, bile acids; Cl_{int}, intrinsic clearance; *C. versicolor*, *Coriolus versicolor*; Ca, calcium; Cl, chlorine; C_{max}, maximum serum concentration; CO_2, carbon dioxide; CK, creatine kinase; CV, coefficient of variation; CREAT, creatinine; CYP450, cytochromes P450; GGT, gamma-glutamyl transpeptidase; GLOB, globulin; GLUC, glucose; HPLC, high-performance liquid chromatography; IVC, individually ventilated cages; K, potassium; k, depletion constant; k_{el}, elimination constant rate; K_i, inhibitor constant; K_m, substrate concentration at half the maximum velocity; MeCN, acetonitrile; Mg, magnesium; Na, sodium; NADPH, nicotinamide adenine dinucleotide; NEFA, non-esterified fatty acids; phosphate; PO_4, phosphate; PSK, polysaccharide krestin, PSP; polysaccharide peptide; QC, quality control; SEM, standard error of mean; $t_{1/2}$, half-life; T_{max}, time to achieve maximum concentration; TAM, tamoxifen; TBIL, total bilirubin; TP, total protein; TRIG, triglycerides; UREA, urea; V_{max}, maximum velocity.

Declarations

Ethics

Approval was endorsed by the Animal Ethics Committee of Western Sydney University (Animal Research and Teaching Proposal (ARTP) Approval Number: A9873).

Acknowledgements

Author's contributions

KC conceived the study. KC, VRN and SN designed the study. SN trained and guided DL in the animal experiments. DL performed the animal experiments. BK performed the HPLC and microsome experiments. VRN provided practical assistance to both DL and BK in the animal and analytical experiments, respectively. BK and VRN analysed the data. VRN drafted the manuscript. HN provided clinical and product guidance to the paper. All authors have read, revised and approved the final manuscript.

Competing interests

Dr Norimoto is the R&D Manager at PuraPharm Corp Ltd Hong Kong. No employees of PuraPharm were directly involved in the experiments, which were done in Australia.

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Data availability statement

The datasets generated and analysed during this study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

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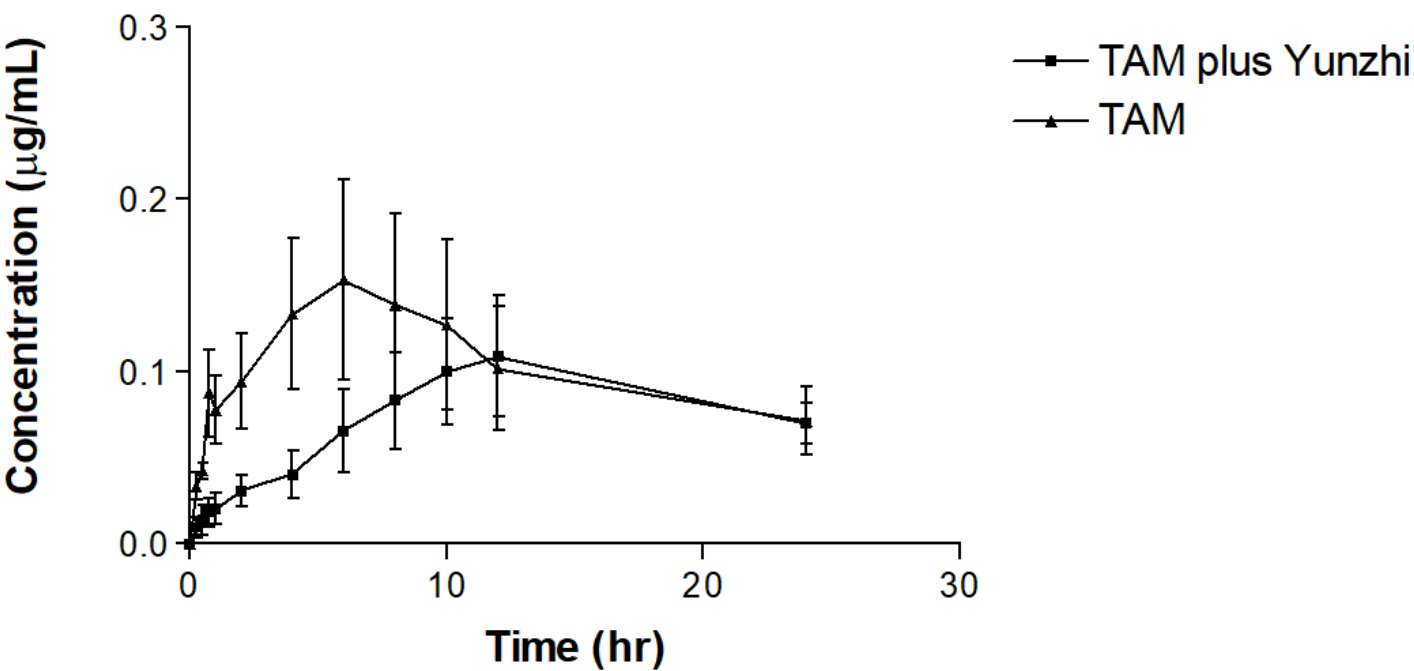
Figures

Figure 1

Mean serum concentration-time profiles of a single oral tamoxifen dose plus extract in rat serum. Tamoxifen (TAM: 20 mg/kg) (▲) on day 8 and with C. versicolor extract pretreatment (340 mg/kg orally for seven days (■)) (TAM plus extract) in female Sprague-Dawley rats. Bars represent the SEM (n=5). Note: timepoints at 10, 12 and 24 hr is n=4 for tamoxifen plus extract.
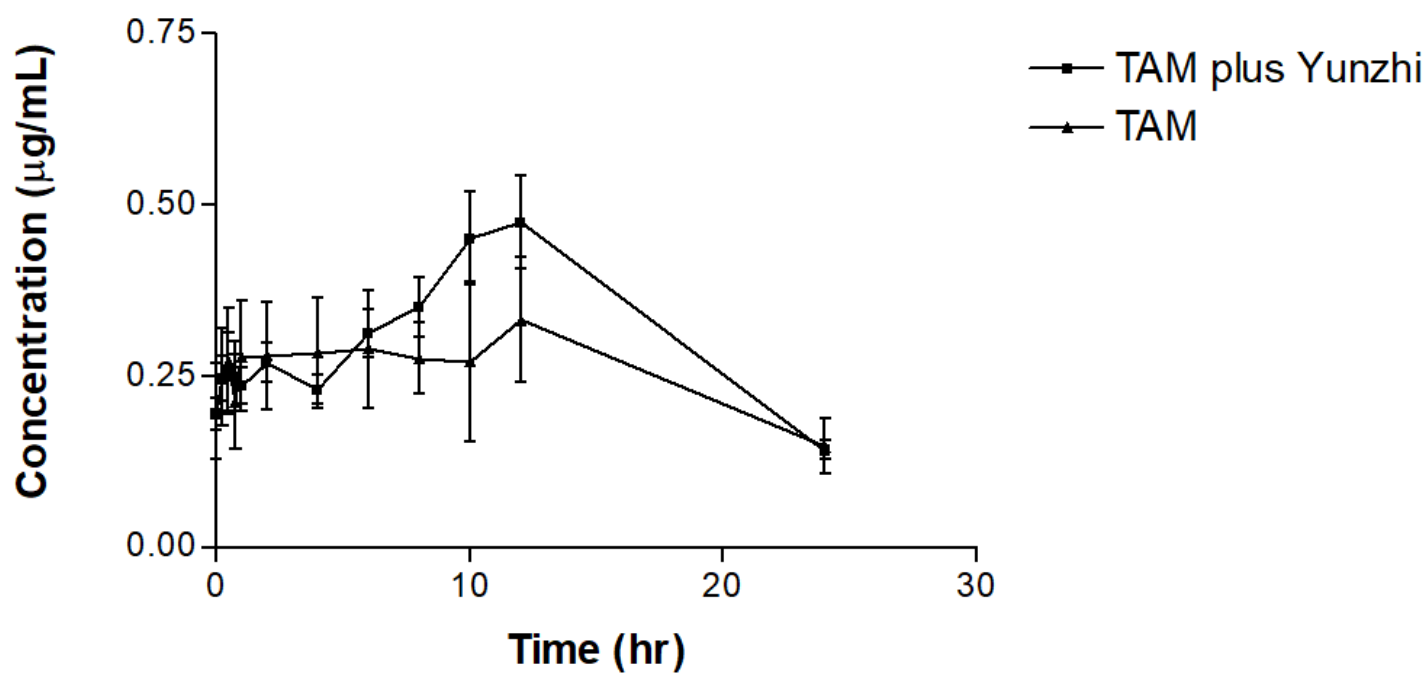
Figure 2

Mean serum concentration-time profiles of repeated oral tamoxifen dose plus extract in rat serum. Tamoxifen (TAM: 20 mg/kg for 13 days) (▲) and with C. versicolor extract pretreatment (340 mg/kg orally on days 6-12) (■) (TAM plus extract) in female Sprague-Dawley rats. Bars represent the SEM (n=5).