Journal of Coastal Life Medicine

Journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.201414J18

Safety of Malaysian marine endophytic fungal extract S2 from a brown seaweed *Turbinaria conoides*

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PEER REVIEW

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Comments

In this valuable research work, authors have demonstrated the safety of *T. conoides* in a rat model. The activity was assessed based on biochemical parameters, antioxidant enzyme levels in liver homogenate and histopathological observations. According to the *in vitro* and *in vivo* toxicity evaluation of extract S2 of *T. conoides*, it did not induce any toxic effects, which indicated its potential for use as a nutraceutical agent.
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ABSTRACT

Objective: To evaluate the *in vivo* acute toxicity and antioxidant activity of the marine endophytic fungus extract S2 isolated from *Turbinaria conoides*.
Methods: Two doses (100 mg/kg and 400 mg/kg) of the S2 extract were administered to rats orally for acute toxicity and antioxidant test. The body weight, relative weight of six organs, haematological, biochemical and antioxidant properties were investigated on Day 14.
Results: A single oral dose treatment did not cause any mortality or observable adverse effects in rats. No significant variations in the body and organ weights between the control and the treated groups were observed. Heamatological analysis and clinical blood chemistry also did not reveal any toxic effects of the extract. The total white blood cell count and haemoglobin levels were increased. The levels of total serum cholesterol in males treated with 100 and 400 mg/kg were significantly (P<0.05) decreased (1.28 and 1.34 mmol/L respectively) compared to control (1.55 mmol/L) rats. Pathologically, neither gross abnormalities nor histopathological changes were observed. This study showed strong evidence of the non−toxic effects of S2 extract. Furthermore the extract exhibited significant (P<0.05) antioxidant activity through increased levels of superoxide dismutase and glutathione peroxidase enzymes in serum, liver and kidney.
Conclusions: The research findings from the present study showed the potential of marine natural products particularly in Malaysia as a source of bioactive compounds. Marine endophytic fungi as a potential source of anticancer drugs with great potential as they are potent yet safe, thus deserving further extensive investigation.

KEYWORDS
Endophytes, *Turbinaria conoides*, Acute toxicity, Cytotoxicity, Antioxidant

1. Introduction

Over the last two decades marine algae have been found to be a vital source of useful bioactive substances. Studies have demonstrated marine endophytic fungi as excellent source of anticancer, antimicrobial, antioxidantand antiplasmodial compounds[1−3]. *Turbinaria conoides* (*T. conoides*) belongs to the family of Sargassaceae. Traditionally, *T. conoides* is widely used to cure fever in children, as food for human consumption, fertiliser, insectrepellent, antibiotics and

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Foundation Project: Supported by Ministry of Education (MOE) of Malaysia and Research Intensive Faculty (RIF) of University of Technology MARA (UiTM) Malaysia [Grant No. 600−RMI/DANA 5/3/RIF (185/2012)].

Article history:
Received 20 Feb 2014
Received in revised form 16 Mar, 2nd revised form 10 Apr, 3rd revised form 13 May 2014
Accepted 2 Jun 2014
Available online 15 Jun 2014

pesticides[4]. Also, the organic extract of *T. conoides* has been reported to exhibit cytotoxicity against P-388 (mouse lymphocytic leukaemia) and KB (human nasopharyngeal carcinoma) cells[5]. Furthermore, it possesses antihistaminic, antiviral, antifungal, antiplasmodial and antioxidant activities[2,6,7]. Results obtained from previous study supported the earlier traditional claim as a paediatric antipyretic remedy[8].

Recently, from our laboratory 64 endophytic fungi were isolated from Malaysian marine plants and seaweeds and the extracts were tested for cytotoxicity against seven cancer cell lines and antimicrobial activity against four bacteria and two fungi[9]. Preliminary screening revealed that 49 (77%) of the 64 extracts (1 mg/mL) were able to inhibit at least one cancer cell line. We recently reported extract S2 was selective and potent against colon (DLD-1) and lung cancer (NCI-H1299) (IC_{50}< 0.01 μg/mL) but with no toxic effect against fibroblast normal cell line (D551, IC_{50}=789.00 μg/mL). In fact, this extract was significantly more potent than existing anticancer drugs (5-fluorouracil and vincristine). Thus, the present study was extended to evaluate the *in vivo* acute toxicity and antioxidant activity of the extract S2. However, to the best of our knowledge this is the first report on the toxicity activities of a marine endophytic fungus (S2) isolated from *T. conoides*.

2. Materials and methods

2.1. Isolation of endophytes

Endophyte was isolated from brown seaweed from Teluk Kemang Port Dickson, Negeri Sembilan, Malaysia, as described by Strobel[9,10]. The seaweed (S2) was washed under running water, sterilized with 75% ethyl alcohol for 1 min and household bleach (5% NaOCl) for 3 min, drained and immersed in 75% ethyl alcohol again for 30 seconds. Finally, the sample was rinsed with sterile water and cut aseptically into 1 cm long segments. The cut segments were incubated on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) supplemented with chloramphenicol (10 g/L; Sigma-Aldrich, St Louis, MO, USA) at 28 °C until mycelia were observed. Pure culture was isolated, subcultured on PDA free of antibiotics and incubated for 30 d at 28 °C prior to extraction. The stock culture was maintained at the Marine Pharmaceutical Research Group (MaReG) Laboratory, UiTM Puncak Alam, Selangor, Malaysia.

2.2. Extract preparation

The endophytic fungi on PDA was macerated and transferred to a conical flask filled with 100% ethyl acetate and the resultant mixture was stirred overnight at room temperature. The extract was filtered (No. 1 filter papers, 20-25 mm, Whatman, Maidstone, UK), after which sodium sulfate was added to further remove the aqueous layer within the extract. The sodium sulfate was removed by filtration and the organic phase was dried by rotary evaporation. The resultant S2 extract was weighed and dissolved in methanol to evaluate the toxicity. The extract was stored at -18 °C.

2.3. Animals and experimental design

Healthy Sprague-Dawley (SD) rats (age 5-6 weeks) weighing about 150 g were purchased from GIFT Sdn Bhd, Selangor, Malaysia and kept under laboratory conditions. The use of laboratory animals and the study design were approved by the Institution Ethical Committee of the University of Malaya, Kuala Lumpur, Malaysia [Ethics No.PM/07/05/2008 MAA (a) (R)]. Rats were randomly divided into three groups with six males and six females in each group. The rats were deprived of feed for 16 h before administration of the test substances. The rats in the experimental groups were given a single dose (1 mL) of S2 at low (100 mg/kg) and high concentration (400 mg/kg) and the control group was given normal saline (1 mL) once every 15 d. The two doses (low and high) in this study were based on published results[11]. The extract was diluted in normal saline and was administered orally using a ball-tipped intubation needle fitted onto a syringe. The observations of pharmacotoxicological signs, which included changes in the skin and fur, eyes and mucous membrane and unusual behaviour such as moving backwards and imbalance in locomotion, were made at 10, 30, 60 and 120 min and 4 h after dosing during the first day and daily thereafter for 15 d. The rats were weighed once per day until Day 15. The rats were deprived of feed for 16 h before being killed on Day 15.

2.3.1. Body weight

The rats were weighed before the commencement of the dosing and then daily throughout the 15 days of the study. The groups' mean body weights were calculated.

2.3.2. Blood collection

Blood samples were collected before and during the treatment (Week 1) through the retro-orbital plexus while at the end of the experiment blood was collected through cardiac puncture for the haematological and biochemical analysis. Blood samples were collected in two types of tubes: one with anticoagulant (ethylene diamine tetraacetic acid) and the other without any additives. The anticoagulant blood (ethylene diamine tetraacetic acid) was sent immediately for haematology analysis. Blood without any additives was centrifuged at 3000 r/min for 15 min at 4 °C to obtain clear

supernatant (serum) which was then transferred into sterile tubes and stored at −80 °C for further biochemical analyses.

2.3.3. Organ weight

At the end of the experiment after collecting the blood, the rats were sacrificed by cervical displacement and the selected organs were removed and weighed immediately. At postmortem, the rats were dissected and careful examination of the organs liver, heart, spleen, colon, kidney and lung were carried out. Each organ was removed and weighed in grams (absolute organ weight). The percentage of relative organ weight (ROW) was then calculated as follows:

%ROW=Absolute organ weight (g)/body weight of rat (g)×100

The liver and kidney samples were immediately excised and one gram of tissue was homogenised in 9 mL phosphate-buffered saline at pH 7.4 using a Potter Elvehjem homogeniser with a teflon pestle. The homogenates were stored at −80 °C until further used for analyses.

2.3.4. Haematology

Hematological analysis was performed using an automatic hematological analyzer. Parameters included: red blood cell (RBC), white blood cells (WBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW) and platelets count.

2.3.5. Biochemical assay

The biochemical parameters included glucose, calcium, total cholesterol, creatine, phosphate, protein, bilirubin, gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were analysed by using standard diagnostic biochemical kits (Instrumentation Laboratory, Italy) with autoanalyzer (ILab 300 Plus Clinical Chemistry System, Italy).

2.3.6. Histopathology examination

After weighing, part of the organs (liver, heart, spleen, colon, kidney and lung) was dried by blotting with filter paper and used for histopathology study. For histopathology study, the tissues were separately fixed with 10% formalin solution for 48 h, dehydrated with absolute alcohol, cleaned with xylol and infiltrated with molten paraffin wax at 50 °C. The samples were fixed as wax blocks with correct morphological orientations. The wax blocks were sectioned using a microtome. Suitable sections were then placed on glass slides. The wax was cleared with xylol and absolute alcohol, then progressively rehydrated with gradually increasing dilutions of alcohol and then stained with eosin. They were then once again dehydrated, cleared with xylol, mounted with di-N-butyl phthalate in xylene (DPX) and covered with cover slips. The sections were examined carefully under light microscope at both low and high power. Abnormal histopathological changes for each of the organs were carefully recorded[12,13].

2.3.7. Antioxidant assays

After weighing, immediately one gram of liver and kidney was washed and homogenised in ice-cold saline water (9 mL) to prepare 10% (w/v) homogenate. The homogenate was then centrifuged at 4000 r/min at 4 °C for 10 min to remove cellular debris and the supernatant was collected for analysis of superoxide dismutase (SOD) and glutathione peroxidase (GPX). The antioxidant assays were carried out using 96 well plate reader, following the procedure of Cayman Chemical assay kit (USA). The above activities were expressed as unit per milliliter (U/mL) for SOD and nanomole per minute per milliliter (nmol/min/mL) for GPX.

2.4. Statistical analysis

Data is expressed as mean±standard error mean (SEM) and analyzed statistically by SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). The homogeneity of the variance was tested using Levene's test. A One-way ANOVA test with Bonferroni *post hoc* analysis was applied. A difference was considered statistically significant at the level of $P<0.05$.

3. Results

The results obtained provide scientific information on the acute effect of extract S2 given in a single dose orally at 100 and 400 mg/kg in male and female SD rats. The extract at both doses did not produce any mortality or alter the behavioral patterns of the rats during the 14-day observation. Overall no deaths were recorded among the groups. The body weight increased gradually throughout the study period in both sexes (Figures 1 and 2).

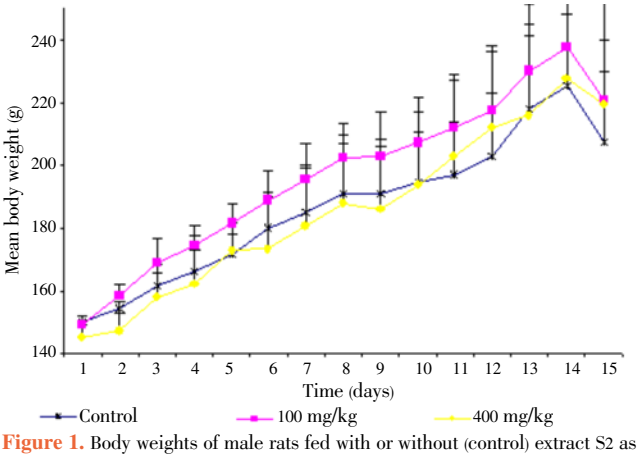

Figure 1. Body weights of male rats fed with or without (control) extract S2 as observed for 15 d. Values are expressed as mean±SEM, n=6.

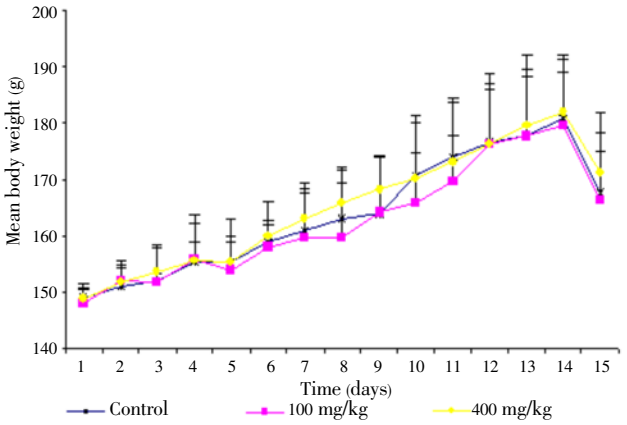

Figure 2. Body weights of female rats fed with or without (control) extract S2 as observed for 15 d. Values are expressed as mean±SEM, n=6.

Table 1 shows the percentage mean weight changes in the rats treated with and without (control) 100 or 400 mg/kg extract S2. All the groups showed progressive weight gain. There were no considerable changes in weight gain (%) of male rats treated with 100 or 400 mg/kg (29% and 31% respectively) as compared to the control (27%). Similar results were observed in weight gain changes (%) of females rats with the extract (9% for 100 mg/kg and 12% for 400 mg/kg) as compared to the control group (11%) (Table 1).

Table 1

Effects of single oral administration of extract S2 at 100 or 400 mg/kg on body weight of SD rats before, during and at the end of the treatment for 14 d.

Rats	Dose	Body weight (g)			% Change over 14 d
	(mg/kg)	Before treatment	Week 1	Week 2	
Male	Control	153.19±1.00	182.22±4.30	208.72±6.10	27
	100	156.00±1.90	194.95±3.10	219.87±4.90	29
	400	152.00±0.40	181.13±5.20	219.87±10.30	31
Female	Control	150.47±1.20	161.85±1.90	168.48±2.00	11
	100	152.00±0.70	160.05±2.20	167.63±3.20	9
	400	151.07±2.20	163.48±1.70	171.62±2.90	12

Values are expressed as mean±SEM (n=6).

The organ weights (% of body weight) of rats body weight are shown in Table 2. There were no significant differences in the relative weights of the liver, heart, spleen, colon, kidney and lung between the treated and control groups. The gross observations also did not show any significant changes in color and texture of the organs from treated animals when compared with the control group.

Table 2

Effect of single dose oral administration of extract S2 on the ROW of liver, kidney, lung, colon heart and spleen of the SD rats treated for 14 d.

Rats	Dose	Relative organ weight (%)*					
	(mg/kg)	Liver	Kidney	Lung	Colon	Heart	Spleen
Male	Control	2.97±0.09	0.79±0.02	0.62±0.03	0.41±0.02	0.41±0.01	0.28±0.01
	100	3.11±0.04	0.78±0.01	0.55±0.01	0.42±0.01	0.38±0.00	0.29±0.01
	400	2.99±0.10	0.79±0.00	0.52±0.01	0.41±0.08	0.40±0.00	0.30±0.01
Female	Control	2.81±0.06	0.73±0.03	0.72±0.02	0.73±0.03	0.42±0.02	0.28±0.02
	100	3.09±0.04	0.74±0.04	0.75±0.01	0.61±0.01	0.36±0.01	0.29±0.03
	400	3.05±0.03	0.73±0.06	0.70±0.02	0.61±0.05	0.35±0.01	0.25±0.01

Values are expressed as mean±SEM, n=6.
*(Weight of organ/body weight)×100

The haematology status after 14 days of oral administration of extract S2 was also assessed. In general, the results showed that the values of WBC (16.05×10^9/L) in females treated with 100 mg/kg of extract S2 significantly increased from that of control rats (Table 3). The MCHC significantly decreased (3 330.67 g/L) at the dosage of 100 mg/kg of extract S2 as compared to the control and the high dose group in female. There was no apparent effect observed for the other parameters HBG, RBC, HCT, MCV, MCH, RDW and platelets in the treated groups as compared to control groups. Meanwhile, the level of total cholesterol also significantly decreased (1.28 mmol/L and 1.34 mmol/L) in both doses (100 and 400 mg/kg, *p.o.*) of the male treated groups compared to the male control group (1.55 mmol/L). The lipid profile showed a decrease in the serum total cholesterol level for male rats in both treated groups (Table 4).

Biochemical parameters measured in the present study (Table 4 and Table 5) showed that the values of glucose, calcium, bilirubin and phosphorus in the blood of all the treated groups even at a high dose (400 mg/kg) were not affected as compared to the control groups. Renal toxicity was not induced by the extract in female rats and this was clearly evident through the creatinine level measured (Table 4). In male rats however, the level of creatinine in the serum (of rats fed 400 mg/kg) was significantly reduced (11.33 μmol/L as compared to control 20.72 μmol/L).

Table 3

Haematological values of female rats treated with extract S2 during acute toxicity and control.

Groups	HGB (g/L)	HCT (%)	RBC ($\times10^{12}$/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)	WBC ($\times10^9$/L)	Platlet count ($\times10^9$/L)
Control	132.50±1.61	0.40±0.00	6.76±0.02	57.00±0.58	18.67±0.33	342.00 ± 1.24^a	16.33±0.79	11.17 ± 0.38^b	1 550.00±41.47
100 mg/kg	132.16±1.53	0.39±0.02	6.91±0.09	56.50±0.85	18.63±0.23	330.67 ± 2.62^b	18.17±1.02	16.05 ± 0.78^a	1 619.33±61.93
400 mg/kg	133.50±0.76	0.40±0.01	6.85±0.06	58.72±0.48	18.67±0.21	334.88 ± 2.42^a	16.58±0.54	11.07 ± 0.18^b	1 625.17±43.09

Values are expressed as mean±SEM, n=6. Data within a column with different superscripts differ significantly (P<0.05).
HGB: Haemoglobin; HCT: Haematocrit; RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean cell haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RDW: Red cell distribution width; WBC: White blood cell.

Table 4

Effect of oral administration of the extract S2 on serum biochemical parameters of male and female rats (n=6).

Rats	Dose (mg/kg)	Glucose (mmol/L)	Calcium (mmol/L)	Inorganic phosphorus (mmol/L)	Total cholesterol (mmol/ L)	Creatinine (μmol/L)
Male	Control	6.81±0.10	2.33±0.02	2.66±0.00	1.55±0.03[a]	20.72±2.80[a]
	100	6.05±0.10	2.41±0.03	2.81±0.10	1.28±0.05[b]	17.33±2.00[a]
	400	6.47±0.80	2.29±0.05	2.78±0.20	1.34±0.04[b]	11.33±0.60[b]
Female	Control	8.72±0.16	2.46±0.04	2.36±0.20	2.03±0.14	21.17±1.14
	100	8.71±0.25	2.51±0.07	2.57±0.14	2.13±0.12	21.83±0.79
	400	8.35±0.22	2.61±0.03	2.36±0.09	1.80±0.47	20.50±1.89

Values are expressed as mean±SEM. Data within a column with different superscripts differ significantly (P<0.05).

In the liver profile test, the mean value of the liver enzymes—ALT, AST and ALP—in all the treated groups was slightly changed, however it was not significantly different when compared to the control groups (Table 5). The mean value of serum GGT was significantly lower (1.42 U/L) at the high dose (400 mg/kg) compared to the control group (2.00 U/L) as well as 100 mg/kg (2.67 U/L). However the liver test profile for female rats remained unchanged.

The antioxidant activity was measured and the effects of extract S2 in the liver and kidney on SOD activities are shown in Table 6. The expressions of SOD in the tissues of both the treated groups generally increased when compared to the control group. In male rats exposed to the high dose of extract S2 (100 and 400 mg/kg), the activities of SOD in kidney were significantly increased by 10% (860.96 U/mL) and 41% (1095.97 U/mL) respectively as compared to the control group (777.98 U/mL). In contrast, in female rats treated with 400 mg/kg, the liver and kidney SOD increased (P<0.05) by 8% and 31% respectively compared to the respective control group. The percentage elevation of SOD levels at a lower dose (100 mg/kg) measured 7% and 9% in liver and kidney respectively.

The changes in the activity of GPX were similar to those observed in SOD. At the dose 100 mg/kg, the GPX level was significantly higher (167.65 nmol/min/mL) as compared to the control group (159.78 nmol/min/mL). Meanwhile in the kidney, the enzyme activity was detected higher in rats treated at 400 mg/kg of extract as compared to the lower dose and control groups. In contrast for females treated with 100 mg/kg of extract S2, the liver GPX activity of both treated groups significantly decreased (P<0.05) by 10% and 7% respectively as compared to the control group. However the expression of kidney GPX enzyme of females treated with 400 mg/kg of extract S2 remained unchanged and within the range of the control group (Table 6).

Histological characterisation of treated organ was undertaken by haematoxylin and eosin (H&E) staining to assess the cytoprotective properties of extract S2 on rats. The administration of extract at both doses (100 and 400 mg/kg) showed a normal morphology without any abnormal changes in any of the organs examined. The photomicrographs for each of the organ tissues for treated and control groups are shown in Figures 3 and 4. The liver showed normal architecture with lobular and central veins surrounded by cords of hepatocytes (Figure 3a). No abnormalities such as necrosis, infiltration, oedema and conjunctions, which are the signs of hepatotoxicity, were observed. In kidney tissue, the control and treated rats showed normal glomeruli and tubules (Figure 3b). Heart, spleen, lung and colon tissue of treated rats also showed no significant difference. None of them were observed to have abnormalities such as oedema and necrosis (Figures 3 and 4).

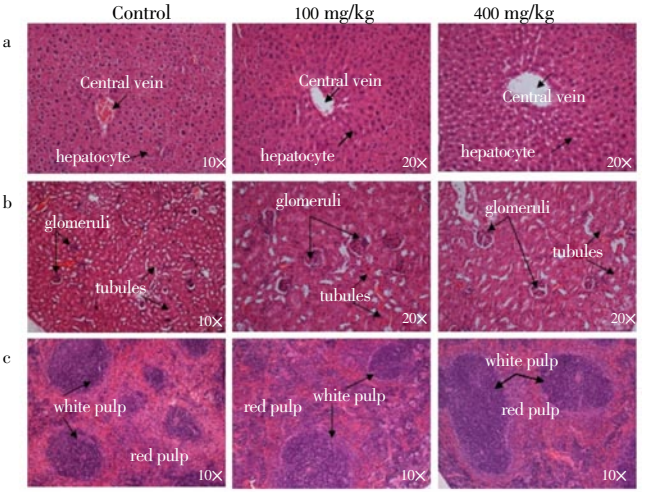

Figure 3. The cross section of liver, kidney, spleen tissue, in control and treated groups of rats (H&E). A: Liver; B: Kidney; and C: Spleen. ×10 and ×20 magnifications. No significant damage was detected in the treatment groups.

Table 5

Effect of oral administration of the extract S2 on the liver profile of male and female rats.

Rats	Dose (mg/kg)	Liver profiles					
		Bilirubin total (μmol/L)	Protein total (g/L)	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)
Male	Control	1.78±0.40	59.17±0.80	53.83±2.00	135.17±20.10	293.67±22.00	2.00±0.00[ab]
	100	1.67±0.52	57.83±1.30	62.00±1.70	131.17±11.30	359.33±38.20	2.67±0.50[a]
	400	1.67±0.82	54.83±3.90	58.67±5.30	128.83±11.30	290.50±10.20	1.42±0.20[b]
Female	Control	2.84±0.48	63.16±0.74	64.33±1.00	99.00±6.50	149.33±12.00	5.50±0.60
	100	2.34±0.56	68.33±2.85	62.33±0.90	130.67±17.60	181.67±13.80	5.33±0.30
	400	2.83±0.31	67.17±0.98	62.00±3.60	116.67±10.30	213.50±25.00	5.75±0.10

Values are expressed as mean±SEM, n=6. Data within a column with different superscripts differ significantly (P<0.05).
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphate; GGT: Gamma-glutamyl transferase.

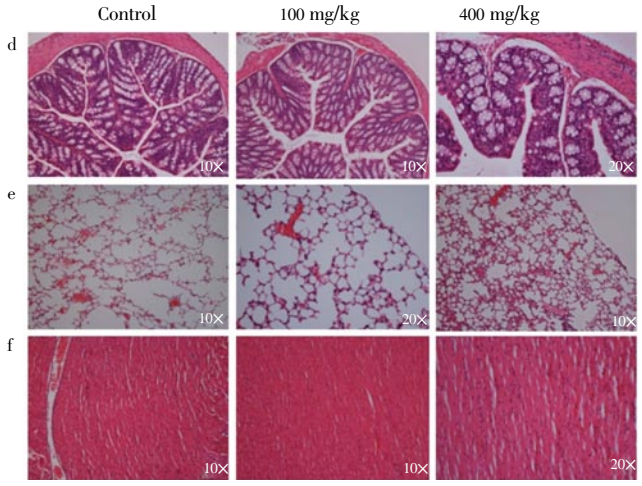

Figure 4. The cross section of colon, lung and heart tissue, in control and treated groups of rats (H&E). D: Colon; E: Lung; F: Heart tissue. ×10 and ×20 magnifications. No significant damage was detected in the treatment groups.

Table 6

Effect of oral administration of the extract S2 on the antioxidant enzymes of male and female rats.

	SOD (U/mL)		GPx (nmol/min/mL)	
	Liver	Kidney	Liver	Kidney
Male				
Control	1186.90±2.90^{b}	777.98±1.90^{c}	159.78±0.47^{b}	172.13±0.81^{a}
100	1207.1.0±0.90^{a}	860.96±2.90^{b}	167.65±1.29^{a}	107.38±0.47^{b}
400	1192.08±2.40^{b}	1095.97±3.40^{a}	163.64±1.21^{b}	173.65±1.35^{a}
Female				
Control	1233.50±5.59^{c}	869.26±3.00^{c}	167.42±1.9^{a}	155.93±8.50
100	1319.46±1.73^{b}	951.66±0.50^{b}	149.88±2.0^{b}	154.80±2.73
400	1366.92±3.88^{a}	1142.92±1.80^{a}	155.52±1.54^{b}	149.99±2.98

Values are expressed as mean±SEM, n=6. Data within a column with different superscripts differ significantly (P<0.05).

4. Discussion

Endophytic fungi from marine algae are poorly documented when compared to mangrove and estuarine plants (Jones *et al*. 2008). To date only few research studies have reported on the bioactivities of marine endophytic fungi, particularly from Malaysia. The endophytic fungal extract isolated from *T. conoides* has been reported to have anticancer properties against a panel of cancer cell lines[9]. The toxicity of the S2 extract must be determined before any further test is employed. If the extract is toxic, it is not worth to proceed to the next level of experiments. A single oral administration of extract S2 at two doses (100 and 400 mg/kg) against SD rats was evaluated after 14 d. The acute toxicity study of extract S2 indicated no changes in the animal behavior immediately after administration and throughout the experiment.

In the present study, the body weight of the treated and control rats gradually increased from Day 1 to Day 14 and no significant difference was found between the treated and control groups of both sexes, which also indicated that extract S2 was not toxic. Toxicity of chemical compounds in experimental animals is often associated with the loss of body weight which leads to death[14,15]. The results showed that the extract did not cause any deaths, thus indicating that it was not toxic even at a high dose of 400 mg/kg. The increase of organ weight (absolute or relative) can be used as a sensitive indicator of organ toxicity[16]. However in this study, the relative organ weight of liver, heart, spleen, colon, kidney and lung in the treated group showed no significant differences from the control rats.

Haematology and clinical biochemistry values in the present study were within the range of values published by other researchers[17]. An appropriate dose of extract in this present study (100 mg/kg) may have some beneficial effects on the blood system. The increase in the WBC count emphasizes the beneficial effect of the extract on the general well being of the animals and its importance in body defense mechanism[18]. In contrast, the decrease of MCHC values as observed in this study may be associated with iron deficiency. However, the haemoglobin (HGB) and haematocrit (HCT) mean values were within the normal range of the control group. Therefore, the decrease of MCHC in the female of low dose group could be due to the experimental variations and not treatment related.

Biochemical parameters measured in the present study showed that all of the values tested in the blood of all the treated groups were not affected as compared to control groups. The serum protein levels in all the treated rats were not significantly different from those in the control group, suggesting that renal function was not impaired[19]. High concentration is related to renal clearance indicating renal dysfunction. The decrease of the serum creatinine levels in the present study in treated male rats and the unchanged creatinine values in the female rats suggested no kidney damage, particularly by a renal filtration mechanism[20]. Thus, it was evident that the extract at the employed doses did not cause renal impairment or kidney.

The results obtained in this study were supported by other findings where compounds present in seaweed have the ability to reduce blood cholesterol level and posses cardioprotective effect[21,22]. The capability of S2 in reducing total cholesterol level in this study might be associated with the capability in reducing cardiovascular risk factors. This effect can be determined by measuring the levels of high density lipoprotein and low density lipoprotein cholesterol in the treated animals. This preliminary data suggested that extract S2 might also act as a hypolipidemic agent. However, further evaluation is necessary to look at the hypolipidemic effects of extract S2.

AST and ALT are critical enzymes in biological processes; consequently they are considered specific indicators for

hepatic dysfunction and damage. In this study, there were no alterations in enzymatic activity of ALT and GGT from treated rats, suggesting a normal function of the liver and kidneys[23]. The increase of these enzymes activities could indicate liver function impairment and cardiac damage[24-26]. In this study, none of the enzymes tested for the liver profile in treated groups showed significant difference compared to the control groups. Therefore, based on the ALT and AST levels, it appeared that no hepatic damage occurred. This result was also supported by the histological study and was in line with *in vitro* preliminary finding which showed that extract S2 was not toxic to the normal liver cell line (WRL-68). The IC_{50} of WRL-68 (30.00±0.02 μg/mL) was significantly higher compared to the other cancer cell lines tested. S2 extract was also not toxic to normal cell Detroit 551[9]. The content of ALP in the serum of treated groups was not significantly different compared with the control groups. Thus, it seems that S2 did not significantly (P>0.05) affect liver function in the treated rats.

The GPX and SOD are major antioxidant enzymes, which are regarded as the first line of the antioxidant defense system against reactive oxygen species generated *in vivo* during oxidative stress. The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. The group of animals treated with extract S2 (100 and 400 mg/kg, *p.o.*) significantly elevated the liver and kidney tissues SOD level in both sexes. SOD is the only enzyme that catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant mechanism. Moreover, the enzyme GPX plays a pivotal role in H_2O_2 catabolism and the detoxification of endogenous metabolic peroxides and hydroperoxides which catalyses glutathione. Interestingly, extract S2 facilitated the enzyme GPX activity in liver and kidney of male rats as well as in liver of female rats. Histopathology examination showed none abnormalities in six organs of the treated animals.

In conclusion, the present data suggests that S2 did not induce any toxic effects through *in vitro* and *in vivo* toxicity study. However sub-acute and chronic toxicological evaluations need to be carried out to extend the toxicological profiles of present extract. Malaysian marine endophytic fungus from *T. conoides*, therefore, is a good potential source of bioactive compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We would like to thank Ministry of Education (MOE) of Malaysia and Research Intensive Faculty (RIF) of University of Technology MARA (UiTM) Malaysia Grant No. 600-RMI/DANA 5/3/RIF (185/2012) for providing the funds to support this research.

Comments

Background

Marine endophytic fungi are good sources of compounds pocessing anticancer, antimicrobial, antioxidant and antiplasmodial activities. *T. conoides* belongs to the family of Sargassaceae has a long history of application in traditional medicine as a cure for fever in children, as food for human consumption, fertiliser, insect repellent, pesticide, and antihistamic, antiviral, antifungal, antiplasmodial and antioxidant agent.

Research frontiers

The present research work described safety of Malaysian marine endophytic fungal extract from a brown seaweed *T. conoides*. Authors showed the potential of natural marine products particularly in Malaysia as a source of new and novel therapeutic entities. They concluded that marine endophytic fungi could be a good potential source of anticancer drugs with great potential as they are potent yet safe, thus deserving further extensive investigation.

Related reports

Different preparations of *T. conoides* have been reported as rich sources of antifungal metabolites including steroids, β-D-mannuronic acid, *etc*.

Innovations and breakthroughs

T. conoides is well known for antimicrobial activities against a wide array of microorganisms. In the present study, authors have demonstrated safety of *T. conoides* in a rat model by oral administration according to the data of the body weight, relative weight of six organs, and haematological, biochemical and antioxidant properties.

Applications

According to the literature, safety of *T. conoides* for biological systems has been a matter of controversy. Results of the present work indicate that *T. conoides* could be a good potential source of anticancer drugs with great potential as it was shown to be safe in a rat model.

Peer review

In this valuable research work, authors have demonstrated the safety of *T. conoides* in a rat model. The activity was assessed based on biochemical parameters, antioxidant enzyme levels in liver homogenate and histopathological observations. According to the *in vitro* and *in vivo* toxicity evaluation of extract S2 of *T. conoides*, it did not induce any toxic effects, which indicated its potential for use as a nutraceutical agent.

References

[1] de Oliveira ALL, de Felício R, Debonsi HM. Marine natural products: chemical and biological potential of seaweeds and their endophytic fungi. *Rev Bras Farmacogn* 2012; **22**: 906-920.

[2] Spavieri J, Allmendinger A, Kaiser M, Itoe MA, Blunden G, Mota MM, et al. Assessment of dual life stage antiplasmodial activity of British seaweeds. *Mar Drugs* 2013; **11**: 4019-4034.

[3] Flewelling AJ, Johnson JA, Gray CA. Antimicrobials from the marine algal endophyte *Penicillium* sp. *Nat Prod Commun* 2013; **8**: 373-374.

[4] Erdmann AM, Bason D. List of algal species in Komodo national park. In: Erdmann AM, editor. *A natural history guide to Komodo National Park*. Indonesia: The Nature Conservancy; 2004, p. 207.

[5] Shanmugam SK, Kumar Y, Yar KMS, Gupta V, De Clercq E. Antimicrobial and cytotoxic activities of *Turbinaria conoides* (J. Agardh) Kuetz. *Iran J Pharm Res* 2010; **9**: 411-416.

[6] Kumar SS, Kumar Y, Khan MS, Gupta V. New antifungal steroids from *Turbinaria conoides* (J. Agardh) Kutzing. *Nat Prod Res* 2011; **24**: 1481-1487.

[7] Kumar SS, Kumar Y, Khan MSY, Anbu J, Sam KG. Acute toxicity study and antipyretic effect of the brown algae *Turbinaria conoides* (J. Agardh) Kuetz. *Afr J Tradit Cmplement Altern Med* 2009; **6**: 233-240.

[8] Kumari SS, Rao SVS, Misra S, Murty US. Antifungal activity of *Turbinaria conoides* and evaluation for the effective concentration against the infection of *Beauveria bassiana* in silkworm larvae. *Res J Microbiol* 2011; **6**: 115-123.

[9] Ariffin SA, Davis P, Ramasamy K. Cytotoxic and antimicrobial activities of Malaysian marine endophytic fungi. *Botanica Marina* 2011; **54**: 95-100.

[10] Strobel GA. Endophytes as sources of bioactive products. *Microbes Infect* 2003; **5**: 535-544.

[11] Ruela HS, Leal ICR, de Almeida MRA, dos Santos KRN, Wessjohann LA, Kuster RM. Antibacterial and antioxidant activities and acute toxicity of *Bumelia sartorum* Mart., Sapotaceae, a Brazilian medicinal plant. *Rev Bras Farmacogn* 2011; **21**: 86-91.

[12] Ray RS, Ghosh B, Rana A, Chatterjee M. Suppression of cell proliferation, induction of apoptosis and cell cycle arrest: chemopreventive activity of vanadium *in vivo* and *in vitro*. *Int J Cancer* 2007; **120**: 13-23.

[13] Adak M, Gupta JK. Acute and subacute toxicity study with hypolipidemic agent of α-alkyl substituted indan-1-acetic acids in mice. *Res J Med Med Sci* 2009; **4**: 345-350.

[14] Parveen S, Das S, Kundra CP, Pereira BM. A comprehensive evaluation of the reproductive toxicity of *Quassia amara* in male rats. *Reprod Toxicol* 2003; **17**: 45-50.

[15] Etuk EU, Igbokwe V, Ajagbonna OP, Egua MO. Toxicology studies of a Nigeria commercial polyherbal product in albino rats. *Res J Med Plant* 2009; **3**: 52-60.

[16] Capasso R, Izzo AA, Pinto L, Bifulco T, Vitobello C, Mascolo N. Phytotherapy and quality of herbal medicines. *Fitoterapia* 2000; **71**: S58-S65.

[17] Ogbonnia SO, Nkemehule FE, Anyika EN. Evaluation of acute and subchronic toxicity of *Stachytarpheta angustifolia* (Mill) Vahl (Fam. Verbanaceae) extract in animals. *Afr J Biotechnol* 2009; **8**: 1793-1799.

[18] Abdullah NR, Ismail Z, Ismail Z. Acute toxicity of *Orthosiphon stamineus* Benth standardized extract in Sprague Dawley rats. *Phytomedicine* 2009; **16**: 222-226.

[19] Ogbonnia SO, Mbaka GO, Anyika EN, Osegbo OM, Igbokwe NH. Evaluation of acute toxicity in mice and subchronic toxicity of hydroethanolic extract of *Chromolaena odorata* (L.) King and Robinson (Fam. Asteraceae) in rats. *Agric Biol J North Am* 2010; **1**: 859-865.

[20] Crook MA. *Clinical chemistry and metabolic medicine*. London, UK: Hodder Arnold; 2006, p. 426.

[21] Boopathy NS, Kathiresan K. Anticancer drugs from marine flora: an overview. *J Oncol* 2010; doi:10.1155/2010/214186.

[22] Krishnamurthy M, Selvaraju M, Tamilarasan M. *Turbinaria conoides* (J. Agardh) sulfated polysaccharide protects rat's heart against myocardial injury. *Int J Biol Macromol* 2012; **50**: 1275-1279.

[23] Almeida-Lima J, Dantas-Santos N, Gomes DL, Cordeiro SL, Sabry DA, Costa LS, et al. Evaluation of acute and subchronic toxicity of a non-anticoagulant, but antithrombotic algal heterofucan from the *Spatoglossum schröederi* in Wistar rats. *Rev Bras Farmacogn* 2011; **21**: 674-679.

[24] Mythilpriya R, Shanthi P, Sachdanandam P. Oral acute and sub acute toxicity studies with Kalpaamurthaa, a modified indigenous preparation on rats. *J Health Sci* 2007; **53**: 351-358.

[25] Aragon G, Younossi ZM. When and how to evaluate mildly elevated liver enzymes in apparently healthy patients. *Cleve Clin J Med* 2010; **77**: 195-204.

[26] Pinton S, Luchese C, Nogueira CW. Comparison of the antioxidant properties and the toxicity of p,p′-Dichlorodiphenyl Ditelluride with the parent compound, diphenyl ditelluride. *Biol Trace Elem Res* 2010; **139**: 204-216.