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Original Article

Toxicological studies of *Orthosiphon stamineus* (Misai Kucing) standardized ethanol extract in combination with gemcitabine in athymic nude mice model

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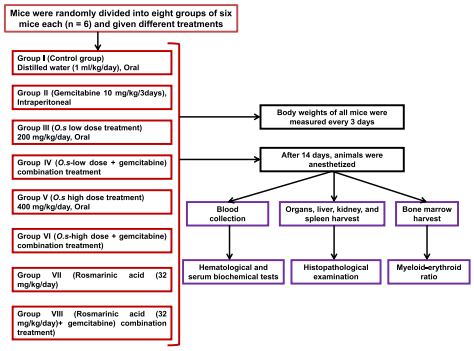
GRAPHICAL ABSTRACT



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ABSTRACT

Pancreatic cancer has the highest mortality rate among cancers due to its aggressive biology and lack of effective treatment. Gemcitabine, the first line anticancer drug has reduced efficacy due to acquired resistance. The current study evaluates the toxicological effects of *Orthosiphon stamineus* (*O.s*) and its marker compound (rosmarinic acid) in combination with gemcitabine. *O.s* (200 or 400 mg/kg/day) and rosmarinic acid (32 mg/kg/day) were administered orally and gemcitabine (10 mg/kg/3 days) intraperitoneally either alone or in combination treatment for fourteen days. Parameters including blood serum biochemistry, hematology, myeloid-erythroid ratio, incident of lethality, and histopathological analysis of liver, kidney, and spleen tissues were studied. Neither, individual drugs/extract nor chemo-herbal combinations at tested doses induced any toxicity and damage to organs in nude mice when compared to control group. Toxicological data obtained from this study will help to select the best doses of chemo-herbal combination for future pancreatic xenograft tumor studies.

Introduction

Cancer is a deadly disease that needs collective efforts to successfully combat and treat. Pancreatic cancer is one of the most aggressive malignant solid tumors which remains the fourth

Abbreviations: O.s., Orthosiphon stamineus.

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leading cause of cancer-related deaths worldwide with an overall survival rate of less than 5% [1]. Surgery, chemotherapy, radiations, and molecular targeted therapies are among the most commonly used options to treat different types of cancers including pancreatic cancer. Although, these therapies have improved survival of cancer patients, unfortunately, majority of these therapeutic modalities have been associated with advent of severe side effects [2].

Gemcitabine, a nucleoside analogue of cytidine is used to treat many cancers including pancreatic cancer [3]. However, its efficacy may be reduced due to multiple adverse reactions and drug resistance [4,5]. The major dose limiting side effects of gemcitabine include hematological toxicities such as thrombocytopenia and neutropenia [6–8]. In addition, combination of gemcitabine with other cancer drugs such as capecitabine, cisplatin, irinotecan, and oxaliplatin may add to its toxicity and reduces its efficacy [9,10].

Herbal products have been utilized for medicinal purposes since ancient times. It is estimated that more than 80% of cancer patients in China, Japan, and other Asian countries use herbs as complementary and alternative medicine (CAM) for the prevention and treatment of different types of cancers [11]. These herbal medicines are now widely accepted as current forms of CAM in cancer treatment in USA and Europe [12,13]. In addition to individual anticancer effects of these herbal products, data from numerous pre-clinical and clinical studies have also highlighted that these natural agents when combined with conventional chemo- or radio-therapies can increase sensitivity of tumor cells towards these treatments, thus improving quality of life and survival time in patients [14,15]. However, this is not always true, as various studies have shown that herbal medicines when combined with conventional chemotherapies, may yield unexpected toxicities and/or enhance toxic potential of standard chemo drugs thus a possible under-treatment seen in cancer patients [16]. Therefore, a thorough understanding of herbal-chemo drugs interactions is urgently needed for proper utilization of herbal drugs in combination with standard chemotherapies to prevent therapeutic failure and advent of toxicities in cancer patients.

Orthosiphon stamineus (*O.s*) is a folklore Asian herbal medicine which is used for the treatment of variety of diseases including inflammation, bacterial infections, urinary tract infections, influenza, rheumatism, jaundice, and angiogenesis-related problems like cancer [17–19]. A decoction made from leaves of *O.s* known as "java tea" is commonly used for general health care needs and fitness [20]. Moreover, safety profile of 50% ethanol extract of *O.s* has already been established globally by numerous research groups in *in vivo* rat models and LD_{50} has been revealed to be more than 5000 mg/kg [21–23]. Phytochemical studies have reported that leaves of *O.s* contain more than 20 phenolic bioactive compounds including rosmarinic acid, eupatorin, pentacyclic triterpenes, betulinic acid, sinesitin, oleanolic acid, ursolic acid, and β-sitosterol respectively. Among these phytoconstituents, rosmarinic acid has been identified as one of the most active compounds in 50% ethanol extract of *O.s* leaves and is responsible for multiple pharmacological activities especially antitumor potency of *O.s* extract [17,24,25]. Antitumor efficacy of 50% ethanol extract of *O.s* against colon has already been established by our research group (Al-Suede et al., 2014). However, to best of our knowledge, no study has reported the anticancer effects of *O.s* 50% standardized ethanolic extract towards pancreatic cancer either alone or in combination with standard chemotherapy drug i.e., gemcitabine.

On the basis of above facts and figures, the present study is designed with an aim to investigate the acute toxicological effect of *O.s,* its major active compound, rosmarinic acid and/or gemcitabine alone and in combination in nude mice. Data from toxicity study is intended to be utilized as a useful tool for choosing the optimal doses for sub-chronic toxicity studies as well as detailed anti-pancreatic cancer studies using different xenograft models.

Material and methods

Plant materials and chemicals

Orthosiphon stamineus as 50% standardized ethanol extract (Catalogue No. 931886-P) was purchased from NatureCeuticals Sendirian Berhad, Kedah DA, Malaysia. The extract was kept in airtight container until further experimentations. Rosmarinic acid (Catalogue No. 536954) was purchased from Sigma-Aldrich, Missouri, USA. Gemcitabine (Catalogue No. S1149) was purchased from Selleckchem, Houston, USA. Both *O.s* extract and rosmarinic acid were dissolved in sterile distilled water and filtered by membrane filter unit (0.22 μm). *O.s* and rosmarinic acid were administrated orally to mice, while gemcitabine was dissolved in phosphate buffer saline (PBS) and injected intraperitoneal to mice.

Animals

The animal study was approved and conducted in strict guidance according to USM Animal Ethics Committee (Reference #: USM/Animal Ethics Approval/2016/(97) (746).

Male athymic nude mice (procured from iDNA, USA) were maintained in filter-top cages under controlled atmospheric conditions at EMAN Testing and Research laboratory, School of Pharmaceutical Sciences, USM. Mice were provided autoclaved food and water and bedding of cages was changed every 48 h.

Experimental design

Treatments

Mice were randomly divided into eight groups of six mice each (n = 6) and given different treatments for 14 days as mentioned in Table 1.

Body weight of all mice was measured every 3rd day. At the end of study, animals were anesthetized with a combination of ketamine and xylazine. Blood samples were collected for hematological and serum biochemical tests. Different body organs including liver, kidney, and spleen were harvested and weighed to observe any changes in organs weights of treated animals compared to control group. Bone marrow was harvested to obtain myeloid-erythroid ratio.

Blood parameters and biochemical tests

Blood samples were used to measure different hematological parameters such as hemoglobin (Hb), total blood count (red blood

Table 1
Different treatment conditions.

No	Group	Treatments
1	Group I (Control group)	Distilled water (1 mL/kg/day), Oral
2	Group II (Gemcitabine)-chemotherapy drugs	Gemcitabine (10 mg/kg/3 days), Intraperitoneal
3	Group III (*O.s* treatment)-low dose	200 mg/kg/day, Oral
4	Group IV (*O.s* combination treatment)-low dose	200 mg/kg/day (Oral) + gemcitabine (10 mg/kg/3 days; Intraperitoneal)
5	Group V (*O.s* treatment) - high dose	400 mg/kg/day, Oral
6	Group VI (*O.s* combination treatment)-high dose	400 mg/kg/day (Oral) + gemcitabine (10 mg/kg/3 days; Intraperitoneal)
7	Group VII (Rosmarinic acid treatment)	32 mg/kg/day, Oral
8	Group VIII (Rosmarinic acid combination treatment)	32 mg/kg/day (Oral) + gemcitabine (10 mg/kg/3 days; Intraperitoneal)

Note: All the treatments were given for a period of 14 days.

cells, white blood cells, and platelets), differential counting of white blood cells, packed cell volume (PCV), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and red cell distribution width (RDW). Serum was used to estimate different liver and kidney function biomarkers such as creatinine, urea, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total bilirubin, total protein, albumin, globulin, albumin/globulin ratio, cholesterol (low and high density cholesterol), triglycerides, and minerals (sodium, potassium, and chloride) respectively.

Histopathological examination

The liver, kidney, and spleen of mice were harvested and fixed in 10% buffered formaldehyde solution and then processed by automated tissue processing machine for histological examination. In the final step tissues from all organs were embedded in paraffin wax to prepare blocks. Tissue sections of 5 μm thickness were cut and hematoxylin and eosin (H&E) stained. Subsequently, they were examined by a pathologist under light microscope.

Myeloid erythroid ratio

Bone marrow was collected from femur bone of mice and processed for cellularity assessment by preparing bone marrow smears. Air-dried smears were then fixed with 100% methanol and stained using a general procedure for Giemsa staining of blood films. Relative percentages of myeloid: erythroid (M: E) ratios were then calculated by observing slides under microscope.

Statistical methods

Prism (GraphPad, USA) and graphing software Excel (Microsoft, USA) were used for statistical analysis. Data was presented as mean ± S.E.M. For parametric data, analysis were performed using one-way analysis of variance (ANOVA) to compare mean values among three or more data sets. The Tukey's honest significant difference (HSD) Post Hoc test was used to assess significant difference from one another. For non-parametric data, analysis were performed using Kruskal-Wallis ANOVA. A value of * $P < 0.05$, ** $P < 0.01$ was considered significant when compared to values in respective control group.

Results

Effect of treatment on mouse body weight and key organs

The average body weight in control group increased by 4.7% when compared with that at start of therapy within the same group (Table 2). Whereas, body weight of animals treated with gemcitabine, *O.s* (low dose), *O.s* (low dose) + gemcitabine, *O.s* (high dose), and *O.s* (high dose) + gemcitabine was decreased by 6.2%, 2.98%, 3.56%, 6.17% and 4.70% respectively (Table 2). On the other hand, a gain in body weight was observed in animals treated with rosmarinic acid (0.4%) alone and in combination with gemcitabine (5.3%) (Table 2). There was significant difference ($P < 0.05$) in average of body weights in all treated groups compared to control group except for group treated with a combination of rosmarinic acid and gemcitabine (Table 2). There was a significant increase ($P < 0.01$) in average of body weight in combination treatment of groups treated with *O.s* (200 mg/kg/day) and rosmarinic acid with gemcitabine compared to group treated by gemcitabine only. The average of body weight of mice treated by *O.s* (400 mg/kg/day) with gemcitabine also increased (1.47%) compared to mice treated by *O.s* (400 mg/kg/day) only (Table 2). However, there was a significant decrease (0.58%) in average of body weight of group treated by *O.s* (200 mg/kg/day) with gemcitabine compared to group treated by *O.s* (200 mg/kg/day) only (Table 2). No statistical difference was observed between group treated with rosmarinic acid alone and group treated with combination of rosmarinic acid and gemcitabine.

No statistical difference was observed between organ weights in control and different treatment groups at the end of the study (Table 3).

Haematological and biochemical parameters

There were no significant changes in Hb levels, total blood cells count, differential counting of WBC, PCV, MCV, MCH, MCHC, and RDW when compared with the corresponding parameters of control group (Tables 4 and 5).

Similarly, no significant changes were found in serum parameters i.e., creatinine, urea, uric acid, AST, ALT, ALP, GGT, total bilirubin, total protein, albumin, globulin, and albumin/globulin ratio of animal groups treated with *O.s* (200 or 400 mg/kg/day) and rosmarinic acid (32 mg/kg/day) alone or in combination with gemcitabine (10 mg/kg/3 days) after fourteen days of treatment when compared with values in control group (Tables 6 and 7). Normal ALT, ALP, and AST levels in serum indicate that there is no damage in hepatocytes. Similarly, urea and total bilirubin levels were also within normal range indicating that no toxic event occurred in kidneys treated with *O.s*, rosmarinic acid, and gemcitabine either alone or in combination treatment.

Lipid and electrolytes profile

The LDL levels were increased and triglycerides levels were decreased in groups treated with 200 mg/kg/day and 400 mg/kg/day of *O.s* in combination with gemcitabine (10 mg/kg/3 days)

Table 2
Effect of different combination treatments on body weights of mice (n = 6).

No	Group	Average of body weights (grams)						*P* value (at 15 days post treatment)
		0-day	3-day	6-day	9-day	12-day	15-day	
1	Control	26.1 ± 1.0	26.7 ± 1.3	26.9 ± 1.0	26.8 ± 0.9	27.1 ± 1.2	27.4 ± 1.0	–
2	Gemcitabine (10 mg/kg/3 days)	27.3 ± 1.2	25.6 ± 1.4	25.5 ± 1.5	24.6 ± 1.7	25.6 ± 1.3	25.6 ± 1.5	(1,2)**, (2,4)**, (2,8)**
3	*O.s* (200 mg/kg/day)	27.6 ± 0.6	26.0 ± 0.7	25.9 ± 0.9	25.4 ± 1.1	26.2 ± 1.2	26.8 ± 1.1	(1,3)**, (3,4)*
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	28.1 ± 0.9	27.0 ± 1.1	26.6 ± 1.2	26.2 ± 1.1	26.3 ± 1.3	27.1 ± 1.5	(1,4)**, (2,4)**, (3,4)*
5	*O.s* (400 mg/kg/day)	25.9 ± 0.7	23.6 ± 0.9	22.9 ± 1.0	23.3 ± 1.3	23.7 ± 1.0	24.3 ± 1.0	(1,5)**, (5,6)**
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	27.4 ± 1.1	26.3 ± 1.5	25.9 ± 0.9	25.7 ± 0.7	26.3 ± 1.0	26.1 ± 1.2	(1,6)**, (5,6)**
7	Rosmarinic acid (32 mg/kg/day)	27.0 ± 0.9	26.5 ± 0.8	26.2 ± 1.0	26.1 ± 0.9	26.4 ± 1.2	27.1 ± 0.9	(1,6)*
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	26.8 ± 1.3	27.4 ± 1.5	26.2 ± 1.4	26.2 ± 1.3	26.7 ± 1.5	28.3 ± 1.2	(2,6)**

Note: O.s: *Orthosiphon stamineus*. Data is presented as mean ± S.E.M. (* = $p < 0.05$, ** = $p < 0.01$, and ns = not significant).

Table 3
Effect of different combination treatments on organ weights of mice (n = 6).

No	Group	Body organs weight (grams)					
		Liver	*P* value	Kidney	*P* value	Spleen	*P* value
1	Control	1.48 ± 0.13	–	0.41 ± 0.03	–	0.11 ± 0.03	–
2	Gemcitabine (10 mg/kg/3 days)	1.57 ± 0.24	ns	0.41 ± 0.05	ns	0.10 ± 0.03	ns
3	*O.s* (200 mg/kg/day)	1.45 ± 0.22	ns	0.41 ± 0.05	ns	0.11 ± 0.03	ns
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	1.45 ± 0.22	ns	0.41 ± 0.05	ns	0.11 ± 0.03	ns
5	*O.s* (400 mg/kg/day)	1.37 ± 0.18	ns	0.39 ± 0.04	ns	0.12 ± 0.02	ns
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	1.43 ± 0.12	ns	0.39 ± 0.04	ns	0.13 ± 0.02	ns
7	Rosmarinic acid (32 mg/kg/day)	1.55 ± 0.14	ns	0.40 ± 0.04	ns	0.13 ± 0.02	ns
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	1.59 ± 0.11	ns	0.41 ± 0.06	ns	0.11 ± 0.02	ns

Note: *O.s*: *Orthosiphon stamineus*. Data is presented as mean ± S.E.M. (* = $p < 0.05$, ** = $p < 0.01$, ns = not significant,). ANOVA is not significant between all treatment groups.

Table 4
Hematological parameters (Part 1) in different treatment groups.

No	Group	Hb g/L	Total RBC 10^12/L	PCV L/L	MCV fL	MCH pg	MCHC g/L	RDW %	Plts 10^9/L
1	Control	125.7 ± 2.0	8.3 ± 1.5	0.42 ± 0.0	48.3 ± 2.0	14.3 ± 1.1	297.7 ± 1.5	21.7 ± 1.6	850 ± 1.1
2	Gemcitabine (10 mg/kg/3 days)	121.7 ± 1.5	8.2 ± 1.7	0.41 ± 0.0	49.0 ± 2.0	14.7 ± 0.5	297.0 ± 2.0	20.8 ± 0.6	1102 ± 1.3
3	*O.s* (200 mg/kg/day)	124.5 ± 2.2	7.9 ± 1.5	0.44 ± 0.1	55.5 ± 2.2	16.0 ± 1.4	285.5 ± 0.7	21.3 ± 1.5	865 ± 1.5
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	127.5 ± 1.9	8.5 ± 1.3	0.44 ± 0.0	52.0 ± 1.4	15.0 ± 0.1	287.5 ± 1.7	18.5 ± 0.5	1038 ± 0.5
5	*O.s* (400 mg/kg/day)	130.0 ± 1.3	8.6 ± 0.9	0.44 ± 0.0	51.0 ± 1.0	15.0 ± 1.4	295.5 ± 2.2	23.0 ± 0.8	905 ± 0.9
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	123.7 ± 1.4	8.7 ± 0.8	0.42 ± 0.0	50.0 ± 1.1	14.7 ± 0.5	295.0 ± 1.8	21.8 ± 1.0	893 ± 1.2
7	Rosmarinic acid (32 mg/kg/day)	129.3 ± 2.0	8.9 ± 1.1	0.43 ± 0.0	48.3 ± 0.1	14.7 ± 0.6	299.0 ± 2.2	20.6 ± 1.4	682 ± 0.7
8	Rosmarinic acid (32 mg/kg/day) +gemcitabine (10 mg/kg/3 days)	126.3 ± 1.7	8.3 ± 1.9	0.44 ± 0.0	53.0 ± 1.9	15.3 ± 0.6	287.0 ± 1.5	19.1 ± 0.8	812 ± 1.7

Note: Hb: Hemoglobin; RBC: Red blood cells; PCV: Packed cell volume; MCV: Mean cell volume; MCH: Mean cell hemoglobin; MCHC: Mean cell hemoglobin concentration; RDW: Red cell distribution width; Plts: Platelets; *O.s*: *Orthosiphone stamineus*; Control: treated with distilled water only. Results are expressed as the mean ± SEM (n = 6). The *P* values in all treated groups were not significant when compared to one another.

Table 5
Hematological parameters (Part 2) in different treatment groups.

No	Group	Total WBC 10^9/L	N (%)	L (%)	M (%)	E (%)	B (%)
1	Control	6.6 ± 1.0	28 ± 1.3	62 ± 1.5	7 ± 0.4	2 ± 0.2	1 ± 0.0
2	Gemcitabine (10 mg/kg/3 days)	6.8 ± 1.9	29 ± 1.6	61 ± 0.9	7 ± 0.7	2 ± 0.1	1 ± 0.0
3	*O.s* (200 mg/kg/day)	6.4 ± 0.9	40 ± 1.9	49 ± 0.5	8 ± 0.3	1 ± 0.1	2 ± 0.1
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	5.6 ± 1.1	55 ± 1.5	37 ± 1.3	5 ± 0.5	2 ± 0.0	1 ± 0.0
5	*O.s* (400 mg/kg/day)	8.6 ± 2.0	57 ± 1.8	31 ± 0.7	9 ± 0.9	2 ± 0.1	1 ± 0.0
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	6.5 ± 1.5	42 ± 0.9	46 ± 1.1	9 ± 1.1	2 ± 0.1	1 ± 0.0
7	Rosmarinic acid (32 mg/kg/day)	5.9 ± 0.9	21 ± 1.5	69 ± 1.2	6 ± 0.9	2 ± 0.1	2 ± 0.1
8	Rosmarinic acid (32 mg/kg/day) +gemcitabine (10 mg/kg/3 days)	4.7 ± 1.2	47 ± 1.9	42 ± 1.0	8 ± 0.6	1 ± 0.1	2 ± 0.0

Note: WBC: White blood cells; N: Neutrophil; L: Lymphocyte; M: Monocyte; E: Eosinophil; B: Basophil; *O.s*: *Orthosiphone stamineus*; Control: treated with distilled water only. Results are expressed as the mean ± SEM (n = 6). The *P* values in all treated groups were not significant when compared to one another.

Table 6
Blood biochemical parameters in different treatment groups.

No	Group	Creatinine mmol/L	Urea mmol/L	Uric acid mmol/L	ALP µ/L	AST µ/L	ALT µ/L	GGT µ/L
1	Control	27.5 ± 1.5	8.0 ± 0.7	0.22 ± 0.0	90.0 ± 0.9	167 ± 0.9	51.0 ± 0.2	<3 ± 0.0
2	Gemcitabine (10 mg/kg/3 days)	27.7 ± 1.9	8.5 ± 1.0	0.20 ± 0.0	84.0 ± 0.6	168 ± 0.4	52.0 ± 0.3	<3 ± 0.0
3	*O.s* (200 mg/kg/day)	27.0 ± 1.3	7.2 ± 1.5	0.20 ± 0.0	70.0 ± 0.8	138 ± 0.7	37.0 ± 0.6	<3 ± 0.0
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	29.5 ± 1.9	8.1 ± 0.2	0.14 ± 0.1	83.0 ± 0.3	142 ± 0.9	44.0 ± 0.9	<3 ± 0.0
5	*O.s* (400 mg/kg/day)	31.5 ± 1.6	8.9 ± 0.9	0.19 ± 0.1	74.0 ± 0.1	128 ± 0.7	38.0 ± 0.3	<3 ± 0.0
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	27.3 ± 1.1	8.4 ± 1.0	0.19 ± 0.0	77.0 ± 0.6	113 ± 0.9	37.0 ± 0.8	<3 ± 0.0
7	Rosmarinic acid (32 mg/kg/day)	26.0 ± 1.7	8.0 ± 0.2	0.18 ± 0.0	74.0 ± 0.9	109 ± 0.9	31.0 ± 0.6	<3 ± 0.0
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	23.7 ± 1.1	7.6 ± 0.4	0.21 ± 0.0	97.0 ± 0.8	140 ± 0.9	48.0 ± 0.7	<3 ± 0.0

Note: ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma Glutamyl transferase; *O.s*: *Orthosiphonstamineus*; Control: treated with distilled water only; Results are expressed as the mean ± SEM (n = 6). The *P* values in all treated groups were not significant when compared to one another.

Table 7
Blood proteins profile in different treatment groups.

No	Group	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	Albumin/Globulin ratio	Total Bilirubin (μmol/L)
1	Control	47.5 ± 0.7	25.5 ± 1.1	22.5 ± 0.4	1.15 ± 0.1	<2 ± 0.0
2	Gemcitabine (10 mg/kg/3 days)	49.3 ± 1.1	27.3 ± 0.5	22 ± 0.9	1.3 ± 0.1	<2 ± 0.0
3	*O.s* (200 mg/kg/day)	50.0 ± 0.9	25.5 ± 0.7	24.5 ± 1.1	1.1 ± 0.2	<2 ± 0.0
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	46.0 ± 1.4	25.5 ± 0.5	20.5 ± 0.9	1.2 ± 0.1	<2 ± 0.0
5	*O.s* (400 mg/kg/day)	51.5 ± 0.5	25.5 ± 0.9	26.0 ± 0.8	1.1 ± 0.4	<2 ± 0.0
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	48.0 ± 0.6	26.3 ± 0.5	21.7 ± 0.9	1.2 ± 0.2	<2 ± 0.0
7	Rosmarinic acid (32 mg/kg/day)	48.7 ± 1.1	28.0 ± 0.9	22.7 ± 0.7	1.2 ± 0.2	<2 ± 0.0
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days)	50.3 ± 0.9	1.0 ± 0.9	22.3 ± 0.5	1.2 ± 0.1	<2 ± 0.0

Note: O.s: *Orthosiphonstamineus*; Control: treated with distilled water only; Results are expressed as the mean ± SEM (n = 6). The *P* values in all treated groups were not significant when compared to one another.

Table 8
Lipids profile in different groups.

No	Group	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)	Total cholesterol/ HDL Ratio
1	Control	2.6 ± 0.0	1.4 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	2.6 ± 0.1
2	Gemcitabine (10 mg/kg/3 days)	2.6 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	2.5 ± 0.1
3	*O.s* (200 mg/kg/day)	2.7 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	2.1 ± 0.2
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3days)	2.6 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	0.8 ± 0.4	2.3 ± 0.1
5	*O.s* (400 mg/kg/day)	2.4 ± 0.2	1.4 ± 0.2	0.8 ± 0.1	0.8 ± 0.0	2.5 ± 0.2
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3days)	2.6 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	2.2 ± 0.2
7	Rosmarinic acid (32 mg/kg/day)	2.6 ± 0.2	1.5 ± 0.1	1.0 ± 0.1	0.91 ± 0.1	2.3 ± 0.1
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3days)	2.6 ± 0.1	1.5 ± 0.3	1.0 ± 0.0	0.8 ± 0.1	2.3 ± 0.1

Note: HDL: High density lipoprotein; LDL: Low density lipoprotein; *O.s*: *Orthosiphon stamineus*; Control: treated with distilled water only; n = 6; Results are expressed as mean ± SEM (n = 6). *P* values in all treated groups were not significant when compared to one another.

(Table 8). However, these changes were not statistically significant when compared to values in control group. The other lipid parameters i.e., HDL, total cholesterol/HDL ratio, and electrolytes were within normal ranges in all groups and no significant changes were observed when compared to control group (Tables 8 and 9).

Histopathology analysis

Histopathological examination of formalin fixed paraffin embedded tissues of liver (Fig. 1), kidney (Fig. 2), and spleen (Fig. 3) of all treatment groups as well as control group revealed normal histology without pathological evidence of inflammation or necrosis. The liver did not exhibit fatty change although there were patchy areas of hepatocyte swelling in animals treated with rosmarinic acid alone and in combination with gemcitabine (Fig. 1G and H). In microscopic view, it is possible to see small and clear vacuoles in cytoplasm.

Erythroid myeloid ratio

The erythroid myeloid ratio was within normal range in all treatment groups when compared to control group (Fig. 4).

Discussion

The use of medicinal plants as complementary therapy has been increasing worldwide and gaining popularity in the developing countries. Numerous studies have indicated that Chinese herbal medicines in combination with chemo- or radiotherapy can be used to enhance the efficacy of and diminish the side effects and complications caused by chemo- and radiotherapies [26]. *O.s* is folklore medicinal herb that is consumed in most of the Southeast Asian countries to treat variety of ailments [23]. The first priority in herbal research is an assessment of the safety profile of herbal products and setting up a criterion for selecting a safe dose in

Table 9
Electrolytes profile in different groups.

No	Group	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)
1	Control	150.3 ± 0.7	5.2 ± 0.9	113 ± 0.7
2	Gemcitabine (10 mg/kg/3days)	151.0 ± 1.1	5.2 ± 0.2	113 ± 1.5
3	*O.s* (200 mg/kg/day)	153.0 ± 0.7	5.0 ± 0.4	113 ± 0.7
4	*O.s* (200 mg/kg/day) + gemcitabine (10 mg/kg/3days)	150.0 ± 0.7	5.2 ± 0.2	113 ± 1.4
5	*O.s* (400 mg/kg/day)	150.0 ± 0.7	5.0 ± 0.3	114 ± 0.1
6	*O.s* (400 mg/kg/day) + gemcitabine (10 mg/kg/3days)	152.0 ± 1.1	5.0 ± 0.3	115 ± 0.9
7	Rosmarinic acid (32 mg/kg/day)	150.0 ± 0.1	4.2 ± 0.2	111 ± 0.5
8	Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3days)	149.0 ± 1.1	5.5 ± 0.9	111 ± 1.5

Note: O.s: *Orthosiphon stamineus*; Control: treated with distilled water only; n = 6; Results are expressed as mean ± SEM (n = 6). *P* values in all treated groups were not significant when compared to one another.

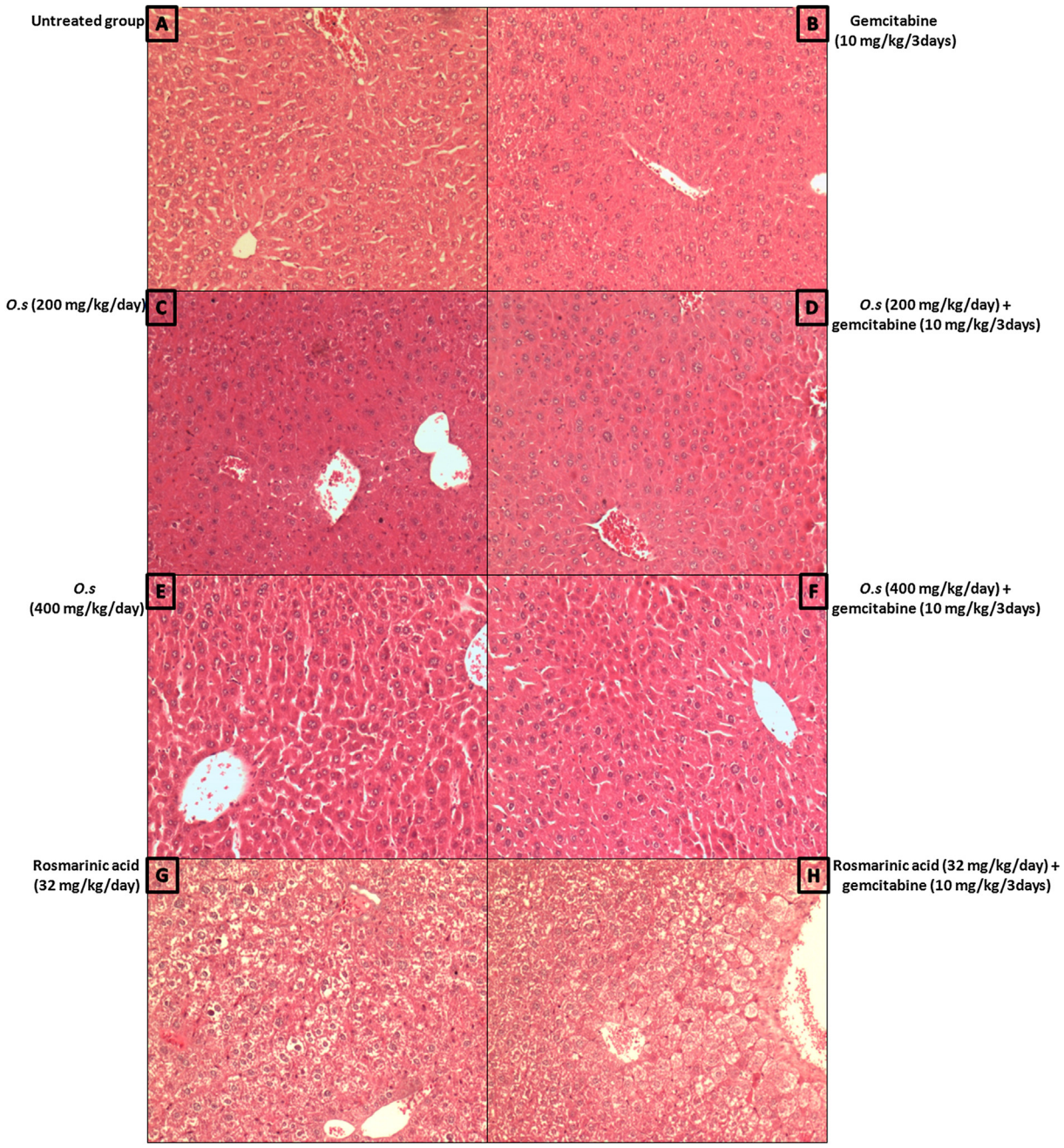

Fig. 1. Tissue sections of mice stained with haematoxylin and eosin. Liver sections showed normal architecture with distinct hepatic cells, sinusoidal spaces, and a central vein in all treatment groups and control; G) and H) a small amount of vacuolar hydropic degeneration. Photos were taken at 100× magnification.

humans [23]. The safety profile of *O.s* has already been established by multiple research groups and data from these studies shows that this herb has LD_{50} values greater than 5000 mg/kg [22,27]. Gemcitabine is a chemotherapy drug used to treat many cancers. However, major dose limiting side effects of gemcitabine are hematological toxicities which often results in dose reduction and or longer intervals between gemcitabine administrations [28]. Chemo-herbal combination treatment is one possible therapeutic option which can be employed to improve disease-free interval and overall survival rate in cancer patients. However, a proper understanding of chemo-herbal combination and data from multiple animal models is required to select the safest combination dose for further clinical studies. Data obtained from our *in vitro* work about the effect of combination treatment ie., *O.s* and gemcitabine on MiaPaCa-2 pancreatic cancer cell lines showed synergistic effect of *O.s* leading to sensitization of cells to gemcitabine treatment (Fig. S1).

In the current study, an attempt is made to select relatively safe doses of *O.s* standardized extract and gemcitabine combination in athymic nude mice pancreatic cancer model for further preclinical anticancer studies. Multiple dose studies are usually helpful in evaluating the safety profile of phytomedicines [29]. Fourteen days data of combination treatment did not reveal any abnormal clinical signs in any of the treatment groups. Animals in all the groups survived and no treatment related mortality occurred during the study. Gross necropsy did not reveal any abnormal pathology in any of the animals. Body weight changes are an indicator of adverse side effects, as the animals that survive

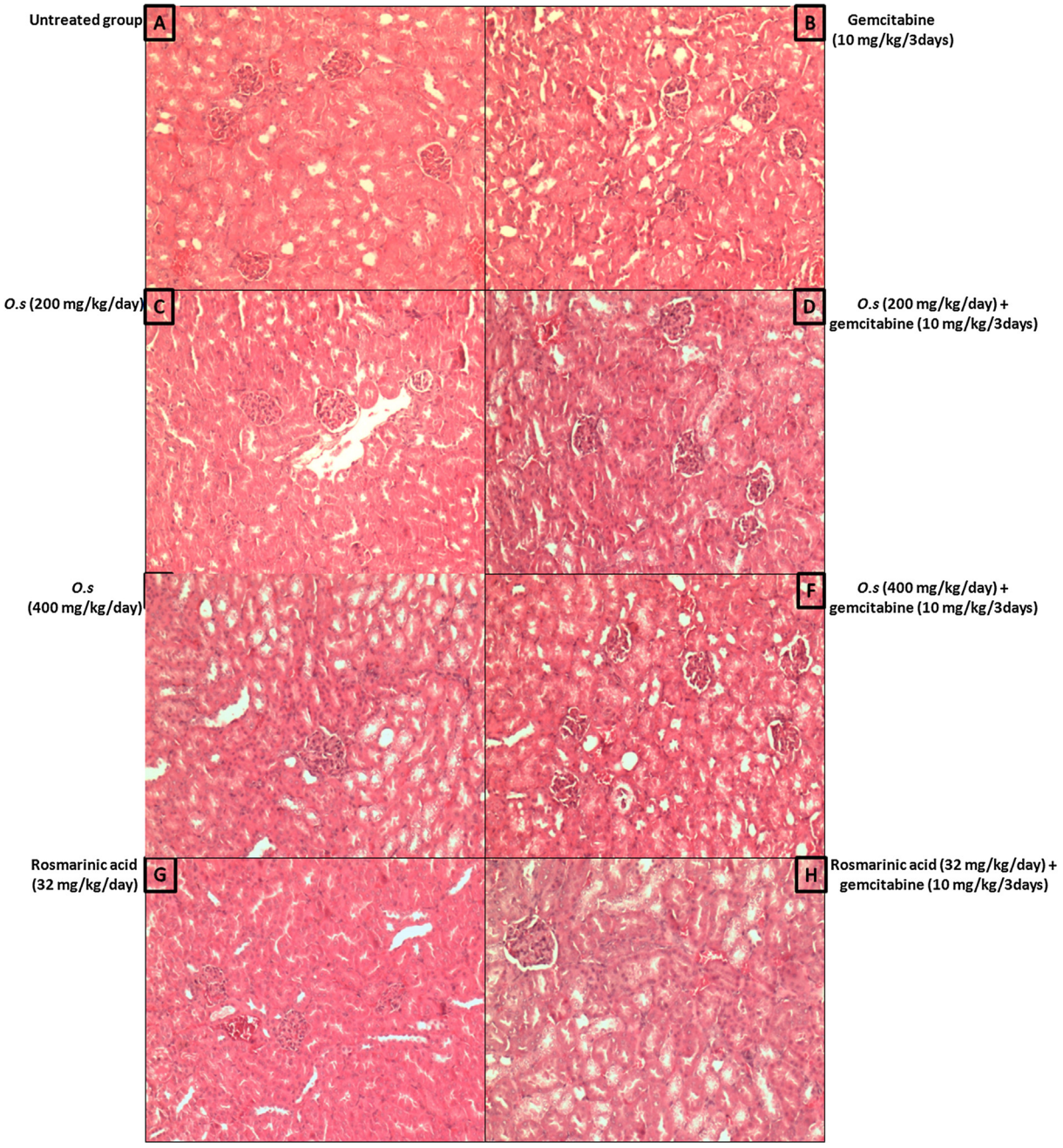

Fig. 2. Tissue sections of mice kidneys stained with haematoxylin and eosin. Tissue sections showed normal glomeruli and tubules in all treated groups and control. Photos were taken at 100× magnification.

cannot lose more than 10% of initial body weight [29]. In the current study loss of body in all the treatment groups was less than 10% indicating relatively safe nature of *O.s* extract gemcitabine combination. Clinical biochemistry and hematological data hold the significant role in determining the toxicity induced by drugs. Blood parameters analysis is relevant to risk evaluation as the hematological system has a higher predictive value for toxicity in humans (91%) when assays involve rodents and nonrodents [30]. Blood forms the main medium of transport for many drugs and xenobiotics in the body and for that matter, components of the blood such as red blood cells, white blood cells, hemoglobin, and platelets are at least initially exposed to significant concentrations of toxic compounds. Damage to and destruction of blood cells are inimical to normal functioning of body [31]. There is no significant alteration in hematological parameters observed, indicating that *O.s* and gemcitabine combination did not affect blood cell production. It also suggests protective potential of *O.s* against gemcitabine-induced hematological malignancies. This data is also supported by normal erythroid/myeloid cells ratio in bone marrow slides of different treatment groups indicating bone marrow protective effects of *O.s* against gemcitabine toxicities. Bilirubin is formed by breakdown of hemoglobin in liver, spleen, and bone marrow. An increase in tissue or serum bilirubin concentrations reflects increased breakdown of RBC (hemolysis) or liver damage [29]. The normal levels of serum bilirubin concentrations in all treatment groups show non-toxic effects of *O.s* gemcitabine combination on hemoglobin metabolic pathways. Kidneys are particularly liable to high doses of drugs as they eliminate many drugs

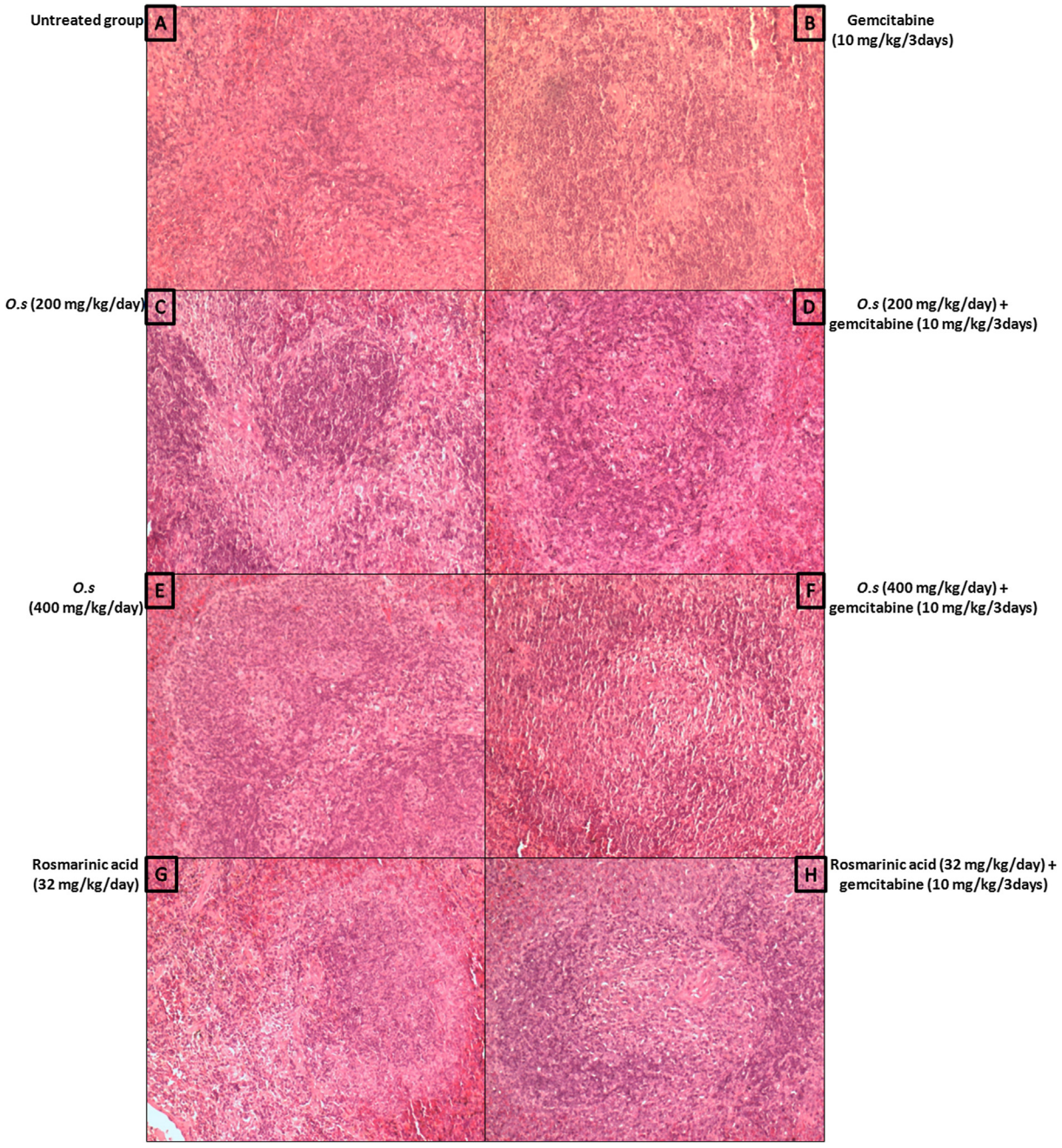

Fig. 3. Tissue sections of mice spleen stained with haematoxylin and eosin. Tissue sections from control and treated groups showed normal red and white pulp. Photos were taken at 100× magnification.

and their metabolites. Serum urea concentration is often considered the more reliable renal function predictor than serum creatinine [32]. In the present study, there were no significant changes observed in urea, creatinine, cholesterol, and albumin parameters between control and different treatment groups thus indicating non-nephrotoxic nature of different chemo-herbal combinations employed. This data is further supported by the normal renal architecture of kidney sections. Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma glutamyl transferases are the most widely used markers for measuring hepatocellular injury during diseases [33]. A significant decrease in serum activities of AST and ALT was observed in all treatment groups except gemcitabine treated animals. Histopathological examination of liver slides showed normal hepatocellular architecture in all treatment groups except rosmarinic acid treated animals where hypoxia in different regions of liver sections can be seen. This might be due to hypoxia (Fig. 1G and H). Tissue hypoxia was seen in some parts of liver section in the groups treated with rosmarinic acid (Fig. 1G and H). According to Kumar study (2000), swelling is formed due to ion imbalance and insufficient homeostasis of cells [34]. He declared that this situation is the first symptom of cellular destruction and although it's hard to notice with a light microscope it is more observable in an organ [34]. In addition, he also mentioned that the color of organ faded and followed by increased weight with turgor and this kind of non-fatal reversible change causes hydropic changes [34]. In our studies, weight of liver and body weights of animals in groups treated with rosmarinic acid alone and in combination with gemcitabine was

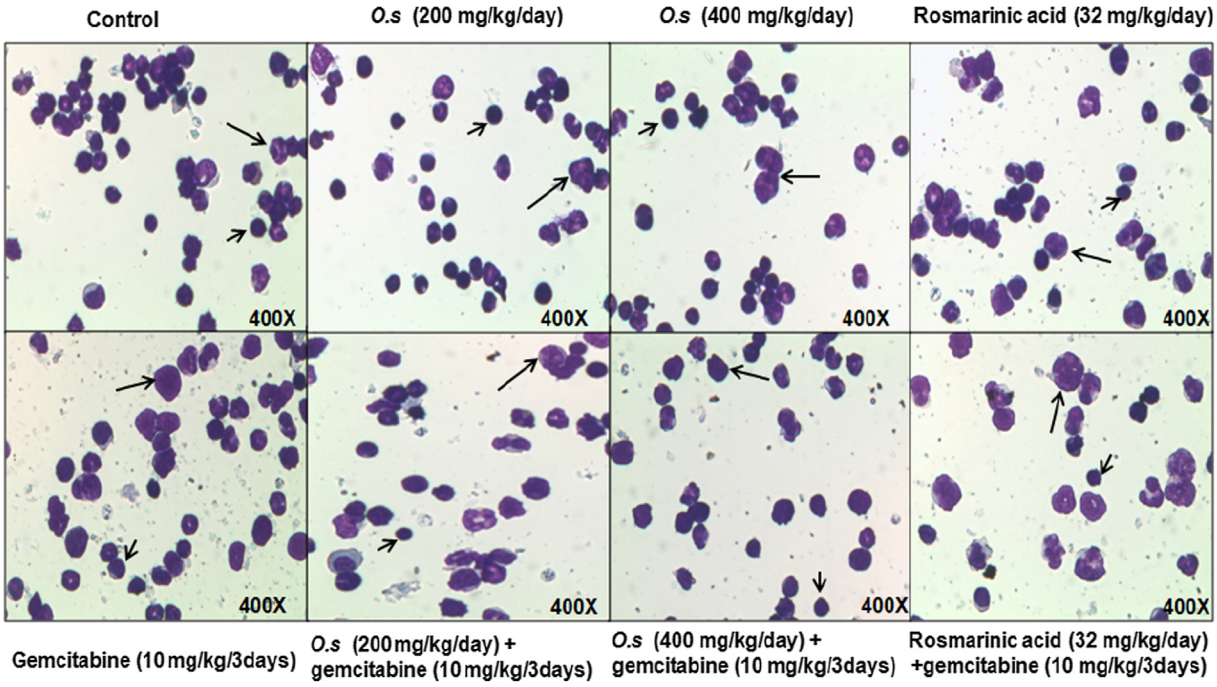

Fig. 4. Bone marrow smears from femur of mice stained with Giemsa stain. The M:E ratio was within the normal range in all the treated mice as compared with the control group. *Note:* Long arrow indicates myeloid cell while short arrows indicate erythroid cells. Photos were taken at 400× magnification.

higher than control group and other treatment groups. However, this increase in weight of liver was not significant when compared to control group.

The recommended human systemic dose of gemcitabine (1000 mg/m^2) was well tolerated with no adverse effects on the bone marrow. However, evidence of mild myelosuppression, with a slight fall in white blood count and platelets was reported with high dose and long term treatment of gemcitabine [35,36]. This study has demonstrated the safety dose of gemcitabine alone and in combination treatment. The bone marrow in treated groups showed normal cellularity with a normal myeloid erythroid ratio. There was no drop in white blood cells and platelets in all treated groups. The antioxidant capability of phenolic compounds in *O.s* is essential to destroy free radicals that exist in human body. This property is also suggested to be palying a pivotal role in the treatment of many diseases including liver cirrhosis [37,38]. It has been reported that *O.s* exhibits radical scavenging activity probably due to the higher concentration of caffeic acid derivatives, especially rosmarinic acid [38].

Conclusions

In conclusion, this study provides preliminary scientific evidence about the safety profile of 50% standardized extract of *O.s* in combination with gemcitabine in an athymic nude mice model. *O.s* extract in combination with standard chemotherapy drug (gemcitabine) was shown to be quite safe and even reduced the incidence of chemo-drug associated liver damage which might be due to its phenolic components. Thus, on the basis of findings of current study, it is proposed that 50% ethanol extract of *O.s* has the potential to be used in combination with gemcitabine to treat pancreatic cancer.

Data obtained from this study will help to select the best dose for future pre-clinical studies. On-going work is being carried out to investigate the effects of *O.s* and gemcitabine combination in pancreatic xenograft tumor model.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jare.2018.05.006.

References

[1] Dahham SS, Tabana YM, Hassan LEA, Ahamed MBK, Majid ASA, Majid AMSA. *In vitro* antimetastatic activity of Agarwood (*Aquilaria crassna*) essential oils against pancreatic cancer cells. Alex J Med 2016;52(2):141–50.
[2] Neoptolemos JP, Palmer D, Ghaneh P, Valle JW, Cunningham D, Wadsley J, et al. ESPAC-4: A multicenter, international, open-label randomized controlled phase III trial of adjuvant combination chemotherapy of gemcitabine (GEM) and capecitabine (CAP) versus monotherapy gemcitabine in patients with resected pancreatic ductal adenocarcinoma. Am Soc Clin Oncol 2016.
[3] Neoptolemos J, Palmer D, Ghaneh P, Valle J, Cunningham D, Wadsley J, et al. ESPAC-4: a multicenter, international, open label randomized controlled phase III trial of adjuvant combination chemotherapy of gemcitabine (GEM) and capecitabine (CAP), versus monotherapy gemcitabine in patients with resected pancreatic ductal adenocarcinoma. Pancreatology 2016;16(3):S63.
[4] Sinn M, Riess H, Sinn B, Stieler J, Pelzer U, Striefler J, et al. Human equilibrative nucleoside transporter 1 expression analysed by the clone SP 120 rabbit antibody is not predictive in patients with pancreatic cancer treated with adjuvant gemcitabine–Results from the CONKO-001 trial. Eur J Cancer 2015;51(12):1546–54.
[5] Sousa Cavalcante L, Monteiro G. Gemcitabine: metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. Eur J Pharmacol 2014;741:8–16.
[6] Al-Nouri ZL, Reese JA, Terrell DR, Vesely SK, George JN. Drug-induced thrombotic microangiopathy: a systematic review of published reports. Blood 2015;125(4):616–8.
[7] Daud AI, Ashworth MT, Strosberg J, Goldman JW, Mendelson D, Springett G, et al. Phase I dose-escalation trial of checkpoint kinase 1 inhibitor MK-8776 as

monotherapy and in combination with gemcitabine in patients with advanced solid tumors. J Clin Oncol 2015;33(9):1060–6.
[8] Thatcher N, Hirsch FR, Luft AV, Szczesna A, Ciuleanu TE, Dediu M, et al. Necitumumab plus gemcitabine and cisplatin versus gemcitabine and cisplatin alone as first-line therapy in patients with stage IV squamous non-small-cell lung cancer (SQUIRE): an open-label, randomised, controlled phase 3 trial. Lancet Oncol 2015;16(7):763–74.
[9] Awasthi N, Zhang C, Schwarz AM, Hinz S, Wang C, Williams NS, et al. Comparative benefits of Nab-paclitaxel over gemcitabine or polysorbate-based docetaxel in experimental pancreatic cancer. Carcinogenesis 2013. bgt227.
[10] Wang-Gillam A, Li C-P, Bodoky G, Dean A, Shan Y-S, Jameson G, et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. Lancet 2016;387 (10018):545–57.
[11] Huebner J, Micke O, Muecke R, Buentzel J, Prott FJ, Kleeberg U, et al. User rate of complementary and alternative medicine (CAM) of patients visiting a counseling facility for CAM of a German comprehensive cancer center. Anticancer Res 2014;34(2):943–8.
[12] Schnipper LE, Davidson NE, Wollins DS, Tyne C, Blayney DW, Blum D, et al. American Society of Clinical Oncology statement: a conceptual framework to assess the value of cancer treatment options. J Clin Oncol 2015;33 (23):2563–77.
[13] Rossi E, Vita A, Baccetti S, Di Stefano M, Voller F, Zanobini A. Complementary and alternative medicine for cancer patients: results of the EPAAC survey on integrative oncology centres in Europe. Supportive Care Cancer 2015;23 (6):1795–806.
[14] Qi F, Zhao L, Zhou A, Zhang B, Li A, Wang Z, et al. The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer. Biosci Trends 2015;9(1):16–34.
[15] Yehya AH, Asif M, Tan YJ, Sasidharan S, Majid AMA, Oon CE. Broad spectrum targeting of tumor vasculature by medicinal plants: An updated review. J Herbal Med 2017.
[16] Alissa EM. Medicinal herbs and therapeutic drugs interactions. Ther Drug Monit 2014;36(4):413–22.
[17] Al-Suede FSR, Farsi E, Ahamed MKB, Ismail Z, Majid ASA, Majid AMSA. Marked antitumor activity of cat's whiskers tea (*Orthosiphon stamineus*) extract in orthotopic model of human colon tumor in nude mice. J Biochem Technol 2014;3(5):170–6.
[18] Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. J Burn Care Res: official publication of the American Burn Association 2010;31(1):158–75.
[19] Geng L, Chaudhuri A, Talmon G, Wisecarver JL, Wang J. TGF-beta suppresses VEGFA-mediated angiogenesis in colon cancer metastasis. PLoS ONE 2013;8 (3):e59918. https://doi.org/10.1371/journal.pone.0059918.
[20] Othman N, Ya'acob M, Abdul-Rahim A, Othman MS, Radzi M, Hizam H, et al. Embracing new agriculture commodity through integration of Java Tea as high Value Herbal crops in solar PV farms. J Clean Prod 2015;91:71–7.
[21] Chin J, Abas H, Sabariah I. Toxicity study of *orthosiphon stamineus* benth (misai kucing) on Sprague dawley rats. Trop Biomed 2008;25(1):9–16.
[22] Mohamed EAH, Lim CP, Ebrika OS, Asmawi MZ, Sadikun A, Yam MF. Toxicity evaluation of a standardised 50% ethanol extract of *Orthosiphon stamineus*. J Ethnopharmacol 2011;133(2):358–63.
[23] Shafaei A, Esmailli K, Farsi E, Aisha AFA, Abul Majid AMS, Ismail Z. Genotoxicity, acute and subchronic toxicity studies of nano liposomes of *Orthosiphon stamineus* ethanolic extract in Sprague Dawley rats. BMC Complement Altern Med 2015;15:360. https://doi.org/10.1186/s12906-015-0885-z. PubMed PMID: PMC4604773.
[24] Shin MR, Kang SK, Kim YS, Lee SY, Hong SC, Kim EC. TNF-α and LPS activate angiogenesis via VEGF and SIRT1 signalling in human dental pulp cells. Int Endod J 2015;48(7):705–16.
[25] Tabana YM, Al-Suede FSR, Ahamed MBK, Dahham SS, Hassan LEA, Khalilpour S, et al. Cat's whiskers (*Orthosiphon stamineus*) tea modulates arthritis pathogenesis via the angiogenesis and inflammatory cascade. BMC Complement Altern Med 2016;16(1):480.
[26] Sellers RS, Mortan D, Michael B, Roome N, Johnson JK, Yano BL, et al. Society of toxicologic pathology position paper: organ weight recommendations for toxicology studies. Toxicol Pathol 2007;35(5):751–5.
[27] Shafaei A, Esmailli K, Farsi E, Aisha AF, Majid AMSA, Ismail Z. Genotoxicity, acute and subchronic toxicity studies of nano liposomes of *Orthosiphon stamineus* ethanolic extract in Sprague Dawley rats. BMC Complement Altern Med 2015;15(1):360.
[28] Keane MP, Belperio JA, Arenberg DA, Burdick MD, Xu ZJ, Xue YY, et al. IFN-gamma-inducible protein-10 attenuates bleomycin-induced pulmonary fibrosis via inhibition of angiogenesis. J Immunol 1999;163.
[29] Chitra B, Ramaswamy RS, Suba V. T oxicity evaluation of Pūrṇa Cantirotaya Centūram, a Siddha Medicine in Wistar Rats. Int Sch Res Notices 2015;2015:10. https://doi.org/10.1155/2015/473296.
[30] Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharm 2000;32(1):56–67.
[31] Adeneye AA, Ajagbonna OP, Adeleke TI, Bello SO. Preliminary toxicity and phytochemical studies of the stem bark aqueous extract of *Musanga cecropioides* in rats. J Ethnopharmacol 2006;105(3):374–9.
[32] Shatoor AS. Acute and sub-acute toxicity of *Crataegus aronia* syn. *azarolus* (L.) whole plant aqueous extract in wistar rats. Am J Pharmacol Toxicol 2011;6 (2):37–45.
[33] Burdick MD, Murray LA, Keane MP, Xue YY, Zisman DA, Belperio JA, et al. CXCL11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. Am J Respir Crit Care Med 2005:171. https://doi.org/10.1164/rccm.200409-1164OC.
[34] Kumar V, Cotran R, Robbins S. Basic Pathol, WD Saunders Company. Çeviri ed: Çevikbaş U. 2000;1:47–52.
[35] Hamada N, Kuwano K, Yamada M, Hagimoto N, Hiasa K, Egashira K, et al. Anti-vascular endothelial growth factor gene therapy attenuates lung injury and fibrosis in mice. J Immunol 2005:175. https://doi.org/10.4049/jimmunol.175.2.1224.
[36] Ou XM, Li WC, Liu DS, Li YP, Wen FQ, Feng YL, et al. VEGFR-2 antagonist SU5416 attenuates bleomycin-induced pulmonary fibrosis in mice. Int Immunopharmacol 2009:9. https://doi.org/10.1016/j.intimp.2008.10.002.
[37] King TE, Pardo A. Selman M. Idiopathic pulmonary fibrosis. Lancet 2011:378. https://doi.org/10.1016/s0140-6736(11)60052-4.
[38] Tabata C, Tabata R, Kadokawa Y, Hisamori S, Takahashi M, Mishima M, et al. Thalidomide prevents bleomycin-induced pulmonary fibrosis in mice. J Immunol 2007:179. https://doi.org/10.4049/jimmunol.179.1.708.