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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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.

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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 [3]. 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 capectabine, 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 of different types of cancers [12]. These 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 LD50 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 antioxidant 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 antitumor effects of O.s 50% standardized ethanol 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 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 experiments. 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) (946)). 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 cells).

Table 1

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

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, and ns = not significant).

**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 rosmaninic 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 rosmaninic 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 rosmaninic 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 rosmaninic acid alone and group treated with combination of rosmaninic 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, ALT, AST, 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 rosmaninic 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, rosmaninic 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)

| No | Group | 0-day | 3-day | 6-day | 9-day | 12-day | 15-day | P value (at 15 days post treatment) |
|----|-------|-------|-------|-------|-------|--------|--------|-----------------------------------|
| 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 | (1.3) *, (2.4) ** |
| 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 | (1.4) *, (2.4) **, (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 | (1.5) *, (5.6) ** |
| 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 | (1.6) *, (2.6) ** |
| 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) | P value | Kidney | P value | Spleen | P value |
|----|--------------------------------------------|----------------------------|---------|--------|---------|--------|---------|
| 1  | Control                                    |                            |         |        |         |        |         |
| 2  | Gemcitabine (10 mg/kg/3 days)              |                            |         |        |         |        |         |
| 3  | O.s (200 mg/kg/day)                        |                            |         |        |         |        |         |
| 4  | O.s (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |                  |         |        |         |        |         |
| 5  | O.s (400 mg/kg/day)                        |                            |         |        |         |        |         |
| 6  | O.s (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |                  |         |        |         |        |         |
| 7  | Rosmarinic acid (32 mg/kg/day)             |                            |         |        |         |        |         |
| 8  | Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |                  |         |        |         |        |         |

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 fL | 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 | 143 ± 1.1 | 297.7 ± 1.5 | 21.7 ± 1.6 | 850 ± 1.1   |
| 2  | Gemcitabine (10 mg/kg/3 days)              | 121.7 ± 2.0 | 8.2 ± 1.7 | 0.41 ± 0.0 | 49.0 ± 2.0 | 147 ± 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 | 285.9 ± 0.7 | 21.3 ± 1.5 | 865 ± 1.5   |
| 4  | O.s (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |         |         |        |        |        |          |        |             |
| 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 | 259.5 ± 2.2 | 23.0 ± 0.8 | 905 ± 0.9   |
| 6  | O.s (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |         |         |        |        |        |          |        |             |
| 7  | Rosmarinic acid (32 mg/kg/day)             | 129.3 ± 2.0 | 8.9 ± 1.1 | 0.43 ± 0.0 | 48.3 ± 0.1 | 147 ± 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) |         |         |        |        |        |          |        |             |

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: Orthosiphon 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  | 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) |             |       |       |       |       |       |
| 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) |         |       |       |       |       |       |

Note: WBC: White blood cells; N: Neutrophil; L: Lymphocyte; M: Monocyte; E: Eosinophil; B: Basophil; O.s: Orthosiphon 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 µL | AST µL | ALT µL | GGT µL |
|----|--------------------------------------------|-------------------|-------------|------------------|--------|--------|--------|--------|
| 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) |              |         |           |       |        |        |       |
| 5  | O.s (400 mg/kg/day)                        | 29.5 ± 1.9        | 8.1 ± 0.2   | 0.14 ± 0.1       | 83.0 ± 0.3 | 142 ± 0.9 | 440 ± 0.9 | <3 ± 0.0 |
| 6  | O.s (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |              |         |           |       |        |        |       |
| 7  | Rosmarinic acid (32 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 |
| 8  | Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3 days) |         |         |           |       |        |        |       |

Note: ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma Glutamyl transferase; O.s: Orthosiphon 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.
Electrolytes profile in different groups.

Lipids profile in different groups.

Blood proteins profile in different treatment groups.

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

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.

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

| 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 ± 0.9 | 1.3 ± 0.1 | <2 ± 0.0 |
| 3  | 0.s (200 mg/kg/day) | 50.0 ± 0.9 | 25.5 ± 0.7 | 24.3 ± 1.1 | 1.1 ± 0.2 | <2 ± 0.0 |
| 4  | 0.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  | 0.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  | 0.s (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days) | 48.0 ± 0.6 | 26.5 ± 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: Orthosiphon stamineus; Control: treated with distilled water only; n = 6; Results are expressed as 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  | 0.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  | 0.s (200 mg/kg/day) + gemcitabine (10 mg/kg/3 days) | 2.6 ± 0.2 | 1.4 ± 0.1 | 0.9 ± 0.1 | 0.8 ± 0.4 | 2.3 ± 0.1 |
| 5  | 0.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  | 0.s (400 mg/kg/day) + gemcitabine (10 mg/kg/3 days) | 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/3 days) | 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). The P values in all treated groups were not significant when compared to one another.

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  | 0.s (200 mg/kg/day) | 153.0 ± 0.7 | 5.0 ± 0.4 | 113 ± 0.7 |
| 4  | 0.s (200 mg/kg/day) + gemcitabine (10 mg/kg/3days) | 150.0 ± 0.7 | 5.2 ± 0.2 | 113 ± 1.4 |
| 5  | 0.s (400 mg/kg/day) | 150.0 ± 0.7 | 5.0 ± 0.3 | 114 ± 0.1 |
| 6  | 0.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 ± 1.1 | 4.2 ± 0.2 | 111 ± 0.5 |
| 8  | Rosmarinic acid (32 mg/kg/day) + gemcitabine (10 mg/kg/3days) | 149.0 ± 1.1 | 5.2 ± 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). The P values in all treated groups were not significant when compared to one another.
The safety profile of O.s has already been established by multiple research groups and data from these studies shows that this herb has LD₅₀ 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 pre-clinical 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. 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.
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
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. 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.
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/m²) 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.

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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.

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