Acute and Subacute Toxicity of Ethyl Acetate Fraction of *Cochliobolus spicifer* (Nelson) isolated from *Phoenix dactylifera*(Linnaeus) on Balb/c Mice

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Abstract
The aim of this study was to assess the toxicological effects of the ethyl acetate (ETAC) fraction of Cochliobolus spicifer (Nelson) isolated from Phoenix Dactylifera (Linnaeus) using Balb/c mice as an animal model. An acute toxicity study of the C. spicifer EtoAC fraction was carried out in male Balb/c mice using a range of doses of the extract (1,000, 2,000 and 4,000 mg/kg body weight). These doses were injected intraperitoneally and the mice were observed for mortality for 24 hrs after exposure. A sub-acute toxicity investigation was performed using a dose of 125 mg/kg body weight. These doses were injected intraperitoneally at 24 hour intervals for 14 days. Pathological alterations in the kidneys and liver were inspected histologically.

In the acute toxicity study, the Balb/c mice did not show mortality within 24 hrs of exposure. In the subacute toxicity study, blood sugar levels were significantly reduced ($p < 0.05$) at a dose of 125 mg/kg body weight. The microscopic investigation of the kidney architecture of control and treated mice showed a normal cellular appearance. The histopathological study of the liver revealed hepatocytes, infiltration with massive inflammatory cells, dilation in the central vein and cytoplasmic vacuolization. The C. spicifer fraction isolated from P. dactylifera was well tolerated when intraperitonally injected at a highest dose of 4000 mg/kg body weight but toxic when injected for 14 days at a dose of 125 mg/kg.

Keywords: *Cochliobolus spicifer*, *Phoenix dactylifera*, Balb/c Mice, Subacute Toxicity.

Introduction

Endophytes such as fungi can inhabit the leaves, stems and roots of a range of plant hosts asymptomatically and play a significant role in the environment[1]. Endophytes are able to change from mutualistic to pathogenic behaviour based on environmental factors[2-4]. Some endophytes can support their hosts in drought tolerance[5], others can protect against pathogens[6], enhance growth[7] and defend the host against herbivory[8]. Endophytes have been considered to be an excellent source of antioxidant, anticancer, antimicrobial and larvicidal bioactive compounds. This study evaluates the toxicity of an active fraction of *Cochliobolus spicifer* (Nelson) extract isolated from *Phoenix dactylifera*. *C. spicifer* is a dematiaceous hyphomycete normally isolated from plants grown in hot and dry climates. *C. spicifer* is the most frequently seen airborne fungi[9].

P. dactylifera, commonly known as the date palm, is found mostly in the Middle East and North Africa[10]. Aside from being a nutritive food source, the date palm has been used traditionally to treat various diseases[11]. The plant has countless beneficial properties including antioxidant, anti-hyperlipidaemic activity and hepatoprotective and neuroprotective effects[12].

Recently, our laboratory isolated twelve endophytic fungi from *P. dactylifera* plants. Among the extracts tested was an ethyl acetate fraction of *C. spicifer* that was found to show antiproliferative activity against several cancer cell lines as well as toxicity to the *Aedescaspius* and *Culexpipiens* species of mosquitos[13]. Building on this work, in this study, we aimed to evaluate the acute and subacute toxicity of the *C. spicifer* fraction in vivo.

Material and Methods

Isolation, purification and identification of the endophytic fungus: The method used for the isolation, purification and identification of the endophytic fungus was reported in our previously published paper[14]. Briefly, six 1 cm long pieces of leaf were washed with dH_2O (distilled water) and surface sterilized by successive immersion in sodium hypochlorite, 70% ethanol and sterile Milli-Q water. The leaf pieces were placed on the surface of potato dextrose agar (PDA) and incubated at 30 °C. The endophytic fungi were identified based on the morphological characters and structures and were further verified by experts from Assiut University Mycological Centre, Egypt. The deposit number for C. *spicifer* is AMUC 10172.

Preparation of fungal extract: Ten plates were inoculated with approximately 6 mm disks of 4-day old culture and kept at 30ºC for ten days. The ten plates were homogenized and transferred to a flask containing 500 ml EtoAC and placed in a shaking incubator to stir at 30°C for 10 min at 140 rpm with the process being repeated three times with ETAC. The mixture was filtered, centrifuged at 12000 RPM for 5 min (F1) and then transferred to a round flask bottle, dried using

a rotary evaporator at 40ºC and stored at -20ºC until required.

Animal husbandry and treatment: The animals used were male BALB/c mice (25-30 gm) who had been acclimatized for 10 days in the Animal House facility at King Saud University. Standard food and water were provided *ad libitum* and the environment was maintained at room temperature, 25±5 ºC. The animal experiments were performed in accordance and as per the approval of the Animal Care of King Saud University, Saudi Arabia, Riyadh. Animals were divided into five groups with three mice in each: The first group was injected with 10% DMSO, this served as the control.

The second, third and fourth groups were injected with the EtoAC fraction of the C. *spicifer* at doses of 1,000, 2,000 and 4,000 mg/kg b.w. respectively. After vortex, a single injection of C. *spicifer* EtoAC fraction was administered intraperitoneally. The mice were monitored for 24 h for mortality.

In the subacute toxicity studies, a group of ten mice were divided into two treatments; five mice in each cage. Group I served as control while group II served as the experimental group. Mice in group I were injected with 10% DMSO, while mice in the experimental group were injected with 125 mg/kg b.w. of C. *spicifer* EtoAC extract. The treatment was injected intraperitoneally on a daily basis for fourteen days. Blood glucose concentration was estimated by using a glucometer (Accu-Chek® Active test meter) one day after each injection for seven days.

Histological analysis: Twenty-four hours after the last dose of treatment, all the mice were sacrificed by cervical dislocation and their organs liver and kidney were excised and stored in 10% formalin solution. Histopathological examinations were performed on the kidneys and liver from the control and treated groups using the haematoxylin and eosin method.

Statistical analysis: The data were presented as mean ± standard deviation. Data were analyzed by the Student's *t*-test using Microsoft Excel application software for Windows. $p<0.05$ was considered statistically significant.

Result and Discussion

The toxicity of endophyte extracts is in general poorly documented. *C. spicifer* EtoAC fraction isolated from *P. dactylifera* has shown cytotoxic activity against different cancer cell lines as well as larvicidal activity[13]. The toxicity of *C. spicifer* extract needs to be further evaluated. In our study, we evaluated the acute toxicity of *C. spicifer* EtoAC fraction in BALB/c mice after a single i.p. injection at a dose of either 1000, 2000 and 4000 mg/kg b.w.

The doses did not cause mortality to BALB/c mice at the highest dose of 4000 mg/kg. Control mice were treated with 10% DMSO. All EtoAC fractions treated mice remained healthy with no evidence of morbidity throughout the 24 hrs observation period. When the liver and kidney of the animals were examined after sacrifice, no toxic lesions were found from either the EtoAC fraction treated mice or the control animals. Thus, the EtoAC fraction extract was not toxic to BALB/c mice at doses as high as 4000 mg/kg.

In this current study, histopathological observation was carried out on the liver and kidney. The photomicrographs of the kidney of the control and treated groups had normal morphological sections. The microscopic inspection of the kidney architecture of control and treated mice also showed a normal cellular appearance. The cross-sections of distal and proximal tubules and the glomeruli appeared to be normal in the kidneys of both control and treated mice. There was also no interstitial, tubular atrophies or intraglomerular congestion and all the cells were normal with clearly visible nuclei and no bleeding, necrosis or degeneration.

After fourteen days of extract administration (125 mg/kg), however, the livers of the treated mice appeared to have lost their characteristic histological architecture compared to the control group. The histopathological alterations in the treated liver section were further indicated in increased vacuolation in the liver hepatocytes, infiltration with massive inflammatory cells and dilation in the central veins which were almost congested with haemolyzed blood. Inflammatory cells were found to be from different origins with immature neutrophils prominent in different regions of the diseased hepatic tissue (Fig. 3A, 3B).

The control liver sections with normal liver architecture, hepatocytes and central veins are presented in figure 3 to compare with the induced histopathological changes in the treated mice group. The results of our study are similar to those of Pathak et al[15]. In their study, it was found that the injection of crude chloroform extract of different fungal metabolites intraperitoneally to groups of mice resulted in no significant differences between the architecture of the kidneys of untreated and treated mice but that the liver of treated animals showed gross and microscopic evidence of necrosis, cytoplasmic vacuolization and fatty degeneration of varying severities[15].

The experimental Balb/c mice were injected with EtoAC fraction at the dose of 125mg/kg b.w. and their blood glucose levels were determined. The blood glucose levels were examined one day after each injection; that is from day 2 to the 15th day. The control group revealed blood glucose levels between 111 and 150mg/dl whereas the treated group showed levels between 70 to 125 mg/dl. The blood glucose level at the dose of 125 mg/kg for 14 days, therefore showed a significant reduction in the glucose level in the treated group (101.2±10.1) when compared to the control (124±8.7).

Fig. 1: Photo of *C. spicifier* isolated from *Phoenix dactylifera*

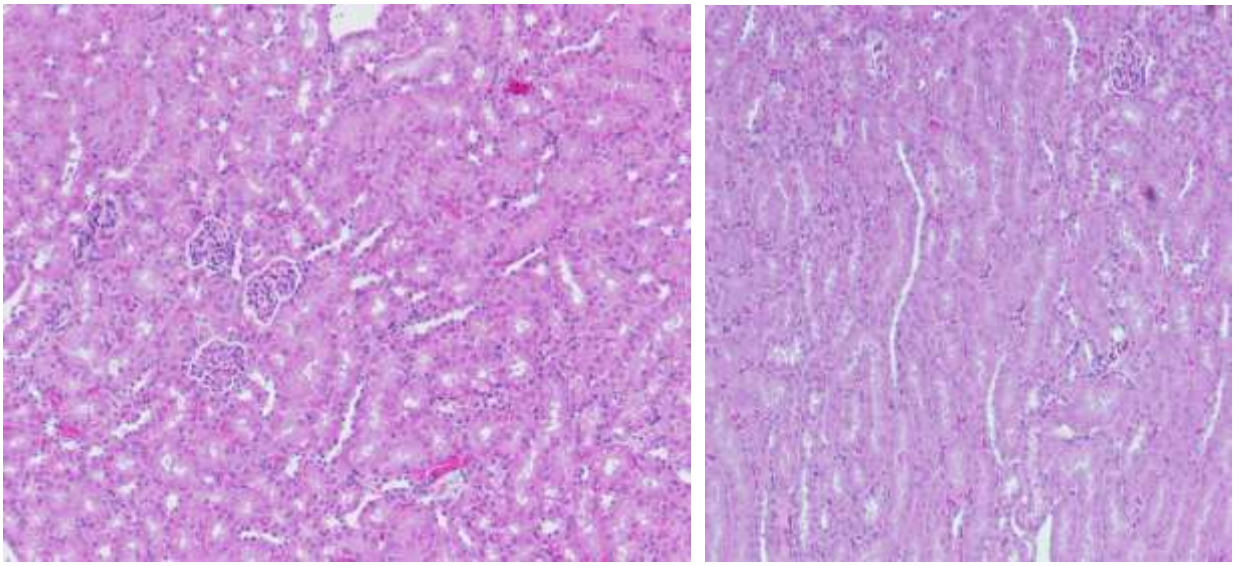

Fig. 2: Photomicrographs of Balb/c mice kindney sections submitted to the toxicity evaluation of the E to AC of *C. spicifier* that was intraperitonially injected at the doses of 125 mg/kg of body weight. 'A' is the kidney of control and 'B' is the kidney of treated Balb/c mice showing normal architecture. (Hematoxylin and Eosin, 100× original magnification).

Conclusion

Additional studies are needed to confirm the toxicity of sub-acute doses of the EtoAC fraction of *C. spicifer* over a longer time period and if the observed changes are reversible. Based on the current results, however, precautions should be practiced when dealing with this extract. In conclusion, the intraperitoneal injection of the EtoAC fraction of *C. spicifer* at the dose tested induced toxicity to hepatic cells but not to renal cells evaluated by microscopic analysis.

Acknowledgement

Authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-028.

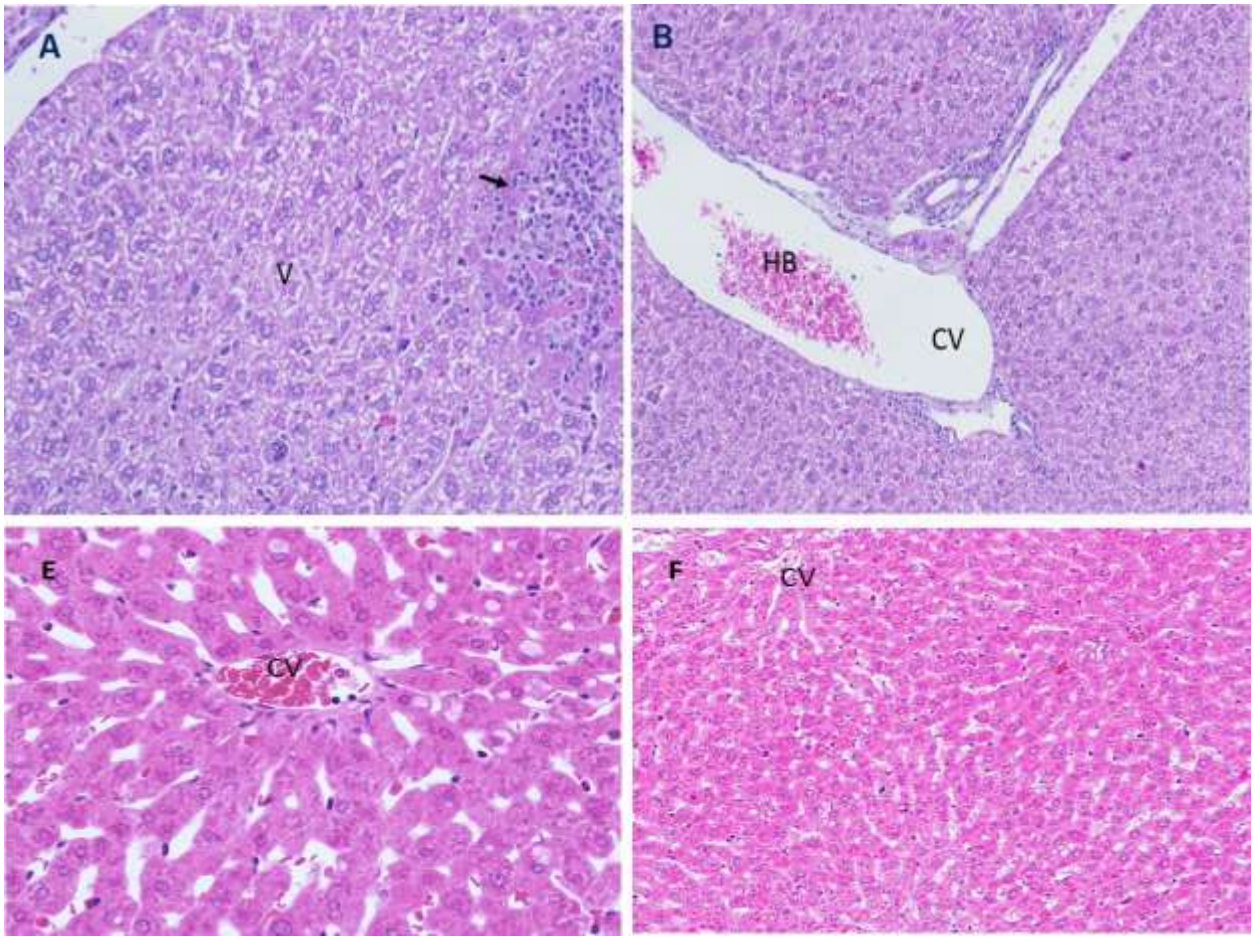

Fig. 3: Photomicrographs of Balb/c mice liver sections submitted to the toxicity evaluation of the EtoACfraction of *C. spicifier* that was intraperitonially injected at the doses of 125 mg/kg of body weight: Representative micrographs showing the histopathological changes in the treated 1 liver sections (A, H&E, 400X; B, H&E, 200X), namely, vacuolation (V) in the haepatocytes, infiltration with massive inflammatory cells (Arrow) and dilation in the central veins (CV) which is congested with hemolyzed blood (HB). For comparison, representative control liver sections (E, H&E, 400X; F, H&E, 200X) with normal liver architecture, hepatocytes and central veins (CV) were inserted.

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(Received 13th June 2016, accepted 09th August 2016)