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Commentary

Comments on "*In Vivo* Toxicological and Microbiological Activity of *Marrubium vulgare* L. on *Candida albicans* Isolated from Nosocomial Infections"

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In the publication by Chabane *et al.*[1], in vivo toxicity and antifungal effects of methanolic extract of *Marrubium vulgare* L. have been tested.[1] In toxicological assessment, the oral dose of 800mg/kg of the extract has been administrated to male Wistar rats for 28 consecutive days. Parameters including weekly body weight, organs weight and histological evaluations of internal organs have also been assessed. In antifungal assessment, two groups of rats have been infected with Candida albicans and then kept treated/non-treated until 10 days. Eventually, blood and the internal organs samples (intestine, colon, liver, spleen, kidney, heart and lung) have been taken and the number of CFU/100 µL of blood or CFU/g wet weight of the tissues have determined. We read the article critically and in this comment we show that the results of this study are not true. The study has serious weakness compromising the interpretation of its findings. We submit the following comments pertain to the experimental methods, histological and antimicrobial reports.

In this work oral acute and sub-acute toxicity assessments have been performed without the use of standard guidelines in toxicological studies. Accordingly, a single dose of the extract (800 mg/kg) has been administrated to male Wistar rats and then daily administrations continued up to 28 days and this group of animals has been considered as both acute and sub-acute experiments. Moreover, in the sub-acute test body weight, organ weight and histological examination have been assessed. We have checked the cited works (references numbers 41-44 of the mentioned article) for the toxicity tests. Unfortunately, we could not find any similarity between the cited works and the mentioned study's method. For example, in the Alwashli et al., study (written in French) which has cited for acute toxicity test, five single doses of an extract (500, 1000, 2000, 3000 and 4000 mg/kg) have been administrated to mice and the animals were checked for 14 days.[2] In addition, in Elberry *et al.*, study which has been cited for 28 days sub-acute test, four doses (100, 250, 500 and 1000 mg/kg) of same extract (methanolic extract of *Marrubium vulgare*) have been orally administrated to rats for 21 days.[3] Chabane et al., claimed that in Elberry et al., study the extract at the dose of 500 mg/kg had not been shown any mortality in diabetic rats; however, Elberry *et al.*, reported that the extract up to 1000 mg/kg did not pose mortality in healthy rats[3]. In toxicological studies, the OECD (Organisation for Economic Co-operation and Development) guidelines are frequently used. Toxicological assessments in experimental animals typically are classified into four classes: acute, sub-acute, sub-chronic and chronic.[4] Based on acute toxicity test, we can have conclusion about the potential toxicity or safety (measuring lethal dose, 50%), and appropriate dosage for sub-acute test. Therefore, high doses of a substance (2000-5000 mg/kg) are usually tested for acute toxicity.[5] In addition, as it has been noted in the discussion part, in study conducted by Paula de Oliveira in 2011, acute toxicity of the methanolic extract of *Marrubium vulgare* leaves (2000 mg/kg) has been tested on female Wistar rats according to a standard protocol with 14 days follow-up.[6] On the other hand, sub-acute toxicity tests are performed as range-finding studies with the aim of choosing dosage levels to be used in further studies.[5] On that account, acute-toxicity method used in the Chabane *et al.*, study is sketchy and the dosage selection for both acute and sub-acute tests is wrong. Moreover, in sub-acute test important parameters such as hematological and biochemical markers have not been evaluated.

Undisputedly, the leading controversial issue presented in this article is the histological report. The first and foremost flaw is the very poor quality of micrographs showing unprofessional slide preparation, staining and capturing. Secondly, erroneous tissue labeling as well as wrong tissue definition was unexpected. For example, in the mentioned manuscript Figure 1 has been addressed as lung tissue micrographs (without scale bar and group name/label) and an artifact has been labeled as an alveoli. It seems that the micrographs probably made from a liver specimen. In order to avoid further verbosity, we have provided clear

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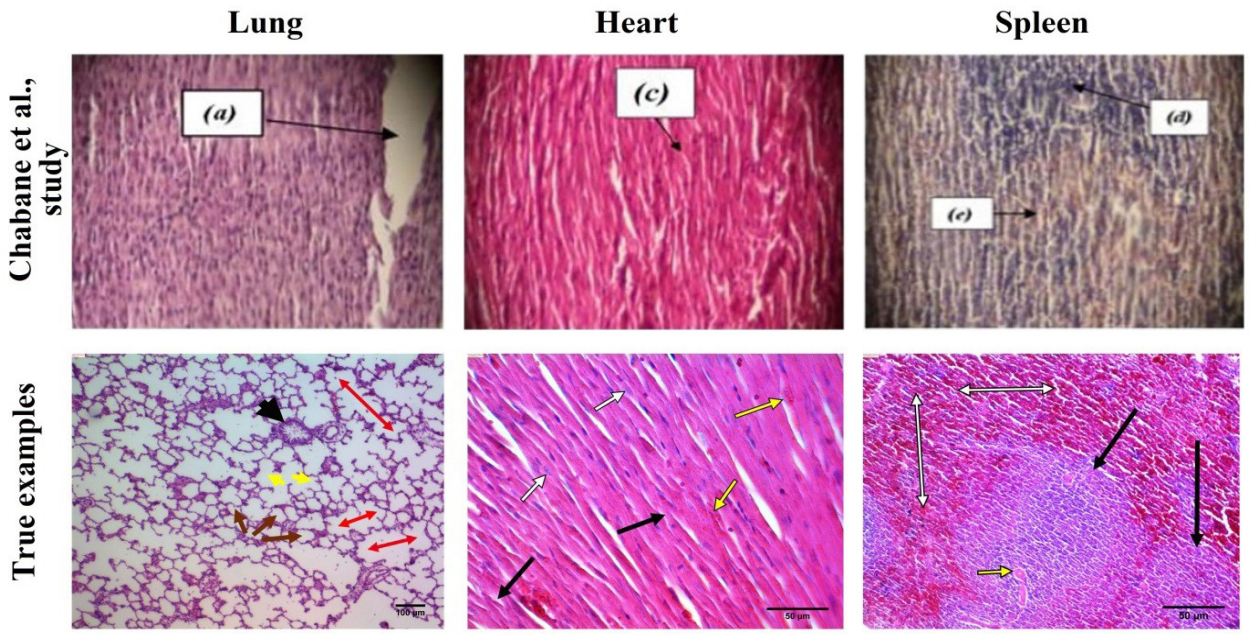

Figure 1. The top row demonstrates micrographs of Chabane et al., study with very low quality and mislabeling whereas; the bottom row represents true ones with clear labeled details. In the true example of lung specimen; bronchiole (black arrow), alveolar ducts (red double-headed arrows), alveolar sacs (yellow arrows) and alveoli (brown arrows) are labeled. In the true example of the heart tissue myocytes with definitive nuclei (white arrows), branching cardiac fibers (black arrows) and capillaries lying intercellular spaces (yellow arrows) are evident. In the true micrographs of spleen red pulp (white double-headed arrows) white pulps (black arrows) and a central arteriole (yellow arrow) are labeled.

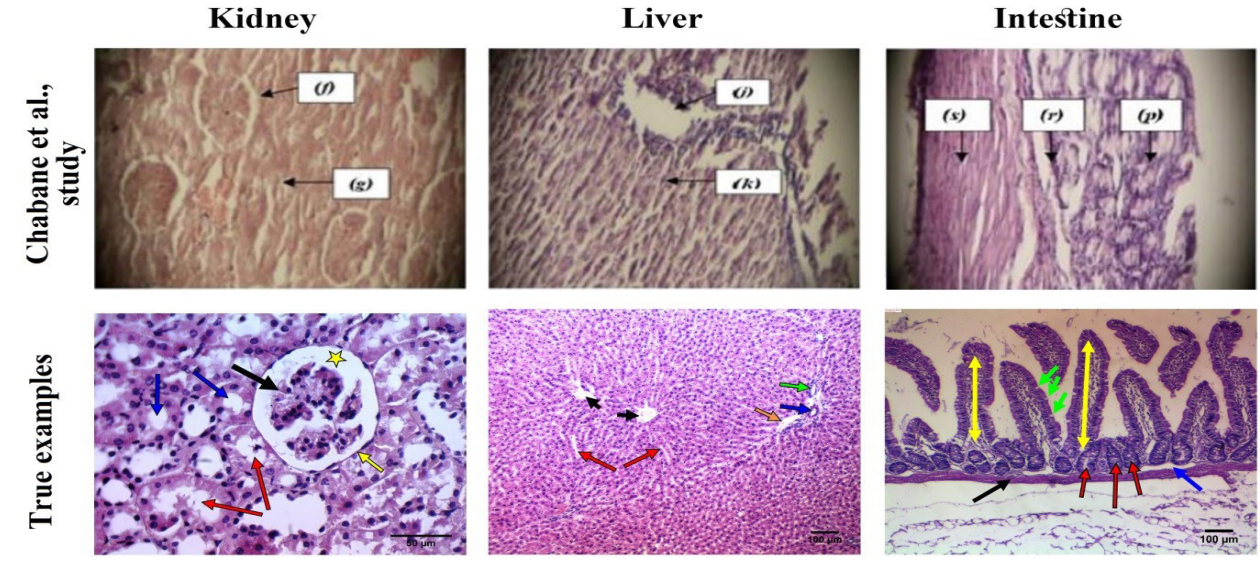

Figure 2. The top row demonstrates micrographs of Chabane et al., study with very low quality, lots of artifacts and mislabeling whereas; the bottom row represents true ones with clear labeled details. In the true example of kidney histological specimen; detail including the glomerulus (black arrow) with definitive parietal layer of Bowman's capsule (yellow arrow), urinary space (yellow star), proximal convoluted tubules (red arrows) and distal convoluted tubes (blue arrows) are labeled. In the true liver histological micrograph; central veins (black arrows), sinusoidal spaces (red arrows) and portal triad structure including bile duct (green arrow), hepatic artery (blue arrow) and hepatic vein (brown arrow) are labeled. In the true histological micrograph of intestine (jejunum) structures including muscularis externa layer (black arrow), sub-mucosa (blue arrow), crypts (red arrows), villi (yellow double-headed arrows) and goblet cells (green arrows) clearly are visible.

and labeled micrographs from our lab to shed some light on this issue (Figures 1 and 2).

Last but not least, another important methodological flaw observed in the Chabane et al., study, is the infection model and microbiological assessment. Briefly, they did not perform any preliminary in vitro test (determination of minimum inhibitory concentration) to support antifungal property of the tested extract. Moreover, they inoculated animals (we could not find the route of inoculation) without any immunosuppression and antibiotic-decontamination

which are necessary for this type of animal models and also have been clearly described in the cited works.[7,8] The authors also claimed that they collected 100 µL of blood sample to EDTA (Ethylenediaminetetraacetic acid) tubes, and then transferred it into petri dishes containing (sabouraud dextrose agar) SDA+0.05% chloramphenicol and incubated them at 37°C for 24-48h. It is well-established that blood specimens must be diluted with culture medium (more than 1:4) in order to neutralize the antimicrobial effects of the serum. Consequently, in direct blood culture (described in the Chabane et al., study) the possibility of the candida growing is low. Another point is the use of EDTA tubes to collect blood samples which can inhibit the growth of numerous organisms such as Candida spp. and therefore its use in microbiological studies is not recommended.[9] Most of Candida spp. are recovered effectively in systems designed for bacteria. Conventional systems for yeast count are pour plate, broth and agar methods, and the standard and perfect techniques are BACTEC and centrifugation-lysis (Isolator) systems in which the time required for candidate growth is 2 days but, if no growth is detected after 5 days of incubation, the culture will be reported as a negative. However, the time required in conventional methods like that used in the mentioned study is 5 days.[10,11] As a result, the incubation period mentioned in this article is scrimpy and in this limited time, it is unlikely that the candida has been grown.

Conflict of Interest

There are neither ethical nor financial conflicts of interest involved in the manuscript.

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