Supplementary information

Materials and Methods

Insect rearing and aqueous extraction procedure

Adult insects of *T. molitor* were obtained from Tenebrio Azteca company (Querétaro, Mexico) and kept at Institute of Agricultural Sciences; Autonomous University of Hidalgo State (ICAP-UAEH), Tulancingo de Bravo, Hidalgo; Mexico. During rearing, insects were fed *ad libitum* and kept in plastic boxes (15 cm×14 cm×9 cm) at room temperature ((25.0 ± 2.0) °C approx.) in a controlled environment of 12 h light/dark photo cycle. Insects were fed with 20 g of wheat bran and provided with 15 g of organic lettuce as the water source, and replaced them every other day. Individuals from larval, pupal and adult life stages (Figs. 1a–1c) were periodically separated based on particular shapes, comparable size, age and cuticle color, and stored at −70 °C for further procedures. Individuals from each life stage were dehydrated in a drying oven (Lumistell HTP-41) at 60 °C for 24 h; ground in a blender (Oster 4655) for 5 min at maximum speed, as previously reported (Siemianowska et al., 2013), and stored at −70 °C for further quantification and beverage preparation. Crude protein amount was determined according to the protocol 957.01 as previously reported (Cunniff, 1999). For quantification, 500 mg of powder from each life stage were graduated to 25 mL of distilled water and mixed for 5 min, centrifuged at 6000 rpm for 15 min at 4 °C, and supernatants were shaking for 1 h at 4 °C. Then, solutions were centrifuged at 4000 rpm for 30 min at 4 °C and supernatants were separated. The highest solubility of each solution was obtained by preparing the biological extracts at 1.2 mg/mL adjusted to different pH values (from 3.0 to 8.0; every 0.5 as the ramp-up value) and then, content of dissolved protein was quantified through the standard Bradford's method (Bradford, 1976). As a result, pH 6.5, 6.0 and 7.0 were selected as the most efficient (Fig. 1d) to dissolve the biological material extracted from larvae, pupae and adult life stages of *T. molitor*, respectively.

Calculation of beverage concentration

Final aqueous extracts concentration was calculated based on the following two considerations. First, the aqueous extract of *Ulomoides dermestoides*, a Tenebrionidae family member, induced anti-inflammatory effects in rodents bearing pleurisy without apparent side effects at doses of 8 and 16 μg/g body weight (BW) (Santos et al., 2010). Second, although the amount of *ad libitum* water intake depends on the mice strains (ranging from (3.9±0.2) to (8.2±0.3) mL daily) (Bachmanov et al., 2002), we have previously observed that whereas in untreated C57BL/6J mice (~25 g BW) water intake is (5.3±0.6) mL, in those subjected to DEN effects (20 mg/kg) consume (3.2±0.7) mL of drinking water daily. Based on these considerations, we decided to evaluate the effects of the lowest dose (8 μg/g BW) previously used

(Santos, et al., 2010), on the mouse hepatocarcinogenesis. For this end, we calculated the final concentration of our aqueous extracts as follows:

$$\frac{(\text{aqueoua extract dose} \times \text{g mouse body weight})}{\text{volume in mL of daily water intake}} = ((8\ \mu\text{g.g} - 1 \times 25\ \text{g}))/(3.2\ \text{mL}) = 62.5\ \mu\text{g/mL}.$$

Thus, if mice subjected to DEN consume (3.2±0.7) mL of drinking water daily, we expected they would intake ~8 μg/g BW from a solution containing 62.5 μg/mL of aqueous extract.

Beverages preparation

Twenty grams of powder from each life stage of *T. molitor* added to 1.0 liter of distilled water and adjusted to their respective pH value, were mixed for 5 min, centrifuged at 6000 rpm for 15 min at 4 °C, and supernatants were shaking for 1 h at 4 °C. Then, solutions were centrifuged at 4000 rpm for 30 min at 4 °C, and supernatants were concentrated (40 mL final volume) by centrifugation at 75 rpm in a rotary evaporator (BÜCHI R-215, Switzerland) at 60 °C. Samples were centrifuged at 6000 rpm for 30 min at 4 °C and then proteins were quantified and solutions were adjusted at 62.5 μg/mL, as the final aqueous extracts concentration. Prepared beverages were aliquoted and stored at –20 °C and then aliquots were daily defrosted to replace the previous one. Beverages were provided *ad libitum* to animals.

Animals

Seven weeks-old C57BL/6J male mice (~20 g) were obtained from the Unit of Production and Experimentation of Laboratory Animals of Center for Research and Advanced Studies of the National Polytechnic Institute (UPEAL-CINVESTAV-IPN; CDMX, Mexico). Animals received proper care in accordance with the Institutional Animal Use and Care Committee of CINVESTAV-IPN and the approved protocol number 0114-14.

Experimental procedure

During experimental procedures, mice were fed *ad libitum* and housed in a controlled environment of 12 h light/dark cycle and temperature at ICAP-UAEH, Tulancingo de Bravo, Hidalgo; Mexico. After one week of acclimation, groups of animals were allowed to drink *ad libitum* either the aqueous extract of *T. molitor* larval, pupal or adult life stage. One week later, animals were subjected to hepatocarcinogenesis induction as previously reported (Fuentes-Hernandez et al., 2019). Briefly, DEN was dissolved in PBS and intraperitoneally injected at 20 mg/kg twice a week for 10 weeks, and euthanized at week 11 (Fig. 2a). Twenty-five animals were randomly divided into five experimental groups: untreated controls (C); DEN (D), DEN plus larva aqueous extract (DL), DEN plus pupa aqueous extract (DP), and DEN plus adult aqueous extract (DA); untreated controls were allowed to drink *ad libitum* tap water and were injected

only with PBS. Euthanasia was performed by exsanguination under isoflurane anesthesia; immediately, pieces of the dissected liver were split for total protein extracts and then stored at −75 °C for further analyses. Other piece was fixed in 4% formalin and embedded in paraffin for histological and immunohistological analyses.

Histological and immunohistochemistry (IHC) analyses

Liver tissues were fixed in 4% formalin, embedded in paraffin, cut into 3-μm–thick, deparaffinized and gradually rehydrated. For histological examination and fibrosis detection, sections were processed by standard hematoxylin and eosin (H&E) and Masson trichrome staining, respectively. Description of histopathological features was performed in a blinded manner by a pathologist and tissue alterations were classified according to the mouse tumors international classification(Mohr, 2001). Immunodetection through IHC analysis was performed after tissue rehydration and antigens unmasking by using Mouse-on-Mouse HRP-Polymer Bundle kit (MM510; BioCare Medical. Concord, CA). Then, primary antibodies against Ki67 (1:300; GTX16667. GeneTex. Irvine, CA) and β-Catenin (1:300; sc7963. Santa Cruz Biotechnol. Santa Cruz, CA); were incubated overnight at 4 °C. After a standard staining protocol using Mouse/Rabbit InmunoDetector HRP/DAB Detection System (BSB 0005; BioSB. Santa Barbara, CA), sections were slightly counterstained with hematoxylin, dehydrated and mounted. Ki67-positive nuclei were quantified as previously reported (Fuentes-Hernandez, et al., 2019); briefly, four randomly selected 20x-field per individual sample (20 pictures per treatment group) were captured using a ZEISS Axio-A1 microscope. Ki67-positive nuclei were quantified using ImageJ 1.5 software(Schneider et al., 2012).

Protein isolation and western blot (WB) analysis

Total protein extracts were prepared as previously reported (Arellanes-Robledo et al., 2018). Briefly, lysis buffer contained 50 mM Tris (pH 8), 150 mM NaCl, 200 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) NP-40, (All from Sigma; St. Louis, MO), Complete and PhosSTOP (Roche, Branchburg, NJ). Lysates were incubated on rocking for 30 min, centrifuged at 14000 rpm for 10 min at 4 °C and stored at −75 °C for further analyses. All procedures were performed at 4 °C to reduce protein degradation. After protein quantification, equivalent amounts of boiled protein in Laemmli's buffer were analyzed through SDS-PAGE and transferred to a PVDF membrane. Antibodies against CYP2E1 (GTX32546) and GSTP1 (SAB3500265) were from GeneTex (Irvine, CA) and from Sigma-Aldrich (St. Louis, MO), respectively. Antibodies against β-Catenin (sc7963) and Cyclin D1 (sc753) were from Santa Cruz Biotechnol (Santa Cruz, CA). Antibody against GAPDH (60004-1-Ig) was from Proteintech (Rosemont, IL). Protein loading was confirmed by reprobing the blots with anti-GAPDH and densitometric analyses were carried out using ImageJ 1.5 software (Schneider, et al., 2012).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software. Data calculations were performed using one-way ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. All experiments included five animals per group. Differences were considered significant when $P<0.05$, and data were expressed as mean±SE.

References

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