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E-mail: jzus_b@zju.edu.cn

Correspondence

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Aqueous extracts from *Tenebrio molitor* larval and pupal stages inhibit early hepatocarcinogenesis in vivo

Armando ZEPEDA-BASTIDA[2]✉, Juan OCAMPO-LÓPEZ[2], Brisa Rodope ALARCÓN-SÁNCHEZ[1,3], Osiris Germán IDELFONSO-GARCÍA[1], Sandra ROSAS-MADRIGAL[4], Diana Ivette APARICIO-BAUTISTA[5], Julio Isael PÉREZ-CARREÓN[1], Saúl VILLA-TREVIÑO[3], Jaime ARELLANES-ROBLEDO[1,6]✉

[1]*Laboratory of Liver Diseases, National Institute of Genomic Medicine-INMEGEN, CDMX 14610, Mexico*
[2]*Institute of Agricultural Sciences, Autonomous University of Hidalgo State-ICAP-UAEH, Hidalgo 43600, Mexico*
[3]*Department of Cell Biology, Center for Research and Advanced Studies of the National Polytechnic Institute-CINVESTAV-IPN, CDMX 07360, Mexico*
[4]*Laboratory of Cardiovascular Diseases, National Institute of Genomic Medicine-INMEGEN, CDMX 14610, Mexico*
[5]*Proteomics Unit, National Institute of Genomic Medicine-INMEGEN, CDMX 14610, Mexico*
[6]*Directorate of Cátedras, National Council of Science and Technology-CONACYT, CDMX 03940, Mexico*

Hepatocellular carcinoma (HCC), which is the most frequent primary liver malignancy, is ranked as the sixth most common cancer and the third leading cause of cancer-related deaths worldwide, with its incidence expected to continue rising. One of the reasons is that most patients are diagnosed at an advanced stage when therapeutic options are ineffective. The development of HCC is attributed to a chronic exposition to either one or a combination of low amounts of different hepatotoxins, such as in hepatitis virus infection, alcohol consumption, aflatoxin from contaminated foods, metabolic factors, and exposure to chemical carcinogens from tobacco smoke (Forner et al., 2018). Integrative studies combining exome sequencing, transcriptome analysis, and the genomic characterization of HCC have shown that these etiological factors may raise the frequency of particular genetic alterations, resulting in intra-tumor heterogeneity that presents a huge challenge for treatment. For example, mutations in the catenin β-1 (*CTNNB1*) gene (a proto-oncogene in the WNT signaling pathway that encodes the β-catenin transcription factor) are strongly associated with alcohol-related HCC, whereas mutations in the telomerase reverse transcriptase (*TERT*) promoter and tumor protein p53 (*TP53*) genes are the most commonly observed in hepatitis B virus (HBV)-associated HCC (Calderaro et al., 2017; Cancer Genome Atlas Research Network, 2017). The above findings emphasize the molecular diversity of HCC and the associations of different etiologies with distinct mechanisms in HCC progression. Consequently, prevention strategies are still attractive for HCC management.

Historically, edible insects have been consumed for thousands of years because of their nutritional and medicinal properties. Insects are rich in proteins, essential amino acids, and unsaturated fatty acids, and some species further contain substantial amounts of minerals and vitamins. The antibacterial and anticancer potential of either the whole insect body or isolated bioactive compounds has also been investigated (Ratcliffe et al., 2011). *Tenebrio molitor* is a coleopteran insect of the Tenebrionidae family, and is considered as a harmful pest of stored grain worldwide (Gao et al., 2018). The development of *T. molitor* includes four life stages: egg, larva, pupa, and adult. The larvae and adults of *T. molitor* are commonly known as yellow mealworms and mealworm beetles, respectively. Owing to their high nutritional value, mealworm beetles have been used as food and medicine in various forms, such as protein powder, oil, chitin, and a whole worm (Gao et al., 2018). Interestingly, the oil extract of *T.*

✉ Jaime ARELLANES-ROBLEDO, jarellanes@inmegen.gob.mx
Armando ZEPEDA-BASTIDA, azepeda@uaeh.edu.mx
Jaime ARELLANES-ROBLEDO, https://orcid.org/0000-0001-7363-4870
Armando ZEPEDA-BASTIDA, https://orcid.org/0000-0003-0572-5206

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molitor larvae has shown both antiproliferative and proapoptotic properties against HCC and colorectal adenocarcinoma in vitro (Wu et al., 2020). Such evidence proves that mealworms contain bioactive components with therapeutic properties, and suggests that the therapeutic potential of mealworms might be dependent on bioactive compounds synthesized at specific life stages of *T. molitor* development.

This study aimed at exploring the therapeutic properties of aqueous extracts of three life stages of *T. molitor*, including larva, pupa, and adult, on the early hepatocarcinogenesis recapitulated by the chronic administration of diethylnitrosamine (DEN). First, individuals from each stage (Figs. 1a–1c) were periodically separated as described in supplementary materials and methods. Based on the highest solubility of each extract, pH 6.5, 6.0, and 7.0 were selected as the most efficient pH (Fig. 1d) to dissolve the biological materials extracted from larvae, pupae, and adults, respectively. The final concentrations were calculated based on previously reported doses and on the ad libitum daily water intake of mice (Materials and methods).

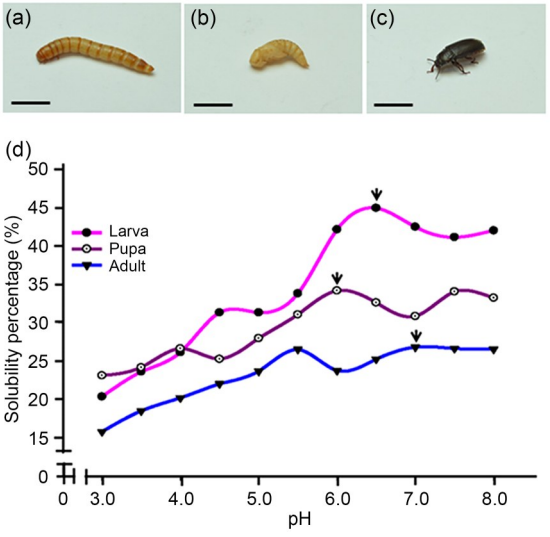

Fig. 1 *Tenebrio molitor* life stages (larva (a), pupa (b), and adult (c)) and the protein solubility percentages at different pH values (d) of aqueous extracts. (a–c) Scale bar=1 cm. The black arrows point out the highest solubility efficiency at a particular pH value, namely, 6.5, 6.0, and 7.0 for larva, pupa, and adult, respectively.

Then, different groups of mice were allowed to drink ad libitum from the aqueous extracts. One week later, animals were subjected to hepatocarcinogenesis induction as previously reported (Fuentes-Hernández et al., 2019). Fig. 2a shows the schematic representation of animal groups subjected to either DEN or DEN plus each aqueous extract. Next, the aqueous extract volumes consumed by animals simultaneously subjected to DEN were recorded. It was found that the mice consumed (3.0±0.4) mL of aqueous extracts daily, which is equivalent to either (186.9±27.0) μg/d per animal or (7.5±1.1) μg/g body weight (BW), a highly similar dose to the expectation (8 μg/g BW; Materials and methods). On the other hand, in contrast to the untreated group, DEN-treated animals did not show BW gain irrespective of *T. molitor* extract consumption (Fig. 2b). Nonetheless, they were significantly lighter from the sixth week of treatment than the control animals. The weight of mice liver relative to BW was not significantly different among the groups (data not shown).

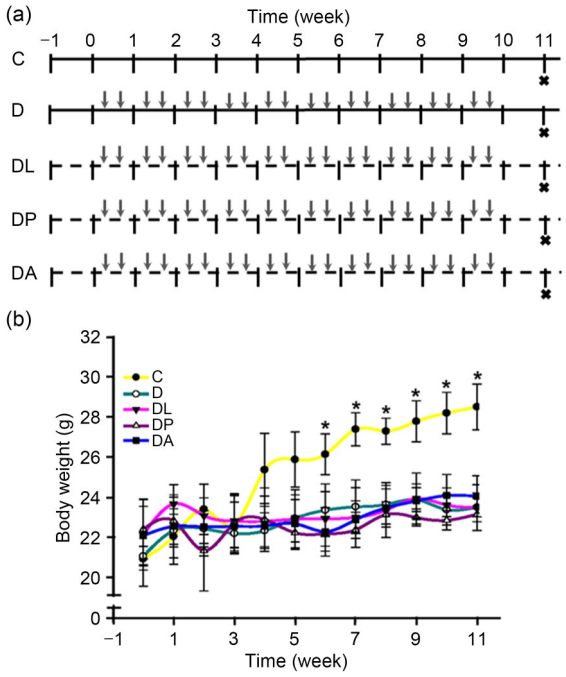

Fig. 2 Schematic representation of diethylnitrosamine (DEN) administration (a) and the recorded body weight of mice (b). Data are expressed as mean±standard error (SE) with n=5. * Statistically significant difference from DEN-subjected animals irrespective of aqueous extract consumption at P<0.05. C, control; D, DEN; DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract.

The histopathological analysis (Fig. 3a) revealed that, while DEN and DEN plus aqueous extract from the adult stage of *T. molitor* induced the disarrangement of hepatocyte cord organization and hepatocyte

hypertrophy, the animals subjected to DEN plus aqueous extracts from larval and pupal stages showed a more similar liver architecture to control animals. The Masson trichrome staining revealed that animals that consumed all three *T. molitor* aqueous extracts did not present collagen deposition in the liver parenchyma. On the other hand, in DEN-treated animals, collagen deposition was observed in both the parenchyma and around the central veins (Fig. 3b). This result indicated that alterations preceding the establishment of HCC, such as liver tissue organization, induction of hepatocytes hypertrophy and fibrogenesis, were prevented by *T. molitor* aqueous extracts, and suggested that these extracts might also inhibit the early molecular alterations associated with the onset of hepatocarcinogenesis induced by DEN.

In order to determine the effects of *T. molitor* aqueous extracts on cytochrome P450 family 2 subfamily E member 1 (CYP2E1), a DEN metabolizer enzyme, and glutathione-*S*-transferase Pi 1 (GSTP1), a well-known hepatocarcinogenesis marker (Fuentes-Hernández et al., 2019), we evaluated their protein status. As shown in Fig. 4a, the western blot analysis revealed that DEN treatment decreased the CYP2E1 protein level by 51% (P<0.001), but it was not modified by any of the three aqueous extracts. As expected, the level of GSTP1 protein was increased by 48% (P=0.009) by DEN treatment. Interestingly, the consumption of larval aqueous extract decreased (P=0.02) this level; however, the pupal aqueous extract showed a non-significant decrement trend of the GSPT1 protein level. Surprisingly, the aqueous extract from adults showed a non-significant increment trend (Fig. 4b). This contrasting result suggested that the effects were different because the aqueous extracts from the three life stages of *T. molitor* contained specific bioactive compounds that might modify the final outcome (Tettamanti et al., 2007).

In order to evaluate the effects of *T. molitor* aqueous extracts on hepatic cell proliferation, we detected the proliferation markers Ki67 and cyclin D1 by immunohistochemistry (IHC) and western blot analyses, respectively. As shown in Figs. 5a and 5b, Ki67-positive nuclei were increased in liver tissues from DEN-treated animals. However, the livers of animals simultaneously subjected to the ad libitum consumption of larval, pupal, and adult aqueous extracts plus DEN showed a lower number of Ki67-positive nuclei. The quantification of Ki67-positive nuclei corroborated this observation. Fig. 5b shows that the number of Ki67-positive nuclei in livers from DEN-treated animals increased (P< 0.0001) by more than 5.50-fold. Of note, *T. molitor* larval and pupal aqueous extracts strongly inhibited the number of Ki67-positive nuclei induced by DEN treatment by 35.5% (P=0.009) and 61.2% (P<0.0001), respectively. The aqueous extract from adults showed a decrement trend (18.1%), but this was not significant. Table 1 shows both the fold change of Ki67-positive nuclei induced by DEN, as well as the percentage of Ki67-positive nuclei inhibited by the aqueous extract effects. While DEN induced a more than 5.57-fold change (P<0.0001) in Ki67-positive nuclei, the aqueous extracts from larvae and pupae reduced them to 3.59-fold

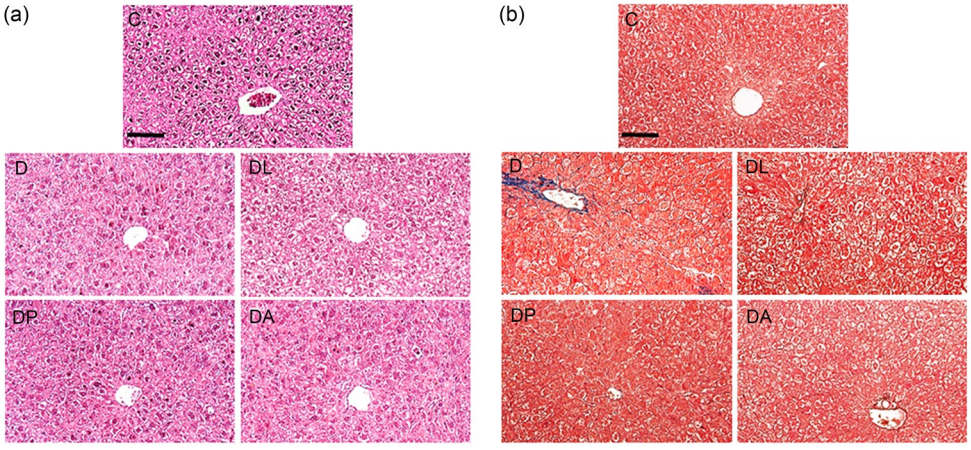

Fig. 3 Liver histopathology of mice subjected to diethylnitrosamine (DEN) and *Tenebrio molitor* aqueous extracts. Hematoxylin and eosin (a) and Masson trichrome (b) staining representative images of mice liver tissues. C, control; D, DEN; DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract. Scale bar=100 μm. Representative images are taken from each group of five animals.

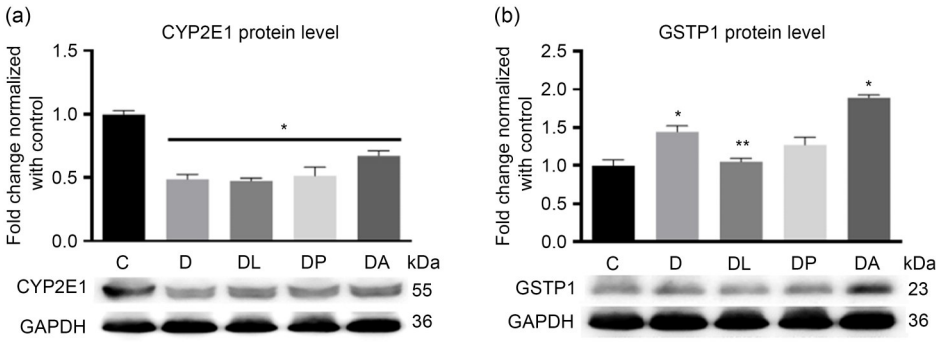

Fig. 4 Effects of *Tenebrio molitor* aqueous extracts on cytochrome P450 family 2 subfamily E member 1 (CYP2E1) and glutathione *S*-transferase Pi 1 (GSTP1) protein levels. CYP2E1 (a) and GSTP1 (b) protein levels detected by western blot analysis were normalized with GAPDH, which was used as housekeeping control. C, control; D, diethylnitrosamine (DEN); DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as mean±standard error (SE) with *n*=5. * Statistically significant difference compared with C at *P*<0.05; ** Statistically significant difference compared with D at *P*<0.05.

Table 1 Fold change of Ki67-positive nuclei per 20× field

Group	Fold change (mean±SE, *n*=5)	Increment vs. C group (fold)	Decrement vs. D group (%)
C	1.00±0.06	0	0
D	5.57±0.62*	4.57	0
DL	3.59±0.16*&	2.59	35.5
DP	2.16±0.17*&	1.16	61.2
DA	4.56±0.42*	3.56	18.1

* Statistically significant difference compared with C at *P*<0.05; & Statistically significant difference compared with D at *P*<0.01. SE, standard error; C, control; D, diethylnitrosamine (DEN); DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract.

(*P*=0.009) and 2.16-fold (*P*<0.0001), respectively, compared to DEN-treated animals. A similar effect was observed upon the determination of cyclin D1. The western blot analysis showed that the larval and pupal aqueous extracts decreased the cyclin D1 protein level by 28.3% (*P*<0.02) and 40.2% (*P*=0.004), respectively (Fig. 5c). We also determined the level of β-catenin, a commonly altered transcription factor in HCC progression (Lecarpentier et al., 2019). Both the IHC and western blot analyses (Fig. 6) showed that DEN treatment increased the β-catenin level by 61.0% (*P*=0.003); however, the larval and pupal aqueous extracts showed a diminution trend of this level, and this was not significant.

Although some biologic drug activity of edible insects, such as anti-microbial, anti-cancer, and anti-angiogenesis, has been identified, their therapeutic potential has been relatively underestimated (Ratcliffe et al., 2011). *T. molitor* is one of the most widely consumed insects worldwide because of both its high nutritional value and medical properties (Nongonierma and Fitzgerald, 2017; Gao et al., 2018). Whether as food or medicine, the typical strategy is to consume the whole body of the adult insect. Nonetheless, during metamorphosis, insects produce a variety of bioactive compounds that, in addition to contributing to tissue remodeling and development, provide them resistance against bacterial and viral infections (Yoo et al., 2007). Thus, stages of *T. molitor* other than adult represent an attractive source of bioactive compounds for better therapeutic outcomes. Therefore, this study determined the effects of aqueous extracts of *T. molitor* larval, pupal, and adult stages on the early hepatocarcinogenesis based on the fact that this insect has been used as anticancer agent in traditional medicine. Besides, certain studies have also evaluated their therapeutic potential in carcinogenic processes (Yoo et al., 2007; Wu et al., 2020).

At the same time, it has been confirmed that DEN is a potent inducer of cell proliferation in mouse liver (Fuentes-Hernández et al., 2019). Although cancer cells might manifest at least ten well-classified physiological alterations, increased cell proliferation is categorized as one of the traits that increases at the onset of carcinogenic processes (Hanahan and Weinberg, 2011). A recent investigation reported that the cytotoxic effect of *T. molitor* larval oil extract is associated with the activation of both antiproliferative and proapoptotic signaling pathways during the progression of HCC as well as colorectal cancer in vitro (Wu et al., 2020). Based on the quantification of Ki67-positive nuclei,

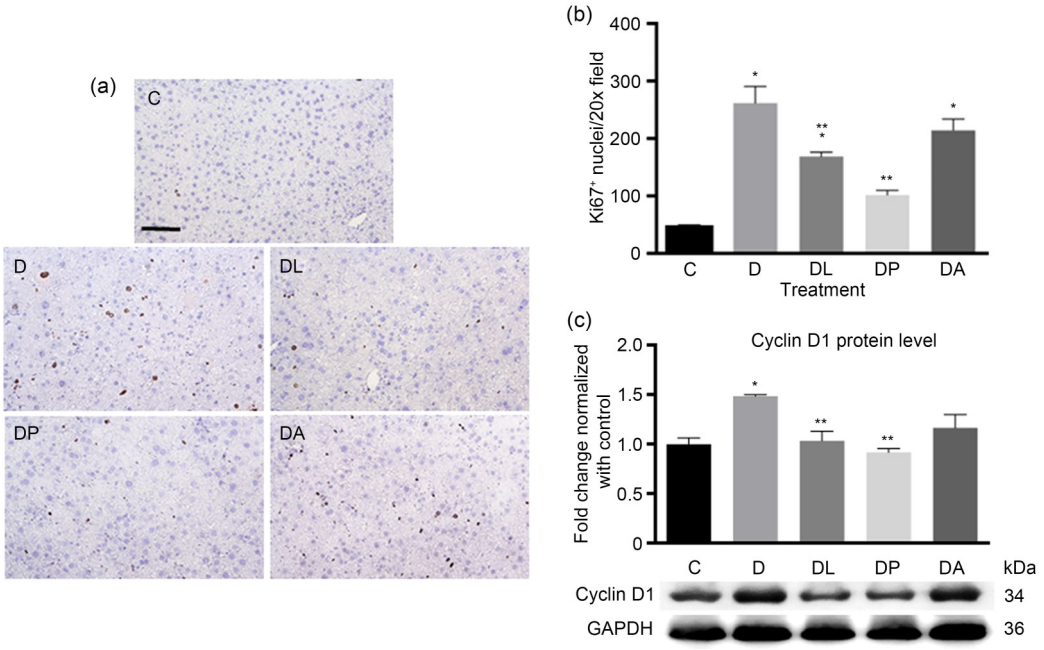

Fig. 5 Effect of *Tenebrio molitor* aqueous extracts on hepatic proliferation induced by diethylnitrosamine (DEN). (a) Immunohistochemistry (IHC) analysis for Ki67 detection. Scale bar=100 μm. (b) Quantification of proliferating cells. (c) The cyclin D1 protein level detected by western blot analysis was normalized with GAPDH, which was used as housekeeping control. C, control; D, DEN; DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as mean±standard error (SE) with n=5. * Statistically significant difference compared with C at P<0.05; ** Statistically significant difference compared with D at P<0.05.

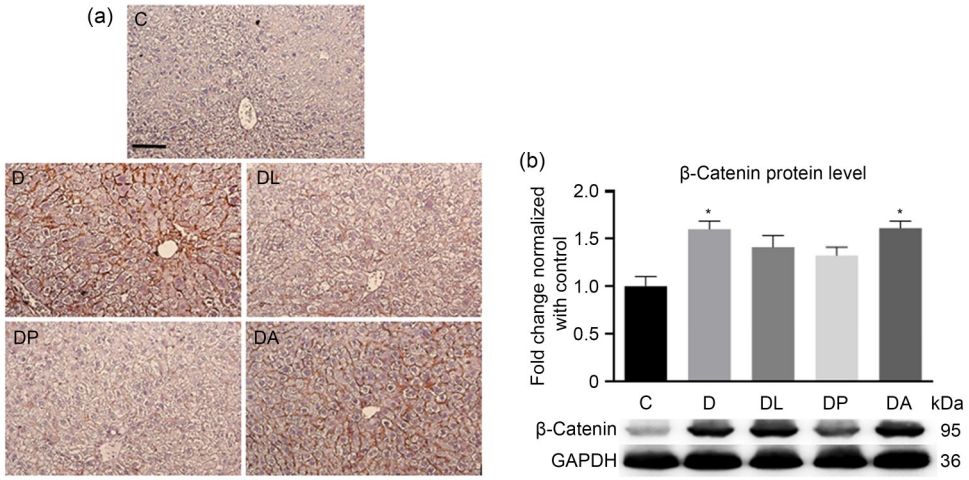

Fig. 6 Effect of *Tenebrio molitor* aqueous extracts on β-catenin nuclear translocation induced by diethylnitrosamine (DEN). (a) Immunohistochemistry (IHC) analysis for detecting β-catenin protein levels. Scale bar=100 μm. (b) β-Catenin protein level detected by western blot analysis was normalized with GAPDH, which was used as housekeeping control. C, control; D, DEN; DL, DEN plus larval aqueous extract; DP, DEN plus pupal aqueous extract; DA, DEN plus adult aqueous extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as mean±standard error (SE) with n=5. * Statistically significant difference compared with C at P<0.05.

we found that altered cell proliferation induced by DEN was inhibited by aqueous extracts from larval and pupal stages of *T. molitor*. Interestingly, the pupal aqueous extract had a stronger inhibition effect on this altered cellular process (Fig. 5 and Table 1). As a result of the antiproliferative effect of larval aqueous

extract, the protein level of GSPT1, an HCC marker, was significantly decreased (Fig. 4b). This phenomenon has also been shown as the anticancer effect of a synthetic compound on the early rat HCC (Arellanes-Robledo et al., 2006), confirming that natural compounds can have as efficient therapeutic effects as those synthesized for targeting specific biological activity.

This underscores the fact that *T. molitor* larvae and pupae have the therapeutic potential of inhibiting altered cell proliferation during early HCC progression in vivo, suggesting that additional bioactive compounds produced during *T. molitor* metamorphosis from larva to pupa might enhance this antiproliferative effect. This proposition is possible since the midgut of insects undergoes a complete renewal process from the stage of pre-pupal instar, involving the activation of programmed cell-death mechanisms, such as autophagy and apoptosis. In addition, the massive proliferation and differentiation of regenerative stem cells take place to generate a new fully functioning epithelium that is maintained throughout the subsequent pupal stage (Tettamanti et al., 2007). A plausible explanation is that during metamorphosis, while insect growth is largely confined to the larval stage, most of the development during ontogeny occurs in the pupal stage; i.e., while the larva only pursues to grow and store energy, the pupa activates molecular mechanisms that might produce transient bioactive compounds, synthesized exclusively at that stage, to finally form the whole insect body (Rolff et al., 2019).

In both rodent models and human HCC, Ki67 has been found to be overexpressed from the onset until the late disease stages (Ueta et al., 2002; Fuentes-Hernández et al., 2019). Moreover, Ki67 overexpression correlates with that of cyclin D1 and β-catenin in human HCC (Ueta et al., 2002). β-Catenin is a transcription factor of the WNT signaling pathway. Canonical WNT/β-catenin signaling is involved in the development of different cancers. Upon its activation, β-catenin transcribes the expression of numerous genes including cyclin D1, which influences the S phase of the cell division cycle (Lecarpentier et al., 2019). In addition to Ki67, this study evaluated the expression of these two proteins. The findings showed that both proteins were induced during early hepatocarcinogenesis; however, while the cyclin D1 (Fig. 5c) was reverted to a similar level as that of the controls, the level of β-catenin (Fig. 6) showed only a diminution trend by both larval and pupal aqueous extracts. Although the level of β-catenin was not significantly reverted as that of cyclin D1, these results indicated that the effects of these two *T. molitor* aqueous extracts on β-catenin were sufficient to significantly decrease the cyclin D1 level, and the number of Ki67-positive nuclei was subsequently reduced. Notably, the *T. molitor* aqueous extracts did not modify the decremented level of CYP2E1 enzyme induced by DEN treatment (Fig. 4a). As confirmed previously (Fuentes-Hernández et al., 2019) and by this study, DEN treatment decreases the CYP2E1 protein level. Thus, our findings suggest that, at least at the early stage of HCC development, *T. molitor* aqueous extracts do not promote the CYP2E1 increment, an enzyme that might participate in HCC progression.

We propose that a future dose-response study might aid in identifying the most efficient dose of aqueous extracts from larvae and pupae of *T. molitor*, and as such achieve the best outcome of this anticancer strategy. Our data also encourage the process of isolating and identifying the specific bioactive compounds with anticancer properties, especially from the pupal stage of *T. molitor*. The present work also introduces a simple formula to calculate the desired dose to be orally administered, which is based on the daily ad libitum liquid intake of mice (Materials and methods).

In summary, we have documented that aqueous extracts from larval and pupal stages of *T. molitor* had better therapeutic properties than those from the adult stage in early hepatocarcinogenesis in mice (Fig. 7). These aqueous extracts blocked the early alterations preceding the establishment of HCC and those associated to the onset of HCC, such as the disarrangement of liver architecture, hepatocyte hypertrophy and fibrogenesis, as well as cell proliferation, by decreasing the levels of both Ki67 and cyclin D1 proliferation markers in the liver of mice chronically exposed to DEN effects. Thus, our results strongly suggest that *T. molitor* larvae, but even more markedly pupae, contain additional bioactive compounds that might be absent in the adult stage, and exhibit therapeutic potential against the early alterations that eventually lead to liver carcinogenesis. It is important to note that, although larval oil extract from *T. molitor* has anticancer activity in vitro (Wu et al., 2020), no studies have demonstrated its anticancer efficacy in an in vivo HCC model. Finally, although several drugs that have been highly promising in vitro failed to exhibit beneficial properties

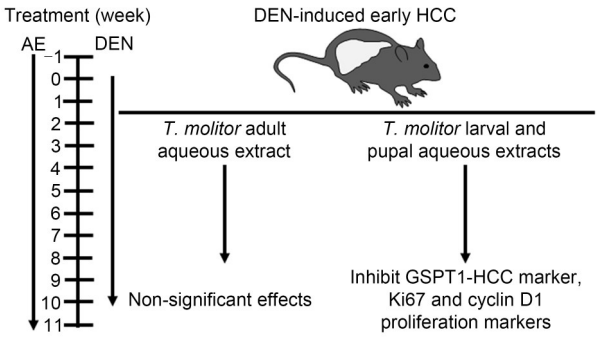

Fig. 7 Schematic representation of the effects of aqueous extracts from different life stages of *Tenebrio molitor* on the early hepatocarcinogenesis. Mice subjected to diethylnitrosamine (DEN) were fed aqueous extracts from three life stages of *T. molitor* ad libitum for ten weeks. The aqueous extract from *T. molitor* adult stage did not significantly affect liver cancer progression; however, those from larvae and pupae inhibited the HCC marker GSTP1 and cell proliferation during the early stage of HCC in vivo. AE, aqueous extract; HCC, hepatocellular carcinoma.

when tested in vivo, our affirmative findings encourage the consumption of *T. molitor* larvae in HCC high-risk populations as a preventive strategy.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Brisa Rodope ALARCÓN-SÁNCHEZ, Osiris Germán IDELFONSO-GARCÍA, Juan OCAMPO-LÓPEZ, Sandra ROSAS-MADRIGAL, and Diana Ivette APARICIO-BAUTISTA performed the experimental research and data analysis. Julio Isael PÉREZ-CARREÓN and Saúl VILLA-TREVIÑO contributed to the study design, writing and editing of the manuscript. Jaime ARELLANES-ROBLEDO and Armando ZEPEDA-BASTIDA contributed to the study conception and design, wrote and edited the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Armando ZEPEDA-BASTIDA, Juan OCAMPO-LÓPEZ, Brisa Rodope ALARCÓN-SÁNCHEZ, Osiris Germán IDELFONSO-GARCÍA, Sandra ROSAS-MADRIGAL, Diana Ivette APARICIO-BAUTISTA, Julio Isael PÉREZ-CARREÓN, Saúl VILLA-TREVIÑO, and Jaime ARELLANES-ROBLEDO declare that they have no conflict of interest.

All animal experimental procedures were performed in accordance with the Institutional Animal Use and Care Committee of CINVESTAV-IPN and the approved protocol (No. 0114-14).

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Supplementary information

Materials and methods