ORIGINAL ARTICLE

Effect of *Garcinia binucao* Crude Leaf Extract Supplementation on Lifespan of *Drosophila melanogaster* Chronically Exposed to Alcohol

Joanne Jennifer E. Tan, MD, PhD,[1] Ourlad Alzeus G. Tantengco, MD, PhD,[1] Nicholas Robert C. Tan, MD,[1] Clyde E. Silverio, MD,[1] Ana Denise V. Sison, MD,[1] Joseph P. Sta. Maria Jr., MD,[1] Karol Ina G. Tablante, MD,[1] Joyce Gillian A. Tiam-Lee, MD,[1] Maria Concepcion C. Sison, MD[2] and Paul Mark B. Medina, PhD[3]

[1]*College of Medicine, University of the Philippines Manila*
[2]*Department of Pharmacology and Toxicology, College of Medicine, University of the Philippines Manila*
[3]*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila*

ABSTRACT

Background. Consumption and abuse of alcohol remains a significant cause of concern worldwide. Furthermore, there is evidence of the association between chronic alcohol use and reduced life expectancy.

Objectives. To study the effects of *Garcinia binucao* extract (GBE) supplementation on lifespan of *Drosophila melanogaster*, in the presence or absence of chronic alcohol exposure.

Methods. *D. melanogaster* was mass cultured and given GBE supplementation in high (1 mg/mL) and low (200 µg/mL) sublethal doses. *D. melanogaster* flies were divided into groups - with and without chronic alcohol exposure, and their respective lifespans were monitored.

Results. In *D. melanogaster* without alcohol exposure, mean lifespan was highest in the control flies (38.15 days), followed by high-dose GBE (34.42 days), low-dose GBE (33.24 days), and DMSO (22.29 days). In *D. melanogaster* chronically exposed to alcohol, the longest mean lifespan was observed in flies treated with high-dose GBE (33.80 days), followed by low-dose GBE (33.63 days), the DMSO group (30.30), and the control group (29.65 days), but the differences were not statistically significant. Comparing groups with and without chronic alcohol exposure, the mean lifespan of the control group chronically exposed to alcohol significantly decreased by 9.51 days ($p < 0.05$). In GBE treatment groups, mean lifespan significantly decreased by 0.82 days in high-dose set-up ($p < 0.05$), and significantly increased by 0.39 days in the low-dose set-up ($p < 0.05$) upon chronic alcohol exposure.

Conclusion. *Garcinia binucao* extract supplementation ameliorated the observed reduction in lifespan of *Drosophila melanogaster* chronically exposed to alcohol.

Keywords: Garcinia binucao, Drosophila melanogaster, chronic alcohol toxicity, lifespan

eISSN 2094-9278 (Online)
Published: March 22, 2024
https://doi.org/10.47895/amp.vi0.5911

Corresponding author: Paul Mark B. Medina, PhD
Department of Biochemistry and Molecular Biology
College of Medicine
University of the Philippines Manila
547 Pedro Gil Street Ermita, Manila 1000, Philippines
Email: pmbmedina@post.upm.edu.ph
ORCiD: https://orcid.org/0000-0001-6116-1818

INTRODUCTION

Chronic consumption of alcohol and its resulting problems remain to be a large cause of concern around the world. The worldwide total consumption according to WHO 2018 data was equal to 6.2 liters of pure alcohol per person 15 years and older.[1] Philippine data show that around 16 million Filipinos aged 20 years or older engage in binge drinking.[2] This is alarming especially since there is evidence on the association of chronic alcohol use and reduced life expectancy.[3] Some studies in the animal model *Drosophila melanogaster* revealed that exposure to ethanol during development resulted in decreased life expectancy.[4,5] This could be likely due to ethanol exhibiting its toxic effect through the transport of neurotransmitters such as biogenic amines and GABA, and the accumulation of acetaldehyde which is highly toxic and inhibits protein synthesis.[6]

There have been several studies on the effects of natural products containing antioxidant molecules that increased longevity. These antioxidant compounds include xanthones, flavonoids, and catechins. *Garcinia mangostana* has been found to contain these highly active antioxidant xanthones.[7] According to previous studies, antioxidant supplements containing xanthones derived from acai, goji berries, mangosteen, and pomegranate increased longevity of *D. melanogaster* flies in the presence of genetically- and chemically-induced oxidative stress.[8,9]

A plant in the same Garcinia genus is, therefore, a prime candidate for investigation for similar compounds. One species of Garcinia that is widely distributed in the Philippines is *Garcinia binucao*. It has been noted for its traditional medicinal properties and potential anti-neurotoxic properties.[10,11] Separate research studies have extracted a plethora of biologically active compounds from the *G. binucao* fruit. However, no studies have been conducted to test the neuroprotective properties of the *G. binucao* extracts. Previous studies showed that compounds extracted from *G. mangostana* (a related species) have dose-dependent neuroprotective effects points to the possible antioxidant and neuroprotective properties of *G. binucao* extracts.[11–13]

In addition, many neurotoxicity and neurodegenerative studies that screen for the potential protective properties of biologically active compounds use *D. melanogaster* as the model organism.[14–16] *D. melanogaster* are ideal subjects to use because they are relatively easy to handle, they have a short lifespan (40 to 120 days), they reproduce rapidly, and 75-77% of human disease-related genes have functional orthologs in the fly genome.[14,17] Since its central nervous system operates on the same fundamental principles as those of humans, this makes them ideal subjects for studies into the nervous system.

Given the current prevalence of chronic alcohol consumption resulting in ethanol- induced toxicity, the potentially anti-neurotoxic properties of *G. binucao* offers a possible solution. This study presents the beneficial effects of *G. binucao* extract (GBE) supplementation on the lifespan of *D. melanogaster*, an established animal model in studying the neurotoxicity of chronic ethanol exposure.

Table 1. Operational Definitions

Conceptual Variable	Operational Definition
Chronic alcohol exposure	Daily exposure of flies to ethanol for five minutes. This was done by closing the lid of the vials with ethanol-soaked cottons for five minutes daily.
Protection	Increase in lifespan compared with alcohol-induced reduced lifespan
Supplementation	Daily feeding of flies with same sweet potato-yeast media diet but with included specific concentration of *Garcinia binucao* extract

MATERIALS AND METHODS

Operational Definition of Terms

Table 1 shows the operational definitions of some terms used in this paper.

Specimen Collection and Crude Extraction

Specimen collection and crude extraction were done as described in a previously published paper.[10] A voucher specimen was prepared and submitted to the Botany Division of the National Museum of the Philippines for taxonomic identification. Fresh *G. binucao* leaves were collected from Sitio Pastolan, Tipo, Hermosa, Bataan, and taxonomic confirmation was done at the Botany Division of the National Museum of the Philippines. Crude extraction was done by soaking powdered leaves in 95% ethanol, filtering using Whatman filter paper grade no. 41 and further purified using rotary evaporation.

Drosophila culture and husbandry

D. melanogaster mass culture and media preparation were done as described in a previously published paper.[10] The sweet potato-yeast media consisted of sweet potato, yeast, and agar. *G. binucao* supplementation was done by adding crude *G. binucao* extract (dissolved in 1% DMSO) to the sweet potato-yeast media and mixing evenly to achieve specific final concentrations.

D. melanogaster were anesthetized by pumping CO_2 in the vial through the cotton plugs and then transferring to the fly pad, following standard protocol ensuring minimal exposure and side effects of CO_2 to the flies.[12] A stereo-microscope was used in sorting and sexing the flies.

Ethanol-Induced Oxidative Stress and Neurotoxicity

Ethanol-induced oxidative stress and neurotoxicity measurements were done using the same protocol as previously published.[10] This standard protocol was chosen due to its established simplicity and practicality as compared with other methods.[4,5,13,14] *D. melanogaster* flies were transferred

into the ethanol exposure vials by tapping groups of about eight flies per vial. Vial plugs flooded with 0.5 mL absolute alcohol were inserted with the ethanol-flooded side facing the flies, and this was done for five minutes daily before returning the flies to their food vials. Sufficient ethanol exposure was assessed daily by confirming sedation of flies in the vials.

Chronic Toxicity Assay

Four different concentrations of GBE, 31.25 µg/mL, 125 µg/mL, 500 µg/mL, and 1 mg/mL were introduced in the diet of *D. melanogaster*. Five groups (4 treatment groups and 1 control group) of 60 adult flies (30 males and 30 females) were prepared and toxicity was determined after 72 hours of treatment by comparing percentage survival of flies per group using one-way ANOVA. Concentrations of *G. binucao* leaf extracts that were determined as sublethal were used in the succeeding survival assay.

Survival Assay

An experimental study design was implemented with eight groups of 180 flies each (90 males and 90 females) included in the assay: control ± ethanol exposure, 1% DMSO ± ethanol exposure, low dose GBE ± ethanol exposure, and high dose GBE ± ethanol exposure. Low dose GBE (200 µg/mL) and high dose GBE (1 mg/mL) groups both had 1% DMSO as solvent. The lifespan of each fly was noted by recording the survivability of flies which includes the number of days from the start of the assay until death.[15] The mean lifespan of all flies was calculated per treatment and analyzed using one-way ANOVA. Kaplan-Meier plots and further statistical analyses were done using OASIS, a free online application for survival analysis.[16]

RESULTS

Chronic toxicity of GBE on *D. melanogaster*

Different concentrations of GBE ranging from 31.25 µg/mL to 1 mg/mL did not show chronic toxic effects in *D. melanogaster* after 72 hours (Figure 1). There is no significant difference in the percentage survival of flies in the control group and those treated with different concentrations of *G. binucao* following Dunn's multiple comparison test of one-way ANOVA ($p>0.05$).

Since no chronic toxic effects were observed in all tested concentrations in the first 72 hours, all were considered sublethal. A low dose and a high dose were selected arbitrarily to be used in the survival assay. The highest concentration tested, 1 mg/mL, was assigned as the high dose, and a five-fold dilution, 200 µg/mL was assigned as the low dose.

Effect of GBE on *D. melanogaster* lifespan with and without chronic alcohol exposure

In flies without alcohol exposure (lifespans of the flies ranging from 17 to 41 days), the longest mean lifespan was observed in the control flies (38.15 days), followed by high-dose GBE (34.42 days), low-dose GBE (33.24 days), and DMSO (22.29 days) (Figure 2). There was a significant reduction of lifespan in the high- and low-dose, and DMSO treatments versus the control treatment ($p < 0.05$).

In flies exposed to ethanol, the longest mean lifespan was observed in flies treated with high-dose GBE (33.80 days), followed by low-dose GBE (33.63 days), the DMSO group (30.30), and lastly the control group (29.65 days). However, the differences were found to be not statistically significant. Furthermore, it was observed that the lifespan of the control

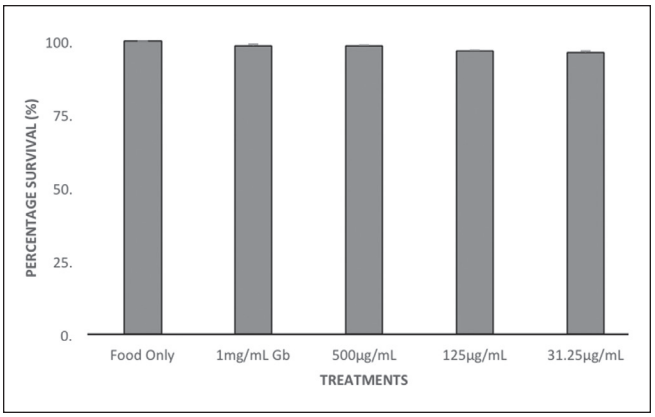

Figure 1. Chronic toxic effects of four different GBE concentrations ranging from 31.25 µg/mL to 1 mg/mL against *D. melanogaster* after 72 hours of supplementation (N=60 per group).

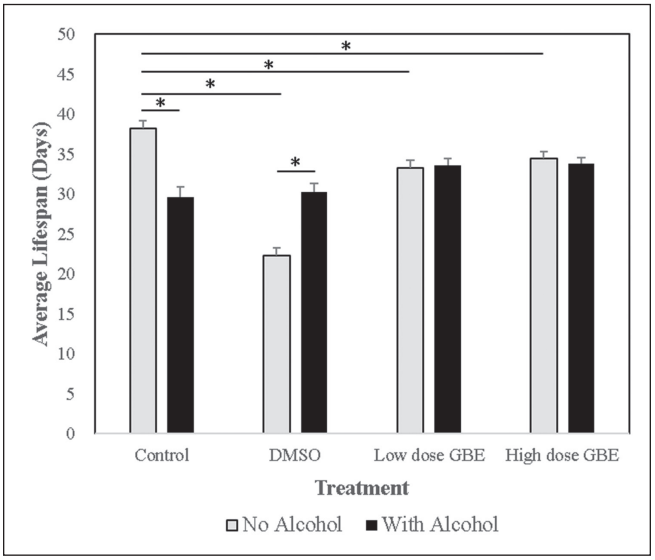

Figure 2. Average lifespans of *D. melanogaster* comparing control (food only) and the treatment groups: 1% DMSO, low-dose GBE (200 µg/mL extract in 1% DMSO), and high-dose GBE (1 mg/mL extract in 1% DMSO), in the presence and absence of chronic alcohol exposure (N=180 per group).

flies exposed to ethanol significantly decreased by 9.51 days ($p < 0.05$) compared to those not exposed to ethanol. In the DMSO group, the lifespan increased significantly by 8.01 days ($p < 0.05$). In flies treated with GBE, mean lifespan significantly decreased by 0.82 days in high-dose set-up and significantly increased by 0.39 days in the low-dose set-up ($p < 0.05$).

DISCUSSION

Chronic alcohol exposure has been known to decrease life expectancy in humans. The effects of alcohol in life expectancy and potential drug candidates to counter this observed reduction in lifespan are currently being studied using animal models such as *D. melanogaster*. [17,18] In this study, GBE supplementation was done and its effects on the lifespan of *D. melanogaster* with and without chronic alcohol exposure were determined.

Without chronic alcohol exposure, the average lifespans of *D. melanogaster* treated with low dose and high dose GBE were lower compared with the control flies. Looking into the results further, it should be noted that these average lifespans are higher compared with *D. melanogaster* treated with DMSO alone. DMSO, which was used to dissolve GBE, has been shown to have toxic effects when used in high concentrations in some studies. However, despite using a DMSO concentration lower than the toxic concentrations published in literature, we still observed toxic effects in this study.[19,20] It may then be hypothesized that the extract has a protective effect against the toxic effect of DMSO on *D. melanogaster*.

DMSO can solubilize a wide range of polar and nonpolar molecules. It has been used ubiquitously due to its apparent low toxicity and has several *in vitro* and *in vivo* applications, bioassays, most particularly in drug discovery.[21,22]

While the use of DMSO is recognized, this study along with other previous studies demonstrated the toxicity of DMSO even at low concentrations. DMSO concentration limits for different experimental endpoints should be further studied to avoid confounding the results of experiments.[19,20]

With chronic alcohol exposure, the observed toxic effect of alcohol on lifespan of *D. melanogaster* were ameliorated by GBE supplementation, as shown by a lower magnitude of lifespan reduction among flies supplemented with GBE compared with flies without chronic alcohol exposure. Furthermore, the average lifespans of control flies and flies treated with DMSO with chronic alcohol exposure were not significantly different. This gives rise to another hypothesis that exposure to ethanol may have neutralized the toxic effects of DMSO, possibly by interactions or chemical reactions between DMSO and ethanol, leading to less toxic byproducts or metabolites.

Previous researches indicated that DMSO and ethanol affect the toxicity of each other.[23,24] DMSO enhances the elimination of ethanol from the body and it also removes the odor of alcohol from the respiratory air. This may explain the improvement of lifespan between the control flies without chronic alcohol exposure and control flies with chronic alcohol exposure.[23,24]

Although both low-dose and high-dose GBE had protective effects against life-shortening effects of both DMSO and chronic alcohol exposure, the difference of the effects between the two doses were not significant. It should be noted that the doses were selected arbitrarily as mentioned previously. Further optimization and trials for doses of GBE, either lower or higher than tested in this study, should be done in order to determine the ideal dose that will exhibit the greatest protective effect.

GBE supplementation seemed to have a protective effect against the life-shortening effects of chronic alcohol exposure to *D. melanogaster*. This protective effect may be attributed to the antioxidant activity and the phytochemicals present in GBE as shown in a previous study.[10] Several studies have shown that flavonoids and terpenoids can increase lifespan across different animal models including Drosophila.[25,26] Flavonoids can induce brain perfusion and stimulate angiogenesis and have been demonstrated to increase lifespan in flies and mice.[27,28] Naphthoquinones, on the other hand, have been shown to increase longevity by engaging a specific adaptive cellular stress response pathway in *C. elegans*.[29]

Further phytochemical analysis, screening for various other compounds, and identifying more specific compounds are highly recommended to identify the active components of the extract. We also recommend increasing the sample sizes in all the behavioral assays to increase the power and significance of the results. Use of a different solvent for crude extraction such as water would be ideal to eliminate the confounding effects brought by the DMSO. To further explore the neuroprotective mechanisms from *G. binucao*, brain dissections and biochemical analyses to quantify the level of antioxidant enzymes can be done. Reverse transcriptase quantitative polymerase chain reaction may also be done to quantify the antioxidant mRNA gene expression in *D. melanogaster* treated with *G. binucao*.

CONCLUSION

G. binucao extract supplementation ameliorated the observed reduction in lifespan of *D. melanogaster* chronically exposed to alcohol. GBE also decreased the toxic effects of DMSO on the lifespan. It is recommended to use a different solvent in the future to further determine the effects of GBE on longevity. Further purification of potential protective compounds from *G. binucao* should be done in future studies.

Acknowledgments

We would like to acknowledge Dr. Raymond Oliva for his inputs in the proposal, and the UP College of Medicine Therapeutics 202 Group 7 AY 2016-2017 (Dr. Frinz Moey Rubio, Dr. Mariale Yasmin Salces, Dr. Jose Manuel Santiago,

Dr. Charity Joyce Santos, Dr. Luis Ysmael Santos†, Dr. Noel Angelo Sarrosa, Dr. Kevin Sese, Dr. Janine May Sta. Maria, Dr. Olin Joseph Tamayo, Dr. Anna Beatrice Tan, Dr. Randell Kelvin Tan, and Dr. Remedios Immarie Teves) for their invaluable assistance in the conduct of all laboratory experiments and support in the completion of this project.

Statement of Authorship

All authors contributed in the conceptualization of work, acquisition and analysis of data, drafting and revising of manuscript, and final approval of the version to be published.

Author Disclosure

All authors declared no conflicts of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Funding Source

This research was funded by the UP College of Medicine and UPM NIH Student Researcher Grant 2016.

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