



Influence of *Basella alba* methanolic extract on alcohol preference in *Caenorhabditis elegans* after acute and chronic alcohol withdrawal

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Abstract. Alcohol disinhibition leads to various social and health problems, especially when abused. One way to reduce alcohol addiction is through withdrawal. Several studies suggest that the active compounds in *Basella alba* (*B. alba*) modulate different hormones and neurotransmitters. In this study, the researchers examined whether *B. alba* methanolic extract (BAME) can revert the alcohol preference of *Caenorhabditis elegans* (*C. elegans*) after acute and chronic alcohol withdrawal. Initially, they exposed the BAME-treated *C. elegans* to ethanol enough to exhibit alcohol preference. They transferred the nematodes to a different plate without ethanol for 30, 60, and 120 minutes, short-term. For long-term alcohol withdrawal, the researchers fed the nematodes with ethanol for 24 hours. They maintained these worms without ethanol for 7 days. They measured the preference index after 1 and 7 days. The researchers found out that BAME-fed *C. elegans* with 1-hour withdrawal demonstrated avoidance of ethanol. However, the 7-day alcohol withdrawn nematodes fed with BAME displayed ethanol preference. In summary, the findings suggest that BAME treatment may provoke alcohol avoidance after acute alcohol withdrawal, but may induce alcohol preference after chronic withdrawal. Further studies are needed to explore the findings of this study.

Key Words: addiction, ethanol, long-term, natural product, short-term.

Introduction. Studies have associated alcohol disinhibition to deviant sexual behavior, violence, property offences, and drug use (George & Norris 1991; Galvani 2004; Vrieze et al 2013). Also, frequent use of alcohol leads to addiction (Vengeliene et al 2008). One approach to managing alcohol addiction is through withdrawal from alcohol (Kosten & O'Connor 2003). However, several studies show the possibility of recurrence of alcohol addiction after withdrawal (Grant et al 2012; Boschloo et al 2012).

A previous study suggests that potential medication for alcoholism should address alcohol-induced neurotransmitter imbalance in the brain (Friedlander et al 2003). Studies have reported that the active compounds of *Basella alba* (locally known as "alugbati") affect various hormones and neurotransmitters (Roth et al 1989; Penney et al 1994; Prajapati et al 2014). Additionally, some studies show that *B. alba* has antidepressant, muscle relaxant, and anticonvulsant properties (Kachchhava 2006; Abhinayani et al 2016). Despite these studies on *B. alba*, its effect on alcohol preference after alcohol withdrawal is still unexplored.

Hence, in this study, the effects of *B. alba* methanolic extract on the alcohol influence in *C. elegans* after short-term and long-term alcohol withdrawal were investigated. Several studies support *C. elegans* as the model organism to study alcoholism because of its simplicity, basic behavioral pattern, and ortholog human genes related to alcohol preference and alcohol withdrawal (Scott et al 2017; Lee et al 2009; Scholz & Mustard 2011).

Material and Method. 1 kg of *B. alba* plants were collected from Los Banos, Laguna, Philippines, in January 2019. The extraction protocol from a previous study with some modifications (Kumar et al 2011) was followed. 100 g of dried and pulverized leaf and stem mixture of the plant was soaked in 1 L of methanol for 12 hours (100 g L⁻¹ methanol). A rotary evaporator was used to remove the solvent from the extract. 1000 mg of extract was dissolved with 1 mL of 1% DMSO and stored at 4 °C until used. The extraction and subsequent experimentations were conducted in the Department of Medical Technology, Institute of Arts and Sciences, Far Eastern University.

The extract was tested for the presence of alkaloids, flavonoids, phenols, saponins, phytosterols, tannin, carbohydrates, proteins, and sugar following the protocol from a previous study (Nas et al 2020a).

Procurement and maintenance of *C. elegans*. The Bristol N2 (wild type) *C. elegans* and *Escherichia coli* (*E. coli*) OP50 were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota (MN, USA). The nematodes were kept on a nematode growth medium (NGM) agar at 25°C, following the protocol from a previous study (Nas et al 2020b). *E. coli* OP50 was maintained in a nutrient agar incubated at 37°C. 100 µL of this strain replenishes the nutrient source of the nematode. The nematode was age-synchronized by collecting eggs laid on NGM plates within 4 hours. The researchers allowed the eggs to hatch and grow until larval stage 4 (L4) before they were used in the experiment.

Sublethal assay. *C. elegans* with *E. coli* OP50 mixed with varying concentrations of BAME (1000, 100, and 10 µg mL⁻¹) were used, whereas 1% DMSO in distilled water serves as the negative control. The worms were transferred to fresh NGM plates every 24 hours. The number of live worms was counted every 24 hours until 72 hours post-L4. Live nematodes were the individuals who move after a light poke. The concentration in which the survival rate was greater than 90% was considered the sublethal concentration (Nas et al 2019).

Short-term withdrawal assay. 30 adult worms were transferred to new NGM plates with 300 mM of ethanol mixed with *E. coli* OP50. The plates were incubated at 25°C for 4 hours. Subsequently, the worms were shifted onto new NGM plates without *E. coli* OP50. The worms were relocated to a new marked-NGM plate with ethanol after 30, 60, and 120 minutes. The NGM plate was marked into four quadrants. 300 mM of ethanol was dispensed on quadrants 1 and 4. The nematodes were placed at the origin of the 4 quadrants. The number of nematodes in each quadrant was counted. The preference index was computed by the following formula (Lee et al 2009):

$$\text{Preference index} = \frac{(Q1_{total} + Q4_{total}) - (Q2_{total} + Q3_{total})}{\text{total no. of nematodes}}$$

Where: Q1, Q2, Q3, and Q4 show the total number of nematodes on that specific quadrant.

Long-term withdrawal assay. The previously mentioned protocol for short-term withdrawal assay was followed with a few modifications. One of these modifications was the incubation of the nematodes for 1 day at 25°C. The worms were transferred to new NGM plates with *E. coli* OP50. The worms were reared in normal physiologic conditions for 7 days. The alcohol preference of the nematodes was observed after 1 and 7 days.

Statistical analysis. In the study, each individual was considered as a replicate. All procedures were repeated twice. In the sublethal assay, the log-rank test was used to determine significant differences between the treatment groups through OASIS version 2 (South Korea). The data of the preference index was presented as mean±SE. GraphPad Prism version 7 (GraphPad Software, CA, USA) was used to compute the significance using the Chi-square test with a statistical significance at p<0.05.

Results and Discussion. Based on the phytochemical screening, BAME tested positive for phenols, flavonoids, tannins, saponin, reducing sugar, starch, carbohydrates, and proteins. *B. alba* methanolic extracts contain carbohydrates, tannins, phytosterols, flavonoids, saponin, and mucilage (Premakumari et al 2010; Kumar et al 2011). Similarly, these metabolites were also present in the extract used in the study.

Sublethal assay. Figure 1 shows that more than 90% of the nematodes treated with 1000 $\mu\text{g mL}^{-1}$ BAME survived under normal conditions after 72 hours from exposure. The survival percentage of the untreated *C. elegans* and those with BAME treatments were comparable after 24, 48, and 72 hours. The results show that BAME in a concentration of 1000 $\mu\text{g mL}^{-1}$ does not pose acute and chronic toxicity to the nematode. Nematodes treated with 1000 $\mu\text{g mL}^{-1}$ BAME have more than 90% survival rate after 3 days, which indicates that the BAME at that concentration is not toxic to *C. elegans*. The researchers considered 90% survival as the benchmark of non-toxicity, which follows a previous study (Nas et al 2019).

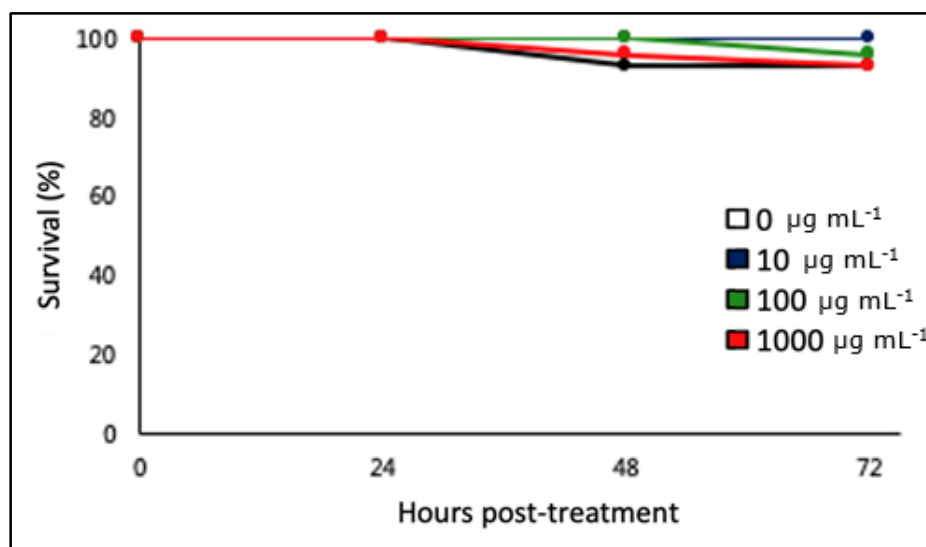


Figure 1. Survival of the *Caenorhabditis elegans* treated with different *Basella alba* methanolic extract concentrations for 72 hours.

***C. elegans* fed with BAME avoids ethanol after short-term alcohol withdrawal.** The researchers observed the ethanol preference of *C. elegans* fed with BAME after withdrawal to alcohol at different intervals, as shown in Figure 2. *C. elegans* fed with 10 and 100 $\mu\text{g mL}^{-1}$ BAME after 60 minutes of alcohol withdrawal exhibited ethanol avoidance. Meanwhile, *C. elegans* fed with BAME and withdrawn to alcohol for 30 and 120 minutes did not display ethanol avoidance.

Several investigations found that *C. elegans* lure toward the scent of ethanol (Lee et al 2009; Scholz & Mustard 2011). A previous study suggests that alcohol initially repels rats, but prolonged exposure shows the opposite effect (Simms et al 2008). Some studies reported that *npr-1* genes influence ethanol preference in *C. elegans* (Davies et al 2004). Importantly, studies show that *npr-1* inhibits acute tolerance to alcohol (Davies et al 2004). Additionally, the *npr-1* gene in *C. elegans* is associated with social feeding and innate immune response (Rogers et al 2003; Styer et al 2008). These observations may indicate that the metabolites present in BAME possibly affect the *npr-1* associated pathway in *C. elegans*.

Interestingly, the researchers only observed the reversal of alcohol preference after the 60-minute interval. This information suggests that the potential target pathway in *C. elegans* may be active for a specific duration only. Studies show that *npr-1* is associated with G-protein coupled receptor (GPCR) neuropeptides, which affect the feeding behavior of the worm (Rogers et al 2003). Some studies propose that the sensory and circuit inputs in *C. elegans* regulate the feeding state and insulin pathway (Gruner et al 2014). However,

the mechanism of how BAME might have influenced these pathways is not yet clear. Thus, this speculation needs further investigations.

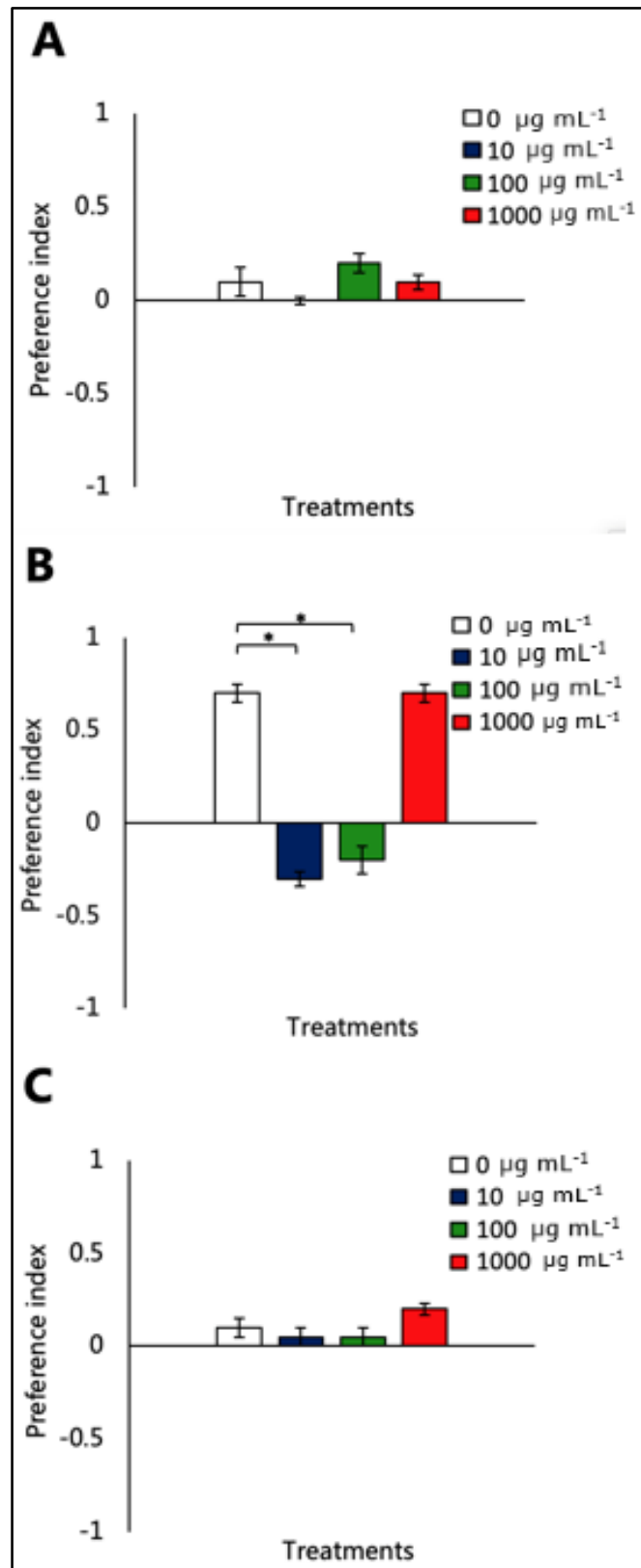


Figure 2. Preference index of *Caenorhabditis elegans* treated with varying concentrations of *Basella alba* methanolic extract on short-term withdrawal from ethanol (A-30, B-60, C-120 minutes), $n=30$, * - significance at $p<0.05$.

***C. elegans* fed with BAME decrease preference to ethanol after long-term alcohol withdrawal.** The researchers examined the ethanol preference of BAME-fed *C. elegans* after 1 and 7 days of alcohol withdrawal. The nematode treated with BAME did not show ethanol avoidance after 1 and 7 days, as shown in Figure 3. Despite this result, they observed that the nematodes fed with different BAME concentrations displayed ethanol preference after 7-day withdrawal.

This section depicts chronic alcohol withdrawal in *C. elegans*. Studies suggest that genes like *slo-1* and *egl-3* influence ethanol preference in *C. elegans* (Mitchell et al 2010; Scott et al 2017). The *slo-1* ion channels are present in motor neurons, sensory neurons, and muscles (Wang et al 2001). Chronic exposure of the *C. elegans* to ethanol impedes the *slo-1* ion channel by down-regulating *slo-1* (Scott et al 2017). Another *slo* family protein, *slo-2*, reverses *slo-1* associated withdrawal behavior in *C. elegans* (Zhang et al 2013). BAME may have affected the *slo* family-related pathway, which possibly caused the ethanol preference. A study suggests that *B. alba* has a central nervous system depressant property (Anandarajagopal et al 2011). This property may have triggered CNS depressant dependence in *C. elegans*.

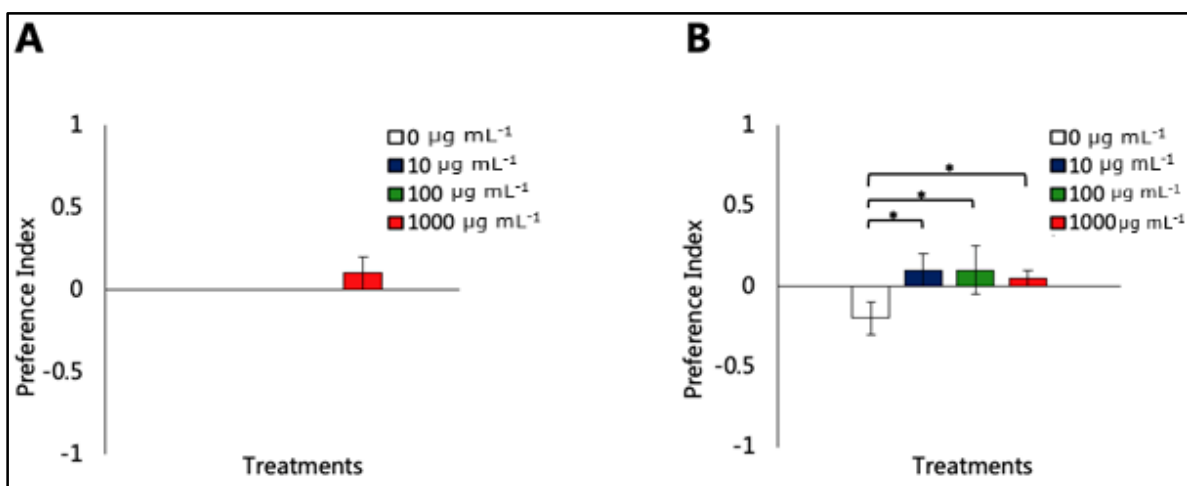


Figure 3. Preference index of *C. elegans* treated with varying concentrations of BAME after long-term withdrawal from ethanol (A-1 and B-7 days post-L4), n=30, * significance at p<0.05.

Conclusions. In this study, BAME displayed potential alcohol avoidance reduction after short-term withdrawal, but not after long-term alcohol withdrawal in *C. elegans*. This decline after the acute alcohol withdrawal may be limited to a specific period in an unclear mechanism of action. Also, the possible mechanism involved in the alcohol preference-induced effect of BAME after chronic withdrawal is still unknown. These insights recommend future investigations to supply the existing knowledge gap in the mechanism involved in alcohol preference reoccurrence.

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