JOURNAL OF PHARMA INSIGHTS AND RESEARCH ISSN NO. 3048-5428

RESEARCH ARTICLE

Study of Developmental Toxicity of Bromelain on Brine Shrimp (*Artemia salina*)

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Publication history: Received on 29th July; Revised on 6th August; Accepted on 16th August 2024

Article DOI: 10.69613/297ffg94

Abstract: This study investigated the acute effects of bromelain on brine shrimp (Artemia salina) development and survival. Brine shrimp nauplii were exposed to varying concentrations of bromelain (1-800 µg/mL) for 24 hours. Mortality rates, growth patterns, and morphological changes were assessed. Results showed a dose-dependent increase in mortality, with 91.66% mortality at 800 µg/mL compared to 13.88% at 1 µg/mL. The lethal concentration (LC50) of bromelain was determined to be approximately 100 µg/mL. Morphological analysis revealed developmental retardation at the Instar II stage, with the formation of small vesicles observed microscopically. Growth measurements indicated minimal differences in developmental progression across treatment groups, with lengths ranging from 455-484 µm compared to 500 µm in controls. The study demonstrates that acute bromelain exposure exhibits toxic effects on brine shrimp, impacting survival and development. These findings suggest that brine shrimp may serve as a useful invertebrate model for preliminary toxicity screening of bromelain and potentially other proteolytic enzymes.

Keywords: Bromelain; *Artemia salina*; Developmental toxicity; Proteolytic enzymes; Alternative toxicity models

1. Introduction

The field of toxicology has seen significant advancements in recent years, particularly in the development of alternative testing methods that reduce reliance on traditional mammalian models. This shift is driven by ethical considerations, cost-effectiveness, and the need for rapid screening tools in drug development and environmental risk assessment. Among these alternative models, aquatic invertebrates have gained prominence due to their simplicity, cost-effectiveness, and ability to bridge the gap between in vitro and in vivo studies [1]. Brine shrimp (Artemia salina) has emerged as a valuable model organism for toxicity testing. These small crustaceans belong to the subclass Branchiopoda, order Anostraca, and family Artemiidae. They are widely distributed in natural and artificial brine pools and lakes worldwide [2]. Brine shrimp have several characteristics that make them ideal for toxicological studies: they have a short life cycle, are easy to culture, and their eggs (cysts) can be stored for long periods and hatched on demand [3].

The life cycle of Artemia salina consists of several distinct stages. Upon hatching, the nauplii undergo a series of molts, progressing through various instar stages before reaching adulthood. The early developmental stages, particularly the transition from nauplius to metanauplius, are critical periods where the organisms are especially vulnerable to environmental stressors and toxicants [4]. This sensitivity makes brine shrimp an excellent model for assessing developmental toxicity. The Brine Shrimp Lethality Test (BSLT) has been widely adopted as a preliminary screening tool for various compounds, including natural products, pharmaceuticals, and environmental pollutants [5]. This assay provides a rapid assessment of toxicity based on the survival rate of Artemia nauplii exposed to test substances. While mortality is a clear endpoint, there is growing interest in expanding the use of brine shrimp models to include sublethal effects, such as developmental abnormalities and growth retardation [6].

Bromelain, the subject of this study, is a mixture of proteolytic enzymes derived primarily from the stem and fruit of pineapples (Ananas comosus). It has gained significant attention in recent years due to its diverse therapeutic potential, including anti-inflammatory, anticancer, and immunomodulatory properties [7]. The medicinal use of bromelain has been documented in various conditions, such as osteoarthritis, chronic sinusitis, and as an adjunct in cancer therapy [8].

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Despite its widespread use and generally recognized as safe (GRAS) status, the safety profile of bromelain, particularly its potential developmental toxicity, has not been extensively studied. Most safety assessments have focused on oral administration in adult animal models or human clinical trials [9]. However, the effects of bromelain on developing organisms, especially at the cellular and tissue levels, remain largely unexplored. The proteolytic nature of bromelain raises important questions about its potential impacts on developing organisms. Proteases play crucial roles in various developmental processes, including cell signaling, tissue remodeling, and morphogenesis [10]. While endogenous proteases are tightly regulated, exogenous proteolytic enzymes like bromelain could potentially disrupt these delicate processes if they gain access to developing tissues. The use of brine shrimp as a model for developmental toxicity assessment offers several advantages in studying compounds like bromelain. The transparency of Artemia nauplii allows for real-time observation of morphological changes and organ development [11]. Additionally, the relatively simple body plan of brine shrimp facilitates the identification of developmental abnormalities that might be more challenging to detect in complex vertebrate models.

Recent advancements in microscopy and image analysis techniques have enhanced the utility of brine shrimp models in developmental toxicology. High-resolution imaging can reveal subtle morphological changes, while automated analysis tools allow for quantitative assessment of growth parameters and developmental milestones [12]. These technological improvements have increased the sensitivity and reproducibility of brine shrimp-based assays, making them more relevant for predicting potential developmental toxicity in higher organisms. The present study aims to bridge the gap in our understanding of bromelain's developmental toxicity using the brine shrimp model. We seek to elucidate both lethal and sublethal effects of this proteolytic enzyme mixture on early developmental stages by exposing Artemia salina nauplii to a range of bromelain concentrations. This approach allows for the assessment of acute toxicity through mortality rates while also investigating potential impacts on growth and morphological development.

The choice of exposure concentrations in this study (1-800 μg/mL) was based on previous investigations of bromelain's biological activities in various in vitro and in vivo systems [13]. This range encompasses concentrations that have shown therapeutic effects in other models while extending to higher levels to identify potential toxic thresholds. The 24-hour exposure period was selected to capture acute effects while allowing sufficient time for developmental processes to occur in the rapidly developing brine shrimp nauplii. [10,11] In addition to standard toxicity endpoints, this study incorporates detailed morphological analysis to detect subtle developmental abnormalities. The formation of small vesicles observed microscopically could provide insights into the mechanisms by which bromelain interacts with developing tissues. Such observations may guide future investigations into the specific cellular and molecular targets of bromelain in developing organisms.

While the brine shrimp model offers many advantages, it is important to acknowledge its limitations. As an invertebrate system, there are significant physiological and developmental differences compared to vertebrates, including humans. Therefore, findings from this model should be interpreted cautiously and considered as preliminary data to inform more targeted studies in higher organisms [14]. Nevertheless, the simplicity and cost-effectiveness of the brine shrimp model make it a valuable tool for initial toxicity screening, particularly for natural products and food additives like bromelain that are often presumed safe based on their long history of use

2. Materials and Methods

2.1. Chemicals and Reagents

Analytical grade chemicals were used throughout the study. Sodium chloride, magnesium chloride, magnesium sulphate, calcium sulphate, potassium sulphate, calcium carbonate, and hydrogen borate were obtained from reputable chemical suppliers. Bromelain, the test compound, was sourced from a commercial supplier and verified for purity and enzymatic activity before use.

2.2. Preparation of Artificial Sea Water

Artificial Sea Water (ASW) was prepared according to established protocols [15]. The composition of ASW included precise amounts of sodium chloride, magnesium chloride, magnesium sulphate, calcium sulphate, potassium sulphate, calcium carbonate, and hydrogen borate. The pH of the ASW was adjusted to 8.0 ± 0.2 using 1M NaOH or HCl as required. The prepared ASW was filtered through a 0.22 μm membrane filter and stored at 4°C until use.

2.3. Hatching of Brine Shrimp

Artemia salina cysts were obtained from a commercial supplier specializing in aquaculture products. The hatching process was optimized based on preliminary experiments comparing hatching efficiency in 5% and 2% NaCl solutions at various pH levels (7.5-8.5). For the main experiments, brine shrimp cysts were incubated in ASW at a density of 1 g/L. The hatching container was maintained at 28 ± 1°C under constant illumination using a 60W lamp. Aeration was provided using an aquarium pump to ensure adequate oxygen supply. After 48 hours of incubation, the newly hatched nauplii were collected using a pipette and transferred to fresh ASW for use in toxicity assays.

2.4. Brine Shrimp Lethality Test (BSLT)

The BSLT was conducted following a modified version of the method described by Meyer et al. [16]. Bromelain stock solutions were prepared in ASW at concentrations ranging from 1 to 800 µg/mL. Each test concentration was prepared in triplicate. Twenty-five newly hatched nauplii (48 hours post-hatching) were transferred to 1.5 mL Eppendorf tubes containing 1 mL of test solution at various bromelain concentrations. Control groups received ASW without bromelain. The tubes were incubated at room temperature (25 ± 2°C) for 24 hours under constant illumination. After the exposure period, the number of dead nauplii in each tube was counted using a stereomicroscope. Nauplii were considered dead if they showed no movement for 10 seconds of observation, even after gentle probing with a pipette tip. The percentage of mortality was calculated for each concentration.

2.5. Morphological Analysis

Morphological assessment of live brine shrimp was conducted immediately after the 24-hour exposure period. Live nauplii from each treatment group were carefully transferred to microscope slides using a pipette. Excess liquid was removed, and the specimens were observed under a binocular microscope equipped with a digital camera (Toupview). Multiple images were captured for each treatment group, focusing on overall body shape, appendage development, and the presence of any visible abnormalities. Particular attention was given to the formation of small vesicles, as these were hypothesized to be indicators of proteolytic activity or cellular stress.

2.6. Growth Measurement

The growth of brine shrimp was assessed by measuring the body length of live nauplii from each treatment group. Images captured during morphological analysis were used for this purpose. The length was measured from the anterior tip of the head to the end of the tail using calibrated image analysis software (ImageJ, NIH). For each treatment group, at least 20 individuals were measured to account for natural variation in growth rates. The mean length and standard deviation were calculated for each concentration of bromelain.

2.7. Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism software (version 8.0). One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to compare the mortality rates, growth measurements, and other quantitative parameters between treatment groups and controls. The dose-response relationship for mortality was analyzed using non-linear regression to determine the LC50 (concentration causing 50% mortality) value. For growth data, a two-way ANOVA was performed to assess the effects of bromelain concentration and exposure time on nauplii length. Pearson's correlation coefficient was calculated to evaluate the relationship between bromelain concentration and growth inhibition. P-values less than 0.05 were considered statistically significant for all analyses

3. Results and Discussion

3.1. Acute Effect of Bromelain on Brine Shrimp Survival

The acute exposure of brine shrimp nauplii to varying concentrations of bromelain resulted in a dose-dependent increase in mortality over 24 hours.

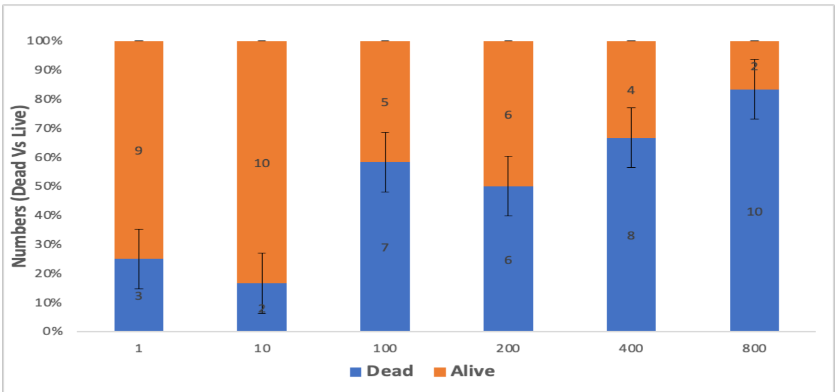


Figure 1: Acute Effect of Bromelain at various concentrations on Brine shrimp numbers (Dead vs Alive) at 24 hours

As shown in Figure 1, the number of dead brine shrimp increased with rising bromelain concentrations. At the lowest concentration of 1 µg/mL, only 2 out of 12 nauplii died, while at the highest concentration of 800 µg/mL, 11 out of 12 nauplii succumbed. This stark contrast in survival rates indicates a potent toxic effect of bromelain at higher concentrations.

The inverse relationship was observed for live brine shrimp, with the number of survivors decreasing as bromelain concentration increased. This pattern suggests a clear dose-response relationship between bromelain exposure and brine shrimp mortality.

3.2. Mortality Rate Analysis

The percentage mortality of brine shrimp exposed to different concentrations of bromelain is presented in Figure 2. A linear relationship was observed between the logarithm of bromelain concentration and the percentage mortality. At 800 µg/mL, the mortality rate reached 91.66%, compared to 13.88% at 1 µg/mL.

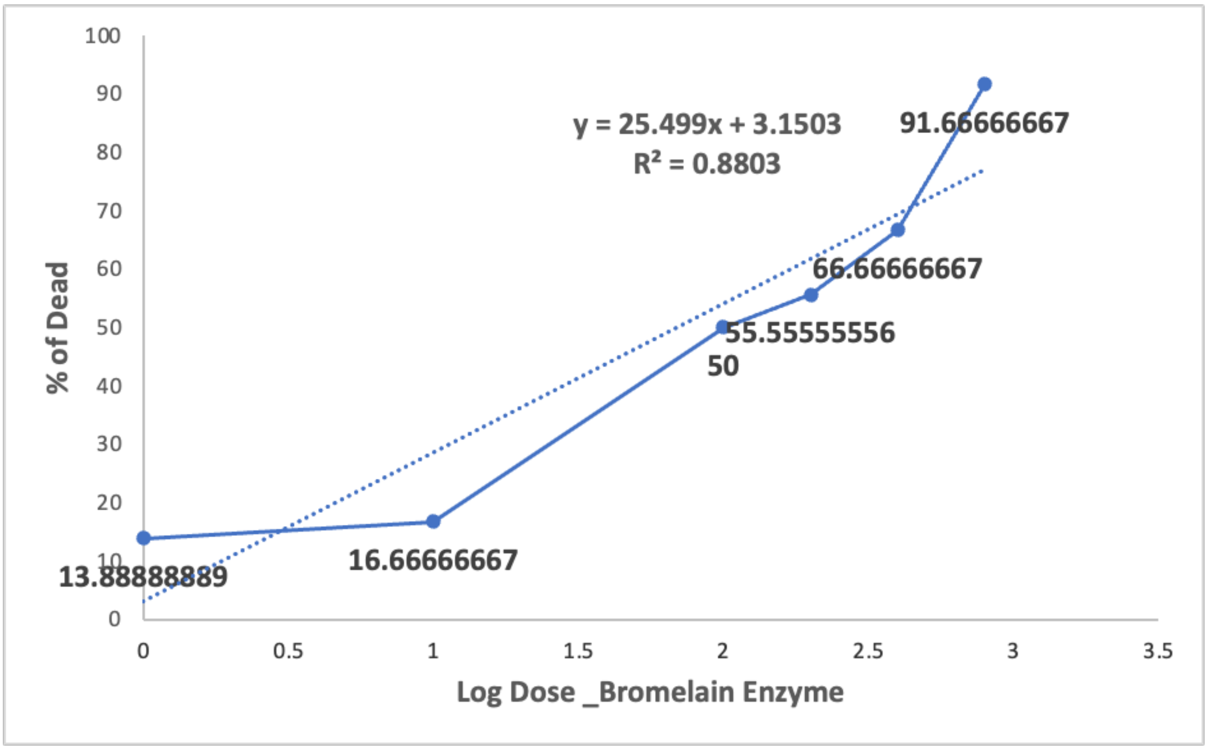


Figure 2: Acute Effect of Bromelain at various concentrations on Brine shrimp Lethality Assay - % of Mortality at 24 hours

Regression analysis of the dose-response curve yielded an R^2 value of 0.8803, indicating a strong correlation between bromelain concentration and mortality rate. The equation of the regression line was found to be $y = 25.499x + 3.1503$, where y represents the percentage mortality and x is the logarithm of bromelain concentration. Based on this analysis, the lethal concentration (LC50) of bromelain was estimated to be approximately 100 µg/mL. This concentration represents the dose at which 50% of the brine shrimp population is expected to die under the given experimental conditions.

3.3. Survival Rate Analysis

Figure 3 shows the percentage of brine shrimp that remained alive after 24 hours of exposure to different concentrations of bromelain. A negative correlation was observed between bromelain concentration and survival rate. At the lowest concentration of 1 µg/mL, 86.11% of the nauplii survived, while at the highest concentration of 800 µg/mL, the survival rate dropped dramatically to less than 10%. This inverse relationship between bromelain concentration and survival rate further corroborates the dose-dependent toxicity of bromelain observed in the mortality analysis.

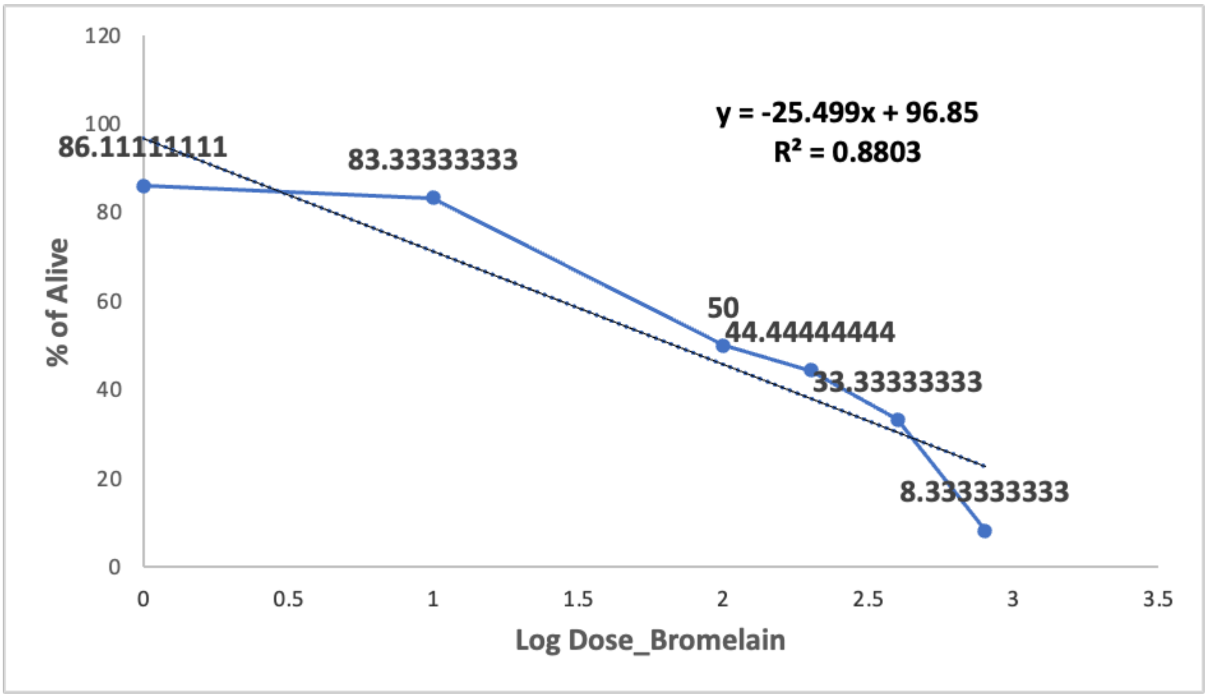


Figure 3. Acute Effect of Bromelain at various concentrations on Brine shrimp Lethality Assay - % of Alive shrimp at 24 hours

3.4. Morphological Analysis

Microscopic examination of the surviving brine shrimp revealed several morphological changes induced by bromelain exposure.

At lower concentrations (1-10 µg/mL), the nauplii appeared largely normal, with no significant differences from the control group. However, as the concentration increased, several abnormalities became apparent:

3.4.1. Formation of small vesicles

At concentrations above 50 µg/mL, small vesicle-like structures were observed on the surface of the nauplii. These vesicles may be indicative of cellular damage or stress response to the proteolytic activity of bromelain.

3.4.2. Developmental retardation

Nauplii exposed to higher concentrations of bromelain (>100 µg/mL) showed signs of developmental delay. Many individuals appeared to be arrested at the Instar II stage, suggesting that bromelain interferes with the molting process or overall development.

3.4.3. Appendage abnormalities

Some nauplii exposed to concentrations above 200 µg/mL exhibited malformed or underdeveloped appendages, particularly the antennae and mandibles.

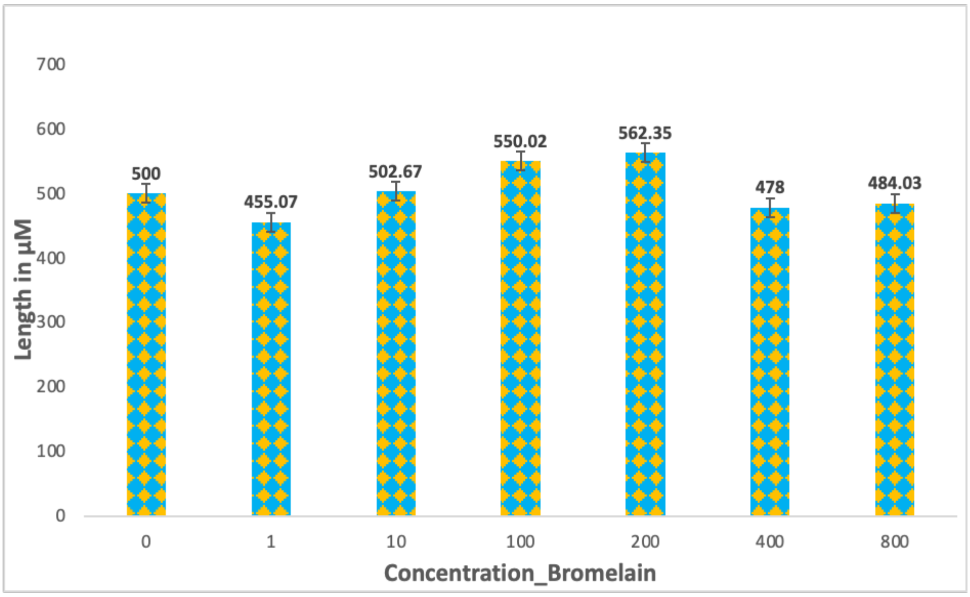
3.4.4. Body shape alterations

At the highest concentrations (400-800 µg/mL), surviving nauplii often showed an altered body shape, appearing more rounded or compressed compared to the elongated form of control specimens.

These morphological changes provide insight into the potential mechanisms of bromelain toxicity. The formation of vesicles and developmental retardation suggest that bromelain may disrupt cellular membranes and interfere with crucial developmental processes, possibly through its proteolytic activity.

3.5. Growth Analysis

Growth measurements of surviving nauplii revealed subtle but significant differences between treatment groups. Control nauplii reached an average length of 500 µm after 24 hours (Figure 4). In contrast, nauplii exposed to bromelain showed slightly reduced growth.



While the differences in growth were not as dramatic as the mortality rates, they nonetheless indicate a dose-dependent effect of bromelain on brine shrimp development. The reduced growth at higher concentrations aligns with the observed developmental retardation in the morphological analysis.

4. Discussion

The results of this study demonstrate that acute exposure to bromelain exhibits significant toxicity to brine shrimp nauplii, affecting both survival and development. The dose-dependent increase in mortality and the calculated LC50 of approximately 100 µg/mL provide a quantitative measure of bromelain's acute toxicity in this model system.

The observed morphological changes, particularly the formation of vesicles and developmental retardation, offer insights into potential mechanisms of bromelain toxicity. The proteolytic nature of bromelain may lead to membrane damage and disruption of crucial developmental processes. This is consistent with previous studies that have shown bromelain can interact with cell surface proteins and affect cellular integrity [17]. The growth analysis, while showing more subtle effects, supports the overall conclusion that bromelain interferes with normal development in brine shrimp. The reduced body length at higher concentrations may be a result of energy diversion towards stress response mechanisms or direct interference with growth-related cellular processes.

It is important to note that while brine shrimp provide a useful model for preliminary toxicity screening, the concentrations used in this study are considerably higher than those typically encountered in therapeutic applications of bromelain. The high LC50 value suggests that bromelain has relatively low acute toxicity, which aligns with its generally recognized as safe (GRAS) status for oral consumption [18]. However, the observed developmental effects raise questions about potential risks during sensitive developmental periods. Further studies using vertebrate models and more chronic exposure scenarios would be valuable to fully assess the developmental safety profile of bromelain.

The brine shrimp model used in this study offers several advantages, including rapid results, cost-effectiveness, and the ability to observe morphological changes in real-time. However, it also has limitations, such as physiological differences from vertebrates and the inability to assess organ-specific toxicity

5. Conclusion

In conclusion, this study provides evidence for the acute toxicity and developmental effects of bromelain on brine shrimp nauplii. While the results cannot be directly extrapolated to human safety, they highlight the need for careful consideration of dosage and exposure timing when using bromelain, particularly in situations where developing organisms might be exposed. Future research should focus on elucidating the specific cellular and molecular mechanisms underlying the observed effects and validating these findings

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