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ASSESSING IN VIVO CYTOTOXICITY: A COMPARATIVE STUDY OF LOCALLY FORMULATED APIARIAN PRODUCTS AND CALCIUM HYDROXIDE

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ABSTRACT:

Introduction: Apiarian products like royal jelly and propolis have gained significant attention in biomedical research due to their diverse chemical composition and reported health benefits.According to literature there have not been enough proven uses of Royal jelly and Propolis in dental studies. Material and Methods: Preparation of test substances was done. Zebra fish was maintained and acclimatised.Experimental groups were divided into 3 with Group A:Control group , Group B;Propolis 155 15mg/ml and Group C:Royal jelly 15% 15mg/ml.All the three groups were observed and assessed by monitoring cytotoxicity with 3 parameters a) Survival rate b)Hatching rate c)Heart rate.Data were collected and given as the mean+_ standard deviation (SD) of triplicates. GraphPad Prism (ver 5.0) was used to do one way ANOVA followed by Tukey Multiple Range Test, with statistical significance levels set at $p< 0.05$ and $p< 0.01$Results: Survival rate was seen higher for Propolis 15% followed by Royal jelly 15% and then the calcium hydroxide when compared with the control group.Hatching rate was seen higher for royal jelly 15% followed by Propolis 15% and then the Calcium hydroxide when compared with the control group. Similarl results were seen for Heart rate where royal jelly 15% showed an increase in heart rate when compared to propolis 15% followed by Calcium hydroxide. **Conclusion**: Royal jelly and Propolis performed better than contemporary pulp capping agents.Survival rate , hatching rate as well as heart rate was higher for Apiarian products compared to Contemporary pulp capping agents.

Keywords: apiarian based products, apitherapy, cytotoxicity , hatching rate,heart rate, indigenous, innovative pulp capping agent , survival rate, zebrafish

1. INTRODUCTION

The field of biomedical research constantly explores natural products for their potential therapeutic properties and their applications in various medical treatments. Apiarian products, derived from bees and beehives, have gained significant attention due to their diverse chemical composition and reported health benefits.(Mizrahi and Lensky, 2013; Jodidio and Schwartz, 2023) One such product is Propolis, a compound commonly found in beehives and known for its antimicrobial and regenerative properties.(Leigh Broadhurst, 2011) However, the safety and cytotoxicity profiles of apiarian products, particularly in comparison to calcium hydroxide, remain to be fully understood.In recent years, calcium hydroxide has been extensively studied for its application in dentistry, particularly in endodontic treatments. It is used as an intracanal medicament due to its ability to disinfect the root canal system and stimulate the formation of new dentin.And used as a pulp capping agent due to its ability to stimulate pulp cells to form

reparative dentin and it's ability to arrest any residual caries progression.(Schröder, 1973)However, as with any medical material, it is crucial to assess the safety of calcium hydroxide, especially regarding its potential cytotoxic effects.(Issarapanichkit, Mahidon and Mahāwitthayālai Mahidon. Faculty of Dentistry, 2003)(Kaur *et al.*, 2015)
Historically, calcium hydroxide has been used most commonly for pulp capping.However, it has certain limitations such as slow healing and tunnel defects.(Schuurs, Gruythuysen and Wesselink, 2000)Apiarian products on other hand have been found to possess anti bacterial, anti inflammatory and regenerative properties making it a promising candidate for use in pulp capping. (Nasri, Jahromi and Aminzadeh, 2022)(Susilowati *et al.*, 2017)(Ioniţă *et al.*, 1990)
Royal jelly is a viscous material which is secreted by by cephalic gland of worker bees. It possesses various pharmacological properties. It can improve wound healing on an exposed dental pulp due to its antioxidant, anti inflammatory, immunomodulatory , antibiotic and tissue regeneration properties.(Leigh Broadhurst, 2011; Susilowati *et al.*, 2017; Botezan *et al.*, 2023)Propolis is a resinous substance obtained from beehives that has anti fungal, anti bacterial, Anti viral, anti tumor and anti inflammatory activity.(Leigh Broadhurst, 2011)
To address this gap in knowledge, we propose a study utilizing the Zebrafish (Danio rerio) model to evaluate and compare the cytotoxic effects of apiarian products with those of calcium hydroxide. Zebrafish have emerged as a powerful model organism in biomedical research, offering several advantages such as genetic similarity to humans, rapid development, and transparent embryos, which facilitate the visualization of internal structures and processes.(Amatruda *et al.*, 2023) They have been widely employed to investigate the cytotoxicity and biological effects of various substances, including pharmaceutical compounds and biomaterials. (McGrath, 2012)(Malta *et al.*, 2022)(Chokkattu *et al.*, 2022)(Ajay Guru *et al.*, 2023)

AIM

The aim of this study is to provide insights into the potential cytotoxicity of apiarian products and assess their comparative effects with calcium hydroxide.Comparing their cytotoxicity to that of calcium hydroxide, a well-studied substance with known regenerative capabilities, will allow for a comprehensive assessment of their safety profiles. Objectives of the study was to assess developmental toxicity with 3 parameters a) Mortality rate b)Hatching rate and c)Heart rate

By exposing zebrafish embryos and larvae to different concentrations of apiarian products and calcium hydroxide, we can analyze the morphological changes, organ development, and cellular responses to evaluate their cytotoxic effects. (McGrath, 2012)(Thorat *et al.*, 2024)This evaluation will involve assessing parameters such as embryonic mortality, malformations, developmental delays, and alterations in cell viability. Additionally, the zebrafish model will enable us to investigate potential underlying mechanisms of cytotoxicity through gene expression analysis and also by histopathological examinations.(Rajeshkumar *et al.*, 2022)

The results of this study will contribute to the existing knowledge on the cytotoxicity of apiarian products, providing a comparative analysis with calcium hydroxide. Understanding the safety profiles of these natural products is crucial for their potential applications in various biomedical fields, including dentistry, wound healing, and regenerative medicine. Furthermore, the findings may guide the formulation of safe and effective therapeutic approaches utilizing apiarian products or their derivatives.

2. MATERIAL AND METHODS

Origin and maintenance of Zebrafish

Complying with the ethical standards and regulations NSK aquarium in Kolathur, Tamil Nadu, sold adult zebrafish (wild type -AB strain, 6 months old). Male and female were separated and kept in our facility in a 10litre glass tank at 28.5 degrees Celsius with a 14/10h light and dark cycle. The fish were given live brain prawns (*Artemia salina*) three times per day. Fish were acclimatised for one month before being used for breeding, and embryos were collected and used in subsequent tests. Unfertilized embryos are discarded, whereas fertilised embryos are transferred to a 6 well plate and cultured in E3 medium.(B S *et al.*, 2023)

Royal jelly 50gm and Crude Propolis 250gms was obtained from Qeenbees Pvt Ltd ,Chennai. Preparation of test solutions i.e Crude Propolis 15%, Royal jelly 15% , calcium hydroxide (Dycal) by diluting to obtain different concentrations for testing was done.

Zebrafish toxicity test

For the toxicity test, 4hpf embryos were employed,and exposure was performed in a 6-well plate containing either E3 medium alone as a control or WL15 peptide at various concentrations: 10, 20, 30, 40, and 50 micrometre.With 3ml of E3 medium, approximately 15 embryos were used per well.Throughout the exposure period (4hpf to 96hpf),the exposure was non-static and was replenished with a new treatment solution every 24 hours. All experiments were carried out in duplicate. Hatching rate, heart rate, and deformities were all measured throughout this time, and the results were published at the end of 96 hpf.On the zebra fish model, three parameters were evaluated. They are as follows: 1) Mortality rate 2)Rate of spawning 3)The probability of survival. After exposure, a viability stain, trypan blue, was employed to determine viability. Data were collected and given as the mean+_ standard deviation (SD) of triplicates. GraphPad Prism (ver 5.0) was used to do one way ANOVA followed by Tukey Multiple Range Test, with statistical significance levels set at $p< 0.05$ and $p< 0.01$. (Guru *et al.*, 2021)

3. RESULTS

In zebrafish larvae (96hpf), all 15mg/ml test solution concentrations did not cause death. (Figure 1; Table 1) At 48hpf, the hatching rate was also calculated. In the control group, 100% of the zebrafish embryos emerged from their chorions and had no effect on the hatching rate. (Figure 2 and Table 2) To test cardiotoxicity, the heart rate of Zebra fish embryos was calculated at 72 hpf. For 1 minute, atrial and ventricular contractions were counted and recorded under a microscope, as were average heart beats per minute. Similarly, 15mg/ml doses had no effect on the heart rate of zebrafish larvae when compared to the control group.(Figure 3 Table 3)

Figure 1, Figure 2, Figure 3

4. DISCUSSION

Calcium hydroxide often used in pulp capping may exhibit cytotoxic effects that may need careful evaluation.Propolis and Royal jelly known for their natural compounds, raise intriguing possibilities for biomedical applications. Antimicrobial and anti-inflammatory effects are found in propolis. (Del Carpio-Perochena *et al.*, 2017)Propolis contains caffeic acid and flavonoids, which reduce the inflammatory response and block the conversion of arachidonic acid to prostaglandins and leukotrienes by inhibiting the lipoxygenase and cyclooxygenase enzymes. Propolis also improves immune system function by inducing phagocytic activity and cell immunity. Because numerous enzyme systems contained in propolis contribute in cell

metabolism cycles and collagen synthesis, it aids in the creation of rigid tissue barriers.(*A Remarkable Hive Product, Propolis: Scientific Data and Suggestions Concerning Its Composition, Properties and Possible Use in Therapeutics*, 1978)(Khurshid *et al.*, 2017)Studies by Lemos et al conducted a study to evaluate cytotoxicity of propolis extract on dental pulp stem cells. Our study results corroborate with the same study. Similarly studies by Widjiastuti et al show less cytotoxicity for Propolis,where he investigated the cytotoxicity test of calcium hydroxide, propolis and calcium hydroxide- propolis combination in human pulp fibroblast.(Widjiastuti *et al.*, 2020)

The Zebra Fish is named because the five uniform, pigmented, horizontal, blue stripes on the side of the body that resemble zebra stripes and extend to the caudal fin.A model organism is a nonhuman species that has been extensively researched, typically because it is simple to keep and breed in a laboratory setting and offers unique experimental advantages.(Bhandari, Bharani and Khurana, 2022)(Holden, Layfield and Matthews, 2013)Valenti et al utilized zebra fish embryo to evaluate cytotoxicity of dental materials. (Valenti *et al.*, 2020)(Francis *et al.*, 2020)Cytotoxicity is caused by the continuous release of calcium hydroxide.Because of the elevated pH, calcium hydroxide produces a necrotic layer.The neighbouring pulp tissue is in charge of pulp healing and the creation of dentin bridges.(Koike *et al.*, 2014)(Schröder, 1973; Schuurs, Gruythuysen and Wesselink, 2000; Valenti *et al.*, 2020)

According to our studies they will contribute to the existing knowledge on the cytotoxicity of apiarian products, providing a comparative analysis with calcium hydroxide. Understanding the safety profiles of these natural products is crucial for their potential applications in various biomedical fields, including dentistry, wound healing, and regenerative medicine. Furthermore, the findings may guide the formulation of safe and effective therapeutic approaches utilizing apiarian products or their derivatives.

In our investigations, the hatching rate and heart rate were found to be normal for royal jelly and propolis when compared to calcium hydroxide, implying that royal jelly and propolis are less cytotoxic than calcium hydroxide. Similarly, when it came to survival rates, propolis and royal jelly outperformed calcium hydroxide and can be regarded less cytotoxic. Future scope can include clinical trials to assess the microbial load in comparison with calcium hydroxide.

The evaluation of factors such as mortality, heart rate, and hatching rate revealed no harm. The heart is one of the essential organs that form and function throughout the development of a zebrafish embryo. Zebrafish larvae have so evolved into a model for studying congenital heart disease, cardiac function, and cardiotoxicity. (Garapati *et al.*, 2022)Propolis and royal jelly had no toxicological reaction in zebrafish larvae.

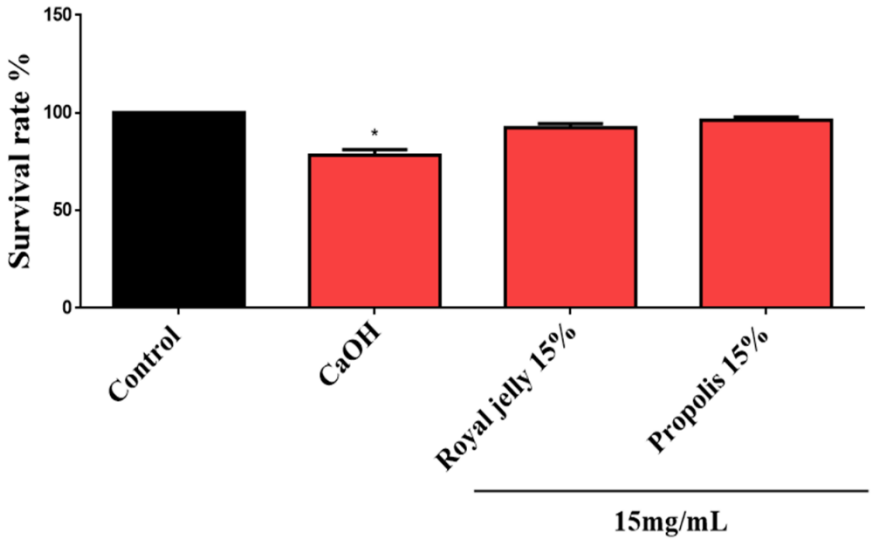

Figure 1: Survival rate in %

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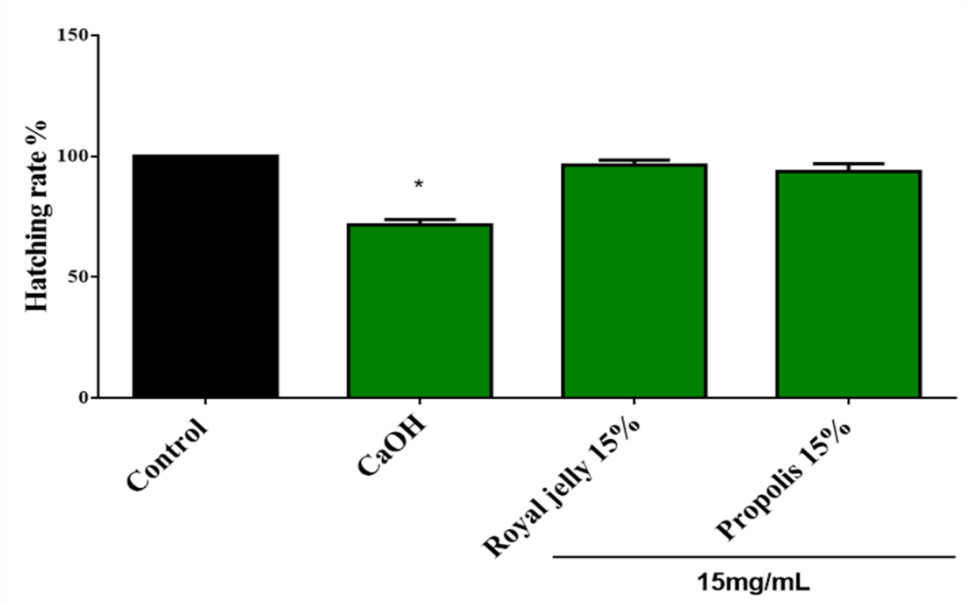
CaOH	Calcium hydroxide

Table 1: Survival rate in %

	Control	Calcium hydroxide	Royal jelly 15%	Propolis 15%
Survival rate in %	100	70	80	90

Graph 2: Hatching rate in %

Graph 2 shows the Hatching rate in %



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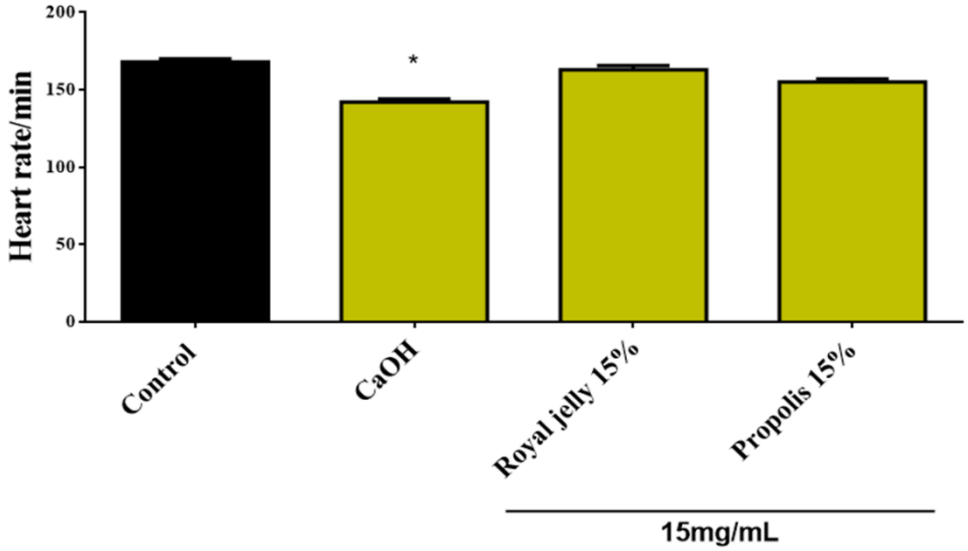
CaOH	Calcium hydroxide

Table 2: Hatching rate in %

	Control	Calcium hydroxide	Royal jelly 15%	Propolis 15%
Hatching rate in %	100	60	90	80

Graph 3 Heart rate in /min
Graph 3 shows the Heart rate in /min



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CaOH	Calcium hydroxide

Table 3 Heart rate /min

	Control	Calcium hydroxide	Royal jelly 15%	Propolis 15%
Heart rate in %	170	140	160	150

5. CONCLUSION

Royal Jelly and Propolis performed better than contemporary pulp capping agent and proved less cytotoxic. Survival rate for Propolis and Royal jelly was normal compared with calcium hydroxide. Hatching rate for Royal jelly and Propolis was normal compared to Calcium hydroxide. Heart rate also showed similar results where Propolis and Royal jelly had a similar heart rate whereas it was less for calcium hydroxide.
In conclusion, this study utilizing zebrafish as a model organism aims to shed light on the cytotoxicity of apiarian products and compare their effects to calcium hydroxide. The findings have the potential to expand our understanding of the safety and potential applications of apiarian products, ultimately contributing to the development of novel therapeutic strategies in biomedical research.
Conflicts of interest: No conflicts of interests
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