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Biocompatibility assessment of chlorhexidine gluconate versus a natural mouthwash containing *Olea europaea* and *Opuntia ficus-indica* in zebrafish embryos

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Abstract

Various chemical solutions are available for oral hygiene and care, but these chemical solutions also cause various side effects in living systems. This study aimed to assess biocompatibility of a conventional chlorhexidine gluconate-based mouthwash versus a natural formulation containing *Olea europaea* leaf and *Opuntia ficus-indica* extracts using the zebrafish embryo model. Zebrafish embryos were exposed to two concentrations (100 ppm and 1000 ppm) of each mouthwash for 72 hours post-fertilization. Developmental properties such as mortality, hatching rate, pericardial edema, and body length were evaluated. When these parameters compared to the control group, mortality rate significantly increased in 1000 ppm Chlorhexidine (CHX) and 100 ppm CHX groups at 24, 48 and 72 hpf ($p<0,05$) while body length significantly decreased in 1000 ppm CHX and 100 ppm CHX groups compared to the control group ($p<0,05$). Biochemical analyses included oxidative stress parameters and acetylcholinesterase (AChE) activity. Biochemical analyses showed that levels of lipid peroxidation were significantly increased in both 1000 ppm and 100 ppm CHX groups compared to the control group ($p<0,0001$; $p<0,001$). Superoxide dismutase (SOD) activity decreased significantly in the 100 ppm CHX group compared to the control group ($p <0,01$). Glutathione S-transferase (GST) activity decreased in the 100 ppm CHX group compared to the control ($p<0,05$) and acetylcholinesterase (AChE) activity was reduced in the 1000 ppm CHX and 100 ppm CHX groups compared to the control group ($p <0,001$; $p <0,0001$). These findings provide evidence regarding the differential biocompatibility of synthetic and plant-based mouthwashes in early developmental models.

Keywords: Chlorhexidine, mouthwash, *Olea europaea*, *Opuntia ficus-indica*, oxidative stress, zebrafish embryos

1. Introduction

Maintaining optimal oral hygiene is essential for preventing a wide range of dental and periodontal diseases. In addition to mechanical plaque control methods such as toothbrushing and flossing, antimicrobial mouthwashes are commonly used for reduce microbial load and improve gingival health [1]. Therapeutic mouthwashes often contain active chemical agents such as chlorhexidine gluconate, exhibit broad-spectrum antimicrobial activity and clinical potency in reducing oral biofilm [2]. Despite these properties, increasing attention has been directed toward the biocompatibility and potential toxicity of this chemical agent, especially when used regularly or over extended periods. Chlorhexidine gluconate mouthwashes have

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been linked to several side effects, including discoloration of teeth and tongue, alterations in taste sensation, and mild irritation accompanied by superficial desquamation of the oral mucosa. These side effects can reduce patient compliance and limit the overall acceptance of chlorhexidine gluconate containing mouthwashes [3–8]. Due to these side effects of chemical structures, there has been an increased interest in alternative oral care products with high biocompatibility. *Olea europaea* (olive) leaf extract and *Opuntia ficus-indica* (prickly pear cactus) have emerged as promising candidates due to their antioxidant, anti-inflammatory, and antimicrobial properties [9–12].

Olea europaea (olive) leaf have been widely used in folk medicine over centuries and have been employed in traditional remedies across various Mediterranean countries [13]. Extracts derived from these leaves have been incorporated into human diets and are known to contain a range of bioactive compounds that may exert beneficial effects such as lowering blood glucose and cholesterol levels, reducing blood pressure, modulating inflammation, and providing antioxidant and antiatherogenic properties [14,15]. *Olea europaea* leaf extract has also been evaluated for its potential use in oral health care. Clinical studies have demonstrated its effectiveness as a mouthwash in reducing gingival inflammation, alleviating chemotherapy-induced oral mucositis, and managing aphthous ulcers, with results comparable to standard treatments such as chlorhexidine and dexamethasone [16,17,9].

Opuntia ficus-indica (prickly pear nopal cactus) a member of the Cactaceae family, possesses significant medicinal potential due to its rich content of bioactive compounds. These include phenolic substances and flavonoids, such as kaempferol and quercetin, which are well-recognized for their antioxidant properties [18–20].

Zebrafish, a freshwater species commonly found in natural habitats and rice fields, were initially utilized as a model organism for embryological and developmental research in the 1930s. Over time, they have become one of the leading model organisms in developmental biology due to notable genetic similarities between the zebrafish and human genomes [21,22]. Their external fertilization and transparent embryos make them particularly suitable for real-time observation in toxicological assessments [22]. Due to all these advantages, in recent years zebrafish embryos has been used as a model organism in the field of dentistry. There are studies about the ingredients of toothpastes, such as fluoride [23], sodium lauryl sulphate [24,25], triclosan [26], sodium benzoate [27], calcium carbonate nanoparticles [28], titanium dioxide [23], silica nanoparticles [29], and ginger in herbal toothpastes [30]. Furthermore, there are researches about dental materials, including Bisphenol A-glycidyl-methacrylate (Bis-GMA) [31], bioceramics [32], and porcelain-to-metal fused crowns [33]. In a different context, zebrafish have also been used in oral radiology studies. A previous study investigated the effects of panoramic X-rays on zebrafish embryos [34], while another recent study examined the potential effects of low-dose diagnostic dental X-rays [35]. Moreover, zebrafish have been used in the field of periodontology to examine the effects of products, for instance gingipain [36]. As a result, zebrafish are frequently chosen in biocompatibility studies due to their rapid reproduction ability and easy adaptation to laboratory conditions. Addtionally they allow monitoring of embryonic development and have high genetic similarity with humans [37].

The aim of this study was to investigate the effects of a conventional chlorhexidine gluconate-based mouthwash and a natural formulation containing *Olea europaea* and *Opuntia ficus-indica* extracts on zebrafish embryos. To this end, oxidant and antioxidant parameters, morphological examinations, mortality and hatching assessments were conducted in zebrafish embryos exposed to chlorhexidine and the natural formulation solution to obtain data on the biocompatibility of plant-based mouthwashes in oral care applications.

2. Material and methods

2.1. Maintenance of zebrafish

Wild-type AB/AB strain zebrafish were maintained under apparently disease-free conditions. Animal husbandry and spawning were performed in accordance with the relevant guidelines and regulations and the protocols approved by the University of Marmara Institutional Animal Care and Use Committee. Fish were kept in an aquarium rack system (Zebtec, Tecniplast, Italy) at 27 ± 1 °C under a light/dark cycle of 14/10-hour and they were fed with commercial flake fish food complemented with live Artemia twice a day. The pH of system water ranges from 6.9 to 7.2. Reverse osmosis water that contains 0.018 mg L^{-1} Instant Ocean™ salt was used for all experiments. After natural spawnings, fertilized embryos were gathered and staged according to their developmental and morphology as described before and were maintained in E3 medium [38].

2.2. Embryo exposure

Two different types of mouthwash were selected according to their different ingredients, Natural solution (NS) and Chlorhexidine (CHX). In this study, the LC50 value was not determined. We conducted experiments with the references we obtained from the literature [3,39,40] and selected doses that were morphologically

close to the control group for both groups. The ingredients of NS and CHX are given in Table 1. Zebrafish embryos were exposed to two different doses, 1000 ppm and 100 ppm at 0-2 hpf (hours post-fertilization) for both solutions and experimental groups were formed (Control, NS 1000 ppm, NS 100 ppm, CHX 1000 ppm, and CHX 100 ppm) (sample size (n): 100 in all groups).

Embryo medium (E3 medium; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) was used as the blank control. Every 24 h solutions were replaced and dead embryos were discarded. The development of embryos were monitored and documented daily under a stereomicroscope and malformations were recorded during the exposure period (Zeiss Discovery V8, Gottingen, Germany) [38]. Embryonic mortality and hatching rate were noted on each day. At the end of 72 hours post-fertilisation (hpf) the exposure period ended and the zebrafish embryos were washed several times using distilled water. The study investigated the effects of exposure to oral care solutions during the embryonic period. Therefore, exposure was initiated between 0-2 hpf after obtaining the embryos and was terminated at 72 hpf. Considering the timeframe considered as the embryonic period up to 120 hpf, it was possible to continue the study until 120 hpf. This is one of the limitations of our study. Since the zebrafish embryos used in this study were less than 5 days old, ethical approval was not required for this study in accordance with the protocol specified by [41].

Table 1. Ingredients of the mouthwashes

Mouthwashes	Ingredients
Natural Solution (NS)	Aqua, glycerin, xylitol, polysorbate 20, sorbitol, phenylpropanol, caprylyl glycol, *Opuntia ficusıindica* extract, *Olea Europaea* extract, maltodextrin
Chlorhexidine (CHX)	Chlorhexidine gluconate, benzydamine hydrochloride, polysorbate 20, sorbitol (70%), propylene glycol, peppermint essential oil, sucralose, ecocool MP (flavor), quinoline yellow, patent V blue, citric acid monohydrate, sodium citrate dihydrate, purified water

2.3. Body length

Body length of the zebrafish embryos were measured in millimeters at the end of 72 hpf. Digital images of zebrafish embriyos and micrometer scale were captured using a stereomicroscope (Zeiss Discovery V8, Gottingen, Germany). Body length was measured from the mouth tip to the tail base and along the body axis utilizing digital images and Kinovea software (0.8.15 version).

2.4. Biochemical assays

At the end of 72 hpf, exposures were terminated, and 3 biological replicates of pools of zebrafish embryos (100 embryos/pool; 3 biological replicates for each group) were homogenized in a tissue lyser for analysis of biochemical parameters. The supernatant, separated by centrifugation, was then aliquoted and stored at -20°C until the experiment was performed.

2.4.1. *Total protein assay*

Total protein levels of the samples were measured by Lowry method [42]. In this method, proteins first react with copper ions in an alkali medium and then they are reduced by Folin reagent. The absorbances are determined at 500 nm. The total protein levels were calculated and used to present the results per protein.

2.4.2. *Lipid peroxidation (LPO) assay*

Yagi's method was applied to evaluate LPO in the embryos. Malondialdehyde (MDA) is the product of LPO reacts with thiobarbituric acid (TBA) and the absorbance of the resulting pinkish color is determined at 532 nm spectrophotometrically using $1.56\times10^5\ M^{-1}\ cm^{-1}$ as the extinction coeffcient. LPO is expressed in the form of MDA equivalents as nmol MDA/mg protein [43].

2.4.3. *Glutathione-s-transferase (GST) assay*

The activity of glutathione-S-transferase (GST) was determined spectrophotometrically at 340 nm and the absorbance of the product formed by GSH and 1-chloro-2,4-dinitro-benzenin (CDNB) conjugation was evaluated [44].

2.4.4. *Superoxide dismutase (SOD) assay*

Superoxide dismutase (SOD) activity was determined using the method based on the ability of SOD to increase the effect of riboflavin-sensitized photo-oxidation of o-dianisidine. Superoxide activity is produced by illuminating the reaction mixture containing O-dianisidine dihydrochloride and riboflavin by the light of a fluorescent lamp. The oxidation of O-dianisidine is sensitized by riboflavin and enhanced by SOD and the increase is linearly dependent on the concentration of SOD. Absorbances at 0 and 8th min of the illumination were measured using a spectrophotometer at 460 nm and the net absorbances were calculated. The results were expressed as U/mg protein [45].

2.4.5. *Acetylcholinesterase (AChE) assay*

The acetylcholine esterase activity of the samples was measured by Ellman method [46]. Acetylcholine esterase produces thiocholine, which combines with 5.5′-dithiobis (2-nitrobenzoic acid) to produce a yellow hue. The resulting yellow color was measured at 412 nm using a spectrophotometer, and the absorbance was determined, and AChE activity was calculated.

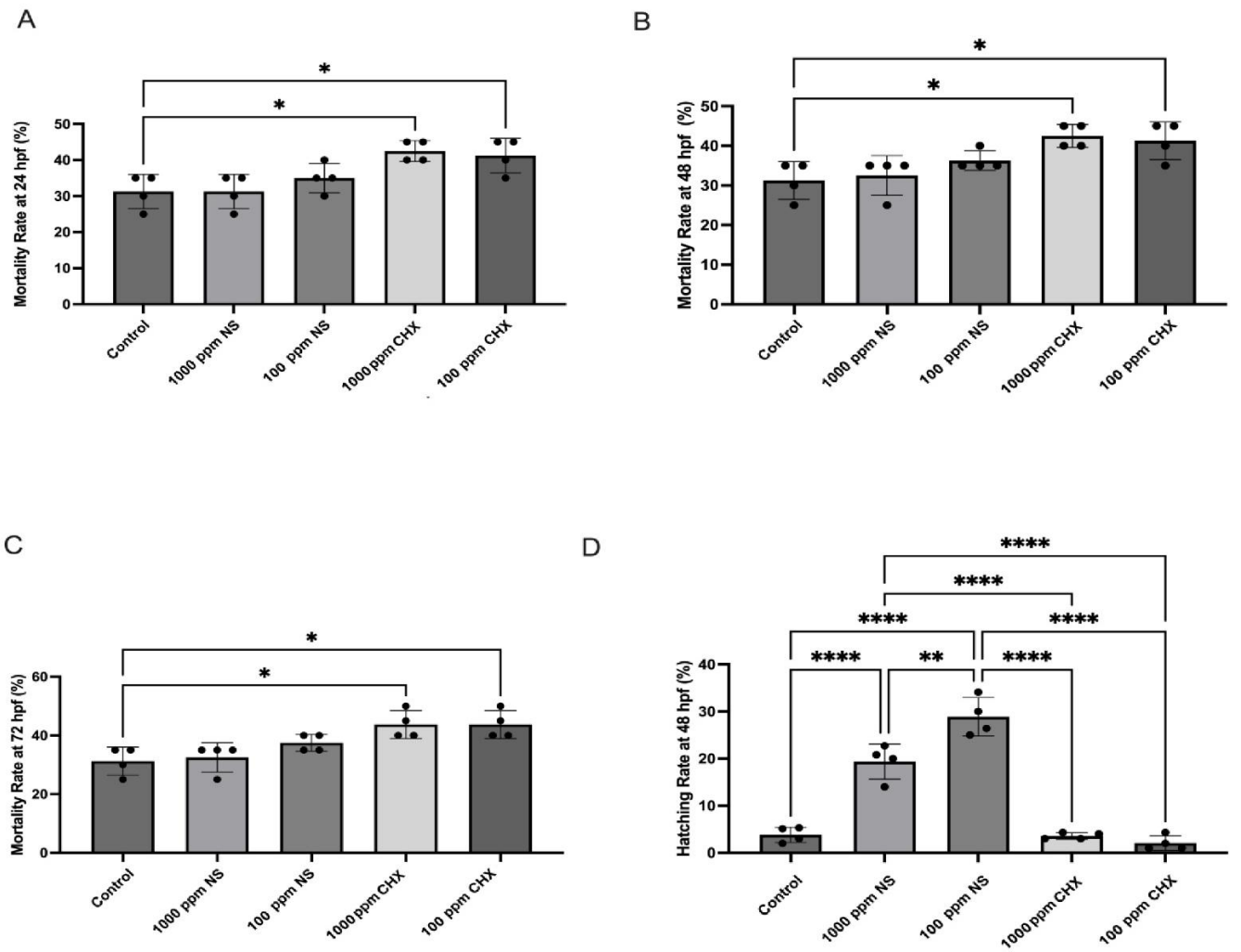

Figure 1. Embryonic mortality rate at 24, 48, 72 hpf and hatching rate at 48 hpf of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. Sample size (n): 100 in all groups. NS: Natural Solution, CHX: Chlorhexidine.
*p<0,05; **p<0,01; ***p<0,001; ****p<0,0001

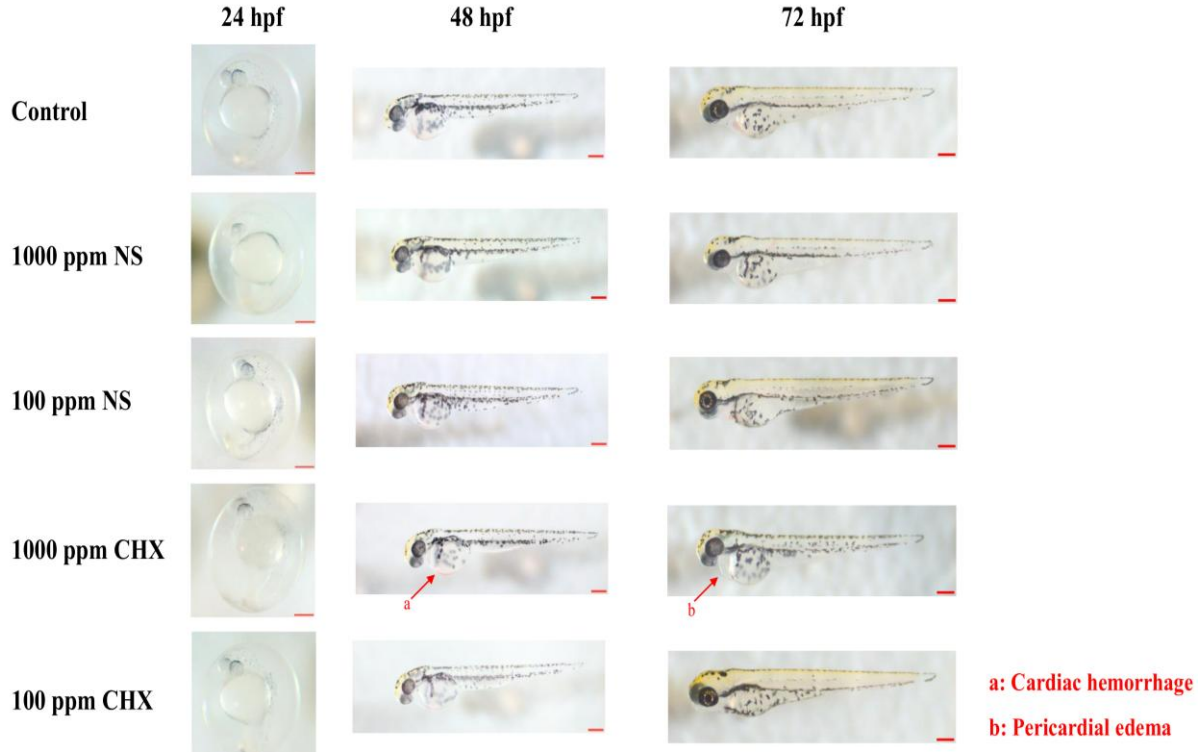

Figure 2. Representative images of the zebrafish embryos in control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. NS: Natural Solution, CHX: Chlorhexidine.

2.5. Statistical analysis

The effects of NS and CHX on zebrafish embryos were assessed using one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used as the post-hoc test for comparisons. Statistical analysis was performed using GraphPad Prism 10, and $p<0.05$ was considered significant.

3. Results

3.1. Embryonic mortality rate and hatching rate

Embryonic mortality rate at 24, 48, 72 hpf and hatching rate at 48 hpf of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups are presented in Fig. 1. Compared to the control group mortality rate significantly increased in 1000 ppm CHX and 100 ppm CHX groups at 24, 48 and 72 hpf (p<0,05). Hatching rate of the 1000 ppm and 100 ppm NS groups increased significantly compared to the control, 1000 ppm CHX, and 100 ppm CHX groups (p<0,0001). Moreover hatching rate of the 100 ppm NS group was significantly higher than the 1000 ppm NS group (p<0,01).

3.2. Results of developmental analyses

The representative images of the zebrafish embryos are given in Fig. 2. Cardiac hemorrhage and pericardial edema were observed in 1000 ppm CHX group at 48 and 72 hpf.

3.3. Results of body length

Body length of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups were measured by Kinovea software (0.8.15 version) at 72 hpf (Fig. 3). Body length significantly decreased in 1000 ppm CHX and 100 ppm CHX groups compared to the control group (p<0,05). However, there was no significant change in the body lengths of the NS groups compared to the control group.

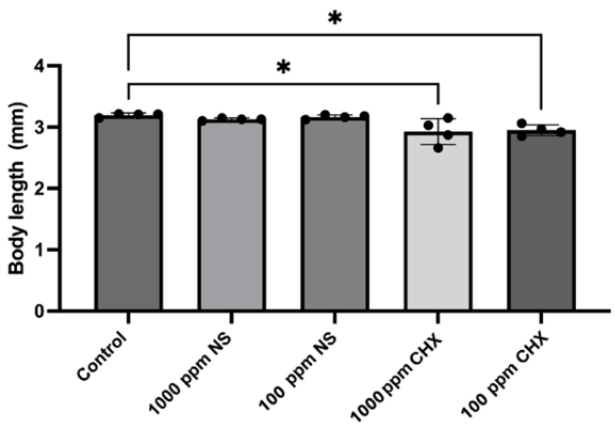

Figure 3. Body length of control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. Sample size (n): 10 in all groups. NS: Natural Solution, CHX: Chlorhexidine. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001

3.4. Results of oxidant-antioxidant analyses

Fig. 4(A) shows the outcomes of lipid peroxidation analyses for the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. Malondialdehyde (MDA) concentrations were significantly increased in the 1000 ppm CHX and 100 ppm CHX groups compared to the control group (p<0,0001; p<0,001 respectively). MDA concentrations of the 1000 ppm CHX group were significantly higher when compared to the 1000 ppm NS and 100 ppm NS groups (p<0,0001). Also MDA concentrations of the 100 ppm CHX group were significantly higher when compared to the 1000 ppm NS and 100 ppm NS (p<0,0001).

The results of SOD activity of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups are shown in Fig. 4(B). SOD activity were significantly decreased in the 100 ppm CHX group compared to the control group (p<0,01) and SOD activity were significantly increased in the 100 ppm NS group compared to the control group (p<0,05). Compared to the 100 ppm NS group SOD activity was significantly decreased in the 1000 ppm CHX and 100 ppm CHX groups (p<0,01; p<0,0001 respectively). SOD activity was significantly lower in the 100 ppm CHX group than in the 1000 ppm CHX groups (p<0,05). On the other hand, SOD activity in the 1000 ppm NS group was significantly higher than in 100 ppm CHX group (p<0,01).

Fig. 4(C) shows the outcomes of GST activity for the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. GST activity was significantly decreased in the 100 ppm CHX groups compared to the control group (p<0,05). GST activity of the 1000 ppm CHX group was significantly higher when compared to the 100 ppm NS and 100 ppm CHX group (p<0,05; p<0,01 respectively).

The results of Acetylcholine esterase (AChE) analyses of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups are shown in Fig. 4(D). AChE activity was significantly decreased in the, 1000 ppm CHX and 100 ppm CHX groups groups compared to the control group (p<0,001; p<0,0001 respectively). AChE activity in the 1000 ppm NS group was significantly higher compare to 1000 ppm CHX and 100 ppm CHX groups (p<0,01). AChE activity of the 100 ppm NS group was significantly higher compared to 1000 ppm CHX and 100 ppm CHX groups (p<0,01).

4. Discussion

Every dental material intended for use in the oral cavity is tested according to criteria established by regulatory and professional bodies such as the American Dental Association (ADA), the Food and Drug Administration

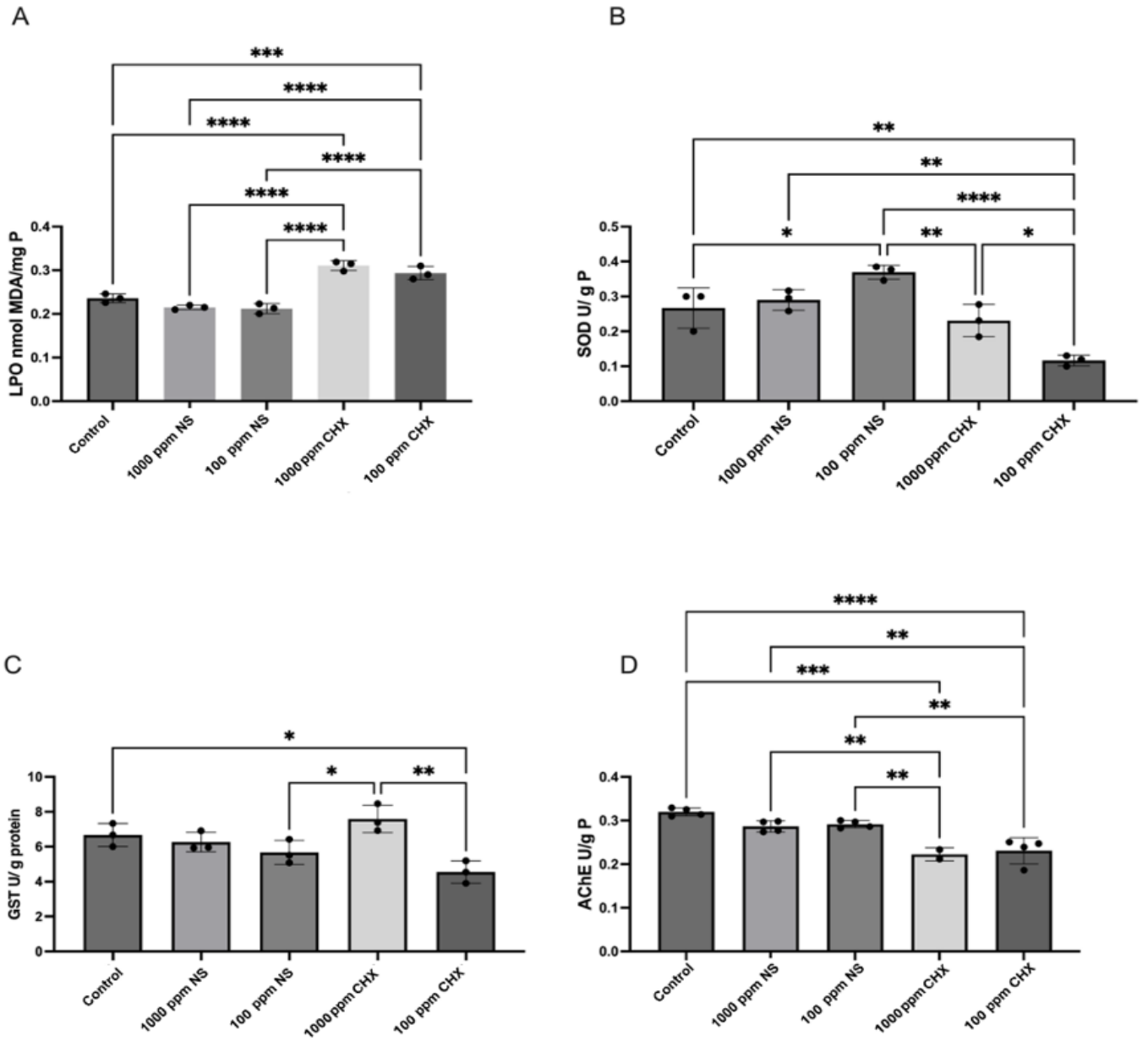

Figure 4. (A) Malondialdehyde (MDA) levels as an index of lipid peroxidation (LPO); (B) Superoxide dismutase (SOD) activities; (C) Glutathione S-transferase (GST) activities; (D) Acetylcholine esterase activities (AChE) of the control, 1000 ppm NS, 100 ppm NS, 1000 ppm CHX and 100 ppm CHX groups. Sample size (n): 100 individuals per pool in all groups.
NS: Natural Solution, CHX: Chlorhexidine. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001

(FDA), and the International Organization for Standardization (ISO) to regulate the safety evaluation of dental materials. [47].

The literature includes studies on the *in vivo* and *in vitro* analyses of various solutions intended for oral hygiene and care. While in vitro cell culture methods are commonly utilized to screen for cytotoxicity, the zebrafish embryo model has emerged as a viable in vivo alternative to conventional animal testing [38]. Our study aimed to compare the effects of a chlorhexidine gluconate-containing mouthwash and a natural solution formulated with *Olea europaea* and *Opuntia ficus-indica* extracts on zebrafish embryos.

Chlorhexidine (CHX) is a cationic bisbiguanide compound with broad-spectrum antimicrobial activity. However, CHX, though widely used for its antimicrobial properties, presents several significant biological disadvantages. *In vivo* studies have shown that chlorhexidine gluconate can cause epithelial disruption and inflammatory infiltration in oral tissues, raising concerns about its biocompatibility with mucosal surfaces [3]. Moreover, neurodevelopmental toxicity has been demonstrated in zebrafish larvae, where chlorhexidine gluconate exposure disrupted oligodendrocyte maturation and delayed myelination, leading to locomotor deficits during early development [39]. From a different perspective, chlorhexidine gluconate is classified as an FDA Category B agent, indicating that animal studies have not demonstrated fetal harm; however, in the absence of adequate and well-controlled studies in pregnant women, potential risks to the human fetus cannot be ruled out. Therefore,

its use during pregnancy should be limited to clearly justified indications [49,50].

As a result of these concerns regarding tolerability and developmental toxicity, alternative mouthwash formulations with improved safety profiles are of increasing interest. *Olea europaea* leaf extract has emerged as a promising candidate due to its anti-inflammatory and antioxidant properties. Clinical studies have reported that *Olea europaea*-based formulations may reduce gingival inflammation and systemic inflammatory biomarkers such as C-reactive protein and alkaline phosphatase [9]. Similarly, *Opuntia ficus-indica*, a phytochemically rich plant containing flavonoids, polyphenols, and betalains, has been shown to exhibit anti-inflammatory and antioxidative effects, contributing to tissue protection under oxidative stress conditions [10,51]. These characteristics suggest that both *Olea europaea* and *Opuntia ficus-indica* may serve as more biocompatible ingredients in oral care products, especially in contexts where minimizing systemic or developmental toxicity is a priority.

LPO serves as a marker of increased free radical levels. Its elevation is a critical indication of cell membrane damage, which could be initiated by the administration of chlorhexidine [52]. Consistent with our study, Newton et al. (2004) demonstrated that the LPO level increased in vitro, as chlorhexidine gluconate promotes Fe^{2+} release from human ferritin, triggering iron-dependent lipid peroxidation and mitochondrial dysfunction. These findings suggest that chlorhexidine may exert pro-oxidant effects through metal ion interaction and mitochondrial destabilization. Similarly, in another study, chlorhexidine exposure was associated with increased reactive oxygen species (ROS) generation and enhanced LPO, which is in line with our findings [53]. Although no direct studies have evaluated our specific formulation, several investigations have demonstrated that *Olea europaea* and *Opuntia ficus-indica*, which are among the constituents of the natural solution used in our study, exhibit antioxidant properties and contribute to the inhibition of LPO [12,54]. Similarly, no statistically significant difference was observed in LPO levels of the embryos exposed to the natural solution group and the control group in the present study. It is also important to note that certain model organisms, such as zebrafish, can rapidly activate endogenous defense systems, such as superoxide dismutase and glutathione peroxidase, upon exposure to mild oxidative stress, which may buffer measurable increases in LPO [55].

In addition to increased oxidative stress parameters, in our study, a decrease in SOD activities, which are anti-oxidant defense system components, was detected in embryos given chlorhexidine. In addition to increased oxidative stress markers, in our study, a decrease in SOD activity was detected in embryos given 100 ppm CHX. This observation is consistent with previous *in vivo* findings, where CHX exposure resulted in reduced SOD expression, indicating a potential suppression of endogenous antioxidant mechanisms during tissue response [56]. The decrease in SOD activity in CHX-exposed embryos may lead to depletion of the activity of SOD, the first defense enzyme of the antioxidant system, resulting from excessive ROS production, and this may be associated with mitochondrial membrane instability. [52,57].

In contrast to chlorhexidine, several studies have reported that *Olea europaea* leaf extracts enhance SOD activity under oxidative stress conditions, likely due to their high content of polyphenolic compounds such as oleuropein and hydroxytyrosol, which support endogenous antioxidant defense systems [58]. Similarly, *Opuntia ficus-indica*, another key component of the natural solution used in our study, has also been reported to increase SOD activity in various *in vivo* models, further supporting its antioxidant potential [59]. These results are consistent with the outcomes of our study, where embryos exposed to the natural solution at a concentration of 100 ppm exhibited increased SOD activity compared to the control group. This elevation in SOD levels suggests that the natural components of the solution, including *Olea europaea* and *Opuntia ficus-indica*, may contribute to the reinforcement of endogenous antioxidant defenses, in line with previous reports in the literature.

A previous study has demonstrated that chlorhexidine increases GST activity in liver and kidney tissues, likely as a response to oxidative stress-induced detoxification demands [60]. In our study, there was a dose-dependent increase in GST activity in the group exposed to 1000 ppm chlorhexidine compared to the 100 ppm chlorhexidine group. However, GST activity decreased in the 100 ppm chlorhexidine group compared to the control group.

Although the exact pathway remains unclear, it is biologically plausible that chlorhexidine-induced LPO leads to the accumulation of electrophilic lipid peroxides, which are known substrates for GST. As a result, GST activity may increase as part of a non-genomic, enzyme-substrate driven detoxification response [61,62]. Similarly, in the study conducted by Çoban et al. (2014), which investigated the antioxidant effects of *Olea europaea* leaf extract in aged rats, no statistically significant difference in GST activity was observed, consistent with our findings regarding the natural solution group. In addition, no direct study has yet reported the specific effects of *Opuntia ficus-indica* on GST activity [49]. Although *Olea europaea* and *Opuntia*

ficus-indica are well-documented for their antioxidant properties, the absence of a statistically significant alteration in GST activity in our natural solution group may be explained by several biological factors. *Olea europaea* and *Opuntia ficus-indica* mainly exert their antioxidative effects by supporting earlier-acting antioxidant enzymes such as SOD, rather than directly activating phase II detoxification enzymes like GST [64,65]. It is also possible that the amount or absorption of the active compounds in the natural solution was not strong enough to cause a clear increase in GST activity, especially when compared to the stronger oxidative stress caused by high-dose chlorhexidine.

When evaluating the morphological alterations of zebrafish embryos, we observed pericardial edema and cardiac hemorrage in the 1000 ppm chlorhexidine exposed group. Furthermore, a significant reduction of AChE activity was observed in the 1000 ppm and 100 ppm chlorhexidine groups, which was also reflected in the reduced body length. This association is consistent with previous research showing that AChE plays a critical role during early zebrafish development. Hanneman and Westerfield (1988) reported that AChE activity markedly increases during somitogenesis, a period essential for proper body axis elongation and musculature development [66]. Interference with AChE function during this stage disrupts cholinergic signaling at neuromuscular junctions, potentially leading to impaired motor neuron patterning and muscle fiber differentiation [67]. Furthermore, decreased AChE activity has been associated with neurodevelopmental delays and morphological abnormalities, including reduced body length, as shown by studies on organophosphate exposure and genetic knockdown models [68]. These findings suggest that the reduction in AChE activity observed in our chlorhexidine-treated groups may have led to neuromuscular dysfunction and subsequent deficits in somatic growth. In contrast, no statistically significant differences were observed in either AChE activity or body length between the natural solution group and the control group, suggesting that the natural components may have preserved cholinergic function and normal developmental progression. Interestingly, phytochemicals derived from *Olea europaea* have shown notable AChE inhibitory activity *in vitro*. Romero-Márquez et al. (2024) identified compounds such as oleuropein as potent inhibitors. Likewise, Khizrieva et al. (2021) reported strong inhibition in subcritical water extracts [69,70]. In contrast, our *in vivo* findings revealed no significant change in AChE activity with the olive-based natural formulation, suggesting potential differences in bioavailability or system-specific effects between *in vitro* and zebrafish models. While our study showed no significant effect of the natural solution on AChE activity, *Opuntia ficus-indica* extracts have demonstrated consistent AChE inhibition. This may be due to their higher concentrations of active flavonoids and betalains, which directly target cholinergic enzymes [71]. Additionally, the mode of administration and bioavailability likely differ; most *Opuntia ficus-indica* studies use concentrated extracts with enhanced systemic exposure [11]. In contrast, our formulation may act through non-cholinergic pathways or lack sufficient levels of AChE-targeting compounds. In addition, the absence of a significant difference in body length between the natural solution groups and the control group in our study may be attributed to the unchanged AChE activity. This interpretation is supported by previous zebrafish studies demonstrating that AChE activity is closely linked to early developmental processes [68,72].

5. Conclusion

Our findings suggest that the natural formulation has a lower risk of adversely affecting development in zebrafish embryos and exhibits a better oxidative stress profile compared to chlorhexidine. On the other hand, the lack of behavioral analyses to support our findings on AChE activity and the lack of in vitro microbiological analyses of the solutions are among the limitations of our study. As a conclusion, our results support the potential use of plant-based oral care products during embryogenesis, but further *in vivo* and clinical studies are needed to confirm their safety and efficacy.

Author contributions statement

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Disclosure statement

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References

[1] P. Axelsson, B. Nyström, J. Lindhe, The long-term effect of a plaque control program on tooth mortality, caries, and periodontal disease in adults, J Clin Periodontol, 31(9), 2004, 749–757.

[2] C.G. Jones, Chlorhexidine: Is it still the gold standard?, Periodontol *2000*, 15(1), 1997, 55–62.

[3] Y.L. Hsieh, J.C. Yao, S.C. Hsieh, N.C. Teng, Y.T. Chu, W.X. Yu, et al., The in vivo toxicity and antimicrobial properties for electrolyzed oxidizing (EO) water-based mouthwashes, Materials, 13(19), 2020, 4299.

[4] A. Santos, Evidence-based control of plaque and gingivitis, J Clin Periodontol, 30, 2003, 13–16.

[5] C.A. Gürgan, E. Zaim, I. Bakirsoy, E. Soykan, Short-term side effects of 0.2% alcohol-free chlorhexidine mouthrinse used as an adjunct to non-surgical periodontal treatment: A double-blind clinical study, J Periodontol, 77, 2006, 370–384.

[6] J.A. Helms, M.A. Della-Fera, A.E. Mott, M.E. Frank, Effects of chlorhexidine on human taste perception, *Arch Oral Biol*, 40, 1995, 913–920.

[7] P. John, D. Cunha, Peridex (Chlorhexidine Gluconate 0.12% Oral Rinse), *RxList*, 2020.

[8] G. Pizzo, R. Guiglia, M. Imburgia, I. Pizzo, M. D'Angelo, G. Giuliana, The effects of antimicrobial sprays and mouthrinses on supragingival plaque regrowth: A comparative study, J Periodontol, 77, 2006, 248–256.

[9] L.S. Hasan, I. Ibrahim Latif, S.H. Mohammed, Efficacy of olive leaf extract mouthwash on clinical and inflammatory parameters of gingival inflammation in relation to chlorhexidine in acute gingivitis patients, Diyala J Med, 22(2), 2022.

[10] N.F.R. Mohamed, R.V. Geetha, P.S. Ganesh, In vitro evaluation of antimicrobial activity of Opuntia ficus-indica seed oil, *J Pharm Res Int*, 33(47B), 2021, 490–496.

[11] Y. Kwon, Y. Liao, B. Koo, H. Bae, J. Zhang, E.H. Han, et al., Ethanolic extract of Opuntia ficus-indica var. saboten ameliorates cognitive dysfunction induced by cholinergic blockade in mice, J Med Food, 21(10), 2018, 971–978.

[12] R. Zaman, E.S.S. Tan, N.A. Bustami, et al., Assessment of Opuntia ficus-indica supplementation on enhancing antioxidant levels, Sci Rep, 15, 2025, 3507.

[13] R. Gucci, L. Lombardini, M. Tattini, Analysis of leaf water relations in leaves of two olive (Olea europaea) cultivars differing in tolerance to salinity, Tree Physiol, 17, 1997, 13–21.

[14] N.E. Sedef, S. Karakaya, Olive tree (Olea europaea) leaves: potential beneficial effects on human health, 67(11), 2009, 632–638.

[15] O. Benavente-García, J. Castillo, J. Lorente, A. Ortuño, J.A. Del-Rio, Antioxidant activity of phenolics from Olea europaea L. leaves, Food Chem, 49, 2000, 2480–2485.

[16] K.M. Ahmed, The effect of olive leaf extract in decreasing the expression of two pro-inflammatory cytokines in patients receiving chemotherapy for cancer: A randomized clinical trial, Saudi Dent J, 25(4), 2013, 141–147.

[17] Z. Atai, M. Ansari, N. Torabi, Efficacy of olive leaf extract in the treatment of minor oral aphthous ulcers, Am J Infect Dis, 3(1), 2007, 24–26.

[18] N. Chougui, A. Tamendjari, W. Hamidj, S. Hallal, A. Barras, T. Richard, R. Larbat, Oil composition and characterisation of phenolic compounds of Opuntia ficus-indica seeds, *Food Chem*, 139(1–4), 2013, 796–803.

[19] H. Chahdoura, P. Morales, J.C.M. Barreira, L. Barros, V. Fernández-Ruiz, I.C.F. Ferreira, L. Achour, Dietary fiber, mineral elements profile and macronutrients composition in different edible parts of Opuntia microdasys (Lehm.) Pfeiff and Opuntia macrorhiza (Engelm.), LWT Food Sci Technol, 64(1), 2015, 446–451.

[20] V. Saxena, G. Mishra, A. Saxena, K. Vishwakarma, A comparative study on quantitative estimation of tannins in Terminalia chebula, Terminalia belerica, Terminalia arjuna and Saraca indica using spectrophotometer, Asian J Pharm Clin Res, 6(3), 2013, 148–149.

[21] S. Ali, D.L. Champagne, H.P. Spaink, MK. Richardson, Zebrafish embryos and larvae: a new generation of disease models and drug screens, Birth Defects Res C Embryo Today, 93(2), 2011, 115–133.

[22] G.E. Karaman, E. Emekli-Alturfan, S. Akyüz, Zebrafish: an emerging model organism for studying toxicity and biocompatibility of dental materials, Cell Mol Biol, 66(8), 2020, 41–46.

[23] P. Stachurski, W. Świątkowski, A. Ciszewski, K. Sarna-Boś, A. Michalak, A short review of the toxicity of dentifrices—zebrafish model as a useful tool in ecotoxicological studies, Int J Mol Sci, 24(18), 2023, 14339.

[24] S. Meşeli, G. Kaplan, D. Cansız, Ü.V. Üstündağ, İ. Ünal, E. Emekli-Alturfan, F. Yanıkoğlu, D. Tağtekin, The biocompatibility of sodium lauryl sulphate on developing zebrafish embryos, Experimed, 11(2), 2021, 67–72.

[25] G.E. Karaman, İ. Ünal, M. Beler, F.D. Üstündağ, D. Cansız, Ü.V. Üstündağ, E. Emekli-Alturfan, S. Akyüz, Toothpastes for children and their detergent contents affect molecular mechanisms of odontogenesis in zebrafish embryos, Drug Chem Toxicol, 47(1), 2022, 15–25.

[26] R. Oliveira, I. Domingues, C. Koppe Grisolia, A.M. Soares, Effects of triclosan on zebrafish early-life stages and adults, Environ Sci Pollut Res, 16(6), 2009, 679–688.

[27] H. Gaur, S. Purushothaman, N. Pullaguri, Y. Bhargava, A. Bhargava, Sodium benzoate induced developmental defects, oxidative stress and anxiety-like behaviour in zebrafish larva, Biochem Biophys Res Commun, 502(3), 2018, 364–369.

[28] M. d'Amora, F. Liendo, F.A. Deorsola, S. Bensaid, S. Giordani, Toxicological profile of calcium carbonate nanoparticles for industrial applications, Colloids Surf B Biointerfaces, 190, 2020, 110947.

[29] H. Yi, Z. Wang, X. Li, M. Yin, L. Wang, A. Aldalbahi, N.N. El-Sayed, H. Wang, N. Chen, C. Fan, Silica nanoparticles target a Wnt signal transducer for degradation and impair embryonic development in zebrafish, Theranostics, 6(11), 2016, 1810–1820.

[30] S. Meşeli, Ü.V. Üstündağ, P.S. Ateş, İ. Ünal, E. Işık Alturfan, D. Tağtekin, F. Yanikoglu, The biocompatibility of a ginger-containing herbal toothpaste on developing zebrafish embryos, J Dent Indones, 30(3), 2023, 150–156.

[31] A.G. Kramer, J. Vuthiganon, C.S. Lassiter, Bis-GMA affects craniofacial development in zebrafish embryos (Danio rerio), Environ Toxicol Pharmacol, 43, 2016, 159–165.

[32] H. Makkar, S.K. Verma, P.K. Panda, E. Jha, B. Das, K. Mukherjee, M. Suar, In vivo molecular toxicity profile of dental bioceramics in embryonic zebrafish (Danio rerio), Chem Res Toxicol, 31(9), 2018, 914–923.

[33] L. Zhao, J. Si, Y. Wei, S. Li, Y. Jiang, R. Zhou, B. Liu, H. Zhang, Toxicity of porcelain-fused-to-metal substrate to zebrafish (Danio rerio) embryos and larvae, Life Sci, 203, 2018, 66–71.

[34] A. Karagöz, M. Beler, B.D. Altun, İ. Ünal, D. Cansız, H. Gündüz, A.A. Alturfan, E. Emekli-Alturfan, Ş.E. Yalçınkaya, Panoramic dental X-ray exposure leads to oxidative stress, inflammation and apoptosis-mediated developmental defects in zebrafish embryos, J Stomatol Oral Maxillofac Surg, 124(6), 2023, 101661.

[35] B.Y. Kollayan, et al., Effects of low-dose ionizing radiation on the molecular pathways linking neurogenesis and autism spectrum disorders in zebrafish embryos, Drug Chem Toxicol, 2024, 1–10.
[36] G. Gündüz, M. Beler, İ. İsmail, D. Cansız, E.E. Alturfan, K.N. Kose, Gingipain injection affects intestinal oxidant–antioxidant status and alkaline phosphatase in overfed zebrafish, Experimed, 13(2), 2023, 80–85.
[37] S. Meşeli, D. Tağtekin, E. Emekli Alturfan, Zebrafish, A Model Organism For Research in Dentistry, Selcuk Dent J, 2025;12(1):144-8.
[38] M. Westerfield, The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish, University of Oregon Press, Eugene, OR, 1995.
[39] E.K. Choi, B.M. Choi, Y. Cho, S. Kim, Myelin toxicity of chlorhexidine in zebrafish larvae, Pediatr Res, 93(4), 2023, 845–851.
[40] A.S. Valan, J. Krithikadatta, R. Eswaramoorthy, A. Guru, Comparative Analysis of the Cytotoxic Effects of Modified Triple Antibiotic Hydrogel: Insights From Experimental Models, *Cureus*, *16*(6), 2024, e62662.
[41] S. Louhimies, Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, Altern Lab Anim, 30(Suppl 2), 2002, 217–219.
[42] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J Biol Chem, 193, 1951, 265–275.
[43] K. Yagi, Assay for blood plasma or serum, Methods Enzymol, 105, 1984, 328–331.
[44] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases: the first enzymatic step in mercapturic acid formation, J Biol Chem, 249, 1974, 7130–7139.
[45] A.A. Mylroie, H. Collins, C. Umbles, J. Kyle, Erythrocyte superoxide dismutase activity and other parameters of copper status in rats ingesting lead acetate, Toxicol Appl Pharmacol, 82, 1986, 512–520.
[46] G.L. Ellman, K.D. Courtney, V. Andres Jr, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem Pharmacol, 7, 1961, 88–95.
[47] M.H. Tabatabaei, F.S. Mahounak, N. Asgari, Z. Moradi, Cytotoxicity of the ingredients of commonly used toothpastes and mouthwashes on human gingival fibroblasts, Front Dent, 16(6), 2019, 450–457.
[48] Y. Ha, Y. Kim, J. Choi, I. Hwang, J.-Y. Ko, H. K. Jeon, Y.-J. Kim, Evaluation of cytotoxicity, genotoxicity, and zebrafish embryo toxicity of mixtures containing *Hyssopus officinalis, Morus alba, Engraulis japonicus,* and 27 other extracts for cosmetic safety assessment, Mol Cell Toxicol, 17, 2021, 221-232.
[49] U.S. Food and Drug Administration (FDA), Peridex (chlorhexidine gluconate) oral rinse – prescribing information, 2013.
[50] NHS, Pregnancy, breastfeeding and fertility while using chlorhexidine, 2024.
[51] E. Benramdane, N. Chougui, P.A.B. Ramos, N. Makhloufi, A. Tamendjari, A.J.D. Silvestre, et al., Lipophilic compounds and antibacterial activity of Opuntia ficus-indica root extracts from Algeria, Int J Mol Sci, 23(19), 2022, 11161.
[52] A.P.N. Newton, L.L. Cogo, C.M.R. de Oliveira, C.C. Kanunfre, J. Freitas-Astúa, New data on biological effects of chlorhexidine: Fe^{2+} induced lipid peroxidation and mitochondrial permeability transition, Toxicol Lett, 151(3), 2004, 407–416.
[53] D. Biswas, M. Tiwari, V. Tiwari, Molecular mechanism of antimicrobial activity of chlorhexidine against carbapenem-resistant Acinetobacter baumannii, PLOS ONE, 14(10), 2019, e0224107.
[54] F. Abdellah, K. Hamden, T. Silarbi, N. Ayad, R. Benaraba, Antioxidant activity and protective effect of olive leaf extracts (Olea europaea L.) against lipid peroxidation, Phytothérapie, 21(6), 2023, 259–267.
[55] F. Saputra, M. Kishida, S.Y. Hu, Oxidative stress induced by hydrogen peroxide disrupts zebrafish visual development by altering apoptosis, antioxidant and estrogen related genes, Sci Rep, 14(1), 2024, 14454.
[56] K. Ukaegbu, D. Foyle, X. Luan, E. Schneiderman, E.P. Allen, J. Plemons, et al., The effect of an antioxidant gel compared to chlorhexidine during the soft tissue healing process: An animal study, J Periodontol, 95(11), 2024, 1086–1096.
[57] Y.C. Li, Y.H. Kuan, T.H. Lee, F.M. Huang, Y.C. Chang, Assessment of the cytotoxicity of chlorhexidine by employing an in vitro mammalian test system, J Dent Sci, 9(2), 2014, 130–135.
[58] R. Pennisi, I. Ben Amor, B. Gargouri, H. Attia, R. Zaabi, A. Ben Chira, M. Saoudi, A. Piperno, P. Trischitta, M. P. Tamburello, M. T. Sciortino, Analysis of antioxidant and antiviral effects of olive (*Olea europaea* L.) leaf extracts and pure compound using cancer cell model, Biomolecules, 13, 2023, 238.
[59] I. Besné-Eseverri, M. A. Martín, G. Lobo, M. P. Cano, M. P. Portillo, J. Trepiana, Antioxidant and anti-inflammatory effects of *Opuntia* extracts on a model of diet-induced steatosis, Antioxidants, 13, 2024, 1416.
[60] S.Z. Moussa, S.A. El Meadawy, H.A. Ahmed, M. Refat, Efficacy of Chelidonium majus and propolis against cytotoxicity induced by chlorhexidine in rats, 2007.
[61] H.J. Forman, H. Zhang, A. Rinna, Glutathione: Overview of its protective roles, measurement, and biosynthesis, Mol Aspects Med, 30(1–2), 2009, 1–12.
[62] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int J Biochem Cell Biol, 39(1), 2006, 44–84.
[63] J. Çoban, S. Öztezcan, S. Doğru-Abbasoğlu, I. Bingül, K. Yeşil-Mizrak, M. Uysal, Olive leaf extract decreases age-induced oxidative stress in major organs of aged rats, Geriatr Gerontol Int, 14(4), 2014, 996–1002.
[64] H. Jemai, A. Mahmoudi, A. Feryeni, I. Fki, Z. Bouallagui, S. Choura, M. Chamkha, S. Sayadi,, Hepatoprotective effect of oleuropein-rich extract from olive leaves against cadmium-induced toxicity in mice, Biomed Res Int, 2020, 4398924.
[65] N. Hfaiedh, M.S. Allagui, M. Hfaiedh, A.E. Feki, L. Zourgui, F. Croute, Protective effect of cactus (Opuntia ficus indica) cladode extract upon nickel-induced toxicity in rats. Food Chem Toxicol. 2008, 46(12),3759–3763.
[66] E. Hanneman, M. Westerfield, Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish, J Comp Neurol, 276(2), 1988, 244–255.
[67] D. Yang, H. Lauridsen, K. Buels, L.H. Chi, J. La Du, R.L. Tanguay, Chlorpyrifos-oxon disrupts zebrafish axonal growth and motor behavior, Toxicol Sci, 121(1), 2011, 146–159.
[68] C. Gravato, F.R. Abe, D.P. de Oliveira, A.M.V.M. Soares, I. Domingues, Acetylcholinesterase (AChE) activity in embryos of zebrafish, Methods Mol Biol, 2240, 2021, 119–124.
[69] J.M. Romero-Márquez, M.D. Navarro-Hortal, T.Y. Forbes-Hernández, A. Varela-López, J.G. Puentes, C. Sánchez-González, et al., Effect of olive leaf phytochemicals on the anti-acetylcholinesterase, anti-cyclooxygenase-2 and ferric reducing antioxidant capacity, Food Chem, 444, 2024, 138516.
[70] S.S. Khizrieva, S.N. Borisenko, E.V. Maksimenko, N.I. Borisenko, V.I. Minkin, Study of the composition and anti-acetylcholinesterase activity of olive leaf (Olea europaea L.) extracts obtained in subcritical water, Russ J Phys Chem B, 15(8), 2021, 1286–1290.
[71] D. Butera, L. Tesoriere, F. Di Gaudio, A. Bongiorno, M. Allegra, A.M. Pintaudi, et al., Antioxidant activities of Sicilian prickly pear (Opuntia ficus-indica) fruit extracts and reducing properties of its betalains: Betanin and indicaxanthin, J Agric Food Chem, 50(23), 2002, 6895–6901.

[72] E. Teixidó, E. Piqué, J. Gómez-Catalán, J.M. Llobet, Assessment of developmental delay in the zebrafish embryo teratogenicity assay, Toxicol In Vitro, 27(2), 2013, 469–478.