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RESEARCH ARTICLE



Synthesis of salicylic acid from wintergreen oil by green chemistry overcomes its cytotoxicity in keratinocyte cells and teratogenicity in zebrafish embryos

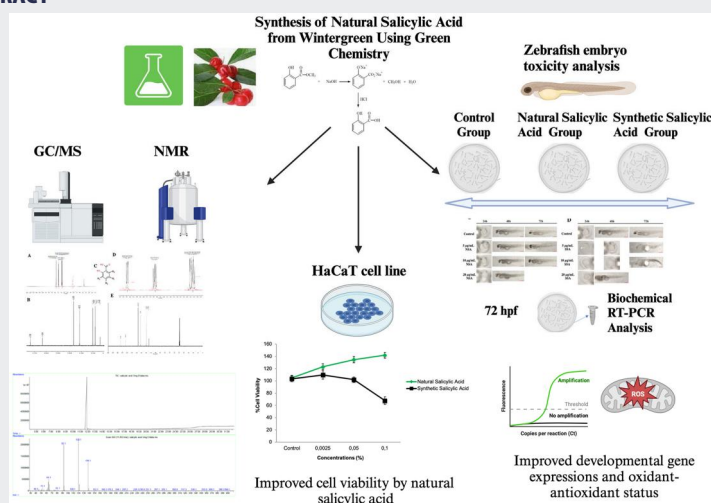
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ABSTRACT

Salicylic acid topical is used to treat variety of skin conditions. However, salicylic acid in these products is generated through industrial synthesis and has been shown to negatively impact fetal development and cause congenital abnormalities. We hypothesized that teratogenic effects reported in salicylic acid can be prevented by naturally synthesizing salicylic acid from wintergreen oil using green chemistry method. For this purpose, we investigated the effects of natural salicylic acid (NSA) synthesized from wintergreen oil using green chemistry and synthetic salicylic acid (SSA) on keratinocyte cell (HaCaT) proliferation and zebrafish embryo development. NSA structures were analyzed by ¹H NMR, ¹³C NMR, and GC/MS methods. Percentage inhibition against HaCaT cell was determined by MTS assay. xCelligence system was used for cellular activities. Zebrafish embryos were exposed to NSA and SSA for 72 h post-fertilization. Lipid peroxidation, nitric oxide, sialic acid, glutathione-S-transferase, catalase, and superoxide dismutase were evaluated using biochemical methods. Expressions of *nqO1*, *gfap*, *bdnf*, *vtg*, *egr*, *cyp1a*, and *igf2* were determined by RT-PCR as developmental indicators. MTS and RT-cell analysis showed increased cell viability by NSA, whereas SSA decreased cell viability. NSA beneficially affected zebrafish embryo development while SSA exerted deleterious effects through oxidant-antioxidant status, inflammation, and development. Results of our study showed for the first time that synthesis of salicylic acid from wintergreen oil by green chemistry overcomes its cytotoxicity in keratinocyte cells and teratogenicity in zebrafish embryos. This finding is important for drug research on safe topical applications during pregnancy, when preventing exposure to drug and chemical-derived teratogens is vital.

GRAPHICAL ABSTRACT



ARTICLE HISTORY

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KEYWORDS

Salicylic acid; wintergreen oil; green chemistry; zebrafish embryo toxicity; cell proliferation

1. Introduction

Salicylic acid has been commonly used in cosmetic items due to its protective properties and dermatological effects. Salicylic acid topical is used to treat a variety of skin conditions, including acne, dandruff, psoriasis, seborrheic dermatitis of the skin and scalp. On the other hand, salicylic acid included in these products is generated through industrial synthesis and salicylic acid has been shown to negatively impact fetal development and cause congenital abnormalities (Joschko et al. 1993; Singh et al. 2009). In this process, the highly poisonous chemical phenol is employed as a raw ingredient (Özokan et al. 2022). Accordingly, industrial salicylic acid synthesis produces contaminants in the final product that have hazardous consequences.

As a strategy to achieve sustainability at the molecular level, green chemistry has been widely applied in all industrial sectors, including agriculture, domestic products, cosmetics, and pharmacy (Anastas and Eghbali 2010). The most significant objectives of green chemistry is to eliminate or reduce the generation and use of compounds that are harmful to the ecosystem and human health (Roozifar et al. 2021). In the process of synthesis of salicylic acid by green chemistry, the oil of wintergreen is used in the natural synthesis process, which has a methyl salicylate level of over 99%. To get crystal salicylic acid free of harmful contaminants, oil of wintergreen is first hydrolyzed with sodium hydroxide and then further hydrolyzed with hydrochloric acid (Parker et al. 2004; Özokan et al. 2022).

For the health of the fetus and the mother, it is crucial that pregnant women choose topical medications that are safe and effective. To offer safe treatment options during pregnancy, verified research on the use of topical treatments in pregnancy are crucial. For doctors and mothers to make informed treatment decisions, regular data and thorough information on the safety profiles of topical treatments during pregnancy are essential (Putra et al. 2022).

In line with this information, we hypothesized that the teratogenic effects reported in salicylic acid could be prevented by naturally synthesizing salicylic acid from wintergreen oil using the green chemistry method. Accordingly,

this research was conducted to evaluate and compare the effects of natural and synthetic salicylic acid (SSA) on the proliferation of human epidermal keratinocyte cell line (HaCaT) and on zebrafish embryos focusing on oxidant–anti-oxidant status, inflammation, and developmental parameters.

The purity of the salicylic acid was evaluated using GC/MS and the molecular structures were confirmed using nuclear magnetic resonance (NMR). Cell culture analysis was performed using HaCaT cell line to determine how natural and SSA affected cell viability. Teratogenic effects of natural and SSA were evaluated on developing zebrafish embryos through the analysis of lipid peroxidation (LPO), nitric oxide (NO), sialic acid levels and the activities of the antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione-S-transferase (GST). Gene expressions of *igf2*, *nq01*, *cyp1a*, *erg*, *gfap*, and *vtg* were evaluated as biomarkers of development, oxidative stress, biotransformation, cardiac development, neurotoxicity, and estrogenic activity.

2. Materials and methods

2.1. The synthesis of salicylic acid by green chemistry

Natural wintergreen oil was used to synthesize the natural salicylic acid (NSA) through green chemistry and organic synthesis. First step was sodium salicylate synthesis that is followed by crystallization step as described previously (Figure 1) (Özokan et al. 2022).

2.1.1. Optimization of the reaction conditions

Different reaction conditions were used in an effort to determine the most effective and feasible method for producing NSA. Table 1 lists the requirements for reactions.

2.1.2. GC/MS analysis parameters

An excellent method to assess and confirm the purity of a raw material is with GC–MS. The NSA produced was purity-tested using GC–MS analysis using the following parameters for column as HP-5ms Ultra Inert, and dimensions of 30 m × 250 μm × 0.25 μm. The other programs applied

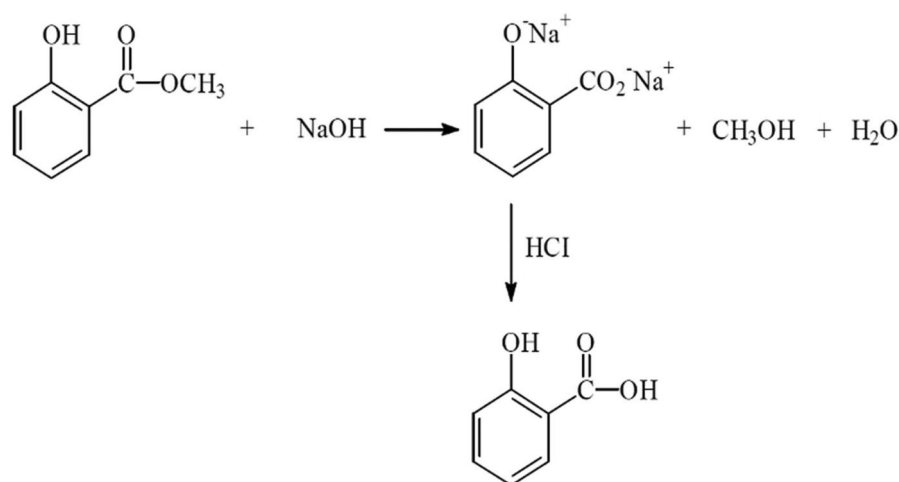


Figure 1. Synthesis of natural salicylic acid using organic synthesis and green chemistry methods.

Table 1. Applied reaction conditions to determine the most feasible and effective method to generate natural salicylic acid.

Condition 1	Reaction temperature	80 °C, 90 °C, 100 °C
Condition 2	Molar ratio of wintergreen oil and sodium hydroxide	1:3, 1:5, 1:7
Condition 3	Molar ratio of sodium hydroxide and water	1:5, 1:7, 1:10
Condition 4	Reaction time	2 h, 3 h, 4 h
Parameter 5	pH (hydrochloric acid precipitation)	1.5, 1.7, 2.0

were: beginning: 60 °C, final temperature: 260 °C, temperature increase rate: 3 °C per minute, analysis duration: 66.6 min; inlet temperature: 250 °C; MS detector temperature: 230 °C; helium flow rate: 1.1 mL/minute; split ratio: 20:1; sample preparation: 5 mg of salicylic acid dissolved in 1.5 mL of methanol; the volume of injection was 1 µL.

2.1.3. The analysis of nuclear magnetic resonance

NMR spectroscopy is a frequently applied method to understand molecular physics, crystals, non-crystalline materials, and organic molecules in solution. In our study, we used NMR to determine the molecular structure of the NSA we obtained, and the spectra were recorded at 25 °C using CD₃OD as a solvent. Chemical shifts are indicated as δ values (ppm) against tetramethylsilane, which is the internal standard, and J values are indicated in Hz.

2.2. Cell culture analysis

2.2.1. Assay for cell viability

To determine how natural and SSA affect cell viability, human epidermal keratinocyte (HaCaT) cell line was used. HaCaT keratinocytes were cultured expansion media containing DMEM (Dulbecco's modified Eagle medium), L-glutamine (2 mM), fetal bovine serum (10%), and penicillin–streptomycin (1%). HaCaT cells were seeded in 96-well plates at 1×10^4 density. After 24 h, the media of the cells were discarded. HaCaT cell line was treated with natural and SSA at different concentrations (0.025, 0.05, and 0.1%) and incubated for 24 h under the standard culture conditions (37 °C and 5% CO₂). Assay for the MTS cell viability was performed in line with the manufacturer's instructions and the plates were incubated for 4 h at 37 °C. Formazan amount in the wells was recorded at 490 nm on a microplate reader. The HaCaT cell line was used as a healthy control cell. Control groups were treated with the DMEM media. Each concentration was prepared in triplicate.

2.2.2. Real-time cell analysis (RTCA)

The HaCaT cell line has been grown in DMEM with 10% FBS and placed on a tissue culture E-Plate to measure the impedance of electricity across interdigitated micro-electrodes on the plate's bottom in order to produce dynamic real-time data. The calculated and shown cell index (CI) represents the measured impedance by the RTCA system, which is proportional to morphology, adhesion, and number of the cells. The device is set up in a CO₂ incubator and is linked via a cable to the analysis and control units, which are placed outside the incubator. Natural and synthetic salicylic acid were prepared at different (0.025–0.05–0.1%) concentrations and applied to the E-plate. The xCelligence technology tracked

the outcomes in real time for ≈ 70 h. Researchers who were blind to the treatments carried out the cell culture analysis.

2.3. Zebrafish embryo toxicity test

Zebrafish (AB/AB strain) were kept in an aquatic rack setup (Zebtec, Tecniplast, Buguggiate, Italy) at a constant temperature of 27 ± 1 °C and with a 14/10-h light/dark cycle. Two times per day, live Artemia were added to commercial flake fish food that was supplied to the fish. The pH of system water ranges from 6.9 to 7.2. All tests were conducted using reverse osmosis water containing 0.018 mg L⁻¹ of Instant OceanTM salt. Following regular spawnings, fertilized embryos were collected and staged in accordance with their previously described development and morphology (Westerfield 1995). As the zebrafish embryos used were no older than five days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (2010), EU Directive 2010/63/EU.

2.3.1. Embryo exposure

Zebrafish embryos were exposed to NSA and SSA (10 µg/L) in well plates for 72 h post-fertilization (hpf). The embryo media served as the blank control. For the toxicity studies using zebrafish embryos, an adequate number of healthy and recently fertilized eggs are needed for each concentration level and control treatment in order to maintain the tested chemicals' accurate dose-response relationships. Zebrafish embryos were randomly assigned to the groups. We used totally six biological replicates of pools of zebrafish embryos (each pool consisted of 50 embryos) for each group that were prepared to have a sufficient RNA pool (50 embryos/pool; three biological replicates for each group) and for the biochemical analyses (50 embryos/pool; three biological replicates for each group).

The exposure solutions were replaced daily with new ones. Using a stereomicroscope (Zeiss Discovery V8, Jena, Germany), developmental parameters were observed daily, and deformities were photographed and recorded. Every 24 h, hatching rates were also recorded. The proportion of hatching embryos to living embryos in each well is known as the hatching rate. For embryo staging, the previously mentioned development indicators – yolk sac, anal pore, pectoral fin, and swim bladder – were used. Researchers who were blind to the treatments carried out assessments of gene expression, biochemical data, and statistical data.

2.3.2. Reverse transcription (cDNA synthesis) and quantitative real-time PCR

Three biological replicates were created as pools of zebrafish embryos for RNA extraction from each group. Fifty embryos

made up each replicate. The Rneasy Mini Kit and Qiacube (Qiagen, Hilden, Germany) were applied in accordance with the producer's instructions. Using RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany), 1 g of total RNA was converted into a single-stranded cDNA. RT-PCRs were carried out using the DNA Master SYBR Green kit (Qiagen, Hilden, Germany). The gene employed as a housekeeping agent was beta-actin. The housekeeping gene was used to normalize the values for the CT technique, which was used to calculate relative transcription levels in a blinded manner (Livak and Schmittgen 2001).

2.3.3. Biochemical parameters related with oxidant-anti-oxidant status and inflammation

Three biological replicates were created as pools of zebrafish embryos in order to get an adequate quantity for the biochemical investigations. Zebrafish embryos were homogenized in 1 mL of PBS for each pool, then quickly centrifuged. The biochemical parameters were determined using the supernatant. Different researchers who were blind to the treatments conducted all biochemical studies and computed the results.

2.3.3.1. Total protein and sialic acid determination. The Lowry technique was used to calculate the total protein level. Using this technique, copper ions are initially used to react with proteins in an alkaline media. After that, it is reduced using phosphomolybdic-phosphotungstic acid reagent (also known as Folin's reagent). A spectrophotometer is used to quantify the blue color's intensity at 500 nm, which is directly proportional to the protein content. The parameters' findings are expressed for each protein (Lowry et al. 1951). Sialic acid levels were determined according to the method of Warren (1959).

2.3.3.2. Lipid peroxidation determination. The spectrophotometric evaluation of the pinkish color produced by the reaction between malondialdehyde (MDA), a byproduct of LPO, and thiobarbituric acid (TBA), was done using the Yagi method. LPO was calculated using the extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol MDA/mg protein (Yagi 1984).

2.3.3.3. Nitric oxide determination. The Miranda method was used to assess the levels of NO. In this process, vanadium(III) chloride reduces nitrate to nitrite. Following the formation of nitrite in an acidic medium, complex diazonium compound was created when sulfonyl amide reacted with N-(1-naphthyl) ethylenediamine dihydrochloride. The color complex at 540 nm was measured using a spectrophotometer, and the findings were computed and displayed as nmol NO/mg protein (Miranda et al. 2001).

2.3.3.4. Glutathione-S-transferase determination. Through the spectrophotometric measurement of the absorbance of the product generated by the conjugation of GSH and 1-

chloro-2,4-dinitro-benzene (CDNB), the activity of GST was determined (Habig and Jakoby 1981).

2.3.3.5. Catalase determination. CAT activities were determined by using the method of Aebi (1984) that is based on the transformation of hydrogen peroxide (H_2O_2) into water by the effect of CAT enzyme. The transformation was noted by the reduction in the absorbances obtained at 240 nm and the activity of CAT is given as U/mg protein.

2.3.3.6. Superoxide dismutase determination. Superoxide dismutase activities were evaluated through the riboflavin-sensitized photooxidation of ortho-dianisidine (Myloie et al. 1986). Oxidation of ortho-dianisidine through the reaction using riboflavin is induced by SOD. The increase in absorbance is directly proportional to the SOD concentration. Finally, the absorbance of the colored product is determined by using spectrophotometry at 460 nm and the results are expressed as U/mg protein.

2.4. Statistical analysis

Experiments using zebrafish embryos were replicated three times using 50 embryos per trial. Power analysis-G*Power 3.1 (<https://www.psych.uni-duesseldorf.de/abteilungen/aap/gpower3/>) was used to estimate the minimum number of embryos required to produce statistically significant results. The Grubbs test was used to detect and exclude the outliers. The significance level, power, and the effect size were applied as $\alpha = 0.05$, $\beta = 0.8$, and $f = 0.4$ respectively and a minimum of 50 embryos were determined to be needed for each experimental group to obtain a statistically significant sample size. GraphPad Prism 9.0 (GraphPad Software, San Diego, CA) was used for all statistical analyses, and the data were presented as mean standard deviation. One-way ANOVA test was used to compare the results, and Tukey's multiple comparison tests were then performed in a blinded manner. The gathered data were expressed as mean standard deviation. A p value of 0.05 or less was considered significant.

3. Results

3.1. Results of optimization of NSA synthesis reaction conditions

Results of the total 15 trials using the parameters given in Table 1 indicated that optimum parameters with the highest reaction yield of 90% were: reaction temperature of 90°C ; wintergreen oil to sodium hydroxide molar ratio is 1:7; the weight ratio of sodium hydroxide to water is 1:5, the reaction takes three hours, and the pH is 1.5.

3.2. Results of NMR analysis

Results of NMR analysis are given in Figure 2(A-E). ^1H NMR (CD_3OD , 400 MHz) schematization is given in Figure 2(C) as δ : 7.84 (1H, doublet of doublet, H_d), 7.43 (1H, triplet of doublet,

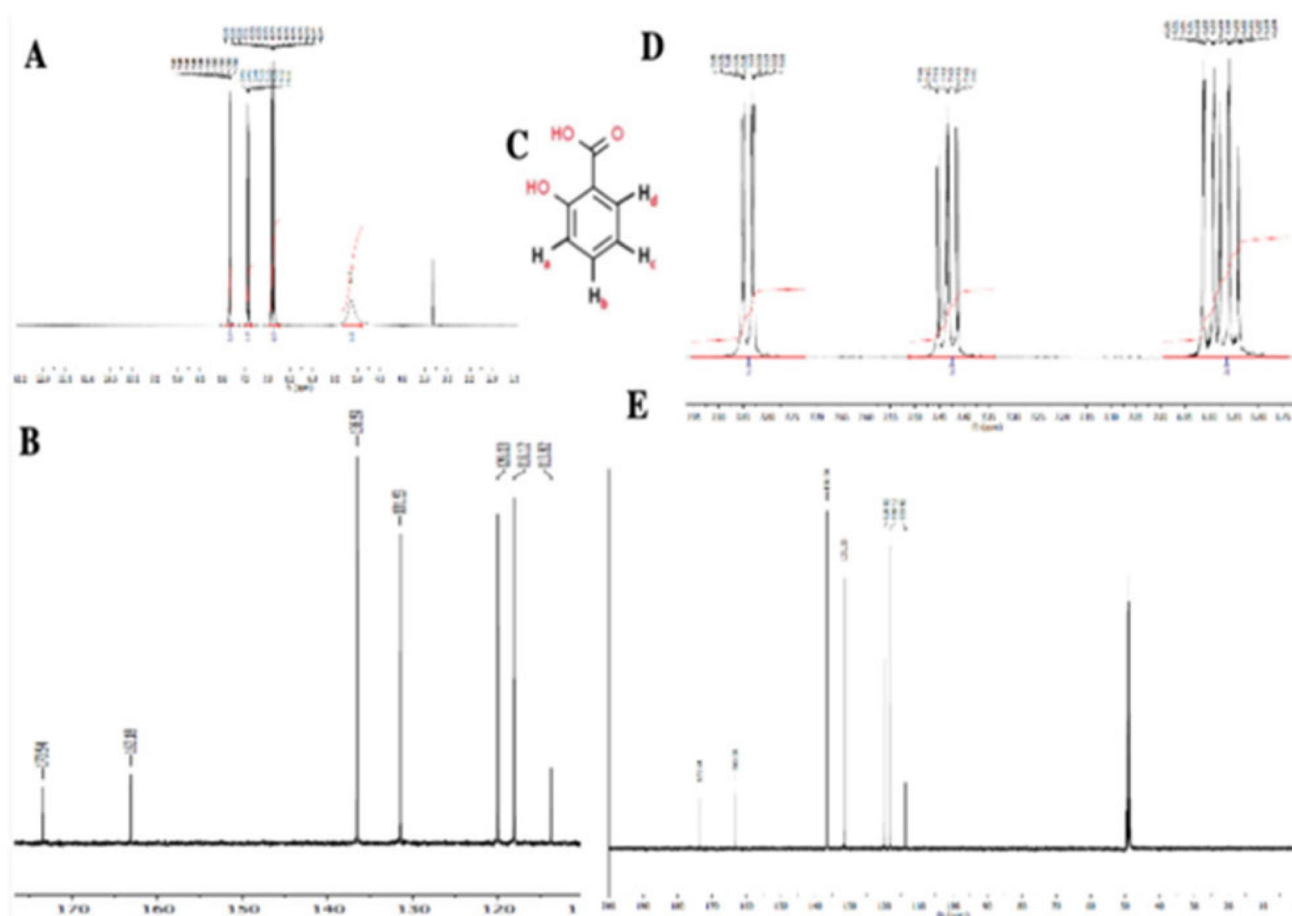


Figure 2. Presentation of ^1H NMR and ^{13}C NMR spectrum results of natural salicylic acid NMR analysis.

H_b), 6.90 (1H, doublet of doublet, H_a), 6.86 (1H, triplet, H_c). $-\text{COOH}$ and $-\text{OH}$ groups of NSA show signal at δ 5.13 ppm (2H, broad singlet). ^1H NMR spectrum of NSA (Plantraction WG 99) is given in Figure 2(A,D). ^{13}C NMR spectrum of NSA (Plantraction WG 99) is given in Figure 2(B,E).

^{13}C NMR (CD_3OD , 100 MHz) δ : 113.82 (C1, C), 118.12 (C3, CH), 120.03 (C5, CH), 131.53 (C6, CH), 136.59 (C4, CH), 163.18 (C2, C), 173.54 (COOH).

3.3. Results of GC/MS analysis

Results of GC/MS analysis revealed salicylic acid retention time as 20.3 min and MS peaks as 138, 120, 92, 64, and 53 (molecular weight). The salicylic acid batches had a purity of 100%. Chromatogram and MS spectrum of NSA (Plantraction WG 99) are given in Figure 3.

3.4. Results of cell culture analysis

3.4.1. Cell viability

To determine whether NSA affects cell viability in HaCaT cell line, we performed MTS cell viability assay. We first confirmed that NSA was not toxic to HaCaT cells since it had no impact on the cell viability at the tested concentrations (Figure 4). On the other hand, SSA led to a dramatic decrease in cell viability within the range of concentrations tested (Figure 4).

3.4.2. Real-time cell analysis

We used RTCA to determine the effects of natural and SSA treatments on human keratinocyte cells on various cellular processes, such as cell proliferation, migration, adhesion, and cytotoxicity. Although 0.1% NSA had a curve similar to the control group, 0.1% SSA led to a dramatic decrease in the slope of the curve (Figure 5).

3.5. Results of zebrafish embryo analysis

Mortality and hatching rates, and the representative images of the zebrafish embryos in the natural and SSA treated groups are given in Figure 6. Mortality rates of the 5 $\mu\text{g}/\text{L}$ and 10 $\mu\text{g}/\text{L}$ SSA treated embryos were significantly higher than that of the NSA treated embryos (Figure 6(A)).

Natural salicylic acid exposed embryos showed similar development to the control group (Figure 6(C)). However, in the case of exposure to SSA, developmental delay characterized by lack of pigmentation and reduced hatching at 48 hpf has been observed (Figure 6(B,D)). In addition, spinal curvature was observed in embryos exposed to SSA at concentrations of 5 and 10 $\mu\text{g}/\text{L}$ at 72 hpf (Figure 6(D)).

3.5.1. Results of biochemical analyses

The oxidant-antioxidant status of the natural and SSA treated zebrafish embryos was determined by the LPO, NO,

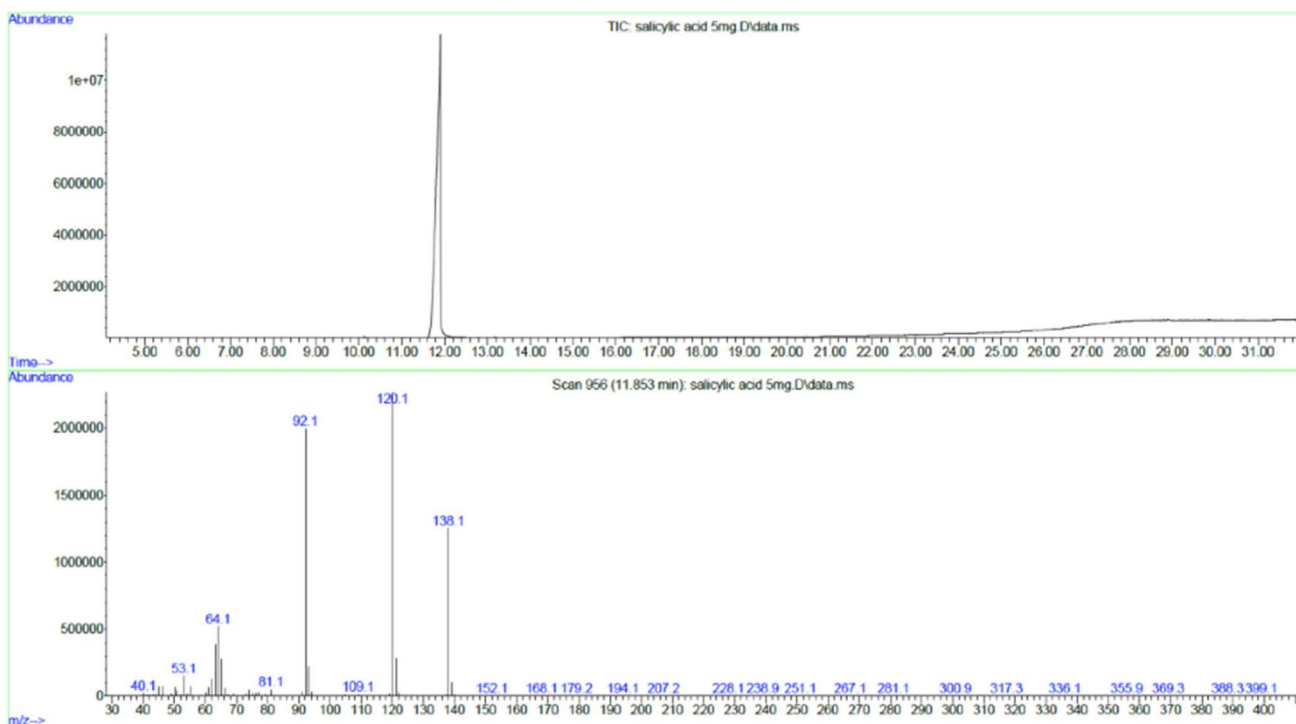


Figure 3. Presentation of chromatogram and MS spectrum of natural salicylic acid (Plantraction WG 99).

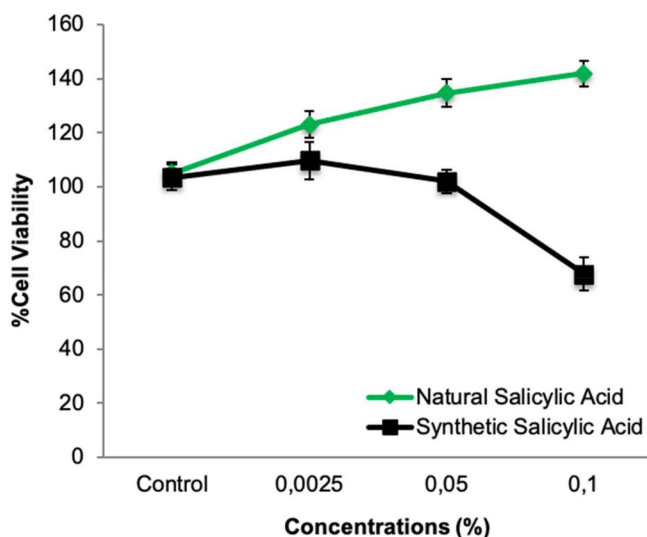


Figure 4. Presentation of cell viability in the presence of natural and synthetic salicylic acid. Results from at least three studies using duplicate samples are shown as mean \pm SD.

SOD, and GST assays. SA was also determined as a marker of inflammatory status. LPO, NO, and SA levels increased significantly in the SSA group when compared with the control group ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively) (Figure 7(A–C)). In the SSA group, NO levels were significantly higher than the NSA group ($p < 0.01$) (Figure 7(B)) and when compared with the control group, there was a significant decrease in the SA levels of the NSA group ($p < 0.05$) (Figure 7(C)).

Although NSA treatment significantly elevated CAT and SOD activities ($p < 0.05$), SSA treatment led to significant decreases in both parameters ($p < 0.01$ and $p < 0.05$,

respectively) (Figure 8(A,B)). In the SSA group, GST activities increased significantly when compared to the control ($p < 0.05$) and to the NSA group ($p < 0.05$) (Figure 8(C)).

3.5.2. Results of gene expression analyses

The expression of *nq01* increased significantly both in the NSA group and SSA groups ($p < 0.001$ and $p < 0.05$, respectively), and the NSA group had significantly higher *nq01* expressions than the SSA group ($p < 0.05$) (Figure 9(A)). The expression of *cyp1a* increased significantly both in the NSA group and SSA groups ($p < 0.05$ and $p < 0.0001$, respectively), and the SSA group had significantly higher *nq01* expressions than the NSA group ($p < 0.001$) (Figure 9(B)). The expression of *vtg* did not change significantly in the NSA and SSA groups indicating that neither compound showed estrogenic activity (Figure 9(C)).

The expression of *erg* was found to be increased significantly in the NSA group ($p < 0.01$); however in the SSA group, its expression decreased significantly compared to the control and the NSA group ($p < 0.001$ and $p < 0.0001$, respectively) (Figure 10(A)). *igf2* expression in the NSA group increased significantly compared to the control and the SSA group ($p < 0.001$) (Figure 10(B)). In the SSA group, *gfap* expressions increased significantly when compared to the control group and the NSA group ($p < 0.01$ and $p < 0.05$) (Figure 10(C)).

4. Discussion

Results of our study showed for the first time that synthesis of salicylic acid from wintergreen oil by green chemistry overcomes its cytotoxicity in keratinocyte cells and

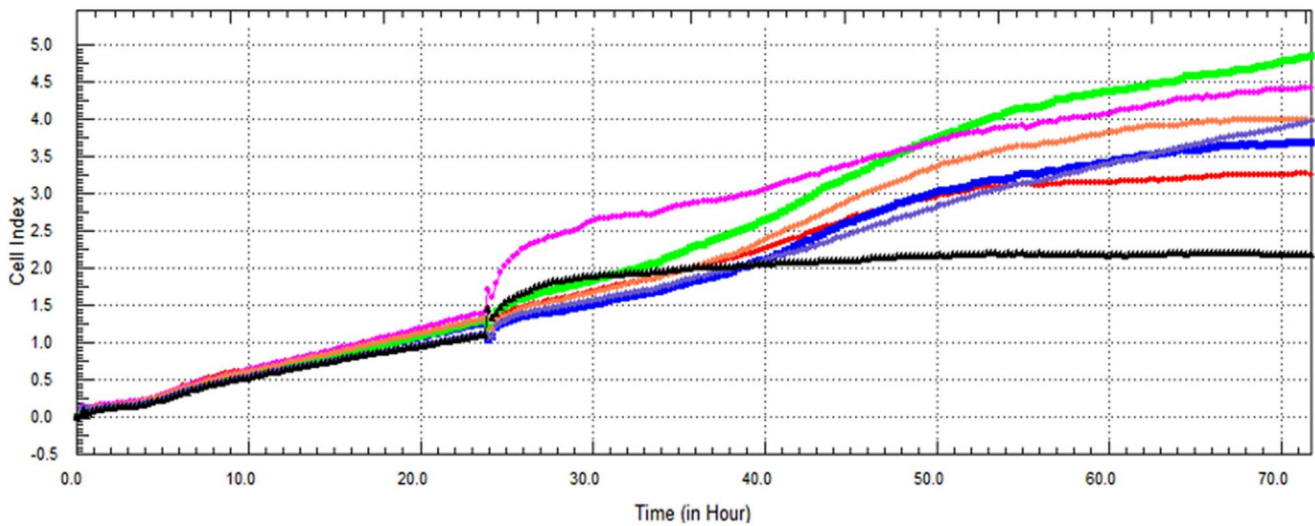


Figure 5. Presentation of the effect of treatments with various concentrations of natural and synthetic salicylic acid on real-time cell analyzer curves generated with human keratinocyte cells. Three duplicate values were used to calculate each data point.

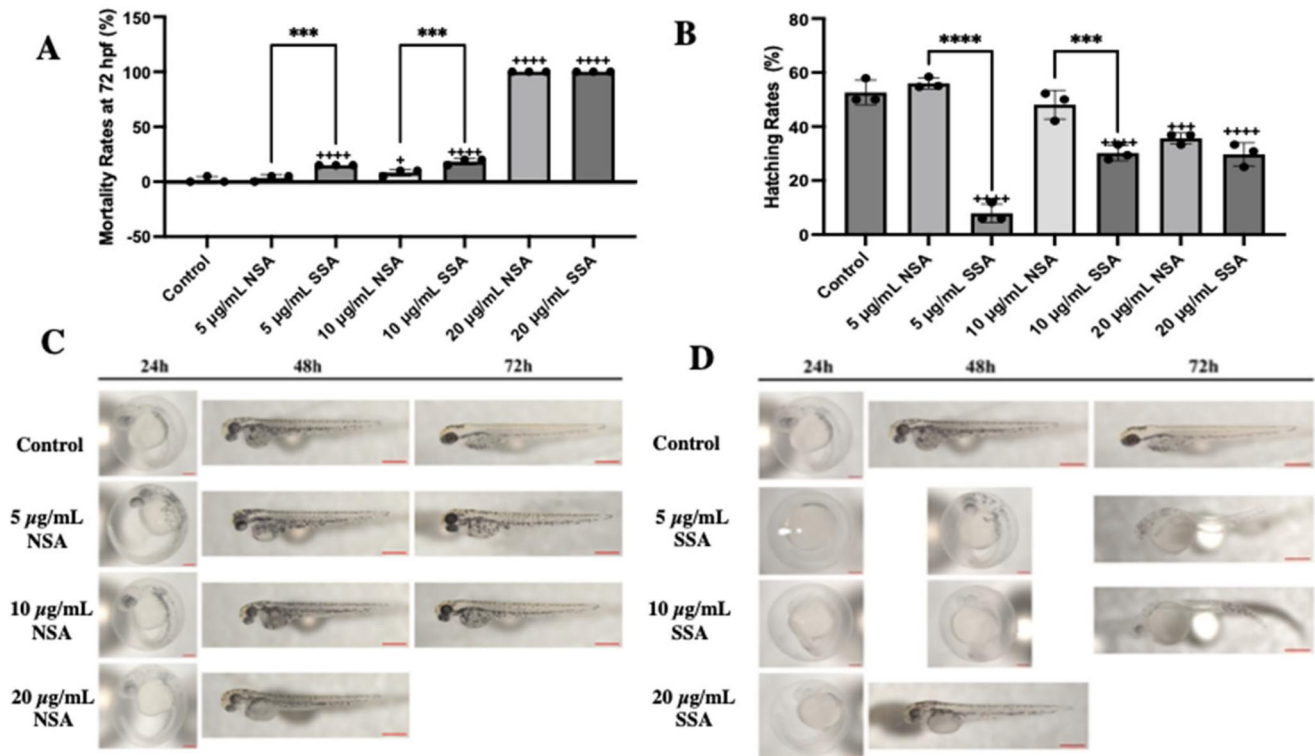


Figure 6. Presentation of (A) mortality rates of the groups, (B) hatching rates of the groups, and (C) representative images of the natural salicylic acid treated zebrafish embryos in the groups. (D) Representative images of the synthetic salicylic acid treated zebrafish embryos in the groups. Data from the three independent studies ($n = 3$, three biological replicates for each group, 50 embryos/pool) are expressed as mean \pm SD.

teratogenicity in zebrafish embryos. This finding is important for drug research on safe topical applications during pregnancy, when preventing exposure to drug and chemical-derived teratogens is vital.

In our study, although SSA negatively affected parameters such as proliferation, adhesion, and migration on human keratinocyte cells, values similar to the control group were obtained in the NSA exposed group. The results of the analyses performed on zebrafish embryos showed that SSA increased mortality rates compared to NSA at both 5 μ g/L

and 10 μ g/L concentrations, while decreasing hatching rates. Moreover, lack of pigmentation and spinal curvature observed in embryos exposed to SSA indicated disruption of normal development as well as developmental delay. Similar to our observations in SSA exposed zebrafish embryos, Singh et al. (2009) reported inhibited growth and developmental parameters and dysmorphologies including abnormal tail curling, neural tube defect, and somite abnormalities in cultured rat embryos exposed to salicylic acid in a concentration-dependent pattern.

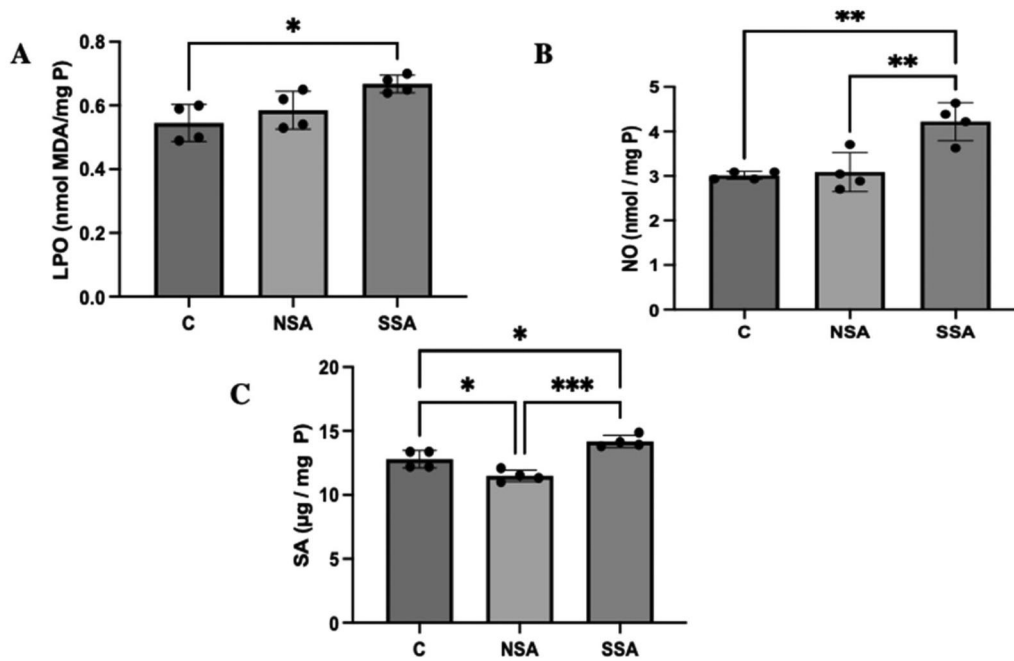


Figure 7. Presentation of (A) MDA levels serve as a measure of lipid peroxidation (LPO); (B) nitric oxide (NO) levels; (C) sialic acid (SA) levels. Data are given as mean \pm SD resulting from the three independent experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. SD: standard deviation.

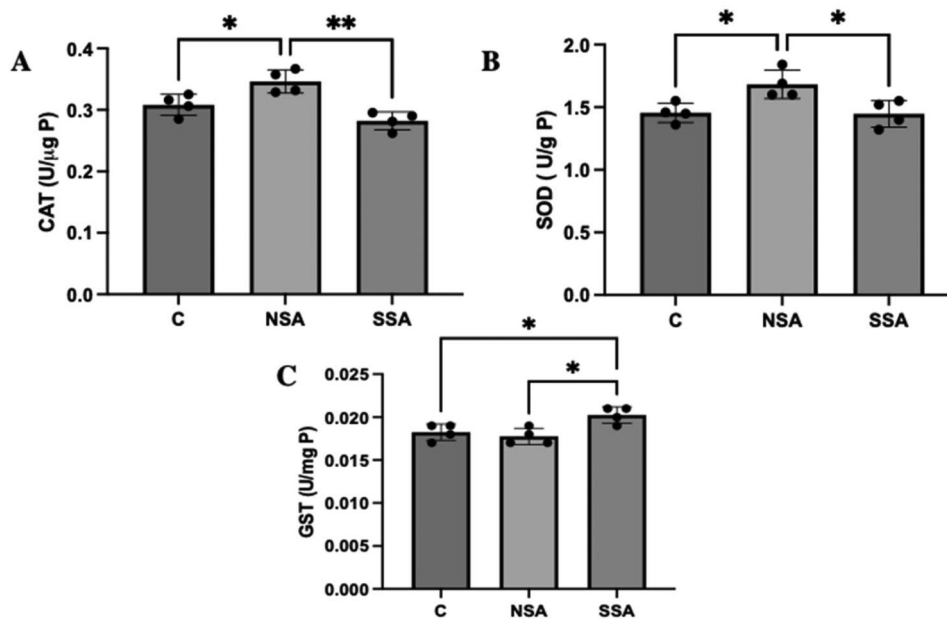


Figure 8. Presentation of (A) catalase (CAT) activities; (B) superoxide dismutase (SOD) activities; (C) glutathione S-transferase activities of the groups. Data are given as mean \pm SD resulting from the three independent experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). * $p < 0.05$; ** $p < 0.01$. SD: standard deviation.

The main IGF ligand controlling prenatal growth in all vertebrates, including humans, is IGF-II. In zebrafish embryos, expression of *igf2a* mRNA is observed in the notochord, during segmentation. The knockdown of *igf2a* gene with morpholino oligonucleotides caused defects in dorsal midline development, delayed segmentation, and also ventral curvature (White et al. 2009). In our study, although there was no change in *igf2a* expression in the SSA group, the upregulation of *igf2a* expression in the NSA group showed that embryo development was

stimulated in this group. While the embryos in the NSA group showed normal and healthy development, the appearance of anomalies such as decreased pigmentation and spinal malformation in the embryos in the SSA group may be associated with the upregulation of *igf2a* expression in the NSA group.

Teratogenicity can be mediated by aberrant patterns of apoptosis, a process required for embryonic modeling. Singh et al. (2009) suggested apoptosis to be involved in the regulation of salicylic acid induced teratogenicity, through

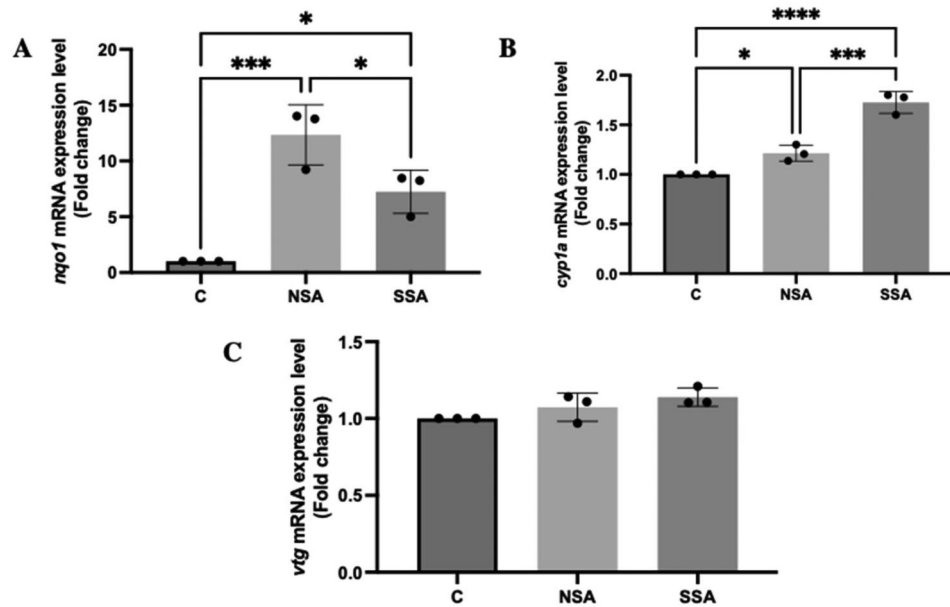


Figure 9. Presentation in a bar graph of the fold change of the RT-PCR-quantified transcripts of (A) *nqo1*, (B) *cyp1a*, and (C) *vtg*. All RT-PCR results are expressed as changes from their respective controls after being normalized to the housekeeping gene -actin. Three studies ($n = 3$, three biological replicates for each group, 50 embryos/pool) were used to calculate the average values. Data presented are mean \pm SD. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. SD: standard deviation.

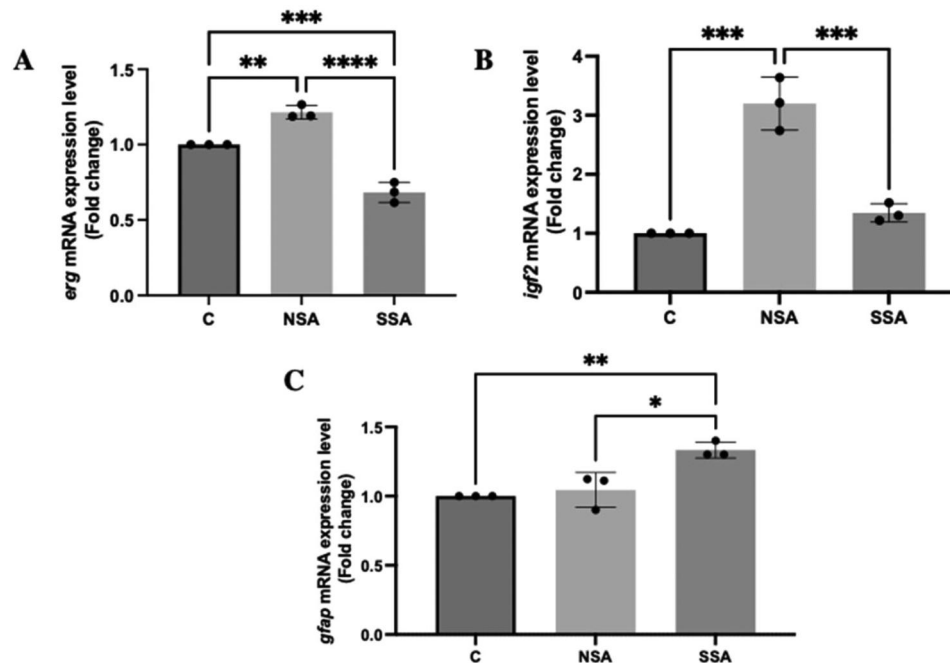


Figure 10. Presentation in a bar graph of the fold change of the RT-PCR-quantified transcripts of (A) *erg*, (B) *igf2*, and (C) *gfap*. All RT-PCR results are expressed as changes from their respective controls after being normalized to the housekeeping gene -actin. Three studies ($n = 3$, three biological replicates for each group, 50 embryos/pool) were used to calculate the average values. Data presented are mean \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. SD: standard deviation.

annexin V binding and DNA fragmentation analysis. 2,3- and 2,5-dihydroxybenzoic acid (DHBA) are the main salicylic acid metabolites and these molecules are known to be teratogenic due to their interactions with free oxygen radicals (Karabulut et al. 2000). Consistent with this information, in our study, when compared to the control, SSA exposure increased the levels of LPO and NO, which are oxidative damage markers, while it did not change the activities of

SOD and CAT, which are antioxidant system components, but increased GST activity.

The development of the embryo is highly sensitive to damage to oxidant molecules due to its low antioxidant capacity (Dennery 2010). Reactive oxygen species act as signaling molecules that trigger the transcription of numerous genes, including those involved in oxygen sensing, cell differentiation, and proliferation. Reduced oxygenation has been

shown to result in decreased fetal growth independent of nutrition, in a chick embryo model (Giussani et al. 2007). Consistent with these studies, in our study, developmental delay and disorders were observed in parallel with increased oxidative stress in the SSA group.

GSTs are enzymes that catalyze the conjugation of glutathione with electrophilic chemicals, especially those formed by biotransformation of foreign xenobiotics (Hayes et al. 2005). A variety of genes coding phase II detoxification enzymes and antioxidant proteins contain the antioxidant response element (ARE), a cis-acting regulatory element (Dhakshinamoorthy et al. 2000). The ARE controls the transcriptional activation of the genes GSTs and NAD(P)H:quinone oxidoreductase (NQO1), which are downstream. While SSA exposure caused an increase in GST enzyme activity, GST activity did not change in the NSA group. This may be due to the fact that NSA does not act as an exogenous xenobiotic. Both NSA and SSA increased *nqo1* expressions indicating the activation of the antioxidant system, which was much more pronounced in NSA group.

The cytochrome P450 (CYP1) family also has critical functions in the biotransformation process of various drugs (Saad et al. 2016). In our study, the fact that SSA caused severe activation of *cyp1a* expression while NSA caused a slight increase in *cyp1a* expression can be considered as an indicator of the weak xenogenous effect of naturally produced NSA. In addition, the statistically significant increase in *cyp1a* expression in the SSA group compared to the NSA group also showed how the activation of xenobiotic metabolism changed in the case of natural production and synthetic production of salicylic acid.

The alpha subunit of a potassium ion channel, which is involved in the electrical activity of the heart, is encoded by the *erg* gene (Vijayaraj et al. 2012; Rossato et al. 2013). When we evaluated the expression of *erg* as a biomarker of cardiac development, we found decreased *erg* expression in the SSA group. This finding is consistent with the morphologically observed developmental anomalies in the same group. On the other hand, NSA treatment was found to enhance the expression of *erg*. Consistent with our findings, SSA exposure caused cardiac abnormalities in the form of primitive 'tubelike' hearts, impaired yolk sac circulation in rat embryos (Joschko et al. 1993).

It is believed that glial fibrillary acidic protein (GFAP), a type III intermediate filament protein that is solely expressed in astrocytes, is a special feature of these cells. The *gfap* gene's expression and the development of the nervous system were linked. In zebrafish, the *gfap* gene was frequently used as a diagnostic for neurotoxicity (Cassiman et al. 2002). Although the NSA treatment did not change *gfap* expressions, SSA treatment caused a significant increase in *gfap* expressions, which might be linked to its neurotoxic effect. This finding is consistent with the study of Joschko et al. (1993) who suggested salicylic acid's teratogenic effects on the development of nervous system in rats. Through cellular and ultrastructural analysis, they showed that the neuroepithelium experienced significant cell death, compared to the mesenchymal cells, which were less affected. Moreover,

Morgan et al. (1997) has shown the activation of GFAP transcription by oxidative stress in cultured glia, and in our study increased *gfap* expression in the SSA treated group is related with increased oxidative stress as evidenced by increased LPO in the same group.

As a conclusion, the results of our analyses in both human keratinocyte cells and zebrafish embryos showed that SSA caused toxic effects when compared to NSA obtained by green chemistry method, pointing to the need to expand the use of green chemistry method in the production of salicylic acid. Accordingly, the previously reported teratogenic effects of salicylic acid can be prevented by the use of salicylic acid produced by the green chemistry method. As a result, although we advocate the need for further studies on the subject, based on the results of our study, we may suggest that NSA obtained by green chemistry method can be a suitable raw material for topical applications to avoid teratogenicity.

Author contributions

Conception/design of study: Gökhan Özokan and Ebru Emekli-Alturfan; data acquisition: Gökhan Özokan, Derya Cansız, Abdulkemil Bilginer, İsmail Ünal, and Merih Beler; data analysis/interpretation: Gökhan Özokan, Derya Cansız, Abdulkemil Bilginer, İsmail Ünal, and Merih Beler; drafting manuscript: Gökhan Özokan and Ebru Emekli-Alturfan; critical revision of manuscript: A. Ata Alturfan and Ebru Emekli-Alturfan; supervision: Ebru Emekli-Alturfan.

Ethical approval

As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (2010), EU Directive 2010/63/EU.

Consent form

All the authors have agreed for authorship, read and approved the manuscript, and given consent to participate.

Disclosure statement

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Data availability statement

Data are available on reasonable request.

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