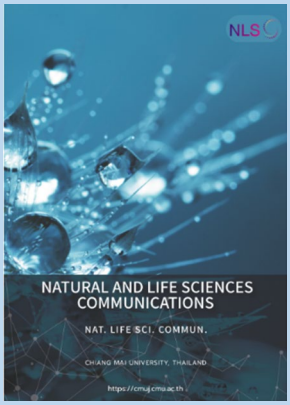
Research article



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The Embryotoxicity of Alpha-Pinene to the Early Life Stages of Zebrafish (*Danio Rerio* Hamilton, 1822)

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ABSTRACT

The Alpha-pinene (AP) is produced by pine trees and other plants. The AP exhibits various biological activities such as the development of antimicrobial and antiviral agents, flavors, fragrances, and fungicidal agents. The toxicity data for AP are limited. This study aimed to determine the embryotoxic effects of AP in zebrafish embryos. 1.5 hpf zebrafish embryos were exposed to different concentrations of AP for 72 hours. The LC_{50} and EC_{50} values for AP were determined as 441.360 mg/L and 367.795 mg/L, respectively. The embryos were not much affected by AP until hatching. In addition, AP showed teratogenic effects at high doses (320 and 640 mg/L). Typical lesions were an absence of somite (≤48 hpf), lordosis, yolk sac deformity, tail abnormality, cardiac edema, and eye shrinkage (≤ 72 hpf). However, survival rates of zebrafish embryos in the 20, 40, and 80 mg/L AP groups were greater than 80 % during the exposure period. Very low teratogenicity for zebrafish embryos was observed in the 160 mg/L AP group. The results show virtually no embryotoxicity and teratogenicity at 20 and 40 mg/L AP concentrations. No delay in developmental was also observed. Therefore, AP can be considered a safe compound in the concentrations.

Keywords: Alpha-pinene, Embryotoxicity, EC_{50}, LC_{50}, Zebrafish

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INTRODUCTION

In recent years, interest in the use of medicinal and aromatic plants has been increasing. These plants contain essential oils rich in biologically active compounds (González-Burgos and Gómez-Serranillos 2012). These compounds can prevent cell damage caused by reactive oxygen by preventing the propagation of the oxidative chain reaction. Alpha-pinene (AP) is a natural compound found in the oils of many coniferous wood species, particularly pine (Zhou et al. 2004). Previous studies have shown that AP has a variety of pharmacological effects. It is also known that AP has a strong inhibitory effect on inflammation in pathological conditions (Bae et al. 2012; Kim et al. 2015; Borges et al. 2019). AP is used in various industrial processes such as the fragrance and flavor industry, pulp and paper industry, pharmaceutical industry, color printing and dye processing (Linares, Fontanille, and Larroche 2009; Rene et al. 2010; Montes, Veiga, and Kennes 2010; Bertouche et al. 2012; Wu et al. 2012). People can be exposed to the compound during the processing, use, storage of softwood or its by-products, and during the use of personal care products, cleaning products, or air fresheners that contain α-pinene as a fragrance component. Human toxicity of α-pinene or terpene mixtures containing α-pinene has been reported as potential respiratory and skin irritation. An animal experiment to determine the general toxicity, reproductive toxicity, or developmental toxicity of α-pinene has not been found in the literature (NTP 2016).

Zebrafish (Danio rerio) has been widely used as a model organism in various fields such as genetics, cancer research, and developmental biology (Nishimura et al. 2016; Aksakal and Sisman 2020). The embryo of zebrafish develops quickly and its development is easy to follow because they are transparent. Therefore, it is a valuable experimental animal for vertebrate development studies (Tran, Facciol, and Gerlai 2016). Developmental processes in zebrafish embryos are very similar to the embryogenesis of other higher vertebrates, including humans (Kimmel et al. 1995). In addition, zebrafish adults and larvae provide great advantages in toxicity studies. In this way, larger vertebrate models such as rats have become less useful in toxicity studies. The zebrafish embryo has been accepted as an alternative model for toxicological evaluation (Tran, Facciol, and Gerlai 2016; Nishimura et al. 2016; Ceylan et al. 2019) and new drug discovery (Falcão et al. 2018; Yang et al. 2018). In this study, embryotoxic and teratogenic evaluations of both medically and naturally exposed AP were determined using zebrafish embryos. There is no previous study of this type in the literature.

MATERIALS AND METHODS

Materials

Alpha-pinene (AP) was obtained from Sigma Aldrich. ((+)-α-pinene; $C_{10}H_{16}$, CAS No. 7785-70-8; Sigma-Aldrich®, USA). Different concentrations of AP were tested. The concentrations (20, 40, 80, 160, 320, and 640 mg/L) were selected according to Turkez and Aydin (2016). Wild-type zebrafish (3-6 months) were purchased from Atatürk University Fisheries Faculty. According to EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the early life stages of animals are not defined as protected (EU 2010). Therefore, it does not fall within the regulatory frameworks regarding animal experiments. Zebrafish embryos/larvae up to 120 hours postfertilization are not free feeding. This study involved the embryo toxicity up to 72 hrs and ethical approval was not required for the study.

Methods

Maintenance, spawning and egg collection

Fish were kept in a glass aquarium providing sufficient space for swimming (1 L per 1 gr fish). Fish were maintained at 26 ± 1°C and 14:10 light/dark cycle. Fish were fed twice a day with tetramin flakes. Eggs were obtained by randomly mating zebrafish in a 3-2 male-female ratio. Glass balls and plastic sieves were used in the hatching aquarium to prevent the eggs from being eaten by the adults. The same dilution water was used for spawning, but no filter. Male and female fish were placed in the brood aquarium at the beginning of the dark cycle. Ovulation and fertilization occur within 30 minutes of the onset of morning light. Ovulation occurred within one hour of the start of the light cycle. About 30-60 minutes after spawning, adult fish were removed. Eggs were collected by siphoning and transferred to Petri dishes containing Hank's solution. Fertilized eggs were detected under the microscope. Fertilized eggs were placed in Petri dishes with test solution.

Fish embryotoxicity test

The six concentrations of AP (20, 40, 80, 160, 320, and 640 mg/L) were prepared as dilution with Hank's solution. Twenty fertilized fish eggs were transferred into 50 mm petri dish containing different concentrations of AP. The control group did not contain the AP, only Hank's solution. The Petri dishes were kept at 26 ± 1°C and 14:10 light/dark cycle. Exposure was initiated within 1.5 hours of fertilization (OECD 2013). The exposure was maintained for 72 hours. All solutions were renewed every 24 hours. The embryos and larvae were observed at 24, 48 and 72 hours. Some teratogenic abnormalities were captured using a microscope with the digital camera. Dead embryos were counted and recorded. According to the data, LC_{50} (median lethal concentration) and EC_{50} (median effective concentration) values were calculated. Teratogenic abnormalities were classified according to the grading reported by Padilla et al. (2011). The Malformation Index (MI) was also proposed by Padilla et al. (2011) and has been applied here with some modifications. This value is a semi-quantitative data. Embryos and larvae of zebrafish were visually graded for malformation at 24, 48, and 72 hours on a grading scale. In the scale, each embryo/larva was evaluated by scoring yes/no or 0 to 4 for various categories (e.g., curved spine, abnormal head, edema). Scores from each category are summarized to give a total malformation index for each embryo/larva. It was accepted that MI value 0-3 was normal; values 4-6 were considered mild abnormality and values above 7-8 clearly deformed fish embryo/larva.

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software program. 72 hours LC_{50} and EC_{50} values were calculated by probit analysis. Evaluation of abnormal embryos and larvae was made using the one-way ANOVA test. All data were given as mean ± standard error. Duncan's test was performed to determine whether any treatment significantly differed from controls or each other and the statistical significance level was accepted as $P < 0.05$.

RESULTS

LC_{50} and EC_{50} values

In embryos exposed to AP for 72 hours, toxicity was particularly dose-related. LC_{50} and EC_{50} values at 72 h were 441.360 mg/L and 367.795 mg/L, respectively. The dose-response curves of AP for 24, 48 and 72 h were indicated in figures 1 and 2.

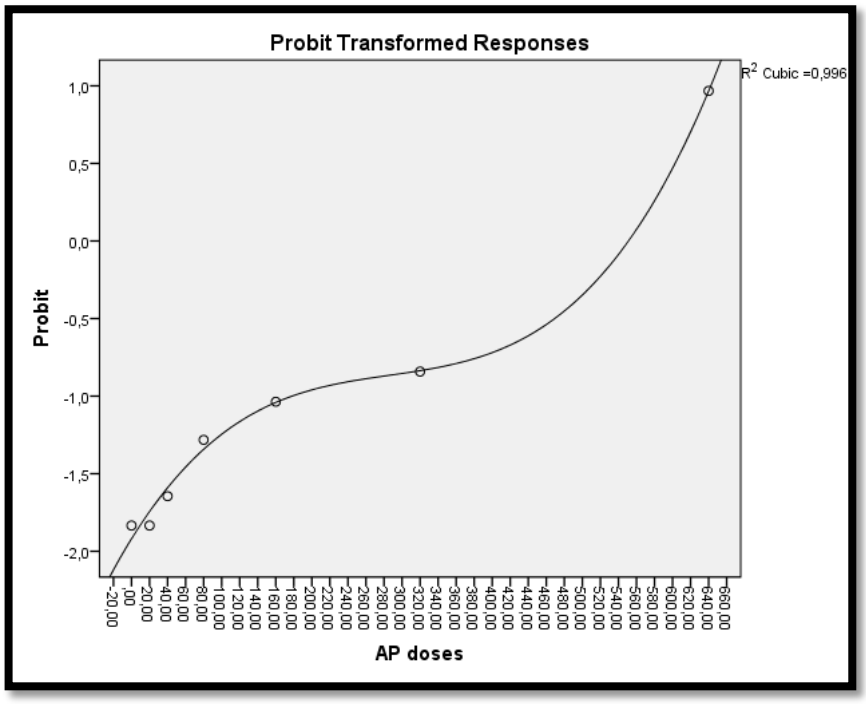

Figure 1. Dose-response curve used for calculation of LC_{50} value for AP (confidence interval 95%).

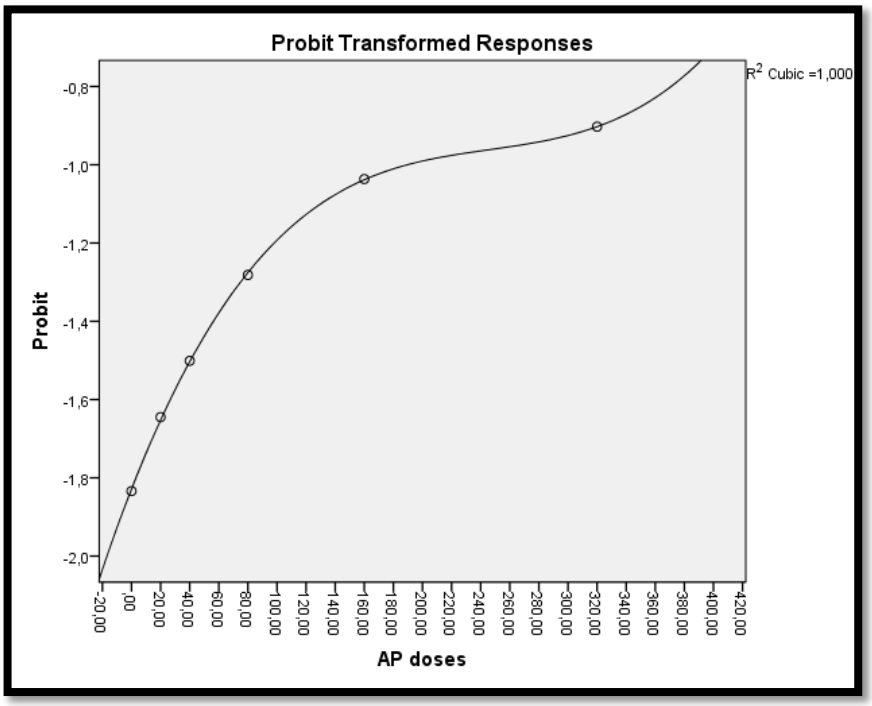

Figure 2. Dose-response curve used for calculation of EC_{50} value for AP (confidence interval 95%).

Mortality rates after 72 hours of exposure were quite interesting. When the deaths in the 20 and 40 mg dose groups were compared with the control, it was understood that there was no significant difference between them. In 80 and 320 mg/L doses, this ratio varies between 10% and 20%. The highest mortality rate was detected in embryos exposed to 640 mg/L AP. Embryotoxic effects appeared similarly. The highest frequency of teratogenic abnormalities was observed in the 320 mg/L AP group. In other groups, this ratio varied between 5% and 10%. The survival

rates of zebrafish embryos in the 20, 40 and 80 mg/L AP groups were greater than 80 % during the exposure period (Table 1).

Table 1. Adverse effects in embryos caused AP at 72-hours exposure.

	Control	20 mg/L	40 mg/L	80 mg/L	160 mg/L	320 mg/L	640 mg/L
∑ Teratogenicity	2	2	3	6	9	12	0
∑ Mortality	2	3	4	6	9	11	60
∑ Affected embryos	4	5	7	12	18	23	60
∑ Normal embryos	56	55	53	48	42	37	0
% Teratogenicity	3.2 ± 0.4	3.2 ± 0.6	5.2 ± 0.6	10.3 ± 0.9	15.0 ± 1.7	20.0 ± 0.8	0
% Mortality	3.5 ± 0.2	5.5 ± 0.7	6.6 ± 0.5	10.3 ± 0.6	15.1 ± 0.9	18.3 ± 0.6	100
% Affected embryos	6.6 ± 1.6	8.3 ± 0.8	11.3 ± 0.9	20.0 ± 0.7*	30.3 ± 2.9*	37.9 ± 1.8	100*
% Normal embryos	93.5 ± 1.7	91.2 ± 2.1	88.4 ± 1.7	80.1 ± 2.5	69.5 ± 4.1	61.3 ± 3.8	0

Note: *Shows differences at $P < 0.05$ level compared to control.

Embryotoxicity results

The embryos were not much affected by AP until hatching. Very low teratogenicity for zebrafish embryos was observed in the 160 mg/L AP group. However, AP showed teratogenic effects at high doses. Typical lesions were an absence of somite (≤48 hpf) (Figure 3), cardiac edema (48 hpf) (Figure 4), lordosis, tail abnormality, yolk sac deformity, cardiac edema, and eye shrinkage (≤ 72 hpf) (Figure 5).

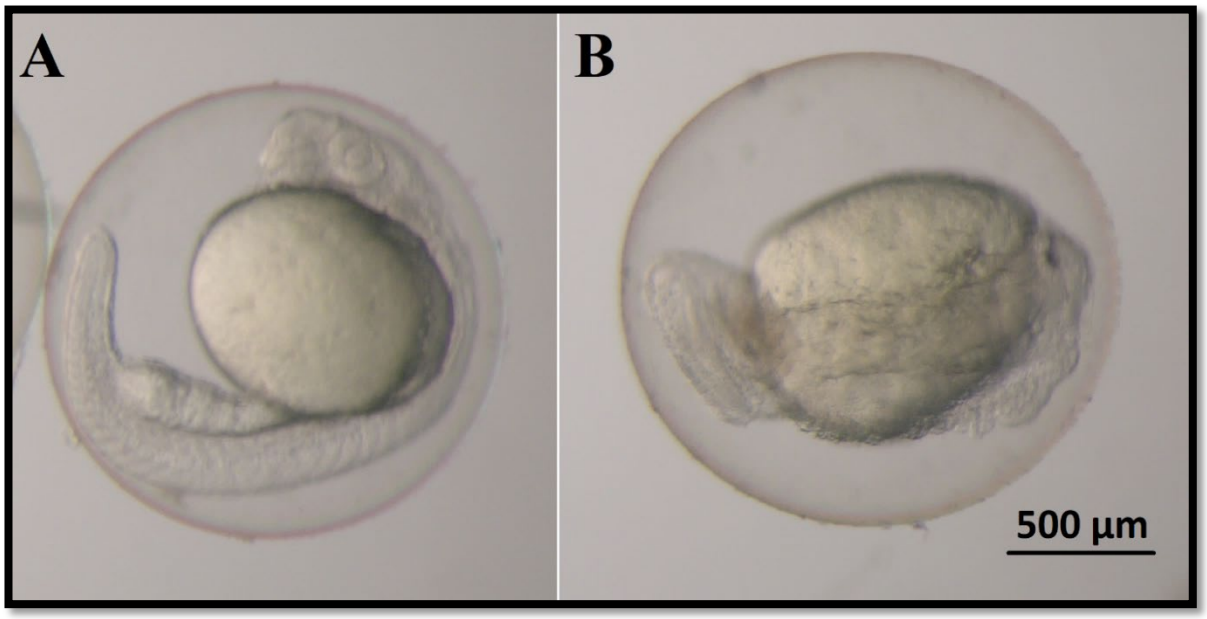

Figure 3. 24 hpf zebrafish embryos. A) normal embryo, B) abnormal embryo exposed to AP; absence of somite.

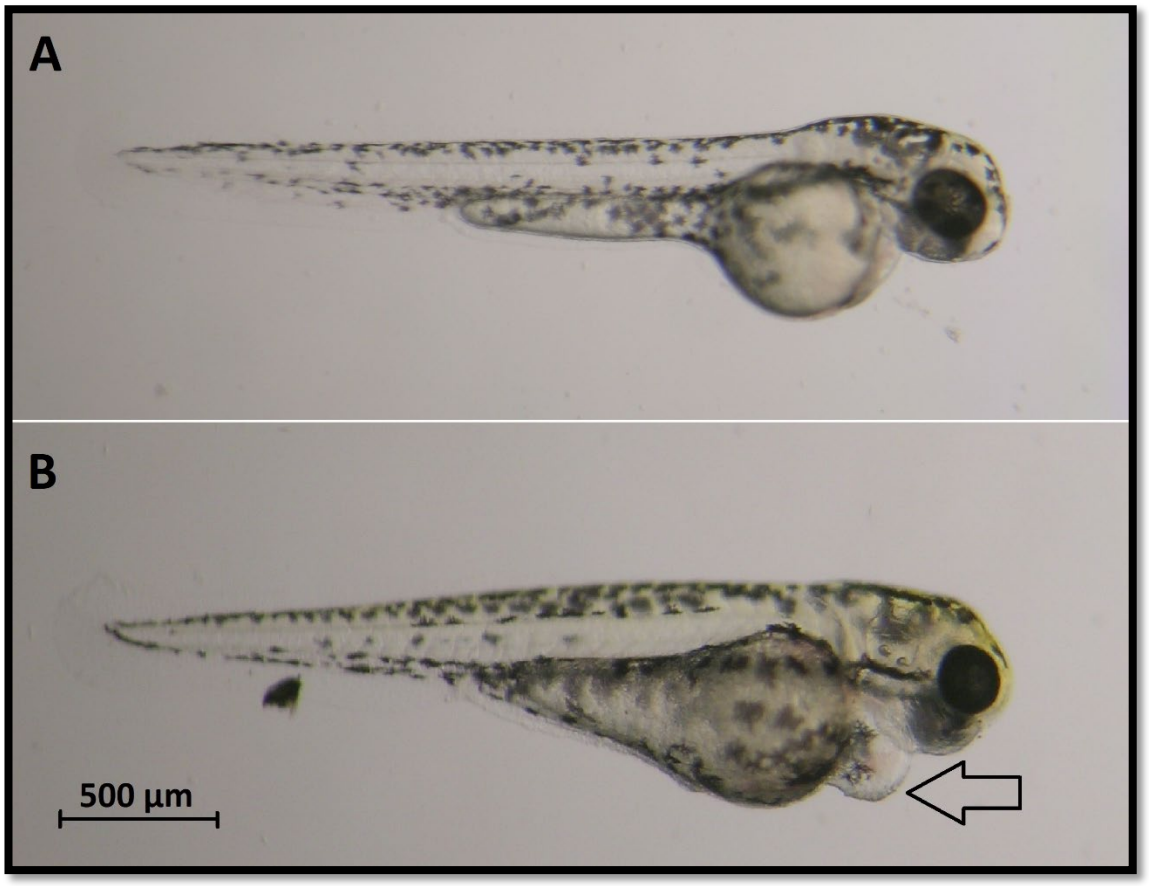

Figure 4. 48 hpf zebrafish larvae. A) normal larva, B) abnormal larva exposed to AP; cardiac edema deformity (arrow).

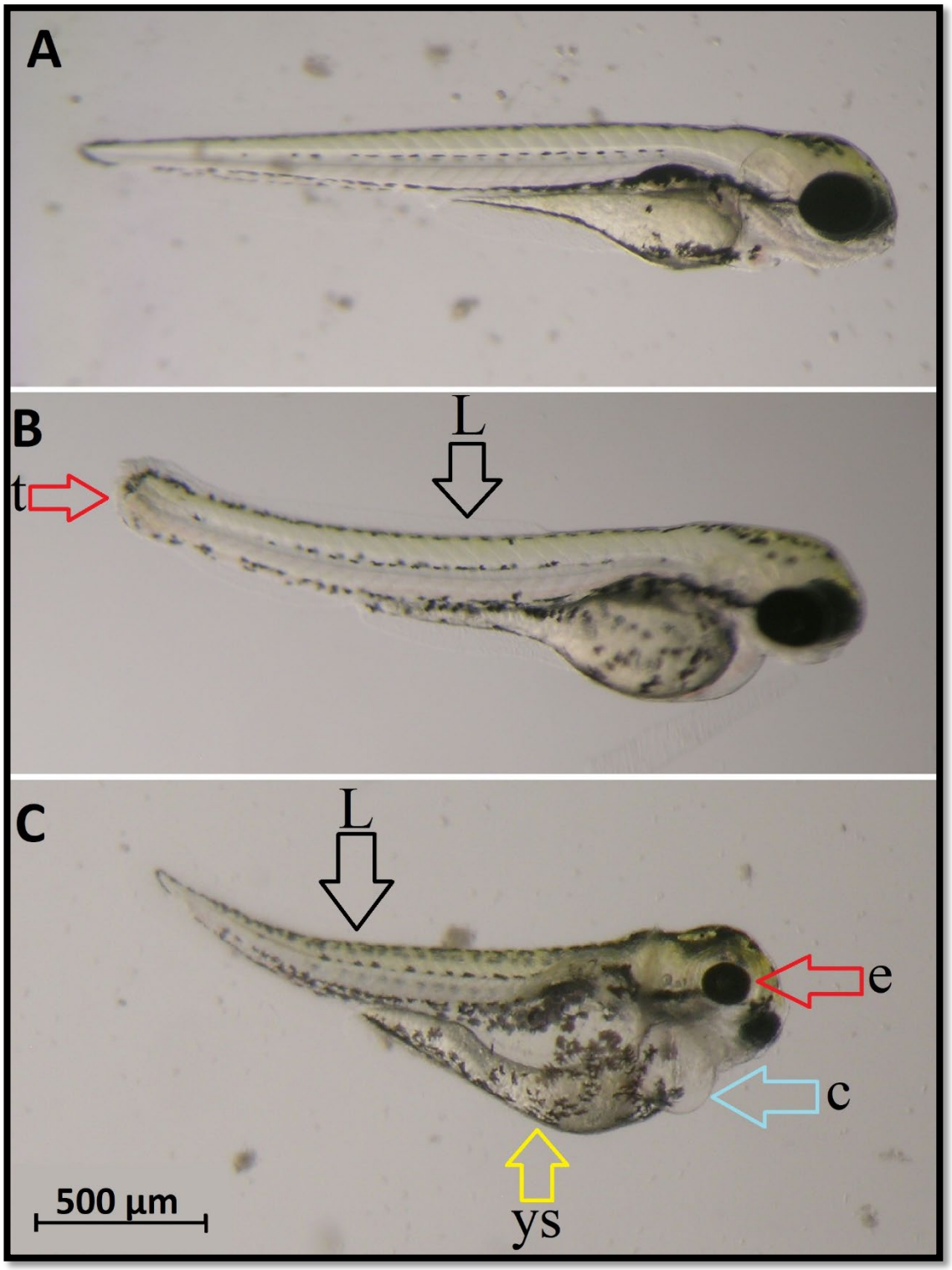

Figure 5. 72 hpf zebrafish larvae. A) normal larva, B) abnormal larva exposed to AP; lordosis (L), tail abnormality (t), C) abnormal larva exposed to AP; yolk sac deformity (ys) cardiac edema (c), eye shrinkage (e), and lordosis (L).

Total malformation index (MI) values calculated for AP were given in Table 2. The MI values for the 20 and 40 mg/L AP doses were not different from the control group. However, MI values increased for other AP concentrations. In particular, MI values for 320 mg/L of AP were above 4, indicating that mild abnormalities were

observed in most embryos and larvae. MI values for 640 mg/L could not be calculated because all embryos died.

Table 2. Total malformation index (MI) values calculated for 72-hour fish larvae exposed to the AP.

Exposure time (hours)	Control	20 mg/L	40 mg/L	80 mg/L	160 mg/L	320 mg/L	640 mg/L
24	0.80 ± 0.02	0.60 ±0. 02	1.06 ± 0.06	0.9 ± 0.01	1.50 ± 0.03	0.90 ± 0.02	0
48	1.30 ± 0.06	1.50 ± 0.04	1.60 ± 0.04	1.90 ± 0.06*	2.50 ± 0.04*	3.30 ± 0.06*	0
72	1.60 ± 0.06	1.30 ± 0.03	1.60 ± 0.01	2.00 ± 0.03*	4.50 ± 0.04*	6.30 ± 0.09*	0

Note: *Shows differences at $P < 0.05$ level compared to control.

DISCUSSION

This is the first investigation for the embryotoxicity and teratogenicity of AP. Safe doses of AP alkaloid are also shown in the zebrafish model. AP at concentrations of 20 and 40 mg/L was not toxic to fish embryos. AP at a concentration of 80 mg/L showed a teratogenic effect of 10.3% and was not considered important in terms of embryotoxicity and teratogenicity. The LC_{50} and EC_{50} for AP were 441.360 mg/L and 367.795 mg/L, respectively. Our findings show virtually no embryotoxicity and teratogenicity at concentrations less than 80 mg/L. The zebrafish embryotoxicity assay is widely used in evaluating the toxicity of different plant extracts, compounds and components. Similar results were obtained in evaluating the toxicity of the active fractions of crude preparations and herbal medicines using the zebrafish embryotoxicity model. In a study, zebrafish embryos were exposed to a series of concentrations of green chireta (*Andrographis paniculata*; a herbal plant recommended various illness) leaves to assess toxicity. It was reported that the LC_{50} value (at 96 hpf) of the leaves was 520 mg/L, and abnormal organ development, enlarged yolk sac, pericardial edema, slow heartbeat, and delayed hatching were recorded in 72 hpf larvae (Ismail et al. 2017). The effect of the extract prepared from the root of the Turmeric plant, which has a lot of pharmaceutical properties, on zebrafish is as follows. The LC50 value (at 72 hpf) was 68.31 µg/mL, and several developmental abnormalities such as kink tail, bend trunk, and enlarged yolk-sac edema were reported in embryos treated with 62.5 µg/mL concentration (Alafiatayo et al. 2019). In another study, the 96 h LC50 of safflower (*Carthamus tinctorius*; a medicinal plant using blood stasis syndrome with dysmenorrhea, amenorrhea, postpartum abdominal pain and mass, and trauma and pain in the joints) to zebrafish embryos was calculated as 345.6 mg/L with delayed hatching rates (Xia et al. 2017). The LC_{50} and EC_{50} values (48 hpf) determined for zebrafish embryos of Martine, a type of alkaloid produced by *Sophora flavescens*, and possessing a variety of pharmacological effects such as antiinflammation, antivirus, antitumor, and antiarrhythmic activities, were 240 and 145 mg/L (Lu et al. 2014). It is seen that the embryotoxic effects of the active fractions of crude preparations and herbal medicines on zebrafish occur at very high doses. From this point of view, we can say that our results are compatible with the studies.

Previous studies have shown that AP has a variety of pharmacological effects. In addition, AP is effective in pathological conditions. It is known that the protective dose range varies in some studies. For example, Khoshnazar, Parvardeh, and Bigdeli (2020) reported that AP at doses of 50 and 100 mg/kg significantly improved sensorimotor function and reduced the volume of the infarct area in the rat brain. The study showed that AP is a promising molecule for attenuation of cerebral ischemia-reperfusion injury. In another study, it was reported that oral administration of AP at doses of 100 and 200 mg/kg for 14 days improved movement

disorder and avoidance memory, accompanied by a decrease in malondialdehyde in the brain and blood (Goudarzi and Rafieirad 2017). A similar dose range gave best results in human cell culture studies. Turkez and Aydin (2016) demonstrated that AP had no mutagenic effects on human lymphocytes over the dose range of 10 to 200 mg/L, and AP exhibited antioxidant properties. AP can reach people in different concentrations depending on the places of use. However, the amount detected in such places is also quite low. Rastogi et al. (Rastogi et al. 2001) reported that they detected AP at 41.3 ± 51.5 ppm concentrations in 39% of 59 occupational and household products. After applying the floor varnish containing AP, the maximum terpene concentration measured in a small chamber was approximately 0.012 ppm (Colombo et al. 1991). Such studies show that the amount of AP released into the air is not high. The above doses are considerably lower than the LC_{50} (441.360 mg/L) and EC_{50} (367.795 mg/L) values obtained in the study.

CONCLUSION

The most abundant monoterpene in the atmosphere is AP. The monoterpene exhibits various biological activities. The embryotoxicity and teratogenicity of AP are not reported, yet. Thus, the current study was used zebrafish embryos to assess the toxicity. Our findings show virtually no embryotoxicity and teratogenicity at concentrations of 20, 40 and 80 mg/L of AP. Overall, results from the study, along with findings from other studies, suggest that AP develops low toxicity.

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AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: T Şişman; data collection: T Şişman; analysis and interpretation of results: T Şişman; draft manuscript preparation: T Şişman, Z Ceylan; language editing: Z Ceylan. All authors reviewed the results and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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