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Effects of cannabis tea on the development of zebrafish embryo and larval behaviour

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Abstract

Cannabis is a known illicit substance that is often used by pregnant women, however its potential impact on the developing embryo is unknown. Thus, this experiment used a zebrafish model to investigate how chronic exposure to cannabis tea affected zebrafish development and behaviour. Zebrafish embryos were exposed to cannabis tea prepared at concentrations of 5, 10 and 15 mg/L for 96 hours. The resulting mortality, hatching duration, morphological abnormalities, startle response, light-dark preference and optokinetic response were then assessed. The cannabis tea had a significant impact on the mortality, hatching duration, startle response and optokinetic response. The tea had a LC_{50} of 11.20 mg/L with mortality increasing with the tea concentration. The hatching duration increased with cannabis exposure ($p = 0.046$). For the startle response, the effect of the cannabis tea was concentration dependent, with higher concentrations reducing the startle response ($p < 0.001$). The optokinetic response initially increased at the lowest cannabis dose, but subsequent increase caused reductions in the response ($p < 0.001$). As for the morphological abnormalities, deformities were only observed in larvae exposed to cannabis. The cannabis tea also showed both anxiolytic and anxiogenic effects on the larvae in the light-dark preference assay. These results were comparable to studies investigating THC and CBD separately. Although more comprehensive research is required to offer a better understanding, these findings aid in our knowledge of how a cannabis whole extract influences development.

Keywords: Cannabis tea; Zebrafish; Chronic exposure; Embryo; Behaviour; Abnormalities

1. Introduction

Cannabis is the most commonly used dependent substance amongst pregnant women with approximately half of those who have used the drug in their lifetime continuing its use during pregnancy [1]. This is attributed to its perceived safety or its use in reducing pregnancy symptoms like nausea [1, 2]. Cannabis, often referred to as marijuana, is derived from parts of the *Cannabis sativa* plant. It has phytocannabinoids as the active compounds and Thompson et al. [1] stated that its two most studied cannabinoids are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Whether inhaled or ingested, cannabis is often used for recreational or therapeutic reasons. For recreational purposes, THC tends to produce a euphoric effect because of its psychoactive properties [1, 3]. Ahmed et al. and Thompson et al. [1, 2] stated that CBD, being non-psychoactive, is responsible for the therapeutic properties of cannabis and has been used as an anxiolytic and even epilepsy treatment. These effects are brought about when the cannabinoids interact with the G-protein coupled receptors of the endocannabinoid system, CB1 and CB2 [1, 2, 3]. CB1 is primarily localised in the central nervous system (CNS) while CB2 is mainly associated with the immune system [1, 2, 3].

CB1 is present in the early stages of mammalian development which suggests that the endocannabinoid system is heavily involved in development/neurodevelopment [3]. Both THC and CBD are able to traverse the placenta [1] suggesting that when pregnant women consume cannabis their embryos will consequently be exposed to the cannabinoids which could impact embryonic development. There have been links between cannabis use and a decreased birth weight as well as more frequent admissions to the neonatal intensive care unit [1, 4]. However, studies

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detailing the effects of cannabis in human pregnancy are limited to being observational or retrospective in nature [1]. Hence, animal models have been used to investigate cannabis toxicity in developing embryos.

The endocannabinoid system is highly conserved in the zebrafish (*Danio rerio*) with the CB1 protein sequence being 70% similar to its human counterpart [3, 5]. Both CB1 and CB2 are present in zebrafish and CB1 has been detected as early as one day post fertilisation [6]. Also, Akhtar et al. [7] established that the CB1 receptor plays a role in the hatching efficiency of zebrafish. This indicates that cannabinoids and their receptors may have developmental functions in zebrafish.

Ahmed et al. [2] documented that exposure to THC during embryonic stages results in abnormal functioning of the CNS and altered locomotor behaviour in a zebrafish model. Also, a study by Carty et al. [8] revealed that CBD produces similar developmental and behavioural toxicities, even at significantly lower concentrations than THC.

A review of literature shows that toxicological effects of CBD and THC on embryonic development have been investigated separately in numerous studies, but very few investigate the effects of a whole extract. One such study done by Licitra et al. [5] showed that a whole cannabis extract had no significant impact on zebrafish hatching and survival rate. They also found that a higher dose of cannabis was able to increase the locomotor activity of the fish. These are contrasting results from those of the publications studying the effects of THC and CBD separately. Therefore, there is a need for more studies on the impact of cannabis whole extracts on embryonic development since typically, cannabis users do not isolate the cannabinoids when using them.

The primary objective of this research was to assess the morphological and behavioural impacts on zebrafish larvae following prolonged exposure to cannabis tea at varying concentrations during the embryonic stage. Additionally, the study sought to investigate whether whole cannabis extracts produce comparable outcomes in zebrafish as those observed when exposed to THC and CBD individually.

2. Material and Methods

2.1. Preparation of solutions, buffers, and cannabis tea

Egg water was prepared using 1.5mL of 40 g/L salt solution to 1 L of distilled water according to the procedure by Westerfield [9]. A drop of methylene blue was added to the litre of salt solution as an antifungal agent [10]. Standardised cannabis tea was prepared by boiling the plant buds in water, with 0.005% DMSO to aid in solubility, following Hazekamp et al. [11]. The 6% methylcellulose solution was prepared according to the methodology by Brokerhoff [12].

2.2. Zebrafish Care and Breeding and Cannabis Exposure

Healthy, adult zebrafish were separated based on sex and placed in two separate tanks during the day to ensure optimal breeding success. In the evening, two males and two females from each tank were removed and transferred to a breeding tank, with a partition separating the male fish from the female fish. The breeding tank was placed in a water bath of 28.5 °C and left over-night in a dark area of the laboratory. The following morning, the breeding tank was removed from the water bath and the partition separating the fish was lifted to allow breeding to commence. Once eggs were observed the fish were removed from the tank. The eggs were then rinsed with 1.5 mL egg water, after which they were placed in a container filled with egg water and incubated at 28°C for 6 hours post fertilization (hpf) until the fertilized eggs had formed their shields. All unfertilized eggs were removed from the tank, and the fertilized eggs were transferred to 24 well plates, with a total of 64 eggs (8 eggs per well) being used per plate. The wells of the plates were filled with 0.005% DMSO, 3mL egg water, and either 5mg/L, 10mg/L, or 15mg/L cannabis tea. The embryos in each well were exposed to each treatment for 96 hours and their development was recorded using a USB microscope connected to a computer display. Treatments were done under static conditions, meaning that neither the control nor treatments were replaced, however the wells were topped daily with their respective treatments to keep evaporation to a minimum. Each treatment was tested one at a time, and each experiment was done in sextuplicate. Replicates were done two at a time, and both 24 well plates were placed in a glass water bath at 28°C with a light source illuminating under each plate.

2.3. Mortality, Hatching and Morphological Analysis

At 5 days post-fertilization (dpf) all deaths were recorded and the LC_{50} was obtained graphically by plotting the proportions of the mortalities, converted to its corresponding probit values, against the log of the cannabis tea concentrations then obtaining the equation of the line. Corrections were made to the proportion of each treatment concentration based on the proportion of deaths occurring 'naturally' that were observed during exposure to only the

egg water. For the surviving larvae, the USB microscope recording of their development was used to calculate average hatching duration between the first and seventh egg hatching in each well. The Morphological abnormalities such as bent axis, bent tails and no tails were then noted and photographed with a microscopic camera.

2.4. Behavioural Assays

2.4.1. Startle Response (Touch)

Startle response (touch) was measured by placing 5 dpf larvae in a clear, shallow cubical container (L 1.2" x W 1.25" x H 0.875") so that their movement could be mainly in the x and y axis. The underside of the tank was covered in opaque white tape so that the larvae could be clearly seen. The larvae were tested one at a time by gently touching their tail with a hairpin to induce an escape response. Using ImageJ software, the speed of each larva was analysed by tracking the distance moved per frame (10 frames per second (fps)).

2.4.2. The Light-Dark Preference Test

A cubical container (L 1.75" x W 1.75" x H 0.875") was divided into two halves and the outside of the container, including the sides and bottom, was covered with opaque white tape and black tape respectively. The container was placed onto a glass stand and a light source was illuminated from underneath. The container was filled halfway with egg water and four 6 dpf larvae were placed in it. The larvae were left for a 5-minute period to allow them to acclimate to the new environment and "choose" a side of the container. Afterwards, the behaviour of the larvae in the container was recorded for a 10-minute period. The results of this analysis were analysed using the ImageJ software which showed the swimming pattern and side preference.

2.4.3. Optokinetic Response

An opaque, white tape was used to cover the perimeter of a depression slide. The slide was filled with 6% methylcellulose, and a 7 dpf larva was placed in the slide. The purpose of the methylcellulose was to constrict the movements of the larvae in the slide. The slide was then placed onto a glass stand and the rotating white circle was projected underneath at 40 fps speed to ensure that the eye movements recorded were in response to a moving object. Eye movements were recorded for a minute, and the rate was calculated manually by dividing the number of movements by the time taken.

2.5. Data Analysis

Video analysis was done with the ImageJ software and statistical analysis was completed with SPSS software. Statistical significance was determined using One-Way Analysis of Variance (ANOVA) paired with Tukey's post-hoc and Games-Howell post-hoc, with statistical significance set at $p < 0.05$. All values were expressed and graphed, using Microsoft Excel, as mean ± standard deviation of the mean (SD).

3. Results

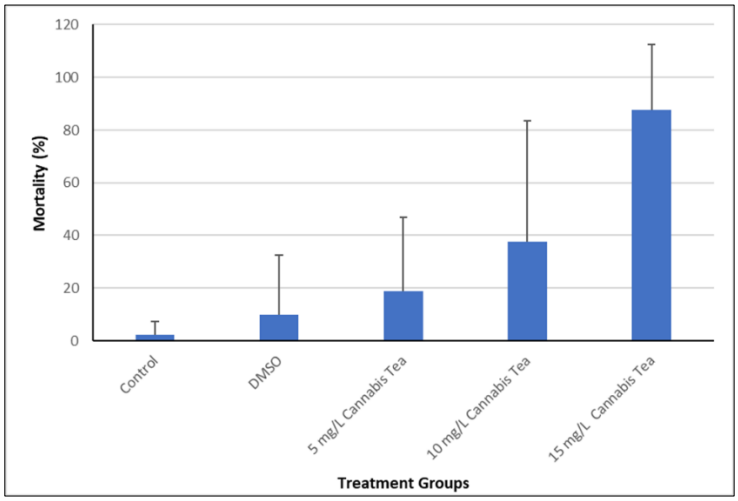

Figure 1 Mean mortality (%) for zebrafish eggs exposed to various treatment for 96 hrs

Table 1 Mean mortality (%) and SD for zebrafish embryos (n = 6) exposed to various treatment for 96 hrs

Treatment Group	Mean Mortality (%)	Standard Deviation (%)
Control	2.08	5.10
DMSO	10.00	22.36
5 mg/L Cannabis Tea	18.75	28.23
10 mg/L Cannabis Tea	37.50	46.10
15 mg/L Cannabis Tea	87.50	25.00

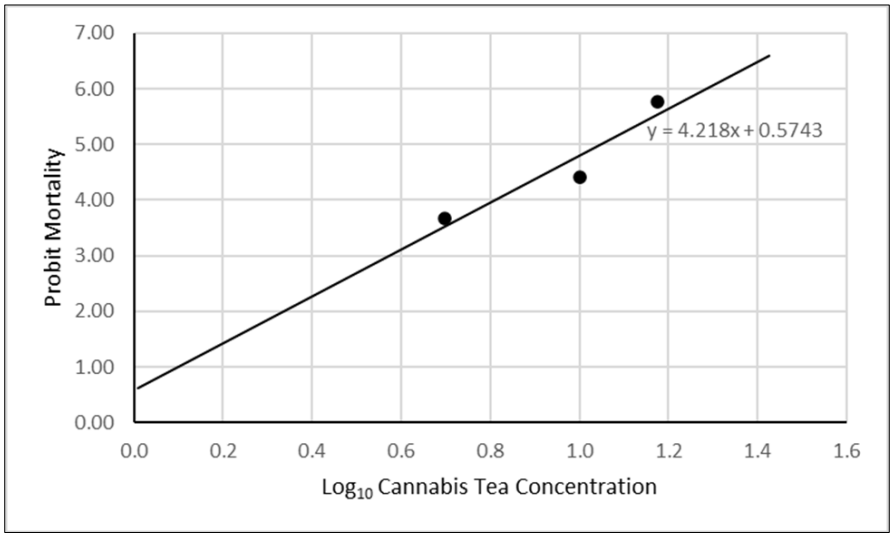

Figure 2 Average corrected mortality (probit units) plotted against the $\log_{10}$ of the cannabis tea dose for LC_{50} determination

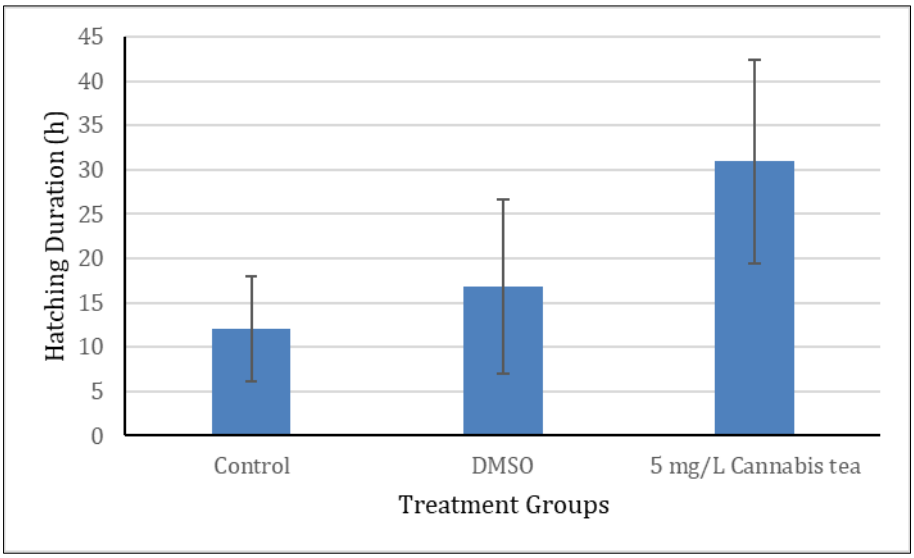

Figure 3 Mean hatching duration against treatment group

Table 2 Mean hatching duration (h) between the first and seventh hatching for zebrafish eggs (n=4)

Treatment Group	Mean hatching Duration (h)	Standard Deviation
Control	12.05	5.95
DMSO	16.80	9.87
5 mg/L Cannabis tea	30.95	11.50
10 mg/L Cannabis tea	-	-
15 mg/L Cannabis tea	-	-

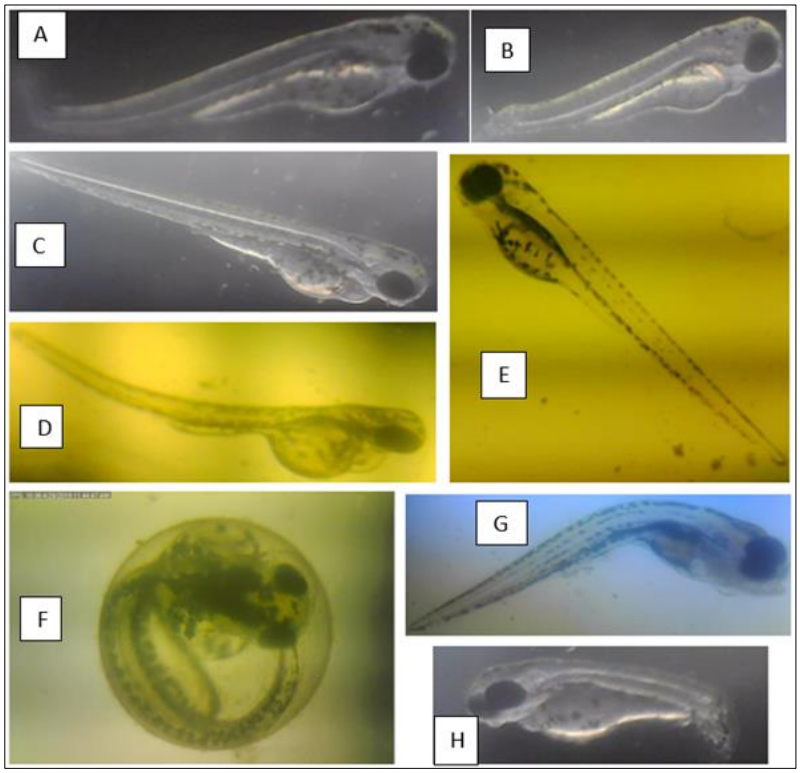

Figure 4 Morphological abnormalities observed in surviving zebrafish larvae after 96 hrs of cannabis tea treatment. Microscopic images taken of zebrafish larvae for the assessment of morphological abnormalities: E, C - normal larvae; F - unhatched larvae; A, D - bent tail; B, H - no tail; G - bent body axis

Table 3 Startle response of zebrafish larvae measured by taking the mean distance travelled (mm) by 5 dpf larvae in response to a touch stimulus in the various treatment groups (n = 6)

Treatment group	Mean Distance Travelled (mm)	Standard deviation
Control	5.13	0.48
DMSO	19.89	1.91
5 mg/L Cannabis tea	16.87	1.57
10 mg/L Cannabis tea	7.22	2.59
15 mg/L Cannabis tea	7.59	3.32

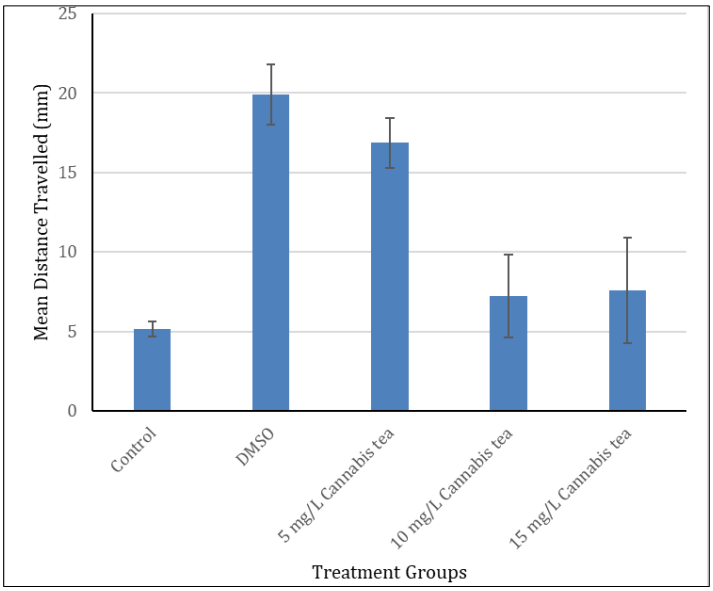

Figure 5 Mean distance travelled (mm) by treated zebrafish larvae in response to a touch stimulus

Table 4 Optokinetic response: mean number of eye movements per minute for 7 dpf zebrafish larvae (n = 6) under a constantly rotating light source

Treatment Group	Mean Number of Eye Movements	Standard Deviation
Control	7.67	1.75
5 mg/L	1.17	1.33
10 mg/L	2.00	1.79
15 mg/L	3.33	2.07

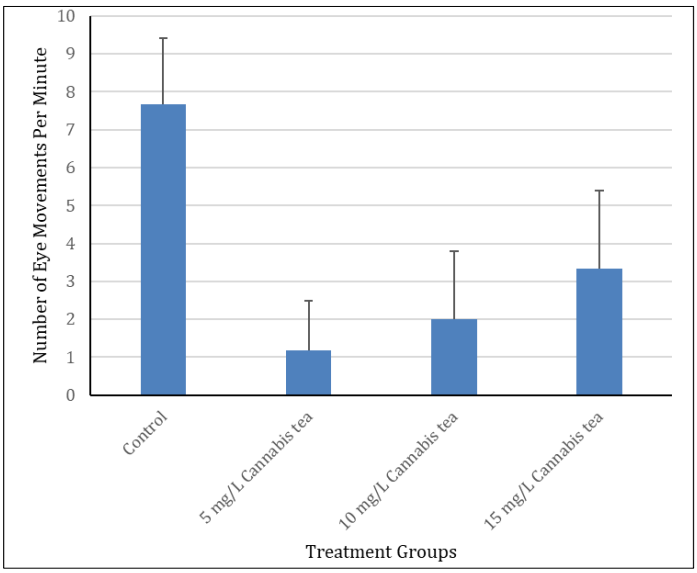

Figure 6 Mean number of eye movements per minute for 7 dpf zebrafish larvae under a constantly rotating light source

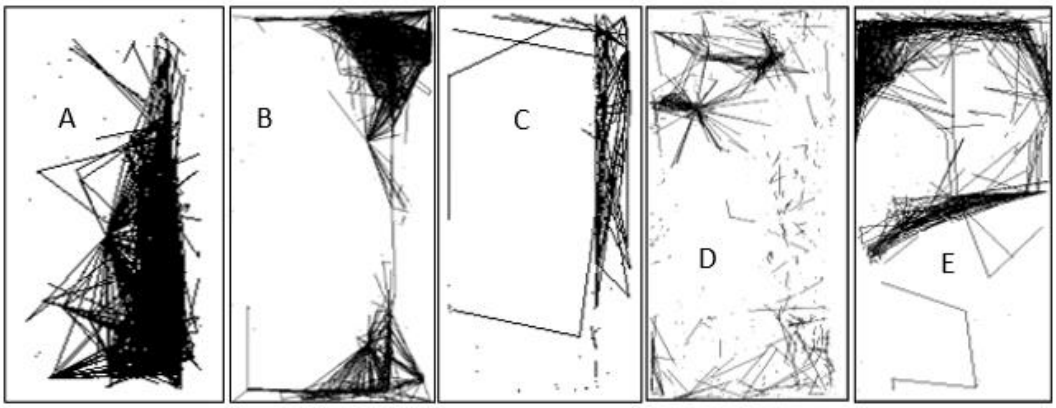

Figure 7 White place behavioral analysis of 6dpf zebrafish larvae. A-E represents the movement displayed in the white area for larvae exposed to the control, vehicle, 5mg/L, 10mg/L and 15mg/L cannabis tea respectively

The results of this study indicate that exposure of the zebrafish larvae to cannabinoids have a significant effect on their mortality, hatching efficiency, startle response and optokinetic response. Increasing concentrations of cannabis tea increased the percentage mortality in 5 dpf larvae after 96 hrs treatment exposure (Figure 1). The 15 mg/L cannabis had a significantly higher mortality than the controls ($p < 0.001$). The LC_{50} was determined from a plot of probit mortality units against the log of the tea concentration and the equation of the line produced a LC_{50} of 11.20 mg/L (Figure 2). Figure 3 shows that the hatching duration of the larvae increased when exposed to DMSO and 5 mg/L of the cannabis tea ($p < 0.05$). At concentrations of 10 mg/L and 15 mg/L cannabis tea the mortality was very high and the majority of the eggs died, thereby preventing hatching analysis. Some of the surviving larvae exposed to cannabis showed various morphological abnormalities that were absent in the controls (Figure 4). The startle response assay indicated that the zebrafish larvae that were exposed to DMSO had a greater startle response than those in the control group (Table 3). It was also noted that the startle response of the larvae decreased as the concentration of cannabis tea increased ($p < 0.001$). Exposure to the cannabis tea resulted initially in decreased optokinetic response (Figure 6). As the concentration of the tea increased from 5 mg/l to 10 mg/L and 15 mg/L, so did the optokinetic response ($p < 0.001$). However, the increase did not bring the response back to normal control levels.

4. Discussion

Given that cannabis is one of the most often used illicit substances by pregnant women, there is a need for research into the possible risks the drug poses to foetuses. This study used a zebrafish model to assess how chronic cannabis exposure affected the developing zebrafish embryo and larvae. Zebrafish eggs at 6 hpf were treated with cannabis and Ahmed et al. [2] noted that at that point in development, the embryo is at the gastrula stage. This is a significant investigative factor, since at this time in human pregnancies the pregnancy could still be undetected, which tends to result in the pregnant woman continuing cannabis use [13].

After zebrafish embryos were exposed to varying concentrations of cannabis tea for 96 hours, the results showed that mortality was proportional to the tea concentration with the 15 mg/L being significantly higher than the controls ($p < 0.001$). This finding is reflected in Ahmed et al. [2] where it was seen that the chance of larval survival decreased with increasing THC and CBD concentrations. Furthermore, the tea had a LC_{50} of 11.20 mg/L. Akhtar et al. and Carty et al. [7, 8] determined that THC had a LC_{50} of 3.37mg/L and 3.65mg/L respectively at 5 dpf. Carty et al. [8] also found that CBD had a LC_{50} of 0.53 mg/L which is about 7 times lower than that of THC. Ahmed et al. [2] had similar findings where CBD had a more severe effect than THC on larval survival. The vast difference in the LC_{50} for this report and those in the previously mentioned studies could be attributed to the method used to prepare the cannabis. Those reports used extracted, pure cannabinoids while this study used a crude cannabis solution prepared by boiling the plant buds in 0.005% DMSO (to aid in solubility). Thus, the solution in this experiment was not as concentrated and the concentration of the dissolved cannabinoids was unknown.

The effect that cannabis has on the hatching of zebrafish larvae was examined during this experiment. Zebrafish eggs were exposed to 5, 10 and 15 mg/L concentrations of cannabis and the duration between the first and seventh egg hatching was recorded. However, there was a high mortality for the 10 and 15 mg/L and so, those results were excluded from the analysis. Hence, the control and DMSO (vehicle) groups were compared to the 5 mg/L group. The data showed that the presence of cannabis significantly increased the hatching time for the eggs ($p = 0.046$). The hatching time was

approximately doubled with cannabis exposure signifying a decreased hatching efficiency. Research by Ahmed et al. and Brigante et al. [2, 14] also reported reduced hatching efficiency for zebrafish eggs exposed to THC and CBD separately. Conversely, Licitra et al. [5] found no difference in hatching rates between their controls and full cannabis extract groups. This disparity might result from the fact that the embryos in this experiment and that of Ahmed et al. and Brigante et al. [2, 14] had initial cannabis exposure starting at an earlier stage of gastrulation, 5-6 hpf, compared to the 24 hpf start used in Licitra et al. [5]. This suggests that cannabis exposure during early gastrulation plays a role in the development of the embryos.

Cannabis treated larvae at 5 dpf were assessed for morphological abnormalities. No abnormalities were observed for the control and DMSO groups, but different abnormalities were observed in the cannabis treated larvae. These morphological abnormalities include bent tails, no tail and bent body axis. Chatzimitakos et al. [15] found that zebrafish larvae exposed to THC also presented with morphological abnormalities, the occurrence of which were dose dependent. Carty et al. [8] also showed that both THC and CBD produced morphological abnormalities in zebrafish larvae after 96 hours exposure to the cannabinoids. Hence, whether using whole cannabis or its individual cannabinoids, there is a likelihood of morphological deformities during development.

A light and dark preference test was used to assess how cannabis affected the anxiety of zebrafish larvae. For this experiment, 6 dpf larvae were considerably active in the white area in the control and DMSO groups (Figure 7). This behaviour signifies high anxiety and correlates to what has been published in existing literatures which have stated that outside of nightfall, zebrafish larvae are aversive to darkness which induces anxiety-like behaviour [16, 17]. Figure 7 also shows that the larval activity was highly concentrated at the edges of the white area (right) that are furthest from the dark area (left), indicating that they are actively trying to avoid the dark area – a characteristic behaviour of zebrafish larvae [16]. Additionally, the DMSO group shows a slightly higher activity level in the white area in comparison to the control. This is in accordance with research that states that concentrations of DMSO above 0.005% increase zebrafish locomotor activity [17]. Larvae exposed to 10 and 15 mg/L cannabis showed a lesser tendency to staying at the edges of the white area. The images (Figure 7) show that moving from 10 to 15 mg/L, there was an increase in activity closer to the dark area. This denotes a reduction in their anxiety-like behaviour and suggests that the cannabis exerted some anxiolytic properties. Previous studies have corroborated that the cannabis component CBD does act as an anxiolytic drug [18, 19]. However, Stoner [20] reported that while low THC levels reduce anxiety, increasing concentrations can heighten it. This is illustrated by the distance the larvae travelled in the white section. At 5 mg/L, there was reduced activity in the white area which implies that the larvae were active in the dark area. The white area activity increased for the 10 then 15 mg/L and signifies that the larvae were more anxious at the higher cannabis concentrations. With the distance travelled in the white area, and also the larvae's tentative venture closer to the dark area, this experiment shows that the cannabis tea can be both anxiolytic and anxiogenic, likely due to the opposing effects of CBD and THC.

Startle effect is a behavioural assay used for testing avoidance behaviour in zebrafish. Avoidance behaviour is usually associated with anxiety. This assay is important as it determines if a drug has affected the sensory or motor neurons. Larvae that were 5 dpf were chosen for this assay because at this stage most of the major body organs have already developed [7], however startle response can be seen as early as 24 hpf [13]. A recent study [2] reported that larvae exposed to cannabinoids for 5 hours during the gastrulation period did not exhibit drastic changes in escape response when exposed to stimuli. The results of this study reported that the zebrafish larvae had statistically significant changes in escape response when exposed to the vehicle, DMSO, as well as cannabis tea. These differences were seen between the larvae exposed to DMSO and 10 mg/L cannabis tea, as well as between DMSO and 15 mg/L cannabis tea ($p < 0.001$). Significant differences were also present between the larvae exposed to 5 mg/L and 10 mg/L cannabis tea, and 5 mg/L and 15 mg/L cannabis tea ($p < 0.001$). When compared to the DMSO group, the larvae exposed to cannabis tea exhibited slow escape response, as shown by lower speeds recorded when exposed to touch stimuli. This was also seen between the groups, as the escape response decreased with the increasing cannabis tea concentration. However, there was no significant difference in the inhibited escape response of larvae exposed to 10 mg/L and 15 mg/L cannabis tea. Overall, this assay displayed that swimming, and the escape response of larvae was affected by the addition of cannabinoid treatment during the developmental stages, suggesting that the cannabis tea exposure affected their motor function.

Optokinetic response (OKR) refers to larvae eye movements in response to a moving object in the immediate field of vision. OKR develops at 4 dpf and is important in feeding methods. At this same stage of development (4 dpf) the swimming bladder of the larvae is also inflated, which allows them to move freely in hunt of prey [21]. For this study, the larvae that were assessed were 7 dpf and thus exhibiting a robust OKR. The OKR assay can be used as a complementary test for the determination of drug effects on the central nervous system (CNS), and isolates larvae with visual mutations [22]. Because of these properties, the assay can adequately assess the effects of cannabinoids on eye movements [21]. The larvae exposed to the cannabis tea treatments had significantly less eye movements than those in

the control group ($p < 0.001$, $p < 0.05$). There was, however, no significant difference in the eye movements of the larvae between the respective treatment groups. It was noted that as the dose of cannabis tea increased, so did the eye movements, but not significantly. Other studies have reported that cannabis in the presence of alcohol results in the slow deterioration of eye movements [23], which was exhibited in this study. Further research needs to be conducted to assess the effect of cannabis on eye movement independent of other substances.

5. Conclusion

The findings of this study indicate that exposure to cannabis tea at varying concentrations (5 mg/L, 10 mg/L & 15 mg/L) during the embryonic stage impacts morphological and behavioural characteristics in zebrafish larvae. The exposure decreased the hatching efficiency of the eggs, and the resulting larvae had varied morphological abnormalities. Further, the exposed larvae to the tea concentrations exhibited a reduction in anxious behaviours. The study results were also comparable to studies assessing effects of THC and CBD individually. Future studies to include a wider variation of concentrations as well as differing THC to CBD ratios can be done to further assess the effects of whole cannabis extracts on morphological and behavioural characteristics of zebrafish.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of ethical approval

This research was conducted after the approval of ethics committee of Department of Basic Medical Sciences, University of the West Indies Mona campus, Kingston, Jamaica.

Author's Contribution

Sanchia Pratt: Carried out all of the experiments (as a part of Master Project at Department of Basic Medical Sciences, University of the West Indies Mona campus, Kingston, Jamaica), Manuscript drafting

Shelby Kyra Kerr: Data analysis and manuscript drafting

Stacey Dana Harris: Statistical analysis and manuscript drafting

Mohammad Kutub Ali: Research supervisor and coordinator, Protocols designer, manuscript reviewer

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