World Journal of Advanced Pharmaceutical and Life Sciences

Journal homepage: https://zealjournals.com/wjapls/

ISSN: 2799-0222 (Online)

(RESEARCH ARTICLE)

Check for updates

Impact of green and black tea extracts on swimming performance and caudal fin regeneration in adult zebrafish (*Danio rerio*) following amputation

Derron Ricardo Taite and Mohammad Kutub Ali *

Department of Basic Medical Science, Faculty of Medical Sciences, University of West Indies, Mona campus, Kingston, Jamaica, West Indies.

World Journal of Advanced Pharmaceutical and Life Sciences, 2024, 07(02), 001–008

Publication history: Received on 02 September 2024; revised on 16 October 2024; accepted on 19 October 2024

Article DOI: https://doi.org/10.53346/wjapls.2024.7.2.0037

Abstract

This study investigates the effects of green tea and black tea extracts on swimming performance and caudal fin regeneration in adult zebrafish (*Danio rerio*) following caudal fin amputation. Zebrafish were treated with either tea extract for 14 days, with applications administered three times daily. Swimming performance was assessed against water currents.

Results indicated that green tea-treated fish exhibited the greatest resistance to water flow, primarily occupying the high-force zone during a 15-minute exercise session, while black tea-treated fish were mostly found in middle and lower zones. Cumulative distribution analysis showed that green tea-treated fish maintained their positions in stronger current zones significantly longer than both black tea and control groups ($p < 0.0001$). Conversely, control fish displayed the highest area of caudal fin regeneration, significantly surpassing both tea-treated groups. Green tea-treated fish showed the least regeneration area, though differences from black tea were not statistically significant.

These findings challenge previous studies suggesting that green tea enhances tissue regeneration. The pro-apoptotic effects of green tea's primary component, epigallocatechin gallate (EGCG), may have limited its regenerative potential despite its antioxidant properties. This research highlights the complex relationship between tea polyphenols, swimming performance, and tissue regeneration, suggesting that while both green and black tea can enhance physiological performance, their effects vary based on the interplay between antioxidative benefits and apoptosis-inducing mechanisms.

Keywords: Zebrafish: Black tea; Green tea: Performance: Caudal fin generation

1. Introduction

Tea, one of the most widely consumed beverages globally, is primarily categorized into black tea and green tea, both derived from the leaves of the Camellia sinensis plant. The processing methods of these teas differ significantly, which influences their chemical composition and associated health benefits. Both black tea and green tea are rich in polyphenols, known for their antioxidant properties. Green tea is particularly abundant in catechins, especially epigallocatechin gallate (EGCG), which has been extensively studied for its health benefits, including anti-inflammatory and anticancer effects [1-2]. In contrast, black tea contains theaflavins and thearubigins, which also exhibit antioxidant properties but differ in their mechanisms of action [3].

Research indicates that green tea consumption is associated with a reduced risk of various cancers, including prostate and gastric cancers. This effect is attributed to green tea's ability to inhibit tumor growth and metastasis [4-5]. The

* Corresponding author: Mohammad Kutub Ali

mechanisms underlying these benefits include the induction of apoptosis, or programmed cell death, which plays a crucial role in cancer prevention. Green tea has been shown to accelerate apoptosis in cancer cells, particularly through the action of EGCG, which can induce cell cycle arrest and promote the death of malignant cells [2,6]. In vitro studies have demonstrated that green tea catechins can inhibit the growth of various cancer cell lines by inducing apoptosis and suppressing tumor metastasis [4].

While black tea also exhibits some anticancer properties, its mechanisms are less understood, and it may not be as effective as green tea in promoting apoptosis in cancer cells [3, 7]. This disparity underscores the need for further research to elucidate the potential health benefits of both tea types.

In addition to their anticancer effects, both black and green tea have been studied for their potential effects on athletic performance. Green tea, due to its high antioxidant content, may enhance endurance and reduce exercise-induced oxidative stress, thereby improving recovery times [8]. The catechins in green tea have been linked to increased fat oxidation during exercise, which can be beneficial for athletes [9]. Conversely, black tea, while also containing caffeine, may provide a more immediate energy boost due to its higher caffeine content, making it advantageous for short-term performance [10]. However, the overall impact of black tea on athletic performance requires further exploration to fully understand its potential benefits.

The use of zebrafish as a model organism offers several advantages for reevaluating the health benefits of black tea and green tea. Zebrafish are transparent during early development, allowing for real-time observation of physiological processes, including tissue regeneration and apoptosis. Their unique regenerative capabilities, particularly in fin regeneration, make them an ideal model for studying the effects of dietary components on health outcomes [3, 11]. Studies have indicated that green tea extracts can enhance tissue regeneration in zebrafish by promoting cellular proliferation and modulating gene expression related to regeneration [11]. The presence of catechins in green tea has been linked to improved regenerative outcomes, potentially through the activation of signaling pathways that facilitate tissue repair [12].

Conversely, while black tea has shown some regenerative properties, its effects are less documented compared to green tea, suggesting that further investigation is warranted [3]. This gap in the literature highlights the need for additional research to better understand how both types of tea influence regenerative processes at the cellular level.

The existing literature, however, raises some inconsistencies regarding the effects of green tea catechins in inhibiting the growth of cancer cell lines and promoting apoptosis [4]. Specifically, the action of EGCG in inducing cell cycle arrest and promoting the death of malignant cells requires further exploration [2, 6]. This complexity underscores the necessity for multiple lines of evidence to clarify the relationships between tea consumption, cancer prevention, and tissue regeneration.

Considering this requirement, the present research aims to re-investigate the effects of black tea and green tea on the regeneration of the caudal fin in zebrafish and their swimming performance against water currents. By utilizing zebrafish as a model organism, this study intends to elucidate the mechanisms by which tea polyphenols influence cellular processes related to regeneration and performance.

2. Materials and Methods

2.1. Materials

Adult zebrafish (*Danio rerio*), averaging 3 cm in length and weighing about 700 mg, were sourced from a local aquarium store. Prior to introducing the fish, 3 g of artificial instant ocean salts were added per liter of deionized distilled water for conditioning. This preparation was done on a large scale (200 L) and allowed it to stabilize over three days. Black tea (10:1) extracts (1g capsule) were obtained from Monoherb, India whereas Green tea (20:1) extracts 300mg capsule containing 50% EGCG, 98% polyphenols and 80% Catechins were purchased from Zazzee Naturals manufacturer, USA.

2.2. Zebrafish Maintenance and Selection

Healthy adult zebrafish were selected using specific criteria and kept in large tanks measuring 45×60×25 cm. They were housed in conditioned water with a conductivity of about 1,500 µS/cm and a pH of 7. The temperature was maintained at 28°C, with continuous aeration provided by an air pump to ensure proper oxygen levels. Water quality was managed through daily two-hour filtration and the addition of aquarium disinfectant. Additionally, 30% of the tank water was replaced daily with Millipore-filtered water containing the appropriate amount of instant ocean salt. The fish were fed three times daily (at 10 am, 2 pm, and 6 pm) with bloodworms, enough to be consumed within five minutes. After ten days of maintenance, zebrafish with similar morphology and length, having equal caudal fin length and exhibiting normal swimming and feeding behaviors, were chosen and housed in pairs separately in 1.5 L Yobiisolut mini aquarium tanks for further study.

2.3. Caudal Fin Amputation

A 1% stock solution of 2-phenoxyethanol was prepared by mixing 5 mL of 100% pure 2-phenoxyethanol with 500 mL of distilled water. For anesthetic use, 6 mL of this stock solution was added to 400 mL of fish water. Individual fish were carefully removed from their tanks and placed in a tank containing a 0.015% 2-phenoxyethanol anesthetic solution. Anesthesia was confirmed by the absence of tail reflex. Each fish was then positioned on a petri dish, dried, and photographed with an Andonstar microscopic webcam to document the uncut caudal fin. Approximately half of the caudal fin was excised using a scalpel, and a photograph was taken immediately afterward [13].

2.4. Tea Preparation and treatment

Each capsule of black and green tea extract was finely powdered using a mortar and pestle, with the following weights prepared per serving: 400 mg of black tea extract and 800 mg of green tea extract, totaling 4 g of dry tea leaves [14]. The tea was prepared by dissolving the powdered extracts in 500 ml of water in a microwaveable ceramic cup, which was then microwaved for 7 minutes. This rapid heating preserves the bioactivity of the polyphenols and enhances solubility [15].

Subsequently, the solution was diluted with an additional 500 ml of fish water in the treatment tank. To transfer fish with caudal fin amputations, a perforated rectangular fish holder was utilized to minimize stress and prevent injury, as illustrated in the accompanying Figure 1. 10 fish in each group were treated with black tea and green tea for 1 hour, three times daily at 9 AM, 1 PM, and 5 PM, over a period of 14 days. Another 10 fish were run parallel mock treatment as control [16].

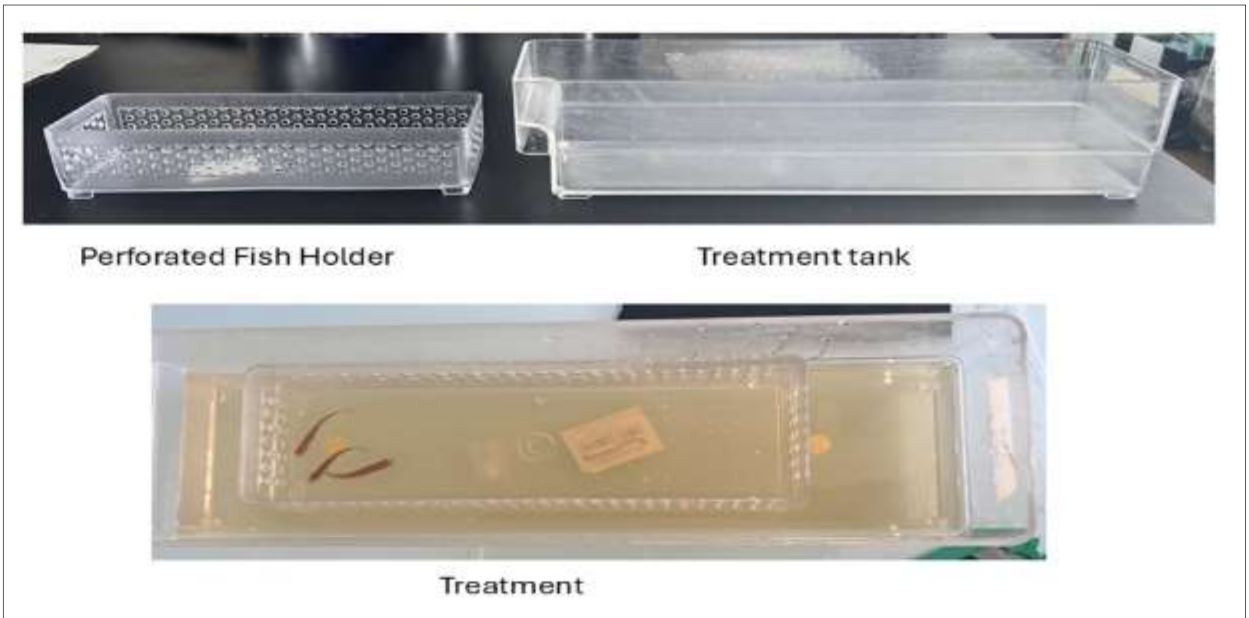

Figure 1 Representative setup of the treatment configuration used in the study

2.5. Recording of Swimming Performance against water current

The setup for assessing swimming performance against a water current consists of two square tanks (15 cm height × 30 cm length × 35 cm width) connected by a rectangular tank (6 cm height × 45 cm length) divided into six 5 cm tunnels (Figure 2). Each tunnel is equipped with a Digiten water flow controller and a sensor flow meter to ensure consistent water currents. Perforated nets at both ends of the tunnels prevent fish from escaping, while a transparent removable glass lid keeps them from jumping between tunnels. The rectangular tank is positioned 5 cm above the surface, allowing water to flow from a pump outlet into one square tank and return to the other through the rectangular tank. Continuous water flow (8 L) circulates between the two square tanks, driven by a Jerepet 800 GPH 30W 16Ft aquarium pump with a speed controller.

Figure 2 Swimming tunnel designed for six individual fish to compete in performance against water current

The swimming tunnels are divided into three zones: (1) Higher Zone (15 cm), where fish experience maximum water force and minimal pulling force; (2) Medium Zone (15 cm), with moderate water force and increased pulling force; and (3) Lower Zone (15 cm), where fish encounter minimal water force but maximum pulling force [17]. Water speeds were calibrated so that 50% of fish occupied the Lower Zone, set as 100% flow speed. Caudal fin-amputated fish from each group (control, black tea, and green tea treated) were given 15 minutes of exercise daily at 50% water flow rate to enhance caudal fin regeneration, as suggested by Fiaz et al., 2014 [18].

On day 14, individual fish from each group were evaluated for performance at 100% pump speed for 15 minutes. The sessions were recorded using a wide-field webcam connected to a computer at 10 fps with Ispy software. The position of the fish in the swimming tunnel was tracked throughout the 15 minutes using the NIH ImageJ wrMTrack plugin, with track point leveling to subtract the fish image. The generated point stack of the entire movie was then resliced into 300 segments (each 0.15 cm) from the water exit point to the water entry point of the swimming tunnel. This resliced stack was subjected to a Z-axis profile plot, where the values obtained for each slice represent the total cumulative position of the fish over the entire 15 minutes of exercise.

2.6. Caudal Fin Regeneration Assay

After a 14-day recovery period following caudal fin amputation, the fish were re-anesthetized. Photos of the regenerated caudal fins were captured, with the boundary of the transparent regenerated fin marked using opaque methylene blue. The areas of the fins before amputation and after regeneration were measured to calculate the percentage of caudal fin regeneration.

2.7. Statistical Analysis

One-way ANOVA with Tukey's multiple comparison tests was performed on the data sets using GraphPad Prism version 6 statistical software. P-values <0.05 were considered statistically significant. Data were expressed as mean ± standard deviation or average percentage ± standard deviation.

3. Results and Discussion

Zebrafish have become a valuable model for studying swimming performance, particularly in response to water currents [19]. Swimming behavior in zebrafish offers insights into their physiological and behavioral responses, which can be influenced by various factors such as exposure to toxic chemicals or food additives [20]. In the current study, zebrafish with caudal fin amputations were treated with green tea and black tea extracts for 14 days, with applications administered three times daily. After the treatment period, their swimming performance against water currents was tested.

Figure 3(a) compares the swimming patterns of control, black tea-treated, and green tea-treated fish during a 15-minute exercise session under the same water force and duration conditions. Green tea-treated fish exhibited the strongest resistance to water flow, frequently remaining in the high-force zone near the water entry point, despite occasionally drifting toward the exit. In contrast, black tea-treated fish mostly occupied the middle and lower water force zones, while control fish largely stayed in the lower zone.

Figure 3(b) presents cumulative average distribution plots along the Y-axis (from low to high water force zones) for 10 fish from each group over the 15-minute session. Green tea-treated fish demonstrated the highest resistance to the water current, maintaining positions in the strongest flow zone significantly longer than both black tea-treated and control fish. These differences in swimming performance were statistically significant ($p < 0.0001$).

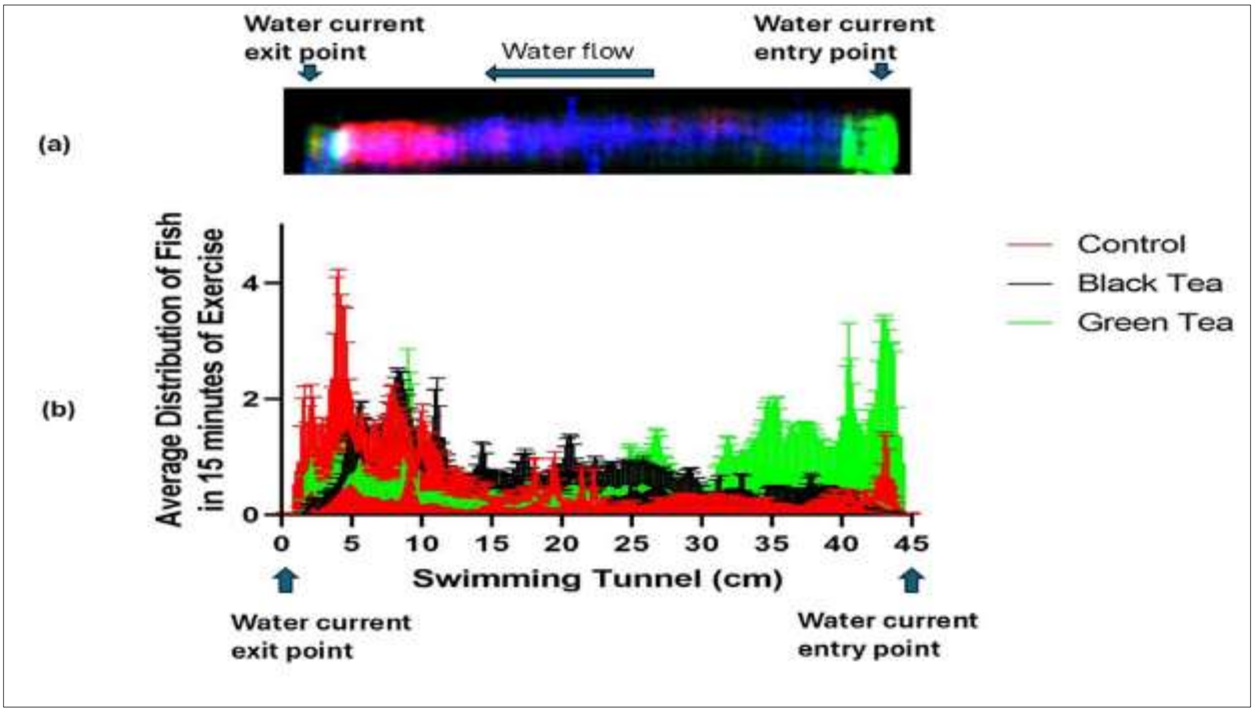

Figure 3 (a) Total distribution of a representative fish in the swim tunnel during a 15-minute exercise session. The distributions for the three groups—Control (red), Black Tea (blue), and Green Tea (green)—are merged and overlapped to illustrate the maximum distribution zone. (b) Graphical representation of the mean ± standard deviation of the performance of 10 fish in the swim tunnel over the entire 15-minute exercise period

Both black and green tea have been studied for their potential impact on athletic performance. Green tea, rich in antioxidants, is known to enhance endurance and reduce exercise-induced oxidative stress, improving recovery [8]. The catechins in green tea also promote increased fat oxidation during exercise, benefiting athletes [9]. Black tea, with its higher caffeine content, may offer a more immediate energy boost, enhancing short-term performance [10]. This study confirms that both green and black tea-treated fish demonstrated greater resilience to water currents compared to controls, likely due to reduced oxidative stress and enhanced fat oxidation.

After 14 days of caudal fin amputation, the control fish exhibited the highest area of regeneration, which was significantly greater ($p < 0.0001$) than that of fish treated with black or green tea extracts. Green tea-treated fish showed the lowest regeneration area, although the difference from black tea-treated fish was not statistically significant. These findings contradict earlier studies [11-12], which suggested that green tea extracts enhance tissue regeneration in zebrafish by promoting cellular proliferation and modulating gene expression. However, green tea's primary component, EGCG, is known to induce apoptosis in various cell types, including stem cells, through mechanisms such as ROS generation and mitochondrial dysfunction [21-22]. EGCG also disrupts mitochondrial function and activates caspase-3, further promoting cell death. This oxidative stress can similarly induce apoptosis in mesenchymal stem cells

[23]. Moreover, green tea polyphenols can inhibit cell proliferation and cause cell cycle arrest in cancer cells, suggesting that their regenerative effects may vary depending on concentration and cellular context [24-25].

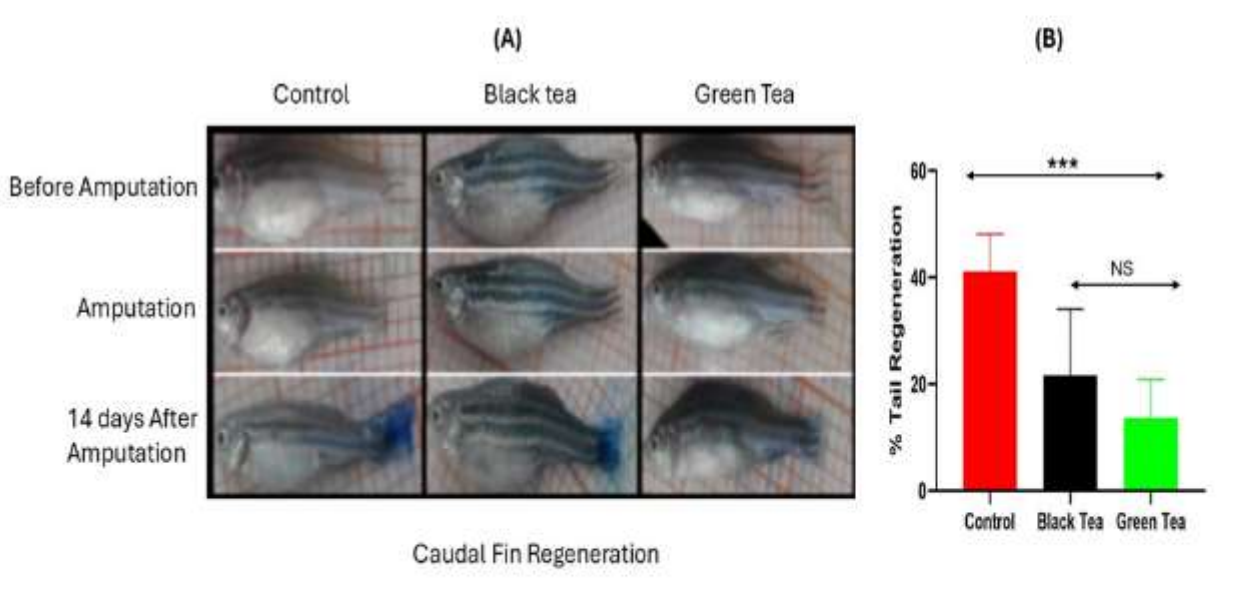

Figure 4 (A) Representative images showing the caudal fin regeneration in zebrafish treated with Control, Black Tea, and Green Tea. (B) Graphical representation of the mean percentage of tail regeneration ± standard deviation for 10 fish in each group

Caudal fin morphology is critical to zebrafish swimming performance, with studies showing that optimal fin length enhances swimming efficiency, especially for predator evasion and navigating water currents [26-27]. In this study, zebrafish treated with green and black tea extracts after caudal fin amputation were tested for their swimming abilities. Green tea-treated fish demonstrated the highest resistance to water current, followed by black tea-treated fish, while control fish remained in lower-force zones. These results align with the extent of caudal fin regeneration observed 14 days after amputation, where control fish had the largest regenerated fin area, but green tea-treated fish still outperformed in swimming endurance.

The superior performance of green tea-treated fish is likely due to its high antioxidant content, particularly EGCG, which helps reduce oxidative stress. For black tea-treated fish, caffeine may offer an energy boost, aiding their swimming capabilities despite having less fin regeneration.

4. Conclusion

In conclusion, this study highlights the differential effects of green tea and black tea extracts on zebrafish swimming performance and caudal fin regeneration. While control fish exhibited the highest area of fin regeneration after 14 days, green tea-treated fish showed superior swimming performance, likely due to the high antioxidant content of green tea, particularly EGCG. EGCG's ability to reduce oxidative stress may have enhanced the fish's endurance against water currents, despite limited tissue regeneration. Black tea-treated fish, although showing less resistance compared to green tea-treated fish, benefited from caffeine's potential short-term energy boost, improving their swimming performance relative to control fish. However, green tea's known pro-apoptotic effects, particularly at high concentrations, might have limited its ability to promote tissue regeneration. These findings suggest that while both green and black tea extracts can enhance physiological performance, their effects on tissue regeneration and endurance may vary depending on the balance between antioxidative and pro-apoptotic mechanisms.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Ethical approval was granted by the Mona Campus Research Ethics Committee (Ref: AN4, 19/20).

Funding Information

We gratefully acknowledge the financial support from the University of the West Indies, Mona Campus, Kingston, Jamaica. We also extend our thanks to Hasina Nicholson for presenting the findings at the Ministry of Health Conference in Kingston, Jamaica.

Authors' Contribution

- **Mohammad Kutub Ali:** Research supervisor and coordinator, data analyser and manuscript drafting.
- **Derron Ricardo Taite:** Research scholar and data collection.

References

[1] Makiuchi, T., Sobue, T., Kitamura, T., Ishihara, J., Sawada, N., Iwasaki, M., Sasazuki, S., Yamaji, T., Shimazu, T., & Tsugane, S. (2016). Association between green tea/coffee consumption and biliary tract cancer: A population-based cohort study in Japan. Cancer Science, 107(1), 76–83. https://doi.org/10.1111/cas.12843

[2] Tuzun, S. (2019). Investigation of the antiproliferative effect of camelia sinensis on liver cancer (hepg2) cell line. International Journal of Academic Medicine and Pharmacy, 1(1), 19-21. https://doi.org/10.29228/jamp.40233

[3] Bag, A. and Bag, N. (2018). Tea polyphenols and prevention of epigenetic aberrations in cancer. Journal of Natural Science Biology and Medicine, 9(1), 2. https://doi.org/10.4103/jnsbm.jnsbm_46_17

[4] Guo, Y., Zhi, F., Chen, P., Zhao, K., Han, X., Qi, M., … & Zhang, X. (2017). Green tea and the risk of prostate cancer. Medicine, 96(13), e6426. https://doi.org/10.1097/md.0000000000006426

[5] Huang, Y., Chen, H., Zhou, L., Li, G., Yi, D., Zhang, Y., … & Yi, D. (2017). Association between green tea intake and risk of gastric cancer: a systematic review and dose–response meta-analysis of observational studies. Public Health Nutrition, 20(17), 3183-3192. https://doi.org/10.1017/s1368980017002208

[6] Hu, X. (2019). Application of a simple and reliable cell proliferation and viability assay in green tea extract-and catechin-induced growth inhibition of cancer cells. Clinical Oncology and Research, 1-6. https://doi.org/10.31487/j.cor.2019.05.01

[7] Hajihossein, F., Khosravi, A., Khosravi, A., & Khosravi, M. (2020). "Anti-Acanthamoeba effect of Camellia sinensis extract (black and green tea) in vitro," Journal of Medicinal Plants, vol. 1, no. 73,pp. 163-170. doi:10.29252/jmp.1.73.163.

[8] Trisha, AT., Shakil, MH., Talukdar, S., Rovina, K., Huda, N., Zzaman, W. (2022). Tea Polyphenols and Their Preventive Measures against Cancer: Current Trends and Directions. Foods. 11(21):3349. doi: 10.3390/foods11213349

[9] Mansoori, A., Khoshdel, A., & Khosravi, A. "A Comprehensive Review on Biological Activity of Green Tea (Camellia sinensis)," Journal of Drug Delivery and Therapeutics, vol. 12, no. 5, 2022, pp. 1-12. doi:10.22270/jddt.v12i5.5613

[10] Ahmad,BO., Mahmoud, K. (2023). "Determination of Caffeine and Few Metals in Halabja Tea and Comparing with Various Tea Samples in Erbil Markets," Journal of Kurdish Chemical Society, vol. 2, no. 9, Article 13291. doi:10.36329/jkcm/2022/v2.i9.13291

[11] Zhang Q, Gao Q, Zhao L, Li X, Wang X, Wang Y, Chen D.(2023). Evaluation of the effect of green tea and its constituents on embryo development in a zebrafish model. J Appl Toxicol. 43(2):287-297. doi: 10.1002/jat.4380.

[12] Miyata Y, Shida Y, Hakariya T, Sakai H. Anti-Cancer Effects of Green Tea Polyphenols Against Prostate Cancer. Molecules. 2019 Jan 7;24(1):193. doi: 10.3390/molecules24010193

[13] Ali, MK., Taite, DR. (2024). Monosodium glutamate disrupts zebrafish sleep-like state likely through activation of peripheral nervous system glutamate receptors. World Journal of Advanced Pharmaceutical and Life Sciences, 07(01), 034–043. url: https://doi.org/10.53346/wjapls.2024.7.1.0034

[14] Yi, M., Wu, X., Zhuang, W., Xia, L., Chen, Y., Zhao, R., … & Zhou, Y. (2019). Tea consumption and health outcomes: umbrella review of meta-analyses of observational studies in humans. Molecular Nutrition & Food Research, 63(16). https://doi.org/10.1002/mnfr.201900389

[15] Peng, P., Wang, L., Shu, G., Li, J., & Chen, L. (2020). Nutrition and aroma challenges of green tea product as affected by emerging superfine grinding and traditional extraction. Food Science & Nutrition, 8(8), 4565-4572. https://doi.org/10.1002/fsn3.1768

[16] Taite, DR., Hewitt, IN., Ali, MK. (2024). The effect of resveratrol and alcohol on zebrafish caudal fin regeneration. World Journal of Advanced Pharmaceutical and Life Sciences, 06(02), 017–022. url: https://doi.org/10.53346/wjapls.2024.6.2.0031

[17] Stephenson, S., Labastide, J., Chambers, S., Peters, S., Plunkett, C., Williams, O., Lindo, RLA., and Ali, MK. (2024). Evaluating Caffeine's Impact on Zebrafish Performance, Swim Coordination, and Glucose Uptake. Journal of arts and science technology – in press

[18] Fiaz, A., Léeon-Kloosterziel, K., Leeuwen, J., & Kranenbarg, S. (2014). Exploring the molecular link between swim-training and caudal fin development in zebrafish (danio rerio) larvae. Journal of Applied Ichthyology, 30(4), 753-761. https://doi.org/10.1111/jai.12510

[19] Usui, T., Noble, D., Fangmeier, M., Lagisz, M., Hesselson, D., & Nakagawa, S. (2018). The french press: a repeatable and high-throughput approach to exercising zebrafish (danio rerio). Peerj, 6, e4292. https://doi.org/10.7717/peerj.4292

[20] Thomas, J. and Janz, D. (2015). Developmental and persistent toxicities of maternally deposited selenomethionine in zebrafish (danio rerio). Environmental Science & Technology, 49(16), 10182-10189. https://doi.org/10.1021/acs.est.5b02451

[21] Magcwebeba, T., Swanevelder, S., Joubert, E., & Gelderblom, W. (2016). In vitro chemopreventive properties of green tea, rooibos and honeybush extracts in skin cells. Molecules, 21(12), 1622. https://doi.org/10.3390/molecules21121622

[22] Magcwebeba, T., Swanevelder, S., Joubert, E., & Gelderblom, W. (2016). Anti-inflammatory effects of aspalathus linearis and cyclopia spp. extracts in a uvb/keratinocyte (hacat) model utilising interleukin-1α accumulation as biomarker. Molecules, 21(10), 1323. https://doi.org/10.3390/molecules21101323

[23] Yagi, H., Tan, J., & Tuan, R. (2013). Polyphenols suppress hydrogen peroxide-induced oxidative stress in human bone-marrow derived mesenchymal stem cells. Journal of Cellular Biochemistry, 114(5), 1163-1173. https://doi.org/10.1002/jcb.24459

[24] Saleh, M., Darwish, Z., Nouaem, M., Fayed, N., Mourad, G., & Ramadan, O. (2023). The potential preventive effect of dietary phytochemicals in vivo. https://doi.org/10.21203/rs.3.rs-2511652/v1

[25] Garcia-Rodriguez, M., Carvente-Juárez, M., & Altamirano-Lozano, M. (2013). Antigenotoxic and apoptotic activity of green tea polyphenol extracts on hexavalent chromium-induced dna damage in peripheral blood of cd-1 mice: analysis with differential acridine orange/ethidium bromide staining. Oxidative Medicine and Cellular Longevity, 2013, 1-9. https://doi.org/10.1155/2013/486419

[26] Wakamatsu, Y., Ogino, K., & Hirata, H. (2019). Swimming capability of zebrafish is governed by water temperature, caudal fin length and genetic background. Scientific Reports, 9(1). https://doi.org/10.1038/s41598-019-52592-w

[27] Blazina, A., Vianna, M., & Lara, D. (2013). The spinning task: a new protocol to easily assess motor coordination and resistance in zebrafish. Zebrafish, 10(4), 480-485. https://doi.org/10.1089/zeb.2012.0860