



**Innovations from Indigenous Knowledge for Using Indian-Almond Leaf
(*Terminalia catappa*) and Herbal Recipes for Rearing and Wound Healing
in Siamese Fighting Fish (*Betta splendens*)**

Saowalak Malawa

**A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Aquatic Science**

Prince of Songkla University

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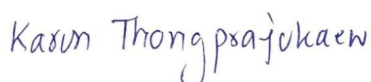
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Author Miss Saowalak Malawa

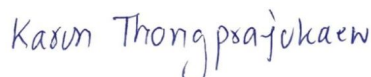
Major Program Aquatic Science

Major Advisor**Examining Committee:**

Digitally signed : 2024.05.30 9:40:56 +07:00
(Professor Dr. Karun Thongprajukaew)



Digitally signed : 2024.05.30 14:06:39 +07:00
.....Chairperson
(Assistant Professor Dr. Suphada Kiriratnikorn)

Co-advisor

Digitally signed : 2024.05.30 9:40:57 +07:00
.....Committee
(Professor Dr. Karun Thongprajukaew)



Digitally signed : 2024.05.30 9:44:49 +07:00
(Assistant Professor Dr. Naraid Suanyuk)



Digitally signed : 2024.05.30 12:37:02 +07:00
.....Committee
(Dr. Nutt Nuntapong)



Digitally signed : 2024.05.30 12:37:01 +07:00
(Dr. Nutt Nuntapong)



Digitally signed : 2024.05.30 9:44:50 +07:00
.....Committee
(Assistant Professor Dr. Naraid Suanyuk)



Digitally signed : 2024.05.31 9:33:26 +07:00
.....Committee
(Associate Professor Dr. Sommai Chiayvareesajja)

The Graduate School, Prince of Songkla University, has approved this Thesis as Partial Fulfillment of the requirements for the Doctor of Philosophy in Aquatic Science



Digitally signed : 2024.05.31 19:20:35 +07:00
(Assistant Professor Dr. Kawinbhat Sirikantisophon)
Acting Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

Karun Thongprajukaew

.....Signature
Digitally signed : 2024.05.30 9:40:59 +07:00

(Professor Dr. Karun Thongprajukaew)

Major Advisor

Naraid Suanyuk

.....Signature
Digitally signed : 2024.05.30 9:44:52 +07:00

(Assistant Professor Dr. Naraid Suanyuk)

Co-advisor

NUTT NUNTAPONG

.....Signature
Digitally signed : 2024.05.30 12:37:04 +07:00

(Dr. Nutt Nuntapong)

Co-advisor

Saowalak Malawa

(Miss Saowalak Malawa)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

Saowalak Malawa

(Miss Saowalak Malawa)

Candidate

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Author	Miss Saowalak Malawa
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ABSTRACT

Breeders and keepers of Siamese fighting fish (*Betta splendens*) often add Indian-almond (*Terminalia catappa*) leaf extract to the water in the aquarium, but there is little information available about the effects of extract concentration on water quality and fish performance. In the current study, the effects of *T. catappa* extract on water quality, growth performance, feed utilization, skin coloration, bubble-nest formation, digestive enzyme activity, muscle quality, whole-body composition, and hematological parameters of *B. splendens* were investigated. Juvenile male fish (2-month-old, 0.80–0.95 g initial weight) were individually reared in 350-mL cylindrical plastic containers. The fish were reared for 8 weeks in four concentrations of *T. catappa* leaf extract (0.125, 0.25, 0.5, and 1 g L⁻¹) and a control group was reared in water without the extract. A leaf extract concentration of 0.25 g L⁻¹ showed no significant effect on growth (specific growth rate = 1.62% body weight day⁻¹), feed utilization (feed conversion ratio = 1.21 g feed g gain⁻¹), viscerosomatic index, muscle quality, whole-body composition, and hematological parameters of reared fish ($P > 0.05$), but did show effects on some of the other metrics that were monitored. Water quality significantly improved during the experiment ($P < 0.05$). Increased skin lightness (L^*) was observed in all experimental groups, but only fish reared in 0.125 g L⁻¹ leaf extract increased skin redness/greenness (a^*), hue (h^*), and chroma (C^*) ($P < 0.05$). Relative to the control, increments in bubble-nest formation frequency (1.70-fold on average) and bubble-nest size (1.52-fold on average) were correlated with the concentration of *T. catappa* leaf extract ($P < 0.05$). Specific activities of protein- and lipid-digesting enzymes were not negatively affected but some modulations were observed in treatments containing leaf extract at 0.125, 0.25, and 1 g L⁻¹. Fish reared in the 0.25 g L⁻¹ treatment exhibited a sparing effect of carbohydrate utilization

indicated by decreased amylase specific activity, increased lipase specific activity, and stable amylase/trypsin ratios. The results suggest that 0.25 g L⁻¹ of *T. catappa* leaf extract is a suitable concentration of the additive during the growing phase of male Siamese fighting fish.

The aggression of Siamese fighting fish (*Betta splendens*) during courtship and reproductive behaviors can result in torn fins. However, the natural regeneration of torn fins has been significantly improved by treating fish with fermented water plants. Half of the caudal fin of male Siamese fighting fish (1.14–1.44 g body weight) was amputated in the vertical plane and then divided the fish into nine experimental groups ($n = 15$ per treatment, each fish serving as an experimental unit). The control groups comprised a negative control (CT 1) of dechlorinated water and a positive control (CT 2) made up of a water fermentation of dried lemongrass (*Cymbopogon citratus*, LL, 1 g L⁻¹) leaf, dried clay (50 g L⁻¹), and salt (5 g L⁻¹). The seven treatments were based on CT 2 which was supplemented with combinations of dried leaf of one, two or all three of the following: Indian-almond (*T. catappa*, IAL, 0.25 g L⁻¹), banana (*Musa sapientum*, BL, 1.5 g L⁻¹), and papaya (*Carica papaya*, PL, 2.5 g L⁻¹). Excised fish was reared for five weeks in its small tank containing a treatment or a control. Fin regeneration was monitored weekly. No mortality occurred, and fin regeneration was statistically improved for the fish reared in combinations of IAL, BL, and PL ($P < 0.05$). Among these alternative treatments, red color intensity was fully redeveloped in fish reared in three herbal treatments ($P < 0.05$) that all contained dried papaya leaf (BL + PL, IAL + PL, and IAL + BL + PL). Therefore, those three treatments effectively enhanced fin regeneration.

The antioxidant activity of fermented herbal extracts in both controls (CT 1 and CT 2) and all treatments was determined. The water in all treatments showed significantly better antioxidant activity than both controls ($P < 0.05$). Water quality and somatic responses of reared fish were monitored. Physico-chemical water parameters improved compared with CT 1, especially total ammonia nitrogen, nitrite, and nitrate ($P < 0.05$). Bubble-nests were superior in height, frequency, area, and number of bubbles in CT 2 and three treatments (CT 2 + BL, CT 2 + BL + PL or CT 2 + IAL + PL) ($P < 0.05$). Adverse effects in some hematological parameters were observed in the treatment that contained BL and PL together. CT 2 and three herbal treatments

improved the antioxidant and physico-chemical properties of the rearing water, and bubble-nest building ability of injured *B. splendens*. Considering growth performance was not caused by herbal treatments. However, the feed conversion ratio significantly increased associated with decreasing amylase-specific activity ($P < 0.05$), whereas protein- and lipid-digesting enzymes remained. Skin coloration coordinates were either reduced or preserved due to herbal treatments. Muscle quality was unchanged, whereas crude protein, crude lipid, and ash contents in whole-body were similar across nine treatments. Whereas entire fish can compensate for growth normally, these findings indicate minor effects of long-term herbal treatments on somatic changes of injured fish.

Keyword: *B. splendens*, Herbal treatment, Injured fish, Fin amputation, Well-being

ชื่อวิทยานิพนธ์ นวัตกรรมการใช้ใบหูกวาง (*Terminalia catappa*) และตำรับสมุนไพรจากภูมิปัญญา

ชาวบ้านในการเลี้ยงและการสมานแผลปลากัด (*Betta splendens*)

ผู้เขียน นางสาวเสาวลักษณ์ มาลาวะ

สาขาวิชา วาริชศาสตร์

ปีการศึกษา 2566

บทคัดย่อ

ผู้เพาะพันธุ์และผู้เลี้ยงปลากัด (*Betta splendens*) มักเติมสารสกัดจากใบหูกวาง (*Terminalia catappa*) ลงในน้ำในภาชนะ แต่มีข้อมูลเพียงเล็กน้อยเกี่ยวกับผลของความเข้มข้นของสารสกัดที่มีต่อคุณภาพน้ำและประสิทธิภาพการเจริญเติบโตของปลา การศึกษาครั้งนี้ได้ตรวจสอบผลของสารสกัด *T. catappa* ต่อคุณภาพของน้ำ การเจริญเติบโต การใช้ประโยชน์จากอาหาร สีผิว การก่อหวอด กิจกรรมของเอนไซม์ย่อยอาหาร คุณภาพกล้ามเนื้อ องค์ประกอบของซาก และพารามิเตอร์ทางโลหิตวิทยาของปลากัด โดยนำปลากัดเพศผู้ระยะวัยรุ่น (อายุ 2 เดือน น้ำหนักเริ่มต้น 0.80–0.95 กรัม) มาเลี้ยงแยกในแก้วพลาสติกทรงกระบอกปริมาตร 350 มิลลิลิตร เป็นเวลา 8 สัปดาห์ ในน้ำที่มีการเติมสารสกัดจากใบหูกวางความเข้มข้นแตกต่างกัน 4 ระดับ (0.125, 0.25, 0.5 และ 1 กรัม/ลิตร) และกลุ่มควบคุมที่ไม่เติมสารสกัดจากใบหูกวาง ผลการทดลองพบว่าปลาที่เลี้ยงในสารสกัดใบหูกวางความเข้มข้น 0.25 กรัม/ลิตร ไม่ได้รับผลกระทบเชิงลบเมื่อพิจารณาจากการเจริญเติบโต (อัตราการเจริญเติบโตจำเพาะเท่ากับร้อยละ 1.62 ต่อวัน) การใช้ประโยชน์จากอาหาร (อัตราการเปลี่ยนอาหารเป็นเนื้อเท่ากับ 1.21 กรัม อาหาร/กรัม น้ำหนักตัว) ดัชนีอวัยวะภายในต่อน้ำหนักตัว คุณภาพกล้ามเนื้อ องค์ประกอบทางเคมีของซาก และพารามิเตอร์ทางโลหิตวิทยา ($P > 0.05$) แต่แสดงผลต่อตัวชี้วัดอื่น ๆ บางส่วนที่ตรวจสอบ ขณะที่คุณภาพน้ำดีขึ้นอย่างมีนัยสำคัญในระหว่างการทดลอง ($P < 0.05$) สีผิวสว่างขึ้น (L^*) ในทุกกลุ่มทดลอง มีเพียงปลาที่เลี้ยงด้วยสารสกัดจากใบหูกวาง 0.125 กรัม/ลิตร เท่านั้นที่เพิ่มค่าความแดงของผิวหนัง (a^*) ค่าฮิว (h^*) และค่าโครมา (C^*) ($P < 0.05$) เมื่อเปรียบเทียบกับปลาในชุดควบคุมพบว่าความถี่ในการก่อหวอด (1.70 เท่าโดยเฉลี่ย) และพื้นที่หวอด (1.52 เท่าโดยเฉลี่ย) เพิ่มขึ้นทุกชุดการทดลองและมีความสัมพันธ์กับความเข้มข้นของสารสกัดใบหูกวาง ($P < 0.05$) กิจกรรมจำเพาะของเอนไซม์ย่อยโปรตีนและไขมันไม่ได้รับผลกระทบในเชิงลบ แต่มีการเปลี่ยนแปลงในชุดการทดลองที่มีสารสกัดจากใบหูกวางความเข้มข้น 0.125, 0.25 และ 1 กรัม/ลิตร ปลาที่เลี้ยงในน้ำที่มีสารสกัด 0.25 กรัม/ลิตร แสดงประสิทธิภาพการใช้ประโยชน์จากคาร์โบไฮเดรตผ่านกิจกรรมของอะไมเลสได้ลดลง เนื่องจากกิจกรรมของไลเปสที่เพิ่มขึ้น และมีอัตราส่วนอะไมเลส/ทริปซินคงที่ ผลการศึกษาทั้งหมดแสดงให้เห็นว่าความเข้มข้นของสารสกัดใบหูกวางที่เหมาะสมสำหรับเลี้ยงปลากัดในระยะการเจริญเติบโต คือ 0.25 กรัม/ลิตร

ความก้าวร้าวของปลากัดระหว่างการเกี้ยวพาราสีและการสืบพันธุ์อาจส่งผลให้ครีบกินขาดได้ อย่างไรก็ตาม การงอกใหม่ของครีบกินเร็วขึ้นอย่างมีนัยสำคัญหากมีการรักษาปลาด้วยน้ำหมักจากพืช การศึกษาในครั้งนี้ได้ตัดครีบกินครึ่งหนึ่งของปลากัดเพศผู้ (น้ำหนัก 1.14–1.44 กรัม) ในระนาบแนวตั้ง

จากนั้นแบ่งปลากัดเป็น 9 กลุ่ม ($n = 15$ /ทรีทเมนต์ ซึ่งปลาแต่ละตัวคือหน่วยทดลอง) ชุดควบคุมประกอบด้วย กลุ่มควบคุมเชิงลบ (CT 1) ที่ใช้น้ำประปาที่ปราศจากคลอรีนในการเลี้ยง และกลุ่มควบคุมเชิงบวก (CT 2) ที่ใช้ใบตะไคร้แห้ง (*Cymbopogon citratus*, LL, 1 กรัม/ลิตร) ดินเหนียวแห้ง (50 กรัม/ลิตร) และเกลือ (5 กรัม/ลิตร) หมักในน้ำ ชุดทดลองประกอบด้วย 7 ทรีทเมนต์ที่เตรียมโดยการหมักน้ำของกลุ่มควบคุมเชิงบวก (CT 2) ซึ่งเสริมด้วยการรวมกันของผงใบแห้งเพียงหนึ่ง สอง หรือทั้งสามอย่าง ได้แก่ ใบหูกวาง (*T. catappa*, IAL, 0.25 กรัม/ลิตร) ใบกล้วย (*Musa sapientum*, BL, 1.5 กรัม/ลิตร) และใบมะละกอ (*Carica papaya*, PL, 2.5 กรัม/ลิตร) เมื่อนำปลาที่ตัดครีบออกมาเลี้ยงเป็นเวลา 5 สัปดาห์ในภาชนะขนาดเล็กที่มีน้ำหมักทั้งชุดควบคุม หรือชุดทดลอง และติดตามการงอกใหม่ของครีบหางทุกสัปดาห์ ผลการทดลองพบว่าไม่มีการตายเกิดขึ้น และการงอกใหม่ของครีบปลาดีขึ้นอย่างมีนัยสำคัญเมื่อเลี้ยงปลาโดยใช้ส่วนผสมของ IAL, BL และ PL ($P < 0.05$) อย่างไรก็ตาม ความเข้มของสีแดงพัฒนาเต็มที่ในครีบปลาที่เลี้ยงด้วยสมุนไพร 3 ชนิด ($P < 0.05$) ซึ่งทั้งหมดมีใบมะละกอแห้ง (BL + PL, IAL + PL และ IAL + BL + PL) ดังนั้น ตำรับดังกล่าวจึงช่วยเพิ่มการงอกใหม่ของครีบได้อย่างมีประสิทธิภาพ

เมื่อตรวจสอบฤทธิ์ต้านอนุมูลอิสระของน้ำในทรีทเมนต์ ผลการศึกษาพบว่าน้ำในตำรับของชุดทดลองทั้งหมดแสดงฤทธิ์ต้านอนุมูลอิสระได้สูงกว่าน้ำในทั้ง 2 กลุ่มควบคุมอย่างมีนัยสำคัญ ($P < 0.05$) เมื่อตรวจสอบคุณภาพน้ำและการตอบสนองด้านร่างกาย พารามิเตอร์ของน้ำทางเคมีกายภาพดีขึ้นเมื่อเปรียบเทียบกับ CT 1 โดยเฉพาะแอมโมเนียไนโตรเจนทั้งหมด ไนไตรท์ และไนเตรต ($P < 0.05$) การก่อหวอดเพิ่มขึ้นเมื่อพิจารณาจากความสูง ความถี่ พื้นที่ และจำนวนฟองใน CT 2 และตำรับที่มี CT 2 + BL, CT 2 + BL + PL หรือ CT 2 + IAL + PL ($P < 0.05$) พารามิเตอร์ทางโลหิตวิทยาได้รับผลกระทบเชิงลบเมื่อเลี้ยงปลาโดยใช้ BL และ PL ร่วมกัน ดังนั้น ตำรับ CT 2 และ 3 ทรีทเมนต์ดังกล่าว ทำให้ฤทธิ์ต้านอนุมูลอิสระและคุณสมบัติทางเคมีกายภาพของน้ำที่ใช้เลี้ยงและความสามารถในการก่อหวอดของปลากัดที่ได้รับบาดเจ็บดีขึ้นเมื่อพิจารณาเกี่ยวกับการเจริญเติบโตด้านร่างกาย ปลาที่เลี้ยงด้วยตำรับสมุนไพรมีการเจริญเติบโตไม่แตกต่างจากชุดควบคุม อย่างไรก็ตาม อัตราการเปลี่ยนอาหารเป็นเนื้อเพิ่มขึ้น ซึ่งเกี่ยวข้องกับการลดกิจกรรมจำเพาะของอะไมเลส ($P < 0.05$) ในขณะที่เอนไซม์ย่อยโปรตีนและไขมันคงที่ สีผิวของปลาลดลงหรือคงที่ คุณภาพของกล้ามเนื้อไม่เปลี่ยนแปลง ส่วนปริมาณโปรตีน ไขมัน และเถ้าในซากมีค่าใกล้เคียงกัน แม้ว่าปลาสามารถชดเชยการเจริญเติบโตได้ตามปกติเมื่อสิ้นสุดการทดลอง แต่ผลการวิจัยนี้บ่งชี้ถึงผลกระทบบางส่วนของ การรักษาด้วยตำรับสมุนไพรในระยะยาวต่อการเปลี่ยนแปลงทางร่างกายของปลากัดที่ได้รับบาดเจ็บ

คำสำคัญ: *B. splendens*, การรักษาด้วยสมุนไพร, ปลาบาดเจ็บ, การตัดครีบ, ความเป็นอยู่ที่ดี

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LIST OF PAPERS AND ABSTRACT PROCEEDINGS

1. **Malawa, S.**, Nuntapong, N., Suanyuk, N., Thongprajukaew, K., 2022. Addition of different concentrations of Indian almond (*Terminalia catappa*) leaf extract to aquarium water resulted in improved water quality and increased bubble nest formation by male Siamese fighting fish (*Betta splendens*) without having any consistent negative effects on growth metrics and blood chemistry. *Aquac. Int.* 30, 3269–3288.
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Fermented water of Thai herbs effectively enhanced amputated fin regeneration in Siamese fighting fish (Betta splendens)

Author: Saowalak Malawa,Nutt Nuntapong,Sugree Waeowannajit,Karun Thongprajukaew

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Kasetsart University Research and Development Institute (KURDI),
Kasetsart University, 50 Ngamwongwan Road, Chatuchak, Bangkok 10900, Thailand
Tel. 66-2-579-5548, 561-1474 e-mail: anres@ku.th | <http://anres.kasetsart.org/>

Reference No. 6501.2104/162

Aquatic Science and Innovative Management Division,
Faculty of Natural Resources, Prince of Songkla University, Songkla 90110, Thailand

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Executive summary

1. Introduction

The Siamese fighting fish (*Betta splendens*) is a globally important ornamental fish prized for its vibrant colors and the pugnacious nature and beauty of the male fish (Monvises et al., 2009; Rahmati-Holasoo et al., 2019, 2020). From 2010 to 2019, this species is one of the two most commercially profitable ornamental fish in Thailand. The United States is the largest market and the biggest importer of Siamese fighting fish from Thailand, followed by China, France, Iran, and Singapore (Sermwatanakul, 2019). The declaration of this fish as a national aquatic animal was not only due to the economic importance of the species, but also to its place in the cultural heritage of Thailand (Sermwatanakul, 2019).

It is common practice to add fallen, reddish-brown Indian-almond (*Terminalia catappa*) leaves to the aquarium water (Chansue and Assawawongkasem, 2008; Nugroho et al., 2016). This plant leaf contains a number of active ingredients, including tannins, flavonoids, and saponins (Nurhidayat et al., 2016). This practice mimics the natural habitat conditions to provide a more suitable captive environment during rearing and before export. Also, this practice has been reported in the rearing of cardinal tetra, *Paracheirodon axelrodi* (Walker, 2004); and Amazon leaf fish, *Monocirrhus polyacanthus* (Ramos et al., 2020). Besides the leaves themselves, extracts of *T. catappa* leaf have been used to reduce water turbidity, pH, and total ammonia (Abe et al., 2019; Nozaleda, 2019; Ramos et al., 2020). Positive effects of *T. catappa* leaf extracts have also been reported on survival, growth performance, and feed utilization of reared fish (Nugroho et al., 2016, 2017; Nurhidayat et al., 2016; Ramos et al., 2020). Additionally, this herb has been associated with improvements in digestive enzyme activities (Malawa et al., 2022) and skin coloration (Ardi et al., 2020; Malawa et al., 2022), but without affecting muscle quality and the whole-body composition in healthy fish (Malawa et al., 2022). On the other hand, excessive concentrations of *T. catappa* leaf extracts adversely affected some water quality parameters (Ikhwanuddin et al., 2014; Mininel et al., 2014).

Inherently, these fish display agonistic behavior in confrontations associated with defending territory, be it between males and females or among individuals of the same gender, resulting in damaged bodies and torn fins (Malawa et al., 2023). Consequently, the breeders raise these fish in solitary captivity, commencing at approximately age 1.5 months (Thongprajukaew et al., 2010a, 2010b), to mitigate the adverse effects of their combative nature. In addition, these injuries can manifest during courtship and reproductive phases (Malawa et al., 2023). In Thailand, immersion in herbal treatment is the preferred and practical treatment to heal external damage in injured fish rather than using antibiotics or chemicals that cause adverse health effects to the fish (via the residues) and in their surroundings (Malawa et al., 2022, 2023). Typically, some herbs, such as the dried leaves of the Indian-almond, banana (*Musa sapientum*), papaya (*Carica papaya*) and lemongrass (*Cymbopogon citratus*) or dried clay or salts, have been incorporated into the recipes used to treat the fish (Malawa et al., 2023). These ingredients have various phytoconstituents, such as tannin, flavonoid and phenolic compounds, differing in quantity and active ingredient type. For example, the dried leaves of the Indian-almond aid in wound healing and act against harmful monogeneans and *Trichodina* spp. (Chitmanat et al., 2005; Ponpornpisit et al., 2007), while the dried leaves of banana, papaya and lemongrass provide anti-bacterial, anti-oxidant and anti-inflammatory activities that help to heal wounds (Maria and Veronica, 2021; Tanmoy et al., 2021; Wijaya et al., 2020), especially those caused during fish fighting. Dried clay and salt are added to help fish recover more rapidly, to heal wounds, to nourish the skin and to reduce pathogen growth (Chiarachavildham, 2016; Puello-Cruz et al., 2010; Putiyanan, 1999).

Although indigenous knowledge of the above ingredients is widely applied by bettas fish farmers in Thailand, there is still a lack of research to support local knowledge for further commercialization. Findings from the present study can help determine the most suitable concentration of *T. catappa* leaf extracts for use in Siamese fighting fish production, support using Thai herbal treatment in betta fish farms, and help develop simple herbal recipes for commercial application in betta fish farming.

2. Objectives

2.1 To investigate the optimal concentration of dried *T. catappa* leaf extract that should be used in rearing male Siamese fighting fish.

2.2 To investigate the ability of Thai herbal recipes to improve the wound healing, well-being, and somatic responses of injured Siamese fighting fish treated with a range of herbal formulations by amputating the caudal fin to mimic the damage caused during mating or fighting contests.

3. Methodology

3.1 Experiment 1

Juvenile male fish (2-month-old, 0.80–0.95 g initial weight) were individually reared in 350-mL cylindrical plastic containers. The fish were reared for 8 weeks in four concentrations of *T. catappa* leaf extract (0.125, 0.25, 0.5, and 1 g L⁻¹) and a control group was reared in water without the extract. A completely randomized design (CRD) was formulated, comprising five alternative treatments with fifteen fish in each. At the end of the 8-week trial, the water quality, fish survival and growth, feed utilization, skin coloration, bubble-nest formation, digestive enzyme activity, muscle quality, whole-body composition, and hematological parameters were assessed.

3.2 Experiment 2

Half of the caudal fin of male Siamese fighting fish (1.14–1.44 g body weight) was amputated in the vertical plane and then divided the fish into nine experimental groups ($n = 15$ per treatment, each fish serving as an experimental unit). The control groups comprised a negative control (CT 1) of dechlorinated water and a positive control (CT 2) made up of a water fermentation of dried lemongrass (LL, 1 g L⁻¹) leaf, dried clay (50 g L⁻¹), and salt (5 g L⁻¹). The seven treatments were based on CT 2 which was supplemented with combinations of dried leaf of one, two or all three of the following: Indian-almond (IAL, 0.25 g L⁻¹), banana (BL, 1.5 g L⁻¹), and papaya (PL, 2.5 g L⁻¹). Excised fish was reared for five weeks in its small tank containing a treatment or a control. The present study

was a complete randomized design with seven treatments and two controls. The assessment was based on fish survival and growth, fin length regeneration, color development in regenerated fins, anti-oxidant activity of fermented herbal water, physico-chemical parameters of rearing water, bubble-nest construction, and blood parameters, feed utilization, skin coloration, digestive enzyme activity, muscle quality, and whole-body composition in injured fish.

4. Results and discussion

4.1 Experiment 1

A leaf extract concentration of 0.25 g L^{-1} showed no significant effect on growth (specific growth rate = $1.62\% \text{ body weight day}^{-1}$), feed utilization (feed conversion ratio = $1.21 \text{ g feed g gain}^{-1}$), viscerosomatic index, muscle quality, whole-body composition, and hematological parameters of reared fish ($P > 0.05$), but did show effects on some of the other metrics that were monitored. Water quality significantly improved during the experiment ($P < 0.05$). Increased skin lightness (L^*) was observed in all experimental groups, but only fish reared in 0.125 g L^{-1} leaf extract increased skin redness/greenness (a^*), hue (h^*), and chroma (C^*) ($P < 0.05$). Relative to the control, increments in bubble-nest formation frequency (1.70-fold on average) and bubble-nest size (1.52-fold on average) were correlated with the concentration of *T. catappa* leaf extract ($P < 0.05$). Specific activities of protein- and lipid-digesting enzymes were not negatively affected but some modulations were observed in treatments containing leaf extract at 0.125 , 0.25 , and 1 g L^{-1} . Fish reared in the 0.25 g L^{-1} treatment exhibited a sparing effect of carbohydrate utilization indicated by decreased amylase specific activity, increased lipase specific activity, and stable amylase/trypsin ratios. The results suggest that 0.25 g L^{-1} of *T. catappa* leaf extract is a suitable concentration of the additive during the growing phase of male Siamese fighting fish.

Another study reported improvement in water quality, survival, growth performance, feed utilization, skin coloration, or health status across several species following rearing in Indian-almond leaf extract at appropriate concentrations: 0.5 g L^{-1} in

the neon tetra fish, *P. innesi* (Ardi et al., 2020); 3 g L⁻¹ in the black tiger shrimp, *Penaeus monodon* (Ikhwanuddin et al., 2014); 0.375 and 0.5 g L⁻¹ in the Siamese fighting fish, *Betta* sp. (Nugroho et al., 2016, 2017); 0.5 and 1.5 g L⁻¹ in the cardinal tetra, *P. axelrodi* (Nurhidayat et al., 2016) and 0.75 g L⁻¹ in the Amazon leaf fish, *M. polyacanthus* (Ramos et al., 2020). Nevertheless, excessive concentrations of these extracts have been detrimental to aquatic fauna. Our findings assessed the effects of *T. catappa* on overall fish performance, which had never been reported in earlier documents. However, the active ingredients, mainly tannins, found in *T. catappa* leaf may be affected by seasonal variation, geographical location, soil fertility, leaf age, extraction solvent, and extraction protocol. Differences in these parameters can lead to differences in observed outcomes in fish.

4.2 Experiment 2

No mortality occurred, and fin regeneration was statistically improved for the fish reared in combinations of IAL, BL, and PL ($P < 0.05$). Among these alternative treatments, red color intensity was fully redeveloped in fish reared in three herbal treatments ($P < 0.05$) that all contained dried papaya leaf (BL + PL, IAL + PL, and IAL + BL + PL). Therefore, those three treatments effectively enhanced fin regeneration. The water in all treatments showed significantly better antioxidant activity than both controls ($P < 0.05$). Physico-chemical water parameters improved compared with CT 1, especially total ammonia nitrogen, nitrite, and nitrate ($P < 0.05$). Bubble-nests were superior in height, frequency, area, and number of bubbles in CT 2 and three treatments (CT 2 + BL, CT 2 + BL + PL or CT 2 + IAL + PL) ($P < 0.05$). Adverse effects in some hematological parameters were observed in the treatment that contained BL and PL together. CT 2 and three herbal treatments improved the antioxidant and physico-chemical properties of the rearing water, and bubble-nest building ability of injured *B. splendens*. Growth performance was not caused by herbal treatments. However, the feed conversion ratio significantly increased associated with decreasing amylase-specific activity ($P < 0.05$), whereas protein- and lipid-digesting enzymes remained. Skin coloration coordinates were

either reduced or preserved due to herbal treatments. Muscle quality was unchanged, whereas crude protein, crude lipid, and ash contents in whole-body were similar across nine treatments.

Observations of a number of fish species have reported varying regeneration times: 24 days for mosquito fish (Johnson and Denton, 1977) and guppy (Kolluru et al., 2006); 14 days for zebrafish (Geraudie et al., 1995; Poleo et al., 2001) and fancy carp, *Cyprinus carpio* (Chansue, 2006); 42 days for goldfish, *Carassius auratus* (Ngamsiri and Laumsomboon, 2021); and 28 days for Nile tilapia (Ngamsiri, 2015). Several studies have improved wound healing and fin regeneration processes using natural products at optimal levels: 0.2 g L⁻¹ of dried Indian-almond leaves for goldfish (Ngamsiri and Laumsomboon, 2021); and 0.03 g L⁻¹ of creat (*Andrographis paniculata*) and dried Indian-almond leaves for fancy carp (Chansue, 2006). Our finding provides more comprehensive information on herbal treatments for amputated fish than previous documents, including details on caudal fin coloration, well-being, and somatic response of injured fish. The leaf age, extraction solvent, extraction protocol, seasonal variation, geographical location, and soil fertility may influence the active ingredients found in each herb, resulting in variations in the observed outcomes in fish.

5. Concluding remarks

4.1 The findings of this work indicated that an aquarium additive of *T. catappa* leaf extract at a concentration of 0.25 g L⁻¹ of water is suitable for rearing male Siamese fighting fish based on observations of water quality, fish growth, feed utilization, skin coloration, bubble-nest creation, digesting enzyme activities, muscle protein, whole-body composition, and hematological parameters. On the other hand, observations of skeletal growth based on body length and condition factor indicated undesirable effects on fish reared in less appropriate concentrations (Experiment 1).

4.2 Male Siamese fighting fish automatically regenerate damaged fins in normal rearing water. Fin length develops primarily followed by the migration of pigmentation. However, findings from the current study demonstrate the ability of

fermented water from Thai herbal formulations to significantly enhance fin regeneration, in terms of both length and color development. All the formulations included dried lemongrass leaf, clay and salt. Fully developed fins were observed in fish reared in fermented water containing the above components with the addition of dried papaya leaf and papaya leaf with dried leaf of either banana or Indian-almond or both (Experiment 2).

4.3 Recuperating injured male Siamese fighting fish in suitable formulations of herbs fermented in water significantly supported the well-being of the fish by improving water quality and water antioxidant activity and by accelerating bubble-nest building. Our findings suggest that the well-being of fish suffering damage to their body and fins could be promoted by rearing them in a water fermentation of 1 g L^{-1} of lemongrass supplemented with 50 g L^{-1} of dried clay and 5 g L^{-1} of salt. This formulation was also effective when the water fermentation included 1.5 g L^{-1} of dried banana leaf, or 0.25 g L^{-1} of dried Indian-almond leaf, or 2.5 g L^{-1} of dried papaya leaf (Experiment 2).

4.4 Although some herbal recipes have reportedly improved fin regeneration and bubble-nest creation in injured male betta fish, some treatments in the current study had negative effects due to lowering feed utilization, as indicated by the feed conversion ratio, amylase specific activity and skin coloration. However, as somatic growth is not a main assessment criterion during fish injury, the current results still endorse using fermented water of Thai herbs for short-term wound healing in betta fish rather than relying on antibiotics or chemical products that can adversely affect aquatic fauna or their surroundings, or both (Experiment 2).

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CHAPTER 1

Addition of different concentrations of Indian-almond (*Terminalia catappa*) leaf extract to aquarium water resulted in improved water quality and increased bubble-nest formation by male Siamese fighting fish (*Betta splendens*) without having any consistent negative effects on growth metrics and blood chemistry

1. Introduction

The Siamese fighting fish (*Betta splendens*) is a globally important species in the ornamental aquarium fish trade. The species is native to Thailand and is found in neighboring Cambodia, Laos, Malaysia, and Vietnam (Monvises et al., 2009; Panijpan et al., 2017). Outside of its native area, it has spread to southeast Australia, Brazil, Colombia, the Dominican Republic, southeast United States, and Singapore (Brammah, 2015). The USA is the largest market for Siamese fighting fish from Thailand (DOF, 2021). Since spectacular coloring, fin characteristics, aggressive behavior, and sexual dimorphism are attractive to keepers of ornamental fish, breeders only market male fish (Goldstein, 2004; Thongprajukaew et al., 2014), which are individually reared in small containers since they exhibit aggressive behavior toward other males (Goldstein, 2004; Monvises et al., 2009).

It is common practice to add fallen, reddish-brown Indian-almond (*Terminalia catappa*) leaves to the aquarium water (Chansue and Assawawongkasem, 2008; Nugroho et al., 2016). The leaves contain a number of active ingredients, including tannins, flavonoids, and saponins (Nurhidayat et al., 2016). This practice mimics the natural habitat conditions to provide a more suitable captive environment during rearing and before export. This practice has also been reported in the

rearing of cardinal tetra, *Paracheirodon axelrodi* (Walker, 2004), and Amazon leaf fish, *Monocirrhus polyacanthus* (Ramos et al., 2020).

Besides the leaves themselves, extracts of *T. catappa* leaf have been used to reduce water turbidity, pH, and total ammonia (Abe et al., 2019; Nozaleda, 2019; Ramos et al., 2020). Positive effects of *T. catappa* leaf extracts have also been reported on survival, growth performance, and feed utilization of reared fish (Nugroho et al., 2016, 2017; Nurhidayat et al., 2016; Ramos et al., 2020). On the other hand, excessive concentrations of *T. catappa* leaf extracts adversely affected some water quality parameters (Ikhwanuddin et al., 2014; Mininel et al., 2014).

Although *T. catappa* leaf and extracts of the leaf are commercially available and widely used by fighting fish culturists, an assessment of the effects of *T. catappa* on overall fish performance has never been reported. Findings from the present study can help determine the most suitable concentration of *T. catappa* leaf extracts for use in Siamese fighting fish production.

2. Objective

To investigate the optimal concentration of dried *T. catappa* leaf extract that should be used in rearing male Siamese fighting fish. The assessment was based on water quality, fish survival and growth, feed utilization, skin coloration, bubble-nest formation, digestive enzyme activity, muscle quality, whole-body composition, and hematological parameters.

3. Materials and methods

3.1 Collection and preparation of leaf extract

Newly fallen *T. catappa* leaves were collected within the Prince of Songkla University campus, Hat Yai, Thailand. Attached dirt was removed and the leaves were immersed in 5 mg L⁻¹ of KMnO₄ for 15 min to eliminate microorganisms. The leaves were then rinsed three times with distilled water. The clean leaves were dried at 60 °C for 12 h, chopped in a blender, sieved, pooled, packed in polyethylene bags, covered with

black plastic bags to exclude light, and then stored at 4 °C until used. The same pooled samples of leaves were used to prepare stocks of extract throughout the experiment.

To prepare the stock, 1 g of leaf sample was macerated in 1 L of distilled water at room temperature for 3 days. The mixture was stirred twice a day. The macerated mixture was passed through a filter cloth and the extract was obtained by passing the filtrate through Whatman paper No. 4 (Chansue and Assawawongkasem, 2008).

The tannin contents of the extract were determined by adding 1 mL of extract to a volumetric flask containing 75 mL distilled water, 5 mL of Folin and Ciocalteu's phenol reagent, and 10 mL of 35% Na₂CO₃. The final volume was adjusted to 100 mL. The mixture was shaken and kept at room temperature for 30 min, and absorbance was spectrophotometrically measured at 760 nm against the linear range of tannic acid standard solution (AOAC, 1990). The average concentration of tannins in the stock (1 g L⁻¹) of leaf extract was 0.14 g L⁻¹. This initial concentration was serially diluted to obtain leaf extract concentrations of 0.125, 0.25, and 0.5 g L⁻¹.

3.2 Fish trial

Solid-red, 1.5-month-old, male Siamese fighting fish were purchased from a local farm in Nakhon Pathom Province, Thailand. They were individually acclimatized for 2 weeks in cylindrical plastic containers measuring 8.3 cm in diameter and 12.5 cm in height. The containers were filled with 350 mL of water, giving a water depth of 8 cm. After acclimatization, the fish were individually weighed immediately prior to the start of the trial to confirm the suitability of their initial body weight (0.80–0.95 g). The fish were then randomly divided into four treatments and a control group, each containing fifteen fish. The fish were reared for 8 weeks in 350 mL of water containing the prepared *T. catappa* leaf extract at concentrations of 0, 0.125, 0.25, 0.5, and 1 g L⁻¹. These concentrations were chosen based on a preliminary study that covered the reported concentrations of *T. catappa* leaf extract used in rearing various fish species (Ardi et al., 2020; Nugroho et al., 2016; Nurhidayat et al., 2016; Ramos et al., 2020). During the present study, the photoperiod was a 12-h light (06.00 to 18.00 h)/12-h dark cycle.

Twice a day at 08.00 and 17.00 h, the fish were given an excess of a commercial floating feed for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). The amount of feed given was adjusted weekly based upon the record of amounts of feed consumed. The proximate chemical composition of the diet on a dry matter basis included 39.4% crude protein, 3.23% crude lipid, 1.97% crude fiber, and 7.72% ash. The procedures for chemical analysis (moisture, crude protein, crude lipid, and ash) are detailed in the whole-body composition data, while crude fiber content was determined based on acid and alkali digestion (AOAC, 2005), using a fiber analyzer (F800; Hanon Advanced Technology Group, Jinan, China). Uneaten food was collected 30 min after feeding, and dried at 60 °C until constant weight was reached. The obtained data were used to calculate the feeding rate (FR), feed conversion ratio (FCR), and protein efficiency ratio (PER).

3.3 Water management and quality determination

Every other day, 80% of the rearing water was changed, and water quality was monitored twice weekly. pH, temperature, dissolved oxygen (DO), conductivity, and turbidity were measured using a multiparameter water quality meter (U-52G; Horiba, Kyoto, Japan). Total alkalinity (by methyl orange indicator), hardness (by ethylenediaminetetraacetic acid (EDTA) titration), and total ammonia nitrogen (by the phenate method) were analyzed based on the procedure of Boyd and Tucker (1992). Nitrite (by colorimetry) and nitrate (by cadmium reduction) were analyzed according to the method of APHA, AWWA, and WPCF (1998).

3.4 Fish sampling and biometrics

At the end of the 8-week trial, the fish were fasted for 12 h and then euthanized with clove oil (75 mg L⁻¹). Body weight and length (total and standard lengths) were recorded for all the surviving fish ($n = 15$ per treatment) before skin coloration was measured. The skin was carefully cleaned with soft blotting paper, and a rapid color measurement was taken from the middle part of the fish body using a

MiniScan EZ (Hunter Associates Laboratory, Reston, VA, USA) calibrated with white and black standards. The color coordinates were automatically recorded in terms of lightness (L^*), redness/greenness (a^*), hue (h^*), and chroma (C^*). The value L^* indicates darkness (0) to brightness (100), a^* indicates the degree of redness (+ a) to greenness (− a), h^* indicates color differences due to wavelength, from red (0°) to blue (270°), and C^* indicates color purity from vividness (+ C) to dullness (− C). Blood samples were taken from the excision of the caudal fin and transferred to a 1% EDTA-coated Eppendorf tube. Samples were collected from all the fish and then pooled from lots of three fish to obtain five pooled samples per treatment ($n = 5$ per treatment). Viscera ($n = 5$ per treatment), digestive tracts ($n = 5$ per treatment), and white muscle ($n = 5$ per treatment) were collected from the same lots of fish to determine the viscerosomatic index (VSI), digestive enzyme activity, and muscle quality, respectively. The whole-body composition analysis used ten fish selected from each treatment after blood collection. The fish were pooled into lots, with the first and second lots comprising three fish and the third lot four fish ($n = 3$ per treatment).

The parameters describing growth performances and feed conversion were calculated as follows:

$$\text{Condition factor (CF)} = 100 \times [\text{body weight (g)}/\text{standard length (cm)}^3]$$

$$\text{VSI (\%)} = 100 \times [\text{wet weight of viscera (g)}/\text{body weight (g)}]$$

$$\text{Specific growth rate (SGR, \% body weight day}^{-1}\text{)} = 100 \times [(\ln W_t - \ln W_o)/(t - t_o)],$$

where W_t = mean weight (g) at day t , and W_o = mean weight (g) at day t_o ;

$$\text{FR (\% body weight day}^{-1}\text{)} = 100 \times C/[W_o + W_t]/2]/t,$$

where C = daily feed consumption (g), W_o = initial body weight (g), W_t = final body weight (g), and t = feeding duration (day);

$$\text{FCR (g feed g gain}^{-1}\text{)} = \text{dry feed consumed (g)}/\text{wet weight gain (g) and}$$

$$\text{PER (g gain g protein}^{-1}\text{)} = \text{wet weight gain (g)}/\text{protein intake (g)}.$$

3.5 Bubble-nest behavior

The bubble-nest production of each fish was photographed from above with a smartphone (iPhone 11 Pro Max, Apple Inc., China). The photographs were taken three times weekly during weeks 6 to 8 of the experiment. The images were transferred to file and each nest area was analyzed (cm^2) by ImageJ software, as described by Bautista et al. (2019). The frequency of bubble-nesting (%) was calculated by dividing the number of aquaria containing bubble-nests by the total number of aquaria in each treatment and then multiplying the quotient by 100.

3.6 Determination of digestive enzyme activities

3.6.1 Enzyme extraction and protein quantification in crude extracts

Frozen digestive tract was extracted in cold 0.2 M Na_2HPO_4 - NaH_2PO_4 buffer (pH 8) at a ratio of 1:15 w/v, using a tissue micro-homogenizer (THP-220; Omni International, Kennesaw, GA, USA). The homogenates were centrifuged at $15,000 \times g$ for 30 min at 4 °C. Supernatants were collected and kept in small portions at -20 °C. The protein concentration of crude extracts (mg mL^{-1}) was assayed using the method described by Lowry et al. (1951) with a protein standard of bovine serum albumin. These protein concentration values were used to quantify the specific activity of digestive enzymes (U mg protein^{-1}).

3.6.2 Digestive enzyme assays

The digestive enzymes were assayed according to the optimal conditions recommended for Siamese fighting fish by Thongprajukaew et al. (2010a, 2010b). These conditions were pH 2 and 40 °C for pepsin (EC 3.4.23.1); pH 8 and 50 °C for trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and α -amylase (EC 3.2.1.1); and pH 8 and 40 °C for lipase (EC 3.1.1.3). All assays were performed within 1 month of enzyme extraction. Pepsin activity was assayed using hemoglobin as the substrate according to the method of Worthington (1993). One enzyme unit (U) of pepsin was defined as an increase of 1.0 in absorbance at 280 nm. The activities of trypsin and chymotrypsin were assayed according

to the method of Rungruangsak-Torrissen et al. (2006), using *N*-benzoyl-*L*-Arg-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the respective specific substrates. The liberated *p*-nitroanilide from both enzymatic hydrolyses was spectrophotometrically measured at 410 nm against the linear range of its standard. Alpha-amylase activity was determined using soluble starch as the substrate according to the method of Areeksere et al. (2004). The liberated product was measured at 540 nm and compared against the linear range of standard maltose. Lipase activity was determined using *p*-nitrophenyl palmitate as the substrate according to the method of Winkler and Stuckmann (1979). The liberated *p*-nitrophenol was spectrophotometrically measured at 410 nm. One U of the last four enzymes above was defined as the amount that catalyzed the conversion of 1 μ mol of substrate per minute. To obtain the amylase/trypsin ratio (A/T ratio), the activity of amylase was divided by the activity of trypsin from the same fish sample.

3.7 Muscle protein synthesis capacity

Concentrations of RNA and protein in white muscle were determined based on the method of Rungruangsak-Torrissen (2007). Briefly, using an ultrasonic processor (VCX; Sonic and Materials Inc., Newtown, CT, USA), 50 mg of frozen muscle was sonicated with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). After sonication, chloroform was added to obtain an upper aqueous phase (RNA) and a lower organic phase (protein). The aqueous phase was precipitated with isopropanol, washed with ethanol, oven-dried, dissolved in sodium acetate, and its absorbance was measured at 260 nm. Similar protocols were applied to the organic phase, except that protein was dissolved with sodium dodecyl sulfate and absorbance was measured at 280 nm. The extinction coefficients for calculating RNA and protein concentrations were $E_{260} = 40 \mu\text{g RNA mL}^{-1}$ and $E_{280} = 2.1 \text{ mg protein mL}^{-1}$, respectively.

3.8 Muscle myosin and actin contents

Myosin, actin, and sarcoplasmic protein in white muscle were detected using differential scanning calorimetry (DSC7; Perkin Elmer, Waltham, MA, USA) within a range of 20 to 100 °C. Approximately 10 mg of muscle was placed in an aluminum pan, sealed with an aluminum lid, and then heated at a rate of 5 °C min⁻¹ against an empty pan. Identification of muscle proteins was based on onset (T_o), peak (T_p), and conclusion (T_c) temperatures from previous reports of muscle of Nile tilapia, *Oreochromis niloticus* (Thongprajukaew et al., 2015). Amounts of native protein were interpreted from the amounts of energy required to completely denature each protein within a specific range of temperature, and the values were automatically converted from the areas under the curves of the thermograms.

3.9 Whole-body composition

Whole-bodies of fish were minced to homogeneity and their proximate compositions, including moisture, crude protein, crude lipid, and ash, were determined as described by the AOAC (2005). The samples were first dried for 24 h in a hot air oven (WOF-155; Wisd Laboratory Instruments, Wertheim, Germany) at 105 °C for moisture analysis. Crude protein was analyzed using the Kjeldahl analyzer (Kjeltec™ 8200; Foss, Hillerød, Denmark). Crude lipid was extracted by petroleum ether using a Soxhlet extraction unit (Soxtec™ 8000; Foss, Hillerød, Denmark). Ash was gravimetrically determined after burning at 600 °C for 2 h in a muffle furnace (E30-HT; Thai Furnaces Engineering, Lampang, Thailand). These procedures were also applied for diet proximate composition.

3.10 Hematological analysis

Red (RBC) and white (WBC) blood cells were manually counted with a hemocytometer under a compound microscope. Hematocrit (Hct) was determined based on the procedure of Blaxhall and Daisley (1973). Hemoglobin (Hb) was determined by measuring the formation of cyanmethemoglobin according to the method of Larsen and

Snieszko (1961). Plasma protein was determined according to the method of Lowry et al. (1951). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\begin{aligned}\text{MCV (fL)} &= 10 \times [\text{Hct (\%)/RBC (} \times 10^6 \text{ cells } \mu\text{L}^{-1})], \\ \text{MCH (pg)} &= 10 \times [\text{Hb (g dL}^{-1})/\text{RBC (} \times 10^6 \text{ cells } \mu\text{L}^{-1})] \text{ and} \\ \text{MCHC (g dL}^{-1}) &= 100 \times [\text{Hb (g dL}^{-1})/\text{Hct(\%)}].\end{aligned}$$

3.11 Statistical analysis

The suitable sample size per treatment was estimated through Program R 3.6.0, requiring the power of test at 0.8. Since the fish were individually reared in isolation, each fish served as an experimental unit. A completely randomized design (CRD) was formulated, comprising five alternative treatments with fifteen fish in each. All data were reported as means and standard errors of means (SEM). The Statistical Package for Social Sciences Version 22 (SPSS Inc., Chicago, USA) was used for all statistical evaluations. Significant differences between means were analyzed by One-Way analysis of variance (One-Way ANOVA) and Duncan's multiple range test (DMRT) at 95% confidence level was used as the *post hoc* test.

4. Results

4.1 Water quality

All the water quality parameters, except temperature (data not shown), were significantly changed depending on the concentration of *T. catappa* leaf extract (Fig. 1, $P < 0.05$). Rearing water containing leaf extract at 0.125 g L⁻¹ showed reduced pH and nitrite and increased DO and hardness, while conductivity, turbidity, alkalinity, ammonia, and nitrate were unchanged compared to the control. Rearing water that contained leaf extract at 0.25 g L⁻¹ showed the same trends in all parameters except for DO, which was unchanged, and ammonia, which was reduced. Rearing water containing leaf extract at 0.5 g L⁻¹ showed decreased pH, ammonia, and nitrite while DO and turbidity increased. pH, conductivity, and nitrogen wastes were significantly reduced in the treatment containing the leaf extract at 1 g L⁻¹ while alkalinity increased relative to control.

4.2 Survival, growth, and feed utilization of reared fish

No mortality occurred over the 8-week trial. Final body weight, SGR, and VSI were not affected by any concentration of *T. catappa* leaf extracts (Table 1, $P > 0.05$). Compared to control, standard length (SL) and total length (TL) were significantly smaller in fish reared in all treatments, except the treatment containing the extract at 0.25 g L^{-1} . As a result, CF significantly increased in the other three treatments relative to control. Significant reductions in FR were observed in fish reared in the treatment containing 0.5 g L^{-1} while FCR and PER did not differ between groups.

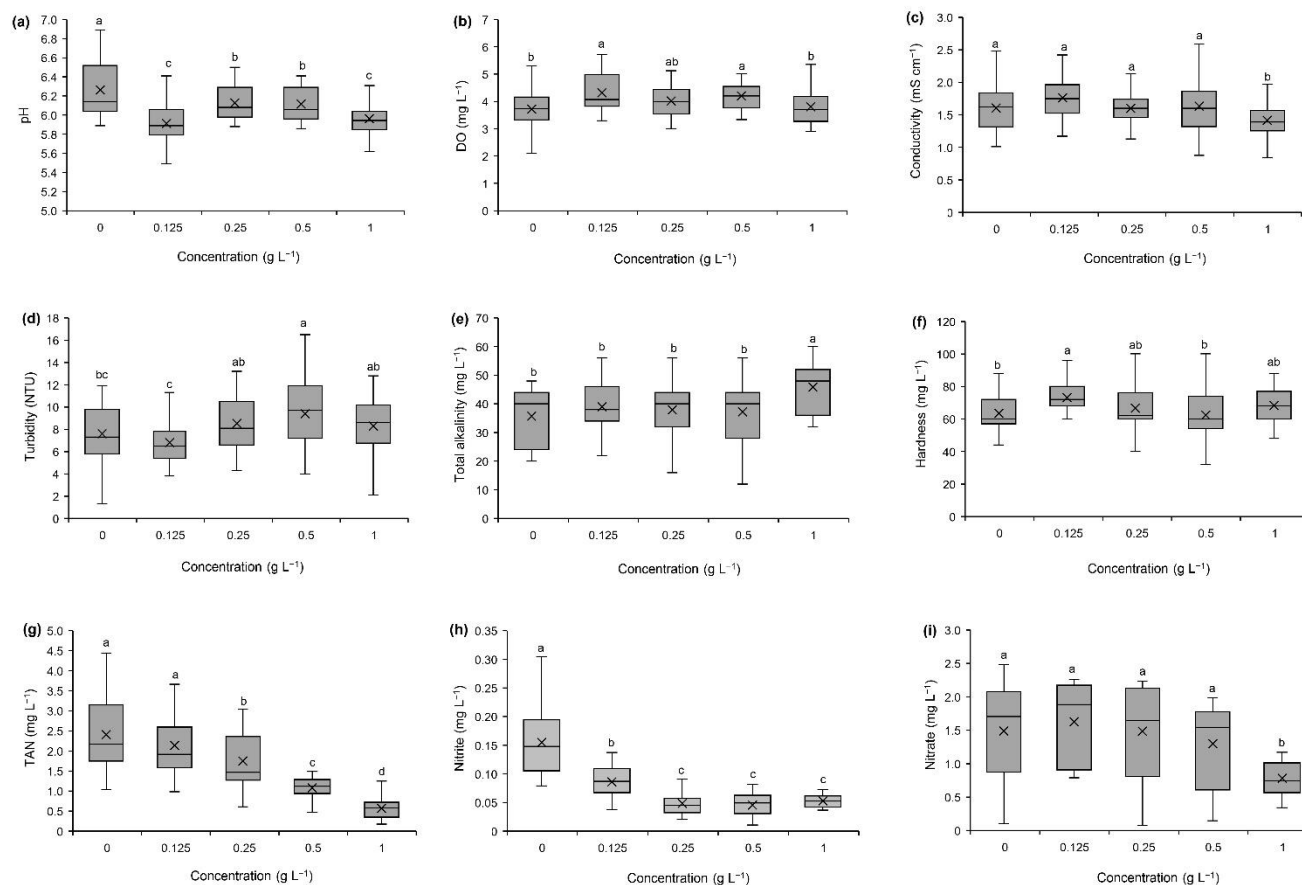


Fig. 1. Boxplots represent water quality parameters during the 8-week experiment: pH (a), DO (b), conductivity (c), turbidity (d), total alkalinity (e), hardness (f), TAN (g), nitrite (h), and nitrate (i). The fish were reared in various concentrations of *T. catappa* leaf extract, and measurements were taken two times a week. DO, dissolved oxygen; NTU, nephelometric turbidity units; TAN, total ammonia nitrogen.

Table 1 Survival, growth performance and feed utilization of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment.

Parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
IBW (g)	0.88 ± 0.01	0.88 ± 0.03	0.87 ± 0.02	0.86 ± 0.02	0.87 ± 0.01	1.000
FBW (g)	2.08 ± 0.12	2.29 ± 0.10	2.24 ± 0.07	2.34 ± 0.05	2.40 ± 0.04	0.091
SGR (% BW day ⁻¹)	1.47 ± 0.11	1.65 ± 0.07	1.62 ± 0.06	1.61 ± 0.07	1.74 ± 0.02	0.158
SL (cm)	4.19 ± 0.04 ^a	3.56 ± 0.07 ^c	4.14 ± 0.03 ^{ab}	4.04 ± 0.02 ^b	4.06 ± 0.03 ^b	<0.001
TL (cm)	8.69 ± 0.08 ^a	7.94 ± 0.07 ^c	8.49 ± 0.06 ^{ab}	8.46 ± 0.08 ^{ab}	8.37 ± 0.12 ^b	<0.001
CF	1.65 ± 0.16 ^c	3.22 ± 0.27 ^a	1.92 ± 0.10 ^{bc}	2.30 ± 0.06 ^b	2.19 ± 0.08 ^b	<0.001
VSI (%)	7.49 ± 1.01	5.68 ± 0.36	5.51 ± 0.69	6.13 ± 1.12	5.60 ± 0.31	0.390
FR (% BW day ⁻¹)	1.92 ± 0.09 ^a	1.79 ± 0.06 ^a	1.77 ± 0.05 ^a	1.35 ± 0.18 ^b	1.64 ± 0.12 ^{ab}	0.008
FCR (g feed g gain ⁻¹)	1.28 ± 0.10	1.10 ± 0.06	1.21 ± 0.08	1.10 ± 0.04	1.07 ± 0.02	0.187
PER (g gain g protein ⁻¹)	1.77 ± 0.17	2.05 ± 0.14	2.03 ± 0.11	2.09 ± 0.09	2.14 ± 0.02	0.292

IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; BW, body weight; SL, standard length; TL, total length; CF, condition factor; VSI, viscerosomatic index; FR, feeding rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

Data are expressed as means ± SEM ($n = 15$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

4.3 Skin coloration

Improved skin coloration (L^* , a^* , h^* , and C^*) was most apparent in fish reared in the 0.125 g L⁻¹ treatment (Table 2, $P < 0.05$). Only partial improvements in color coordinates were observed in the other three treatments, where fish reared in *T. catappa* leaf extract at 0.25 and 0.5 g L⁻¹ showed improvements in the L^* parameter, and fish reared in the extract at 1 g L⁻¹ showed improvements in L^* and a^* parameters.

4.4 Bubble-nest behavior during experimental duration

The characteristics of bubble-nest formation are shown in Fig. 2a. The frequency of bubble-nest formation and bubble-nest size were influenced by the concentration of *T. catappa* leaf extract (Fig. 2b and c, $P < 0.05$). Relative to the control, the frequency of bubble-nest formation was significantly higher in all experimental treatments (Fig. 2b). The areas of bubble-nest were similar between fish reared in 0.125 and 0.25 g L⁻¹ treatments and then increased progressively at concentrations of 0.5 and 1 g L⁻¹ (Fig. 2c).

4.5 Digestive enzyme activities

The concentration of the leaf extract had no effect on pepsin specific activity (Fig. 3a, $P > 0.05$). Significantly increased trypsin specific activity relative to control was only observed in fish reared in the lowest concentration (Fig. 3b, $P < 0.05$). The highest chymotrypsin-specific activity was observed in fish reared in the 0.125 and 1 g L⁻¹ treatments (Fig. 3c). Increased lipase specific activity was found only in fish reared in the 0.25 g L⁻¹ treatment (Fig. 3d). Amylase specific activity was significantly reduced compared to control in all treatments except 0.125 g L⁻¹ (Fig. 3e). The A/T ratio remained unchanged compared to the control only in fish reared in the 0.25 g L⁻¹ treatment. Significant reductions were found in the other three treatments (Fig. 3f).

Table 2 Color parameters of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment.

Color parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
<i>L</i> *	4.00 ± 0.54 ^b	7.45 ± 0.75 ^a	9.13 ± 0.93 ^a	9.79 ± 1.31 ^a	10.6 ± 1.4 ^a	< 0.001
<i>a</i> *	18.4 ± 1.8 ^b	23.8 ± 0.8 ^a	21.5 ± 0.7 ^{ab}	20.7 ± 1.1 ^{ab}	22.0 ± 1.0 ^a	0.025
<i>h</i> *	19.2 ± 1.1 ^{bc}	25.0 ± 1.7 ^a	23.6 ± 1.7 ^{ab}	19.8 ± 2.1 ^{bc}	18.3 ± 1.8 ^c	0.021
<i>C</i> *	19.6 ± 1.9 ^b	25.8 ± 1.0 ^a	23.3 ± 0.7 ^{ab}	22.3 ± 1.3 ^{ab}	23.4 ± 1.2 ^{ab}	0.021

Data are expressed as means ± SEM (*n* = 15 per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference (*P* < 0.05).

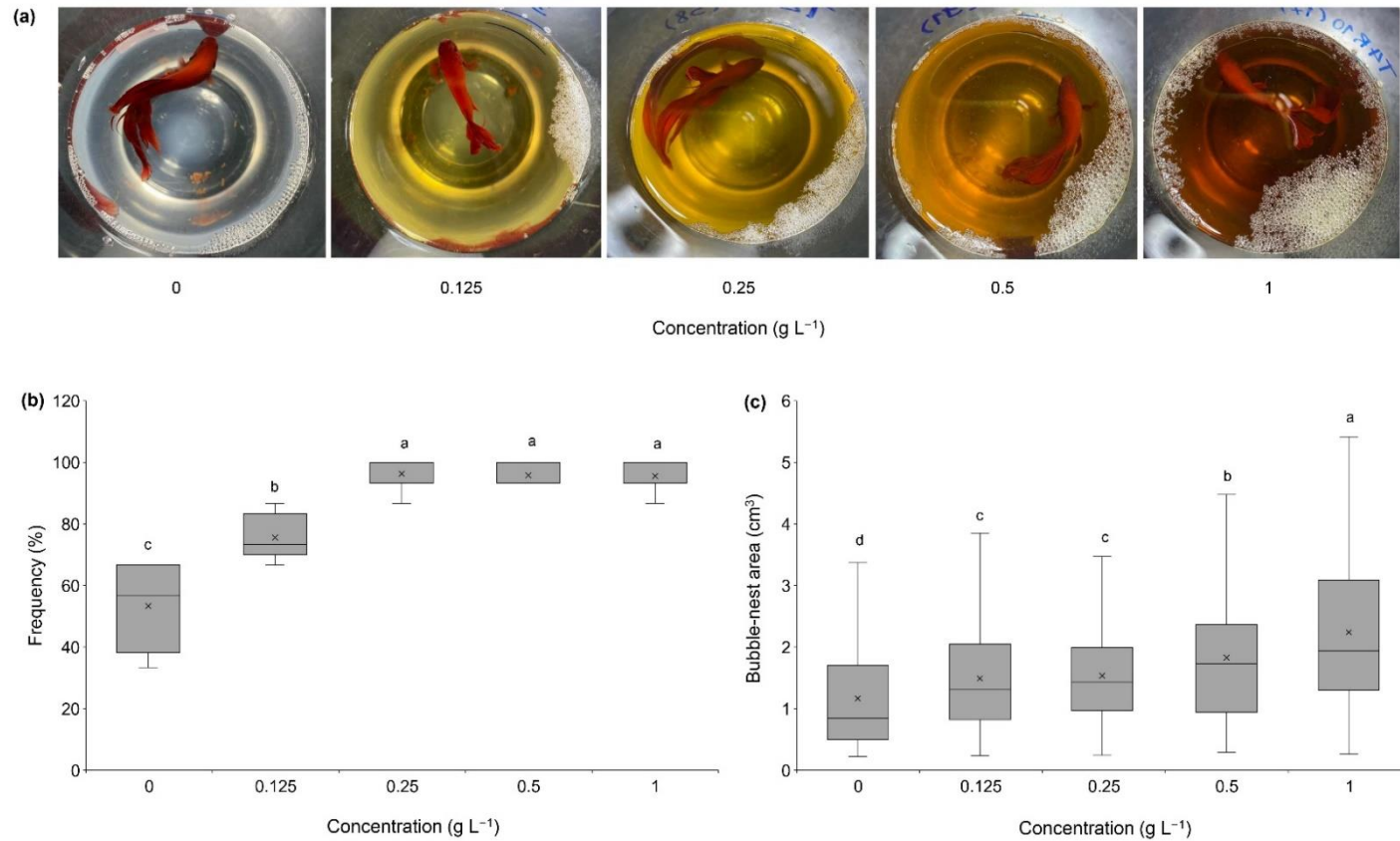


Fig. 2. Characteristics (a) , frequency (b), and area (c) of bubble-nest formation by male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The data on frequency and area were recorded from all fish three times weekly from week 6 to 8. Differences between means were tested with Duncan's multiple range test. Different superscripts indicate a significant difference ($P < 0.05$) between treatments.

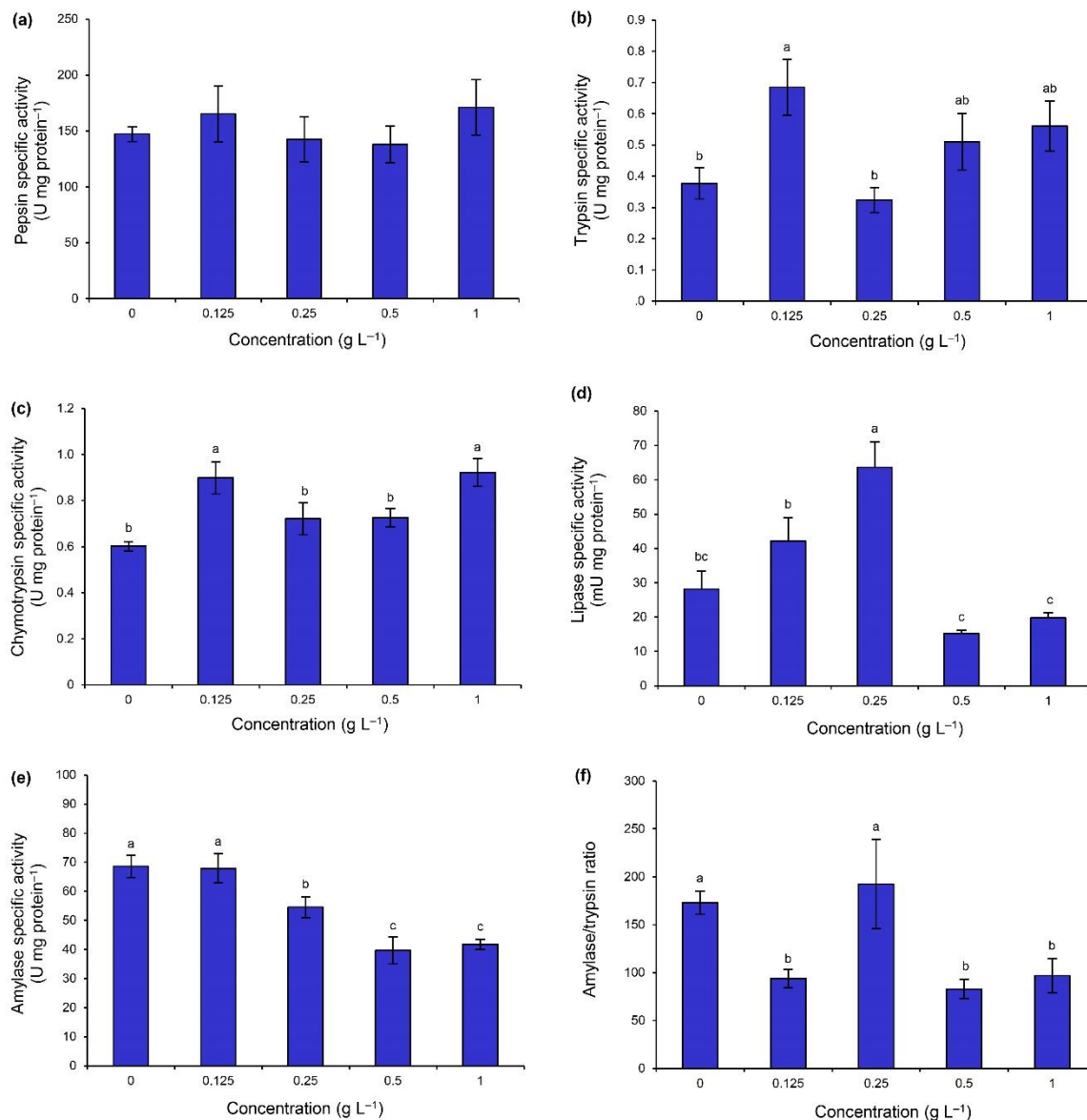


Fig. 3. Specific activities of digestive enzymes were analyzed in male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract: pepsin (a), trypsin (b), chymotrypsin (c), lipase (d), amylase (e), and amylase to trypsin ratio (f). The observed parameters were recorded at the end of the 8-week experiment. Data are expressed as tank means $\pm$ SEM ($n = 5$ per treatment). Differences between means were tested with Duncan's multiple range test. Different superscripts indicate a significant difference ($P < 0.05$) between treatments.

4.6 Protein synthesis capacity, and myosin and actin muscle contents

RNA, protein, and the RNA/protein ratio did not differ between treatments (Table 3, $P > 0.05$). The main muscle proteins, myosin and actin, were found within onset to conclusion temperature ranges of 28.5 to 56.9 °C and 65.4 to 76.4 °C, respectively. Sarcoplasmic protein was found within a range of 79.3 to 91.3 °C (data not shown). According to the thermal responses, no significant differences were present in myosin, actin, and sarcoplasmic protein contents, or their summations or ratios, between the control and all four treatments (Table 3, $P > 0.05$).

4.7 Whole-body composition

No significant differences were found in moisture, crude protein, crude lipid, and ash contents between the control and all four treatments (Table 4, $P > 0.05$). On a wet weight basis, the respective average values were 70.4, 16.9, 5.84, and 4.07%.

4.8 Hematological parameters

RBC, Hct, Hb, MCV, MCH, and MCHC were similar across all groups (Table 5, $P > 0.05$). WBC was significantly higher in fish reared in water containing the leaf extract at 0.25 and 0.5 g L⁻¹, and plasma protein was statistically higher compared to control in fish reared in the treatment containing leaf extract at 0.5 g L⁻¹ (Table 5, $P < 0.05$).

Table 3 The muscle quality of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment.

Parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
Protein synthesis capacity						
RNA (μg g ⁻¹)	6,200 ± 619	7,676 ± 435	7,818 ± 623	7,405 ± 794	6,619 ± 304	0.243
Protein (mg g ⁻¹)	314 ± 23	267 ± 7	292 ± 15	246 ± 15	292 ± 40	0.294
RNA/protein ratio (μg mg ⁻¹)	20.4 ± 3.0	28.8 ± 1.7	27.1 ± 2.6	30.2 ± 2.8	24.7 ± 2.9	0.101
Muscle protein						
ΔH Myosin (J g ⁻¹)	1.11 ± 0.09	1.08 ± 0.16	1.29 ± 0.13	1.20 ± 0.06	1.26 ± 0.05	0.553
ΔH Actin (J g ⁻¹)	0.38 ± 0.11	0.32 ± 0.03	0.35 ± 0.08	0.39 ± 0.07	0.42 ± 0.02	0.866
ΔH Sarcoplasmic protein (J g ⁻¹)	0.11 ± 0.03	0.15 ± 0.03	0.12 ± 0.02	0.22 ± 0.09	0.12 ± 0.02	0.492
ΣΔH (J g ⁻¹)	1.60 ± 0.21	1.54 ± 0.15	1.77 ± 0.21	1.81 ± 0.13	1.96 ± 0.16	0.483
ΣΔH Myosin and actin (J g ⁻¹)	1.49 ± 0.19	1.40 ± 0.15	1.65 ± 0.20	1.59 ± 0.05	1.68 ± 0.07	0.630
ΔH Actin/myosin	0.33 ± 0.08	0.33 ± 0.08	0.27 ± 0.06	0.34 ± 0.08	0.34 ± 0.00	0.943

Data are expressed as means ± SEM ($n = 5$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

Table 4 Whole-body compositions (% of wet weight) of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment.

Composition	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
Moisture	70.9 ± 0.1	69.6 ± 0.5	70.0 ± 0.2	70.5 ± 0.6	70.8 ± 0.9	0.441
Crude protein	16.5 ± 0.1	16.7 ± 0.3	17.3 ± 0.3	17.1 ± 0.3	16.9 ± 0.5	0.525
Crude lipid	5.75 ± 0.31	6.54 ± 0.24	5.71 ± 0.24	5.45 ± 0.06	5.73 ± 0.30	0.086
Ash	4.01 ± 0.08	4.12 ± 0.10	4.13 ± 0.03	4.15 ± 0.19	3.94 ± 0.06	0.804

Data are expressed as means ± SEM ($n = 3$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

Table 5 Hematological parameters of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment.

Hematological parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
RBC (×10 ⁹ cells mL ⁻¹)	2.66 ± 0.09	3.09 ± 0.05	2.78 ± 0.16	2.85 ± 0.10	2.99 ± 0.10	0.145
WBC (×10 ⁷ cells mL ⁻¹)	3.65 ± 0.43 ^b	5.43 ± 0.28 ^{ab}	7.27 ± 1.04 ^a	7.29 ± 0.84 ^a	5.82 ± 0.48 ^{ab}	0.047
Hct (%)	44.3 ± 3.0	31.9 ± 6.7	39.4 ± 3.0	46.0 ± 6.1	40.2 ± 3.0	0.355
Hb (g dL ⁻¹)	11.8 ± 1.7	13.5 ± 0.9	12.3 ± 0.8	12.1 ± 0.5	15.0 ± 0.9	0.210
Plasma protein (g L ⁻¹)	56.2 ± 2.2 ^b	54.3 ± 2.2 ^b	60.2 ± 2.2 ^b	88.0 ± 2.2 ^a	60.5 ± 1.7 ^b	0.021
MCV (fL)	167 ± 12	104 ± 22	143 ± 10	162 ± 20	109 ± 26	0.122
MCH (pg)	44.9 ± 7.6	43.5 ± 2.3	44.8 ± 2.8	48.9 ± 6.0	56.3 ± 6.3	0.434
MCHC (g dL ⁻¹)	26.8 ± 4.1	48.3 ± 10.0	31.6 ± 1.9	31.4 ± 3.4	37.5 ± 2.1	0.095

RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Data are expressed as means ± SEM ($n = 5$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

5. Discussion

5.1 Water quality

A number of water qualities were significantly improved by the various concentrations of *T. catappa* leaf extract. The low water pH produced by all concentrations of *T. catappa* leaf extract represents a significant improvement in water quality since Siamese fighting fish prefer a slightly acidic environment (Nugroho et al., 2016). In studies of Amazon leaf fish larvae, Abe et al. (2019) and Ramos et al. (2020) reported that aqueous *T. catappa* leaf extract at 0.75 g L⁻¹ improved pH and total ammonia levels of rearing water and subsequent survival and growth performances. In a study of cardinal tetra (Nurhidayat et al., 2016), similar results were reported for water treatment with a dose of *T. catappa* leaf at 0.5 g L⁻¹. In general, *T. catappa* leaves contain a lot of tannins, so water pH should gradually decline as the concentration of the extract increases (Ramos et al., 2020).

We found that DO levels remained within an acceptable range of 3 to 5 mg L⁻¹ (Bhatnagar and Devi, 2013) but improved in some treatments. Possibly, tannins reduce the breakdown of organic matter by precipitating proteins and inhibit the secretion of bacterial digestive enzymes (Smith et al., 2005). Water conductivity was also within the desirable range, at 0.1 to 2 mS cm⁻¹ for fish ponds (Stone and Thomforde, 2004), and at 0.5 to 3 mS cm⁻¹ for natural resources (Hem, 1985).

The increased water turbidity we observed conflicts with the report by Nozaleda (2019). The results of our study are possibly due to the use of whole *T. catappa* leaves. *T. catappa* leaves contain pectic substances which can interact with water, so it is possibly more appropriate to use only the leaf blade, without the midrib. Although some significant changes in alkalinity and hardness were observed in the 1 and 0.125 g L⁻¹ treatments, these water qualities would not be toxic to fish and are similar to or lower than those recorded in previous reports (Chansue and Assawawongkasem, 2008; Purivirojkul, 2012; Saekhow et al., 2018).

In the present study, significantly decreased N-wastes were observed in all treatments. Reduced N-wastes were facilitated by hydrogen bridges between OH functional groups of tannins and N-waste (Siebert et al., 1996), which help precipitate the products. Similar reductions in N-wastes due to *T. catappa* leaf extract water additives have been found (Pimolrat et al., 2018; Puttamat and Suwannasarn, 2007).

5.2 Growth, weight-length morphometrics, and feed utilization

Several species have shown significantly improved growth parameters that were dependent on the concentration of *T. catappa* leaf extract used in the treatment. Growth parameters of post-larvae of the black tiger shrimp (*Penaeus monodon*) improved in the presence of *T. catappa* leaf extract at 1 to 4 g L⁻¹ (Ikhwanuddin et al., 2014); growth parameters of juvenile cardinal tetra improved at concentrations of 0.5 and 1.5 g L⁻¹ (Nurhidayat et al., 2016); and growth parameters of Amazon leaf fish larvae improved at a concentration of 0.75 g L⁻¹ (Ramos et al., 2020). In the present study, water treatment with *T. catappa* leaf extract did not significantly affect weight-related parameters (final body weight and SGR) of the Siamese fighting fish at any concentration. Length-related parameters (SL, TL, and CF), however, were significantly influenced by the extract, except in fish reared in a concentration of 0.25 g L⁻¹. Similar changes in weight- and length-related growth parameters were observed in neon tetra fish (*Paracheirodon innesi*) reared in *T. catappa* leaf extract at 0.75 g L⁻¹ (Ardi et al., 2020). It is possible, as reported by Nurhidayat et al. (2016), that the leaf extract might disturb bone development, and if so, the application of *T. catappa* leaf extract during the growing phase of Siamese fighting fish may well be unsuitable. However, the amount of ash in the whole-fish body at the end of the present trial showed insignificant differences across all groups. Therefore, the effect of *T. catappa* leaf extract on bone development and mineral availability requires further investigation.

Regarding feed utilization, treatment with *T. catappa* leaf extract at 0.5 and 1.0 g L⁻¹ produced an undesirable FR while FCR and PER were still acceptable. Since these fish take a long time to consume their feed, these results show that growth performances in the present study were in line with the known behavior of the fish. Theoretically, tannins in *T. catappa* leaf bind to proteins and this protein-binding reduces the digestion of food by inhibiting the activities of digestive enzymes, such as proteases, amylase, and lipase (Maitra and Ray, 2003). Since Siamese fighting fish are carnivorous, they require large amounts of protein in their diet but, although a tannin-rich diet can reduce appetite and digestive efficiency (Maitra and Ray, 2003), the effects of *T. catappa* leaf extract water additive on the secretion of fish digestive enzymes have never been reported. In the present study, the absence of negative effects on the specific activities of protein-digesting enzymes (pepsin, trypsin, and chymotrypsin) indicates normal gastrointestinal functionality in protein digestion.

Although some treatments produced positive results, the adverse effects on growth performance and feed utilization negated them. Fish reared in water containing 0.25 g L⁻¹ of leaf extract exhibited the highest lipase specific activity. This change indicates improved digestion of dietary lipids, and would support the use of *T. catappa* leaf extract in fighting fish farms since live diets comprise high amounts of lipid (Thongprajukaew et al., 2014, 2019). In the 0.25 g L⁻¹ treatment, the A/T ratio was maintained while amylase specific activity decreased. These results indicate normal use of available carbohydrate per unit of protein (Thongprajukaew et al., 2011). Since no negative effects on growth and feed utilization were found in this treatment, it is possible that some flexibility might exist between lipid- and carbohydrate-digesting enzyme activities that contributed a carbohydrate-sparing effect in the studied fish. In other words, the increased digestion of fat reduced the need to digest carbohydrate.

5.3 Bubble-nest formation

T. catappa contains isoflavones, a functional group of phytoestrogens that are structurally similar to animal estrogens (Vadwala and Kola, 2017). Exposing male *B. splendens* to isoflavones (genistein and equol) in a previous study was shown to promote bubble-nest building (Clotfelter and Rodriguez, 2006). In the present study, the increased frequency and area of bubble-nest formation were closely associated with the concentration of leaf extract in the treatment. Increased gonadosomatic indices have been reported after the dietary incorporation of isoflavones (Pastore et al., 2018; Rzepkowska et al., 2020), and a hydroalcoholic extract containing phytoestrogenic compounds (Enayat Gholampour et al., 2020). On the other hand, phytoestrogens can inhibit steroidogenesis, which could interfere in the transition from courtship to parental behavior in fish (Desmawati and Sulastri, 2019; Sisneros et al., 2004), and significant changes in hormones and gonads in male fish are known to reduce aggression (Ofir et al., 2003; Perreault et al., 2003; Stevenson et al., 2011), a chief attraction of *B. splendens* to keepers. The conflicting results reported above were dependent on the concentration of leaf extract and fish species, and we concluded that only the optimal concentration used in the present study is suitable for male Siamese fighting fish.

5.4 Skin coloration, body homeostasis, and general health status

Although improved skin color coordinates were observed in all experimental treatments, the negative effects of the treatments on body length have to be taken into account. Body length was impacted the least in fish reared in water containing 0.25 g L⁻¹ of leaf extract and only this treatment represents a suitable choice. However, information on the mechanisms involved in improving color parameters is limited. It is possible that some active ingredients from *T. catappa*, such as flavonoids, serve as potent tyrosinase inhibitors that inhibit melanogenesis without provoking melanocytotoxicity (Zhu and Gao, 2008). Some elicitors from active ingredients may trigger coloration and since water soluble extracts from *T. catappa* leaf can be used as natural dyes in the

presence of mordants (Chitnis, 2013), direct immersion of fish in the extract might contribute some skin pigmentation.

The absence of significant changes in RNA, protein, and the RNA/protein ratio among all treatments in the present study indicates that normal muscle protein growth occurred even in the presence of the higher concentrations of *T. catappa* leaf extract. Also, no significant changes were observed in the amount of major muscle proteins (mainly myosin and actin) and their proportional ratios, and whole-body compositions. These results indicate that no negative effect on the health status of Siamese fighting fish resulted from the use of *T. catappa* leaf extract during this study.

The health status of aquatic animals can also be monitored through hematological parameters. In our study, fish reared in the 0.25 g L⁻¹ treatment showed significantly increased WBC and those reared in the 0.5 g L⁻¹ treatment showed significantly increased WBC and plasma protein levels. Improved results for WBC have been reported for fighting fish treated with *T. catappa* extract at concentrations above 0.375 g L⁻¹ (Nugroho et al., 2016); and treatment of the same species with *T. catappa* extract at 0.5 g L⁻¹ increased the percentage of monocytes and granulocytes against *Aeromonas hydrophila* (Nugroho et al., 2017). Exposure to 0.5 g L⁻¹ *T. catappa* leaf extract was associated with increased plasma protein concentrations in Tambaqui, *Colossoma macropomum* (Meneses et al., 2020).

Some of the positive effects observed in the current study might be attributed to active ingredients such as tannins, flavonoids, and saponins in *T. catappa* leaves (Nurhidayat et al., 2016). In a previous study of fish (Pandey, 2013), these components increased both the non-specific defense and the specific immune response. Elsewhere, flavonoids were shown to modulate the production of cytokines such as interferon and interleukin, and also enhance T-cell activities (Helal and Melzig, 2011; Lyu and Park, 2005; Nugroho et al., 2016; Xiong et al., 2012). Flavonoids also have antioxidant, antiparasitic, antifungal, and antibacterial activities that prevent and cure disease, and are applied in aquaculture as an alternative to antibiotics (Chansue and Assawawongkasem, 2008; Chitmanat et al., 2005; Purivirojkul, 2012).

6. Concluding remarks

Terminalia catappa leaf and its products are widely used by fighting fish culturists but reports of some negative effects on reared fish have been published. Therefore, we set out to clarify the overall effects of *T. catappa* leaf extract on fish performance. Based on observations of water quality, fish growth, feed utilization, skin coloration, bubble-nest creation, digesting enzyme activities, muscle protein, whole-body composition, and hematological parameters, the findings of this work indicated that an aquarium additive of *T. catappa* leaf extract at a concentration of 0.25 g L^{-1} of water is suitable for rearing male Siamese fighting fish. On the other hand, observations of skeletal growth based on body length and condition factor indicated undesirable effects on fish reared in less appropriate concentrations. Practically, this environmentally friendly leaf extract appears to be most suitable if used occasionally for specific purposes such as improving water quality, prophylactic treatment, or courtship issues. Despite not being appropriate for the whole duration of fish rearing, the correct use of *T. catappa* leaf should yield not only the desirable results mentioned, but also reductions in the use of antibiotics or chemicals that cause side effects and residue problems in the commercial production of aquatic animals. However, the active ingredients, mainly tannins, found in *T. catappa* leaf may be affected by seasonal variation, geographical location, soil fertility, leaf age, extraction solvent, and extraction protocol. Differences in these parameters can lead to differences in observed outcomes in fish.

CHAPTER 2

Fermented water of Thai herbs effectively enhanced amputated fin regeneration in Siamese fighting fish (*Betta splendens*)

1. Introduction

The Siamese fighting fish (*Betta splendens*) is a globally important ornamental fish prized for its vibrant colors and the pugnacious nature and beauty of the male fish (Monvises et al., 2009; Rahmati-Holasoo et al., 2019, 2020). From 2010 to 2019, the species was one of the two most commercially profitable ornamental fish in Thailand. The United States is the largest market and the biggest importer of Siamese fighting fish from Thailand, followed by China, France, Iran, and Singapore (Sermwatanakul, 2019). The declaration of this fish as a national aquatic animal was not only due to the economic importance of the species, but also to its place in the cultural heritage of Thailand (Sermwatanakul, 2019).

Behaviorally, *B. splendens* are bubble-nest builders in which males are responsible for raising eggs and larvae until they can support themselves (Monvises et al., 2009). The courtship and reproductive behaviors (male-female) include combative behavior (male-male or female-female) when fish encounter each other. Threatening poses are struck towards opponents. Body color darkens, gill covers flare, fins are erected, and tails beat. These displays can lead to chasing, biting, butting, and locking jaws. These actions result in damaged bodies and torn fins, especially pelvic and pectoral fins, and sometimes dorsal, anal, or caudal fins (Braddock and Braddock, 1955; Rainwater, 1967; Ramos and Gonçalves, 2019; Simpson, 1968). Therefore, male fish are reared individually in small containers until they reach 1.5 months of age (Thongprajukaew et al., 2010a, 2010b).

In general, fish can completely regenerate injured or lost fins naturally. Previous studies have investigated fin regeneration amputating fins, especially the caudal fin (Pfefferli and Jaźwińska, 2015). Different caudal fin excision sites resulted in divergent reconstruction times/rates. Proximal amputations took longer time than distal amputations to be regenerated and for the fin to regain its original size (length) (Uemoto et al., 2020). Previous studies have reported species-related differences in complete regeneration time: fourteen days for zebrafish, *Danio rerio* (Geraudie et al., 1995; Poleo et al., 2001); twenty-four days for mosquito fish, *Gambusia affinis* (Johnson and Denton, 1977) and guppy, *Poecilia reticulata* (Kolluru et al., 2006); and twenty-eight days for Nile tilapia, *Oreochromis niloticus* (Ngamsiri, 2015). However, the capacity to regenerate fin length has been significantly improved by rearing fish in water containing herbs (Chansue, 2006; Ngamsiri and Laumsomboon, 2021).

B. splendens culturists have always soaked dried Indian-almond (*Terminalia catappa*) leaves in rearing water (Malawa et al., 2022). The properties of this plant for wound healing and eliminating harmful monogenean and *Trichodina* spp. have been demonstrated (Chitmanat et al., 2005; Ponpornpisit et al., 2007). Apart from dried Indian-almond leaves, other herbs commonly used by *B. splendens* culturists in Thailand are dried banana leaves (*Musa sapientum*), papaya leaves (*Carica papaya*), and lemongrass (*Cymbopogon citratus*) leaves. These herbs contain active ingredients with various biological activities that especially help heal wounds caused by fighting (Maria and Veronica, 2021; Tanmoy et al., 2021; Wijaya et al., 2020). As well as these herbs, some *B. splendens* culturists also add clay and salt to rearing water. The first helps the fish recover faster (Chiarachavildham, 2016) while the second helps heal wounds, nourishes the skin, and reduces pathogen growth (Puello-Cruz et al., 2010; Putiyanan, 1999).

Although indigenous knowledge of the above ingredients is widely applied by bettas fish farmers in Thailand, there is still a lack of research to support local knowledge for further commercialization. The results of this study will help develop simple herbal recipes for commercial application in betta fish farming.

2. Objective

To investigate the ability of Thai herbal recipes to improve the healing of wounds of Siamese fighting fish by amputating caudal fin to mimic the damage caused during mating or fighting contests.

3. Materials and methods

3.1 Animal protocols

The transportation, acclimatization, fin amputation, rearing, sampling, and euthanasia of the fish conformed to the “Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes,” National Research Council, Thailand (Application No. U1–06514-2560). This research project was conducted as part of a larger project entitled “Innovations from indigenous knowledge for using Indian-almond leaf (*Terminalia catappa*) and herbal recipes for rearing and wound healing in Siamese fighting fish (*Betta splendens*)”, which was approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Project Code 2564–01-030).

3.2 Preparation of ingredients and fermented water from Thai herbal recipes

The principal herbs of interest included dried Indian-almond (*T. catappa*) leaves (IAL), dried banana (*M. sapientum*) leaves (BL), and dried papaya (*C. papaya*) leaves (PL). While dried lemongrass (*C. citratus*) leaves (LL), dried clay, and salt were secondary ingredients, they were included in every formulation except one of two controls. The leaves were harvested from a few farms within Songkhla Province in southern Thailand. Each leaf type was cleaned, separately soaked in 5 mg L⁻¹ of KMnO₄ for 15 min, and washed three times with distilled water to remove any attached microorganisms and impurities. The cleaned leaves were dried at 60 °C for 12 h, crushed using a grinder (WF-04; Siam Wealth Food Co., Ltd., Nonthaburi, Thailand), sieved, homogenously mixed, packed in polyethylene bags, placed inside black plastic bags to exclude light, and stored at 4 °C until used. The formulations of the Thai herbal recipes are summarized in Table 6. Raw tap water was dechlorinated using sodium thiosulfate

coupled with aeration for three days. Fermentation was performed by soaking ingredients in dechlorinated water in 30 L plastic containers (31 cm width $\times$ 30 cm length $\times$ 49.7 cm height) at room temperature for three days, stirring twice daily. At the end of three days, the mixtures were passed through filter cloth and Whatman No. 4 paper, as described by Malawa et al. (2022).

3.3 Fish acclimatization and fin amputation

Three-month-old, solid-red, male Siamese fighting fish were purchased from a local farm in Nakhon Pathom Province, Thailand. During a two-week acclimatization period, specimens were separately reared in cylindrical plastic containers (9.3 cm diameter $\times$ 12.5 cm height $\times$ 8 cm depth), containing 350 mL of water. At the end of the acclimatization period, each fish was immediately weighed to obtain initial body weight (1.29 ± 0.01 g, $n = 135$). After weighing, the fish were randomly divided into nine treatments of fifteen fish. The acclimatized fish were anesthetized with a 15 mg L^{-1} solution of clove oil and caudal fin lengths were measured with a Vernier caliper. The fin amputation procedure was conducted as described by Kolluru et al. (2006). Half of the caudal fin length was amputated in the vertical plane using a sterilized scalpel (see Fig. 4).

3.4 Fish treatments

After amputation, all the fish were carefully transferred to 350-mL cylindrical plastic containers (9.3 cm diameter $\times$ 12.5 cm height $\times$ 8 cm depth) containing seven different treatments ($n = 15$ per treatment) and reared for 35 days under a 12-h light (06.00 to 18.00 h)/12-h dark cycle. Two controls ($n = 15$ per treatment) were designed: non-fermented water (CT 1), and fermented water containing LL, clay, and salt (CT 2). All of fifteen fish, each kept apart from all others in an individual container. Each fish, therefore, served as an experimental unit. Three treatments were obtained by adding IAL (treatment IA), BL (treatment B), or PL (treatment P) to CT 2. Three more treatments were obtained by adding IAL and BL (treatment IAB), IAL and PL (treatment IP), and BL

and PL (treatment BP) to CT 2. The seventh treatment was obtained by adding IAL, BL and PL (treatment IBP) to CT 2. The formulations of these treatments are summarized in Table 6.

All fish were fed *ad libitum* twice daily at 08.00 and 17.00 h with a commercial floating feed for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). The proximate chemical composition of the feed was analyzed according to AOAC (2005), and the results on a dry matter basis were: $39.4 \pm 0.2\%$ crude protein, $3.23 \pm 0.12\%$ crude lipid, $1.97 \pm 0.18\%$ crude fiber, and $7.72 \pm 0.24\%$ crude ash. Any feed uneaten after 30 min was recovered and dried at $60\text{ }^{\circ}\text{C}$ until a constant weight was achieved. Obtained data were used to calculate the feeding rate, and the amounts of feed given were adjusted weekly according to the rate of consumption. Static system without aeration was conducted over five-week experiment. Every three days, 80% of the rearing water was replaced with water containing fresh fermented water. During the experimental period, the quality of rearing water was monitored twice weekly. The parameters of interest were water temperature ($23.9 \pm 0.1\text{ }^{\circ}\text{C}$), pH (6.51 ± 0.03), conductivity ($5.41 \pm 0.21\text{ mS cm}^{-1}$), total dissolved solids ($3.43 \pm 0.13\text{ g L}^{-1}$), dissolved oxygen ($5.71 \pm 0.06\text{ mg L}^{-1}$), salinity ($3.00 \pm 0.12\text{ g L}^{-1}$), total alkalinity ($129 \pm 5\text{ mg L}^{-1}$), hardness ($255 \pm 9\text{ mg L}^{-1}$), total ammonia nitrogen ($1.28 \pm 0.03\text{ mg L}^{-1}$), nitrite ($0.09 \pm 0.01\text{ mg L}^{-1}$), and nitrate ($0.86 \pm 0.15\text{ mg L}^{-1}$).

Mortality and morbidity were monitored daily over the duration of the experiment. At the end of the trial, all the fish ($n = 135$) were starved for 24 h and then sacrificed by chilling in ice before body weight and total length were measured. Both values were then used to calculate specific growth rates and condition factors. The parameters describing survival, growth performance and feed consumption were calculated as follows:

$$\text{Survival (\%)} = 100 \times (\text{final fish number}/\text{initial fish number}),$$

$$\text{Specific growth rate (\% body weight day}^{-1}\text{)} = 100 \times [(\ln W_t - \ln W_o)/(t - t_o)]$$

$$\text{where } W_t = \text{mean weight (g) at day } t, W_o = \text{mean weight (g) at day } t_o,$$

$$\text{Condition factor} = 100 \times [\text{body weight (g)}/\text{standard length (cm)}^3], \text{ and}$$

$$\text{Feeding rate (\% body weight day}^{-1}\text{)} = 100 \times C / [(W_o + W_t)/2] / t$$

where C = daily feed consumption (g), W_o = initial body weight (g), W_t = final body weight (g), t = feeding duration (day).

3.5 Fin regeneration monitoring

Caudal fins ($n = 15$ per treatment) were photographed every seven days (Nikon D60, Nikon Inc., Thailand). An off-white light-emitting diode (LED) was used as a light source inside a closed box (20 cm width $\times$ 20 cm length $\times$ 26 cm height) with a black background. Each fish in its cylindrical container was placed in the box, and a plastic millimeter grid was placed behind the fish as a reference for measurements. All images were produced with the flash off and automatic white balance turned on. The recorded images were processed by Image J software to calculate caudal fin length. Regeneration (% in length) was calculated from $100 \times [(\text{length on day N (cm)} - \text{length on day 0 (cm)}) / \text{fin length amputated (cm)}]$.

Based on fin regeneration ability, the suitable treatments (IA, IAB, BP, IP, and IBP) were chosen for evaluating red pigmentation development. The same set of images from five alternative treatments were processed with the Image J software, and automatically analyzed for red intensity values. The original images were decomposed and returned numerical values ranging from 0 to 255. All parts of regenerated fins, and the parts of fins that remained after amputation, were individually classified into three zones of red intensity: dark red zone (DRZ), light red zone (LRZ), and transparent zone (TZ). A total red zone (TRZ) and the ratio TRZ: TZ were also calculated. After measuring the length of each zone, the calculation was performed as described for percent regeneration in length. The monitoring of fin regeneration is illustrated in Fig. 5.

Table 6 The composition of traditional Thai herbal recipes used to treat male Siamese fighting fish after caudal fin amputation. The symbols × and ✓ indicate absence and presence of ingredients, respectively.

Treatment	Recipe ingredients					
	IAL (0.25 g L ⁻¹) ¹	BL (1.5 g L ⁻¹) ²	PL (2.5 g L ⁻¹) ³	LL (1 g L ⁻¹) ⁴	Clay (50 g L ⁻¹) ⁵	Salt (5 g L ⁻¹) ⁶
CT 1	×	×	×	×	×	×
CT 2	×	×	×	✓	✓	✓
IA	✓	×	×	✓	✓	✓
B	×	✓	×	✓	✓	✓
P	×	×	✓	✓	✓	✓
IAB	✓	✓	×	✓	✓	✓
BP	×	✓	✓	✓	✓	✓
IP	✓	×	✓	✓	✓	✓
IBP	✓	✓	✓	✓	✓	✓

IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

¹⁻⁶ The herbal recipe concentrations as described by Malawa et al. (2022)¹, Rucksapram (2007)², Banplakatrachong (2022)³, Institute for Innovative Learning (2022)⁴, Chiarachavildham (2016)⁵, and Puello-Cruz et al. (2010)⁶.

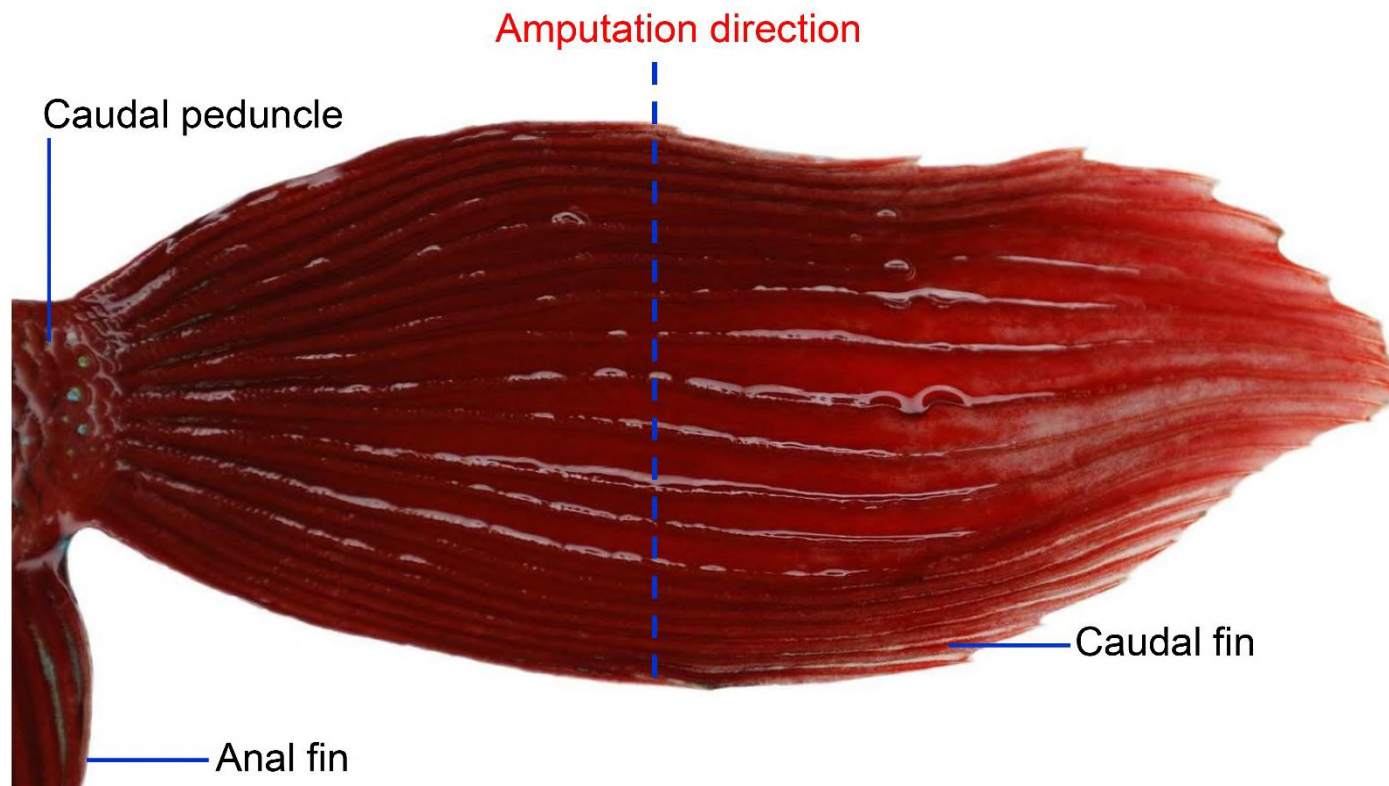


Fig. 4. The amputation of caudal fins for studying regeneration in male Siamese fighting fish.

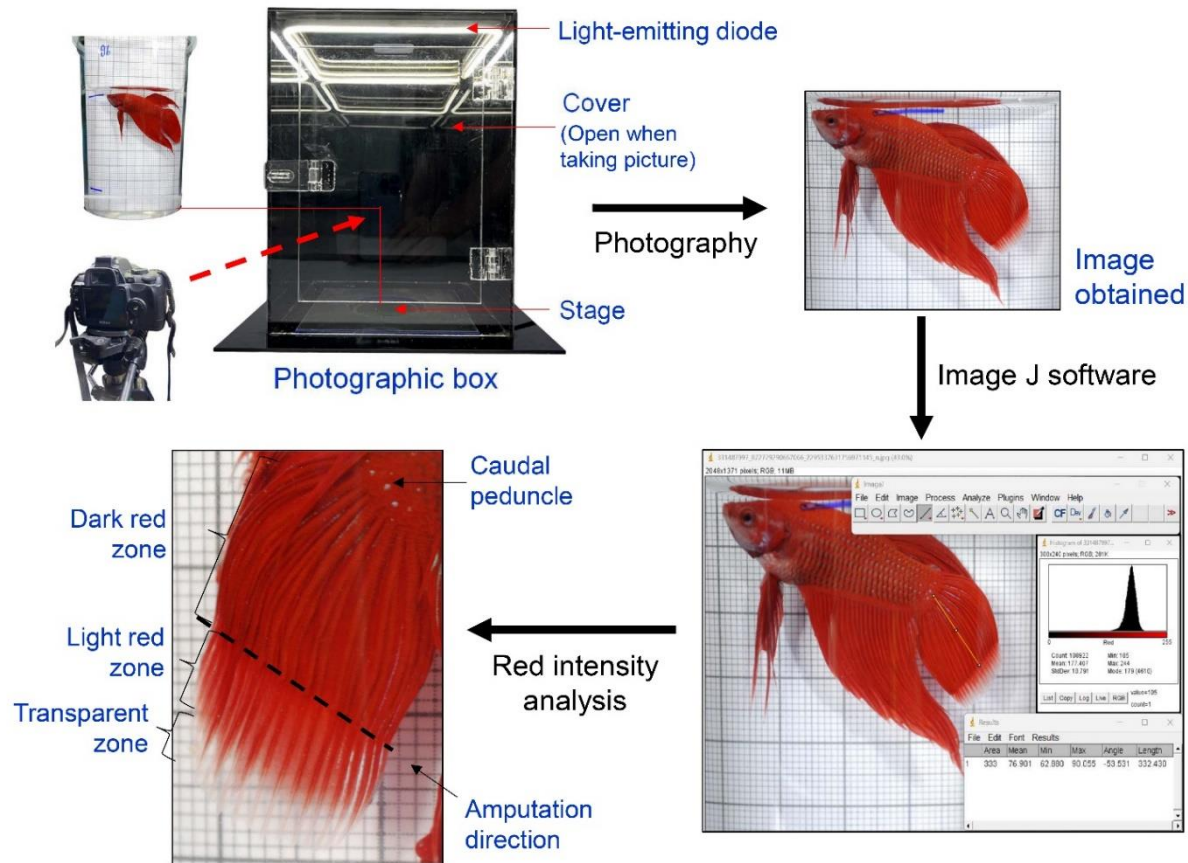


Fig. 5. Weekly monitoring of fin length and color development after caudal fin amputation. The fins were photographed in a closed box. The recorded images were transferred to Image J software to calculate regenerated fin lengths and regenerated color zones. The regenerated fins were divided for color development monitoring into dark red, light red, and transparent zones.

3.6 Statistical analysis

Sample size per treatment was calculated using Program R 3.6.0, requiring the power of test at 0.8. The present study was formulated as a complete randomized design with seven treatments and two controls. The obtained data were expressed as means $\pm$ standard errors of means (SEM). All statistical comparisons were made using the Statistical Package for Social Sciences Version 22 (SPSS Inc., Chicago, USA). Differences among means between treatment groups were subjected to One-Way analysis of variance using Duncan's multiple range test as a *post hoc* test. The hypothesized condition was rejected if the *P*-value was 0.05 or below.

4. Results

4.1 Growth and feed utilization characteristics

No mortality was observed over five weeks after caudal fin amputation ($P > 0.05$, Table 7). The herbal treatments had no effect on growth (specific growth rate), body morphometrics (condition factor), or feed utilization (feeding rate) ($P > 0.05$). The respective average values were 0.46 body weight day⁻¹, 1.23, and 1.82% body weight day⁻¹.

4.2 Fin length regeneration

Among the seven treatments and two controls, approximately half of the original caudal fin length was regenerated within the first week. On day 14, fin length had increased 1.55-fold. Thereafter, regeneration was slower (1.06 to 1.14-fold increases) until day 35. At the end of observation, the length of regenerated fins was statistically higher for fish reared in IA, IAB, BP, IP, and IBP treatments, relative to treatments CT 1 and B ($P < 0.05$, Table 8). However, intermediate results were observed for fish reared in treatments CT 2 and P ($P > 0.05$). Based on third-order polynomial regression equations, the fish in the IA treatment completely regenerated the caudal fin within 30.8 days. The coefficient variation (CV) of 9.78% was relatively low at the end of day 35 (Fig. 6a). Caudal fin regeneration times for fish in other treatments were as follows: IAB 32.3 days

(CV = 11.6%, Fig. 6b), BP 32.7 days (CV = 10.2%, Fig. 6c), IP 32.4 days (CV = 14.2%, Fig. 6d), and IBP 31.3 days (CV = 8.56%, Fig. 6e).

4.3 Color development in regenerated fins

Herbal recipes and rearing times had significant effects on the development of red pigmentation in the regenerated caudal fins ($P < 0.05$, Table 9). As the fins grew in length, color development was most pronounced in the TRZ followed by the LRZ. Color intensity in the TRZ ranged from 87.9 to 100% and the TRZ: TZ ratio ranged from 88:12 to 100:0. TRZ and TRZ: TZ developed faster in the fish reared in BP, IP, and IBP than in IA and IAB ($P < 0.05$). At the end of day 35, the development of red pigmentation was complete in those three treatments ($P < 0.05$).

Table 7 Survival, growth, and feed consumption of male Siamese fighting fish after caudal fin amputation. The measurements were performed at the end of a five-week trial.

Treatment	Parameter					
	Survival (%)	FBW (g)	SGR (% BW day ⁻¹)	FTL (cm)	CF	FR (% BW day ⁻¹)
CT 1	100	1.47 ± 0.05	0.48 ± 0.04	4.88 ± 0.08	1.27 ± 0.04	1.75 ± 0.04
CT 2	100	1.50 ± 0.04	0.43 ± 0.04	5.02 ± 0.04	1.19 ± 0.04	1.83 ± 0.03
IA	100	1.56 ± 0.02	0.53 ± 0.03	5.01 ± 0.05	1.25 ± 0.04	1.81 ± 0.02
B	100	1.52 ± 0.02	0.50 ± 0.04	5.00 ± 0.04	1.22 ± 0.03	1.84 ± 0.02
P	100	1.50 ± 0.03	0.43 ± 0.02	4.78 ± 0.17	1.25 ± 0.02	1.82 ± 0.03
IAB	100	1.53 ± 0.02	0.46 ± 0.03	4.97 ± 0.05	1.26 ± 0.03	1.84 ± 0.03
BP	100	1.42 ± 0.04	0.42 ± 0.03	4.88 ± 0.05	1.23 ± 0.03	1.87 ± 0.02
IP	100	1.53 ± 0.06	0.43 ± 0.03	5.02 ± 0.06	1.23 ± 0.07	1.80 ± 0.04
IBP	100	1.47 ± 0.03	0.46 ± 0.04	4.98 ± 0.03	1.20 ± 0.04	1.84 ± 0.02
<i>P</i> -value	—	0.229	0.250	0.290	0.882	0.205

FBW, final body weight; SGR, specific growth rate; BW, body weight; FTL, final total length; CF, condition factor; FR, feeding rate; IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Data are expressed as means ± SEM (*n* = 15 per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference (*P* < 0.05).

Table 8 Percent regeneration (in length) of caudal fin after amputation, followed by treatment in fermented water from Thai herbal recipes. The data were recorded on days 7, 14, 21, 28, and 35.

Treatment	Percent regeneration				
	Day 7	Day 14	Day 21	Day 28	Day 35
CT 1	42.4 ± 1.6 ^d	72.3 ± 2.5 ^d	75.9 ± 2.1 ^c	90.0 ± 3.5 ^{bc}	95.7 ± 4.0 ^b
CT 2	43.5 ± 1.4 ^d	74.6 ± 1.9 ^{bcd}	78.2 ± 2.0 ^c	90.1 ± 2.3 ^{bc}	99.6 ± 3.0 ^{ab}
IA	50.9 ± 1.4 ^{bc}	80.8 ± 2.3 ^{ab}	85.9 ± 2.1 ^a	98.3 ± 2.5 ^a	108 ± 3 ^a
B	46.4 ± 1.4 ^{cd}	73.5 ± 2.0 ^{cd}	78.4 ± 2.0 ^c	86.8 ± 2.5 ^c	96.2 ± 3.2 ^b
P	48.9 ± 1.4 ^c	75.4 ± 1.6 ^{bcd}	79.2 ± 1.8 ^{bc}	89.7 ± 1.8 ^{bc}	100 ± 3 ^{ab}
IAB	54.0 ± 1.4 ^b	83.3 ± 2.2 ^a	87.3 ± 2.1 ^a	97.2 ± 2.6 ^{ab}	108 ± 4 ^a
BP	50.6 ± 1.2 ^{bc}	79.8 ± 1.7 ^{abc}	84.6 ± 1.7 ^{ab}	94.3 ± 2.0 ^{abc}	108 ± 2 ^a
IP	50.8 ± 1.9 ^{bc}	76.1 ± 2.3 ^{bcd}	81.6 ± 2.3 ^{abc}	92.7 ± 2.5 ^{abc}	107 ± 3 ^a
IBP	58.6 ± 2.1 ^a	77.4 ± 2.0 ^{abcd}	85.0 ± 2.2 ^{ab}	97.5 ± 2.6 ^{ab}	107 ± 3 ^a
<i>P</i> -value	< 0.001	0.003	< 0.001	0.008	0.005

IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Data are expressed as means ± SEM ($n = 15$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

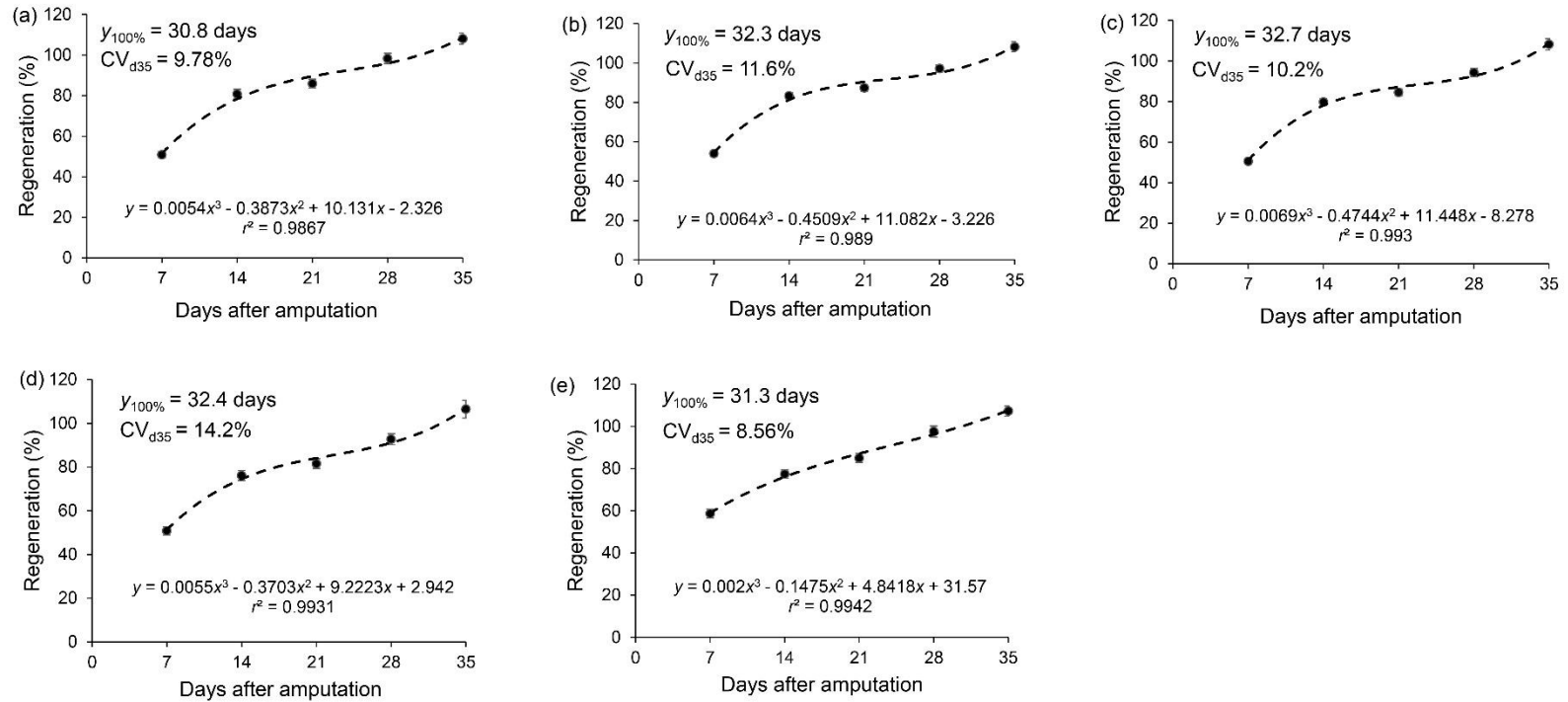


Fig. 6. Caudal fin regeneration in male Siamese fighting fish after amputation. The fish were reared in water fermented with herbs. The water contained dried lemongrass leaf, clay and salt. Added ingredients were dried Indian-almond leaf (a), dried Indian-almond and banana leaf (b), dried banana and papaya leaf (c), dried Indian-almond and papaya leaf (d), and dried Indian-almond, banana, and papaya leaf (e). Each data point was calculated from all individual fish ($n = 15$).

Table 9 Color development (in length) on regenerated caudal fin of male Siamese fighting fish. The values were recorded on day 7, 14, 21, 28, and 35 of the experiment.

Day	Treatment	DRZ (%)	LRZ (%)	TZ (%)	TRZ (%)	TRZ: TZ
7	IA	77.2 ± 0.8 ^a	15.1 ± 0.6 ^b	7.70 ± 0.41 ^a	92.3 ± 0.4 ^b	92:8
	IAB	74.7 ± 0.7 ^b	17.1 ± 0.4 ^b	8.22 ± 0.59 ^a	91.8 ± 0.6 ^b	92:8
	BP	77.8 ± 0.6 ^a	16.3 ± 0.7 ^b	5.91 ± 0.70 ^{ab}	94.1 ± 0.7 ^{ab}	94:6
	IP	78.0 ± 0.8 ^a	17.5 ± 1.1 ^b	4.58 ± 1.01 ^b	95.4 ± 1.0 ^a	95:5
	IBP	75.1 ± 0.7 ^b	20.8 ± 1.1 ^a	4.12 ± 1.21 ^b	95.9 ± 1.2 ^a	96:4
	<i>P</i> -value	0.002	< 0.001	0.002	0.002	
14	IA	66.2 ± 2.3 ^b	21.7 ± 2.5 ^{ab}	12.2 ± 1.0 ^a	87.9 ± 1.0 ^b	88:12
	IAB	72.5 ± 2.5 ^b	17.5 ± 2.9 ^b	10.0 ± 1.8 ^a	90.0 ± 1.8 ^b	90:10
	BP	71.4 ± 1.9 ^b	27.9 ± 1.8 ^a	0.73 ± 0.51 ^b	99.3 ± 0.5 ^a	99:1
	IP	68.3 ± 2.2 ^b	29.0 ± 2.2 ^a	2.70 ± 1.29 ^b	97.3 ± 1.3 ^a	97:3
	IBP	82.3 ± 3.8 ^a	14.3 ± 2.9 ^b	3.47 ± 1.41 ^b	96.5 ± 1.4 ^a	97:3
	<i>P</i> -value	0.001	< 0.001	< 0.001	< 0.001	
21	IA	70.8 ± 1.7 ^b	18.1 ± 2.1 ^{ab}	11.2 ± 1.3 ^a	88.8 ± 1.3 ^b	89:11
	IAB	76.1 ± 2.5 ^{ab}	13.8 ± 3.0 ^b	10.1 ± 2.1 ^a	89.9 ± 2.1 ^b	90:10
	BP	74.8 ± 3.1 ^b	24.1 ± 2.8 ^a	1.04 ± 0.61 ^b	99.0 ± 0.6 ^a	99:1
	IP	71.7 ± 2.9 ^b	25.3 ± 3.1 ^a	3.05 ± 1.40 ^b	97.0 ± 1.4 ^a	97:3
	IBP	84.0 ± 3.8 ^a	13.8 ± 3.3 ^b	2.24 ± 0.95 ^b	97.8 ± 1.0 ^a	98:2
	<i>P</i> -value	0.014	0.010	< 0.001	< 0.001	
28	IA	75.3 ± 2.4	14.5 ± 3.3	10.2 ± 1.9 ^a	89.8 ± 1.9 ^{bc}	90:10
	IAB	79.2 ± 3.3	10.2 ± 3.8	10.1 ± 2.5 ^a	89.9 ± 2.5 ^c	90:10
	BP	78.3 ± 4.7	20.4 ± 4.4	1.34 ± 1.12 ^b	98.7 ± 1.1 ^a	99:1
	IP	75.1 ± 4.3	21.5 ± 4.5	3.40 ± 1.71 ^b	96.6 ± 1.7 ^{abc}	97:3
	IBP	85.8 ± 4.9	13.2 ± 4.8	1.01 ± 0.70 ^b	99.0 ± 0.7 ^{ab}	99:1
	<i>P</i> -value	0.338	0.267	< 0.001	< 0.001	
35	IA	84.0 ± 2.4	8.3 ± 2.6	7.78 ± 1.76 ^a	92.2 ± 1.8 ^b	92:8
	IAB	90.2 ± 2.8	2.4 ± 1.5	7.41 ± 2.23 ^a	92.6 ± 2.2 ^b	93:7
	BP	87.5 ± 4.2	12.5 ± 4.2	nd	100 ± 0 ^a	100:0
	IP	83.2 ± 5.2	16.9 ± 5.2	nd	100 ± 0 ^a	100:0
	IBP	86.5 ± 5.3	13.5 ± 5.3	nd	100 ± 0 ^a	100:0
	<i>P</i> -value	0.753	0.117	< 0.001	< 0.001	

DRZ, dark red zone; LRZ, light red zone; TZ, transparent zone; TRZ, total red zone; IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL; nd, not detected.

Data are expressed as means ± SEM ($n = 15$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

5. Discussion

In this study, the amputated caudal fins of male Siamese fighting fish were treated over 35 days with water-based fermentations of various herbs. At the end of observation, no mortality was found, and growth performance (final body weight, specific growth rate, and final total length), body morphometrics (condition factor), and feed utilization (feeding rate) parameters did not differ across seven treatments and two controls ($P > 0.05$). Unchanged these parameters indicate normal growth and no negative effects due to herbal treatments. No effects of survival due to herbal treatments in amputated fish has also been reported (Ngamsiri and Laumsomboon, 2021), without existing records on growth biometrics (Geraudie et al., 1995; Johnson and Denton, 1977; Kolluru et al., 2006; Ngamsiri, 2015; Poleo et al., 2001). High growth obtained in this study was higher than those previously reported in male Siamese fighting fish (James and Sampath, 2006; Saekhow et al., 2018), suggesting that the amputated fish recovered well under the treatment conditions. In addition, concurrent observations in bubble-nest creating ability and hematological parameters (data not shown) of the same fish set indicate well-being of the fish reared in herbal recipes.

Phytoconstituents contained in the herbs' fermented water, such as tannin, can inhibit bacterial infection (Chansue and Assawawongkasem, 2008; Chitmanat et al., 2005; Purivirojkul, 2012). During the experiment, external signs such as non-bleeding wounds, inflammation, or mucous covering were not observed in all amputated fish. The length of damaged fins was rapidly regenerated in the first week (~50%), and then increased more gradually until the end of the five-week study. Preliminary studies showed that caudal fins of fighting fish regenerated faster when ~50% was amputated rather than ~25%. Divergent reconstruction times/rates were observed among different fin excision sites and fish species. More proximal amputations took longer to regain their original length than distal amputations (Uemoto et al., 2020). Observations of a number of fish species have reported varying regeneration times: 24 days for mosquito fish (Johnson and Denton, 1977) and guppy (Kolluru et al., 2006); 14 days for zebrafish (Geraudie et al., 1995; Poleo et al., 2001) and fancy carp, *Cyprinus carpio* (Chansue, 2006); 42 days for

goldfish, *Carassius auratus* (Ngamsiri and Laumsomboon, 2021); and 28 days for Nile tilapia (Ngamsiri, 2015).

In the current study, fish reared in the formulations containing Indian-almond leaf (IA), Indian-almond leaf and banana leaf (IB), banana leaf and papaya leaf (BP), Indian-almond leaf and papaya leaf (IP), and Indian-almond leaf, banana leaf, and papaya leaf (IBP) completely regenerated fin length within a duration of 31–33 days. Fish reared in CT 1 were calculated to have regenerated their fins in 35.8 days. Fish can completely regenerate injured or lost organs naturally (Pfefferli and Jaźwińska, 2015), but the herbal treatments improved regeneration rates.

Several studies have improved wound healing and fin regeneration processes using natural products at optimal levels: 0.2 g L⁻¹ of dried Indian-almond leaves for goldfish (Ngamsiri and Laumsomboon, 2021); and 0.03 g L⁻¹ of creat (*Andrographis paniculata*) and dried Indian-almond leaves for fancy carp (Chansue, 2006). For Siamese fighting fish, since scientific information is limited, all ingredients (including leaves of Indian-almond, banana, papaya, and lemongrass) were applied based on the limited available documents (Banplakatraying, 2022; Chiarachavildham, 2016; Institute for Innovative Learning, 2022; Malawa et al., 2022; Puello-Cruz et al., 2010; Rucksapram, 2007). These Thai herbs contain phytoconstituents such as tannins, flavonoids, and phenolic compounds that provide anti-oxidant, anti-parasitic, anti-fungal, anti-bacterial, and anti-inflammatory activities (Chitmanat et al., 2005; Maria and Veronica, 2021; Ponpornpisit et al., 2007; Tanmoy et al., 2021; Wijaya et al., 2020). The addition of clay to the water helps fish recover faster (Chiarachavildham, 2016) whereas salt helps heal wounds, nourishes the skin, and reduces pathogenic growth (Puello-Cruz et al., 2010; Putiyanan, 1999). These ingredients promote three main stages of wound healing: they shorten the inflammation phase and accelerate the transition from proliferation to remodeling (Maria and Veronica, 2021; Pastore et al., 2012; Tanmoy et al., 2021; Wijaya et al., 2020).

Caudal fin coloration is consistent with body color. Primarily, regenerated fins will have a light-colored region, or non-pigmented regenerated tissue that requires a pigment progenitor to transfer chromatophores (Huang et al., 2017; Yi et al., 2014). Chromatophores containing pteridine-based pigment cells are color-associated cells derived from the neural crest during embryonic development and determine the differential expression of color in individual fish species. These pigments emerge from the proximal end of the base of the excision and spread distally with fin regrowth (Goodrich and Nichols, 1931; Huang et al., 2017). In the present study, solid-red male Siamese fighting fish were the model, and therefore the focus was on the deposition of erythrophores (Khoo et al., 2012).

The herbal treatments and treatment times affected the development of red pigmentation in the caudal fin, from light to dark red. The regenerated fin was classified into three color zones (dark red, light red, and transparent), and the red coloration developed faster in fish reared in BP, IP, or IBP than in IA and IAB treatments. Some studies have reported that herb extracts added to rearing water stimulated pigmentation. Examples include fermented water from Indian-almond leaf for fighting fish (Malawa et al., 2022) and neon tetra fish, *Paracheirodon innesi* (Ardi et al., 2020). Based on our observations, the three most effective treatments were BP, IP and IBP. The ingredient common to all three was dried papaya leaf. Papaya leaf contains flavonoids that reduce oxidative stress by inhibiting free radicals. They also contain dietary β -carotene that improves skin coloration (García-Chavarría and Lara-Flores, 2013; Das and Biswas, 2020; Van der Salm et al., 2006). Papaya leaf extracts are also used to dye textiles (Rani et al., 2020). Therefore, it is possible that dried papaya leaf might be a mordant that contributes some skin pigmentation to fish immersed in its extract.

6. Concluding remarks

Male Siamese fighting fish automatically regenerate damaged fins in normal rearing water. Fin length develops primarily followed by the migration of pigmentation. However, findings from the current study demonstrate the ability of fermented water from Thai herbal formulations to significantly enhance fin regeneration, in terms of both length and color development. All the formulations included dried lemongrass leaf, clay and salt. Fully developed fins were observed in fish reared in fermented water containing the above components with the addition of dried papaya leaf and papaya leaf with dried leaf of either banana or Indian-almond or both. Findings from the current study could be applied to enhance fin regeneration in betta fish farming.

CHAPTER 3

Rearing water containing fermented extracts of Thai herbal formulations improved well-being of injured Siamese fighting fish (*Betta splendens*)

1. Introduction

The Siamese fighting fish (*Betta splendens*), or betta fish, is a freshwater fish with gorgeous coloration and fascinating fin characteristics. The species originated in Southeast Asia, and is especially associated with the central region of Thailand (Goldstein, 2004; Monvises et al., 2009). Decades ago, the ornamental fish industry in Thailand began breeding and exporting Siamese fighting fish (DOF, 2021). The United States is now the biggest importer of Siamese fighting fish from Thailand, followed by China, France, Iran, and Singapore (Sermwatanakul, 2019).

Since the male fish are bigger, more vibrantly colored, and have longer fins than females (Jaroensutasinee and Jaroensutasinee, 2001; Thongprajukaew et al., 2014), only male fish are of interest to keepers of *B. splendens*, and only male fish are exported. The male betta fish is agonistic and displays to defend his territory or upon encountering opponents. These behaviors dictate the practices used in the commercial production and management of this species. At 1.5 months, male fish are placed in solitary captivity to avoid the negative effects of their aggressive behavior (Thongprajukaew et al., 2010a, 2010b). However, damaged bodies and torn fins often result from the matching of male fish for betting, as well as courtship and reproductive behaviors during mating (Malawa et al., 2023). Secondary infections of wounds by *Mycobacterium* result in clamped fins, fin wasting, dulled coloration, lethargy and loss of appetite (Lichak et al., 2022).

Injured and infected betta fish are traditionally treated in Thailand with fermented herbal extracts. The herbal formulations used are well-established combination

that differ in the quantity and type of active ingredients but typically comprise phytoconstituents such as tannin, flavonoids and phenolic compounds. For instance, the dried leaf of the Indian-almond (*Terminalia catappa*) aids wound healing and combats harmful monogenean and *Trichodina* spp. (Chitmanat et al., 2005; Ponpornpisit et al., 2007). The dried leaf of banana (*Musa sapientum*), papaya (*Carica papaya*), and lemongrass (*Cymbopogon citratus*) provide anti-bacterial, anti-oxidant, and anti-inflammatory activities that help heal wounds (Maria and Veronica, 2021; Tanmoy et al., 2021; Wijaya et al., 2020), especially those caused by fighting. However, the literature about fighting fish contains no report that describes the efficacy of treating wounds with herbal fermentations (Banplakatrachong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Some treatments have included dried clay and salt, added to heal wounds, nourish the skin, reduce pathogen growth and help fish recover faster (Chiarachavildham, 2016; Puello-Cruz et al., 2010; Putiyanan, 1999). Certain herbs are used to improve water quality. Extracts of *T. catappa* leaf, for instance, reduce water turbidity, pH, and total ammonia (Malawa et al., 2022; Nozaleda, 2019; Pimolrat et al., 2018; Puttamat and Suwannasarn, 2007) but excessive concentrations can increase acidity and ammonia (Ikhwanuddin et al., 2014).

Apart from the therapeutic effects of herbal treatments, male betta fish behaviors can be influenced by some herbs that contain phytoestrogens. Males construct bubble-nests and betta fish reared in water containing herbs have been observed to construct significantly bigger bubble-nests (Malawa et al., 2022). In general, male fish, juveniles as well as adults, create a bubble-nest by exhaling mucus-coated bubbles rich in glycoproteins. The quality, size and area of the bubble-nest are essential for supplying oxygen to eggs and larvae, which are nursed by males (Jaroensutasinee and Jaroensutasinee, 2003). Moreover, bubble-nest building capacity plays a vital role in health evaluations. Active bubble-nest builders exhibit lower levels of stress-induced cortisol than non-builders (Agues-Barbosa et al., 2022). Furthermore, herbal treatments can also improve hematological parameters (Malawa et al., 2022; Nugroho et al., 2016, 2017) and stimulate skin coloration in aquatic animals (Malawa et al., 2022).

Although the treatment of Siamese fighting fish with Indian-almond leaf has been reported (Malawa et al., 2022; Purivirojkul, 2012), information regarding the right combination of ingredients and their effects is still unavailable. The caudal fins of male fish were amputated to simulate fins torn in male-female courtship and reproductive behaviors or in male-male combative behavior (Malawa et al., 2023). The most effective herbal formulations used in this study could boost animal welfare and help support betta fish farming.

2. Objective

To assess the well-being of injured fish treated with a range of herbal formulations. The anti-oxidant activity of fermented herbal water, the physico-chemical parameters of rearing water, bubble-nest construction, and blood parameters were used as assessment criteria.

3. Materials and methods

3.1 Animal protocols

The present protocols conformed to the use of animals for scientific purposes approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Project Code 2564-01-030) under a larger project entitled “Innovations from indigenous knowledge for using Indian-almond leaf (*Terminalia catappa*) and herbal formulations for rearing and wound healing in Siamese fighting fish (*Betta splendens*)”.

3.2 Preparation of fermented herbal extract

All the fermented herbal formulations were based on powdered dried leaf of lemongrass (LL, 1 g L⁻¹), dried clay (50 g L⁻¹), and salt (5 g L⁻¹). Additional constituents were powdered dried leaf of Indian-almond (IAL, 0.25 g L⁻¹), powdered dried leaf of banana (BL, 1.5 g L⁻¹), and powdered dried leaf of papaya (PL, 2.5 g L⁻¹). The plant leaves were from farms in Songkhla province in southern Thailand. The leaves

were first immersed in 5 mg L⁻¹ of potassium permanganate (KMnO₄) for 15 min (Carpenter et al., 2001; Noga, 1996) and washed three times with distilled water to remove attached impurities. The clean samples were oven-dried at 60 °C for 12 h, and then finely powdered using a blender (WF-04; Siam Wealth Food Co., Ltd., Nonthaburi, Thailand), sieved, homogeneously mixed, packed in polyethylene bags, and kept at 4 °C until used. Clay was oven-dried after visible impurities were removed, and prepared as described for the components above.

The ingredients and amounts of each ingredient in all the studied herbal formulations are shown in Table 6. Two controls were designed: dechlorinated tap water (CT 1), and a dechlorinated water fermentation of LL, containing clay and salt (CT 2). CT 2 was then used as the basis of seven treatments. Three treatments, respectively labeled IA, B and P, were obtained by adding Indian-almond leaf (IAL), banana leaf (BL), or papaya leaf (PL) to CT 2. Three more treatments, respectively labeled IAB, IP and BP, were obtained by adding IAL and BL, IAL and PL, and BL and PL to CT 2. The seventh treatment, labeled IBP, was obtained by adding IAL, BL and PL to CT 2. To prepare each treatment, the appropriate dried leaf powders were mixed with dechlorinated tap water in 30 L plastic containers (31 cm width × 30 cm length × 49.7 cm height). The mixtures were fermented at room temperature for three days, with stirring twice daily, before being passed through filter cloth and filtered through Whatman No. 4 paper (Malawa et al., 2022). Dried clay was wrapped in filter cloth and placed in each small individual fish tank, except in CT 1. The chosen concentration of each herb in the formulations was based on available data (Banplakatrachong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Amounts of dried clay were estimated from Chiarachavildham (2016). Table salt was directly added to the rearing water of CT 2 and treatments to achieve a final concentration of 5 g L⁻¹ (Puella-Cruz et al., 2010).

3.3 Antioxidant characteristics of fermented herbal extracts

3.3.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free DPPH radical scavenging activity was measured based on the method described by Madhujith et al. (2006) with some modifications. In brief, a DPPH working solution was prepared by diluting a stock solution of 0.2 mM DPPH in 95% ethanol until absorbance was 1.0 ± 0.5 when measured at 517 nm. Then, 3 mL of the working solution were mixed with 100 μ L of CT 1, CT 2, and the seven treatments. Scavenging activity (%) was calculated from the equation $[(A_0 - A_i)/A_0] \times 100$ where A_0 and A_i were the absorbance values of the buffer and sample, respectively.

3.3.2 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

Free ABTS^{•+} radical scavenging activity was determined according to the method of Oladipo and Ibukun (2017). Briefly, stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate in phosphate buffer saline (pH 7.4) were mixed in a ratio of 1:1 and left for 16 h in darkness. The working solution was prepared by adding 5 mL of the mixed stock solutions to 145 mL of distilled water. Scavenging capacity was measured at 734 nm by mixing 900 μ L of working solution with 100 μ L of CT 1, CT 2, and the seven treatments. Scavenging activity (%) was calculated from equation $[(A_0 - A_i)/A_0] \times 100$ where A_0 and A_i were the absorbance values of buffer and sample, respectively.

3.4 Fish preparation and fin amputation

Three-month-old, solid-red male Siamese fighting fish were purchased from a farm in Nakhon Pathom Province, Thailand. They were separately acclimatized in cylindrical plastic containers (9.3 cm diameter $\times$ 12.5 cm height $\times$ 8 cm depth, containing 350 mL of water) for two weeks. The fish were then screened (1.29 ± 0.01 g body weight; 6.67 ± 0.02 cm total length; $n = 135$) and randomly divided into nine treatments of fifteen fish each. The fish were anesthetized with a 15 mg L⁻¹ solution of clove oil (L.B. Science, Songkhla, Thailand) and half the caudal fin was amputated in the vertical plane using a sterilized scalpel (Fig. 7).

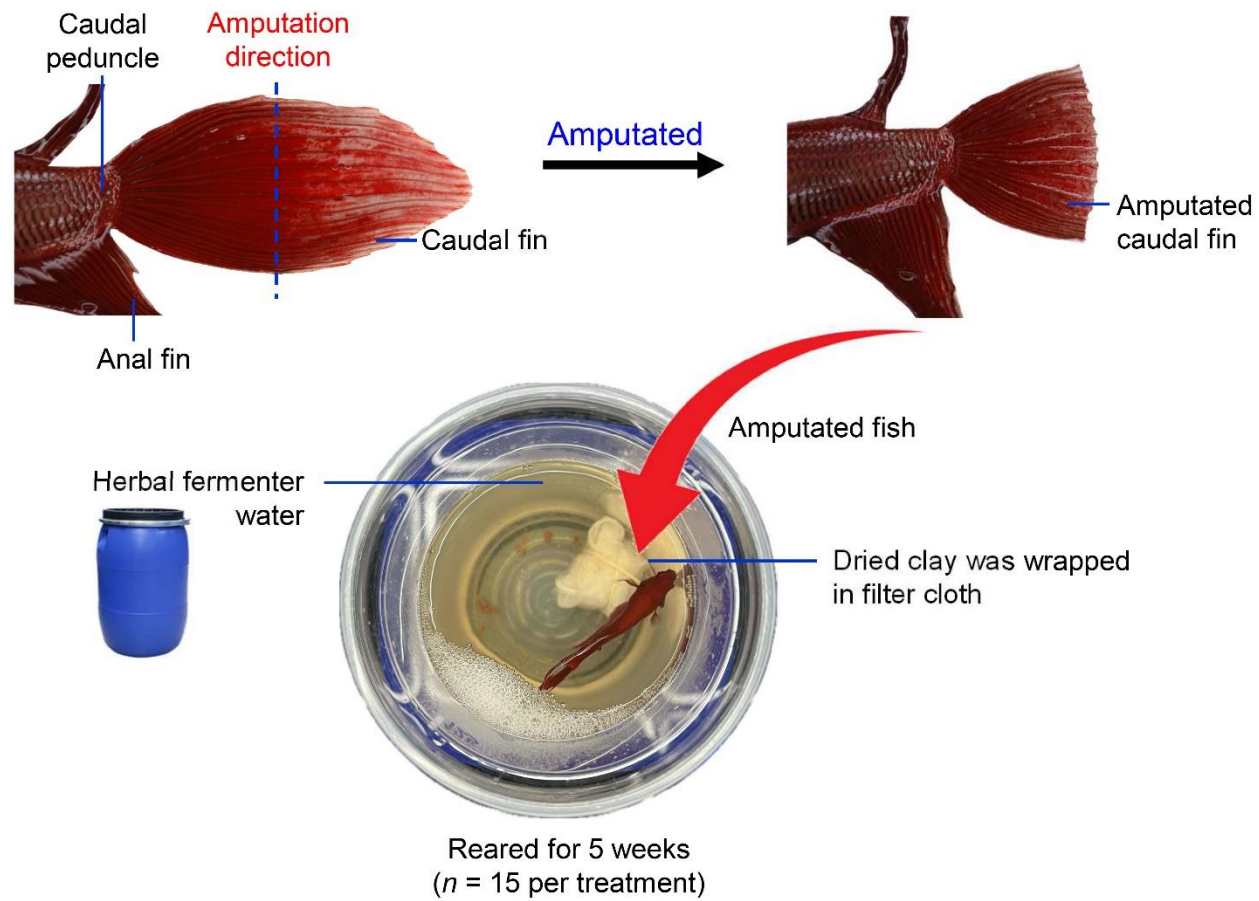


Fig. 7. The diagram shows herbal treatment of amputated fish.

3.5 Herbal treatment trial

A diagram showing the herbal treatment of amputated fish is showed in Figure 7. After amputation, each fish was carefully transferred to a cylindrical plastic container (9.3 cm diameter × 12.5 cm height × 8 cm depth) holding 350 mL of CT 1, or CT 2, or one of the seven treatments (IA, B, P, IAB, BP IP, and IBP). Each fish was reared in isolation from the other fish for five weeks under a 12-h light (06.00 to 18.00 h)/12-h dark light regime. Every three days, 80% of the water in each container was replaced, including fresh control or treatment. Twice daily, at 08.00 and 17.00 h, the fish received a commercial floating feed (39.4% crude protein) for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). After 30 min, remaining feed was collected and dried at 60°C until a constant weight was reached. The data were used to compute the feeding rate, and the amount of feed given was adjusted weekly according to the recorded feed intake. Fish mortality was monitored daily throughout the experiment. At the end of the trial, all the fish ($n = 15$ per treatment) were starved for 24 h and sacrificed by chilling in ice. Body weight was then measured and used to calculate specific growth rates. The parameters describing growth and feeding rate were calculated as follows:

$$\text{Specific growth rate (\% body weight day}^{-1}\text{)} = 100 \times [(\ln W_t - \ln W_o)/(t - t_o)]$$

where W_t = mean weight (g) at day t and W_o = mean weight (g) at day t_o ; and

$$\text{Feeding rate (\% body weight day}^{-1}\text{)} = 100 \times C/[(W_o + W_t)/2]/t \text{ where } C = \text{daily feed consumption (g), } W_o = \text{initial body weight (g), } W_t = \text{final body weight (g) and } t = \text{feeding duration (day).}$$

3.6 Water quality

During the experimental period, the quality of rearing water was monitored twice weekly before the water was changed. Physical and chemical water qualities, including pH, temperature, conductivity, salinity, and total dissolved solids (TDS), were measured using a multi-parameter water quality meter (U-52G; Horiba, Kyoto, Japan). Dissolved oxygen (DO) was determined using a modified Winkler method. Total alkalinity and hardness were measured by titration with sulfuric acid and

ethylenediaminetetraacetic acid (EDTA), using methyl orange and Eriochrome Black-T as respective indicators. Total ammonia nitrogen was measured by using the phenate method. These four parameters were analyzed based on the procedure of Boyd and Tucker (1992). Nitrite (by colorimetry) and nitrate (by cadmium reduction) were examined based on the methods of APHA, AWWA, and WPCF (1998).

3.7 Bubble-nest building behavior

Each cylinder with fish ($n = 15$ per treatment) was placed in a box (20 cm width $\times$ 20 cm length $\times$ 26 cm height) with a black background illuminated by an off-white LED. Twice a week, a top-view photograph was taken with a smartphone (iPhone 13 Pro, Apple Inc., China) to record bubble-nest formations. The frequency of bubble-nest building (%) was calculated by dividing the number of cylinders containing bubble-nests by the number of cylinders in each treatment (15), and multiplying the quotient by 100. The total area of each bubble-nest (μm^2) and the individual bubble area (μm^2) were quantified by Image J software, as described by Malawa et al. (2022). The number of bubbles was computed by dividing the total area of each bubble-nest by the individual bubble area. The Plot Profile tool in Image J was selected to generate a graph between gray values and bubble-nest distance (μm) from each top-view image. Bubble-nest height was expressed as the calculated mean gray value. A diagram showing how bubble-nest parameters were monitored is presented in Figure 8.

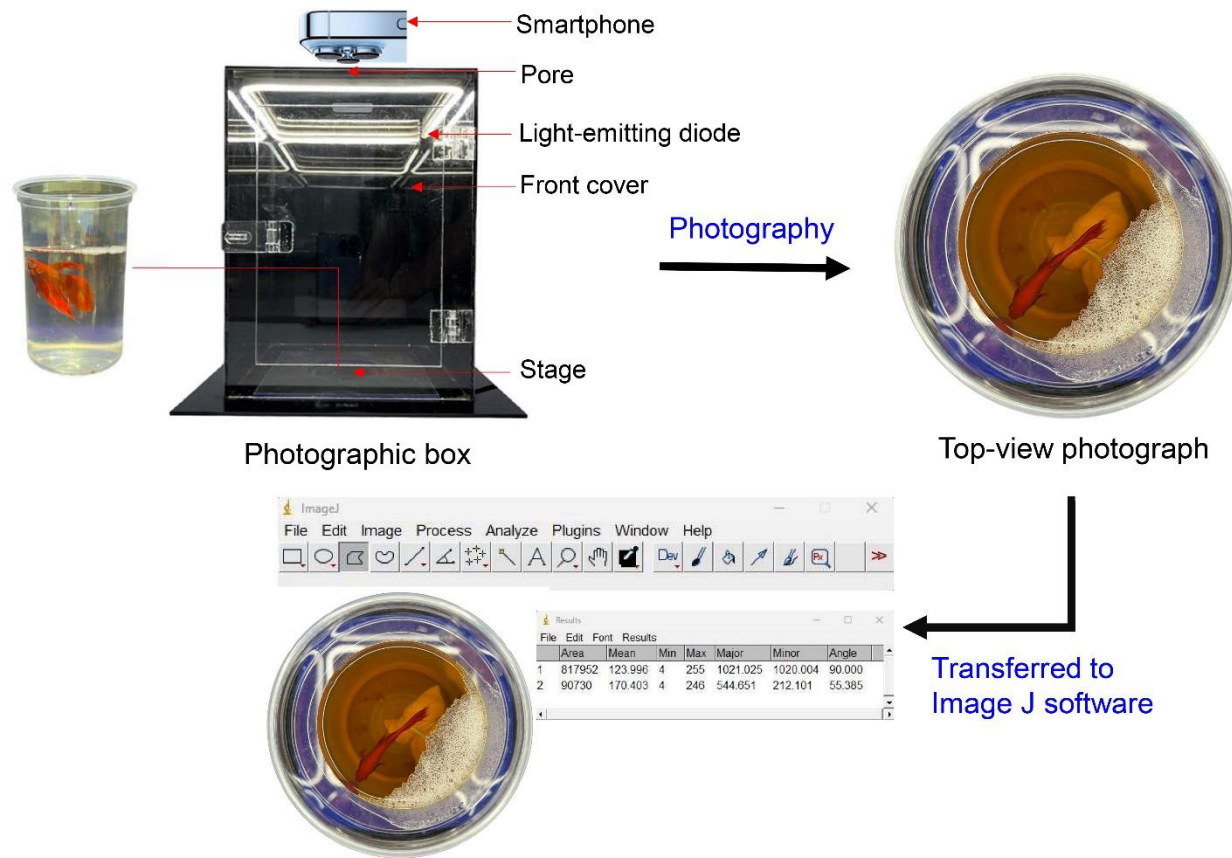


Fig. 8. The diagram shows the monitoring of bubble-nest parameters through Image J software.

3.8 Hematological parameters

Blood samples were taken from the caudal peduncle and samples from three fish were pooled to obtain five pooled samples per treatment ($n = 5$ per treatment). Each pooled sample was placed in an Eppendorf tube coated with 1% EDTA. Red and white blood cells (RBC and WBC) were counted manually on a hemacytometer under a compound microscope at 40 $\times$ magnification (cells mL⁻¹). Hemoglobin (Hb) was analyzed by the cyanmethemoglobin method (Blaxhall and Daisley, 1973). To calculate hematocrit (Hct), blood samples were centrifuged in capillary tubes and Hct % was calculated from the length of the RBC component divided by the length of the total blood sample. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\begin{aligned} \text{MCV (fL)} &= 10 \times [\text{Hct (\%)} / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})], \\ \text{MCH (pg)} &= 10 \times [\text{Hb (g dL}^{-1}) / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})], \text{ and} \\ \text{MCHC (g dL}^{-1}) &= 100 \times [\text{Hb (g dL}^{-1}) / \text{Hct (\%)}]. \end{aligned}$$

3.9 Statistical analysis

The sample size was computed on the R 3.6.0 program to obtain a test power of 0.8. The trial was a completely randomized design comprising eight herbal formulations and a non-herbal control, each of which contained fifteen fish serving as individual experimental units. The obtained data were expressed as means $\pm$ standard errors of means (SEM). All statistical values were performed under the Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, USA). Differences between treatment groups were assessed by One-Way Analysis of Variance (ANOVA) with Duncan's Multiple Range Test as a *post hoc* test. Rejection of hypothesis was based on a *P*-value equal to or lower than 0.05.

4. Results

4.1 Antioxidant characteristics of fermented herbal extract

Significant improvement in radical scavenging activity was observed in all herbal treatments relative to CT 1 and CT 2 (Fig. 9, $P < 0.05$). Relative to controls, treatment B had the lowest DPPH scavenging activity followed by treatment BP (Fig. 9a). High DPPH scavenging activity was observed in treatments IA, P, and IAB, whereas treatments IP and IBP showed intermediate results. The ABTS assay did not show the same inhibition trend as the DPPH assay, except for the higher scavenging activity of the treatments relative to CT 1 and CT 2 (Fig. 9b). The highest inhibition was found in treatment IBP followed by treatments IP and BP. The remaining treatments (P, IAB, IA, and B) exhibited relatively low scavenging activity.

4.2 Chemical and physical qualities of water

Compared with CT 1, water pH, salinity, total alkalinity, and hardness were significantly higher in all herbal treatments while total ammonia nitrogen (TAN), nitrite, and nitrate were lower (Fig. 10, $P < 0.05$). DO levels were the same or higher in all treatments compared to CT 1, except for treatment IBP (Fig. 10, $P < 0.05$). Relative CT 1, the water temperature profile remained similar over the five-week trial (Fig. 11, $P > 0.05$) while conductivity and TDS increased significantly in all treatments (Fig. 11, $P < 0.05$).

4.3 Survival, growth performance, and feed utilization of amputated fish

At the end of the trial, the survival of fish was 100% in all treatments. The treatments had no effect on growth (final body weight = 1.50 ± 0.03 g, final total length = 4.95 ± 0.06 cm, specific growth rate = $0.46 \pm 0.03\%$ body weight day⁻¹) or feed utilization (feeding rate = $1.82 \pm 0.03\%$ body weight day⁻¹) ($P > 0.05$).

4.4 Bubble-nest ability of amputated fish

Top-view photographs of bubble-nests are presented in Figure 12a. Relative to CT 1, significant higher in bubble-nest height, indicated by gray values, were observed in all experimental treatments except IAB (Fig. 12b, $P < 0.05$). The frequency of bubble-nest creation was similar in treatments P, IAB, and IBP (Fig. 13a, $P > 0.05$), while the other remaining treatments raised creating ability significantly ($P < 0.05$). Total bubble-nest area was significantly greater in all treatments relative to CT 1 (Fig. 13b, $P < 0.05$) but individual bubble-nest area was lower compared to CT 1 in all treatments except IA, and in CT 2 (Fig. 13c, $P > 0.05$). The number of bubbles produced was similar in CT 1, IA, and IAB (Fig. 13d, $P > 0.05$) but significantly higher in all other treatments relative to CT 1 ($P < 0.05$).

4.5 Hematological parameters of amputated fish

Hematological parameters were significantly different in all treatments (Table 10, $P < 0.05$), except for WBC. Significantly lower RBC counts were observed in BP and IP treatments ($P < 0.05$) but Hct % in these two treatments remained stable compared to CT 1 ($P > 0.05$). Slightly improved ($P < 0.05$) or constant ($P > 0.05$) Hb, MCHC, MCH, and MCV were observed in all treatments except for treatment IBP, where fish showed a significantly lower MCV ($P < 0.05$).

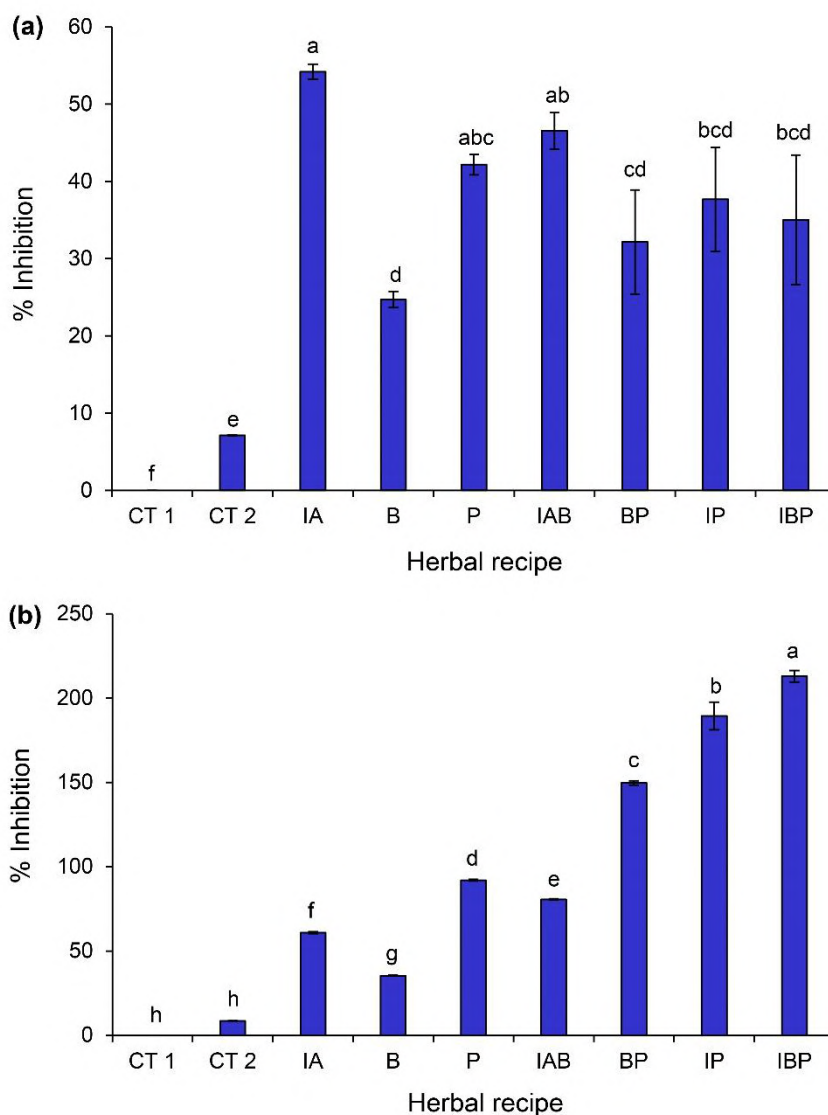


Fig. 9. Column charts show the percent inhibition of free radicals due to water fermentations of various herbal formulations used for rearing male Siamese fighting fish: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (a) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity (b). The assays were performed in triplicate. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

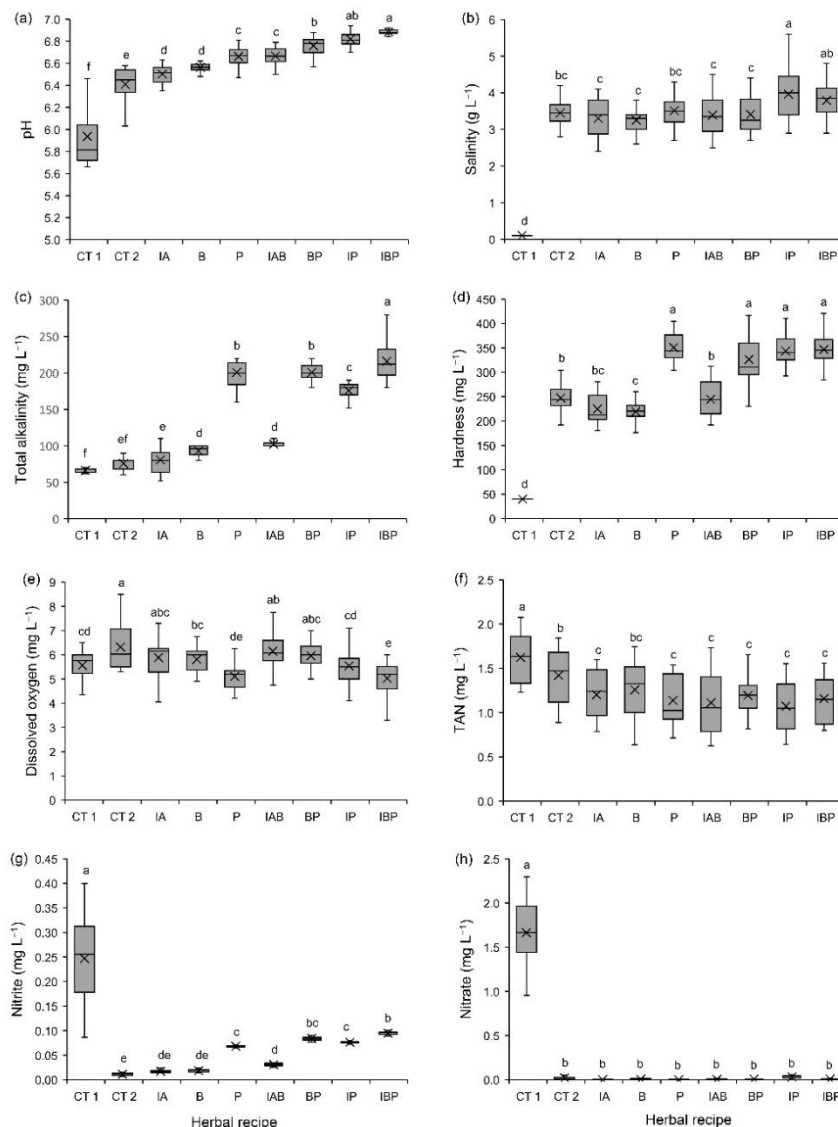


Fig. 10. Boxplots show the chemical characteristics of water fermentations of various herbal formulations over the five-week trial: pH (a), salinity (b), total alkalinity (c), hardness (d), dissolved oxygen (e), TAN (f), nitrite (g), and nitrate (h). Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. TAN = total ammonia nitrogen. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

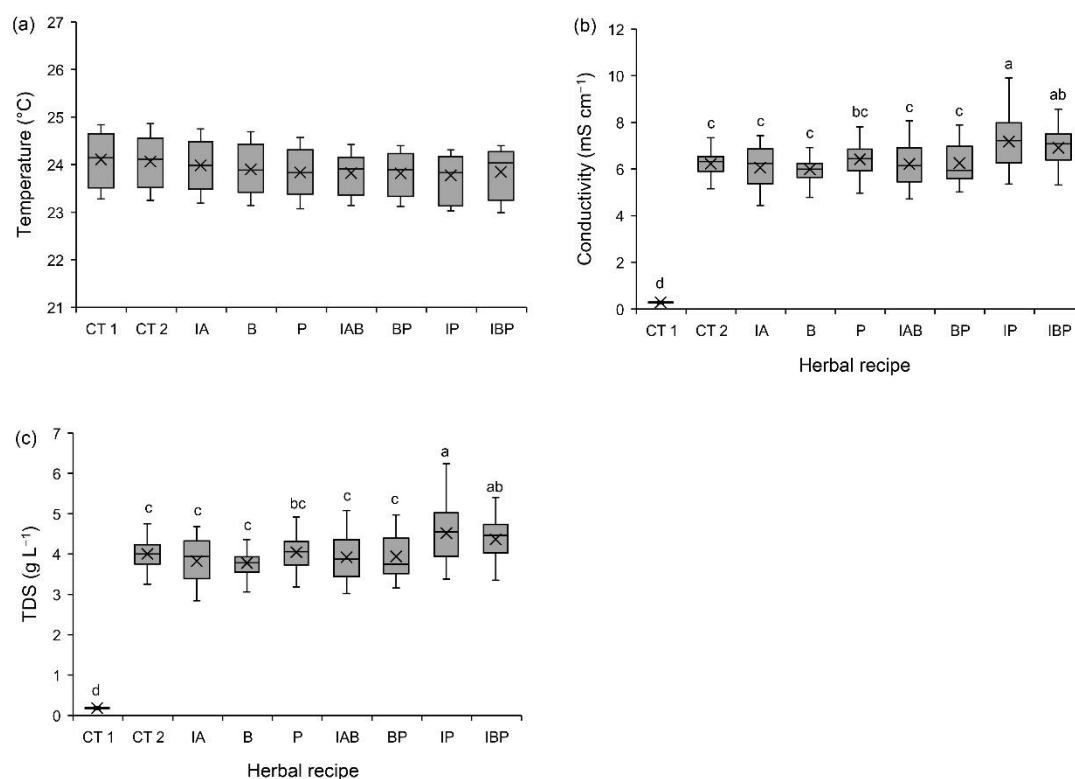


Fig. 11. Boxplots show physical characteristics of water fermentations of various herbal formulations over the five-week trial: temperature (a), conductivity (b), and TDS (c). Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. TDS = total dissolved solid. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

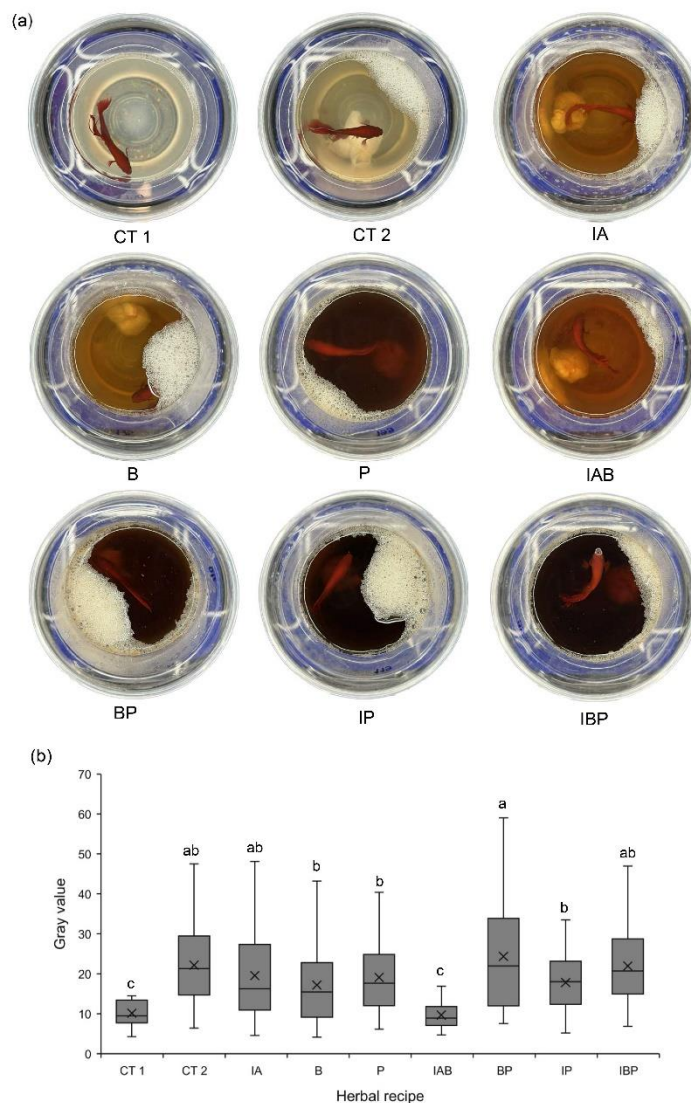


Fig. 12. Representative photographs show the morphological appearance (a) and gray values (b) of bubble-nests of injured male Siamese fighting fish recuperated in water fermentations of various herbal formulations. Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

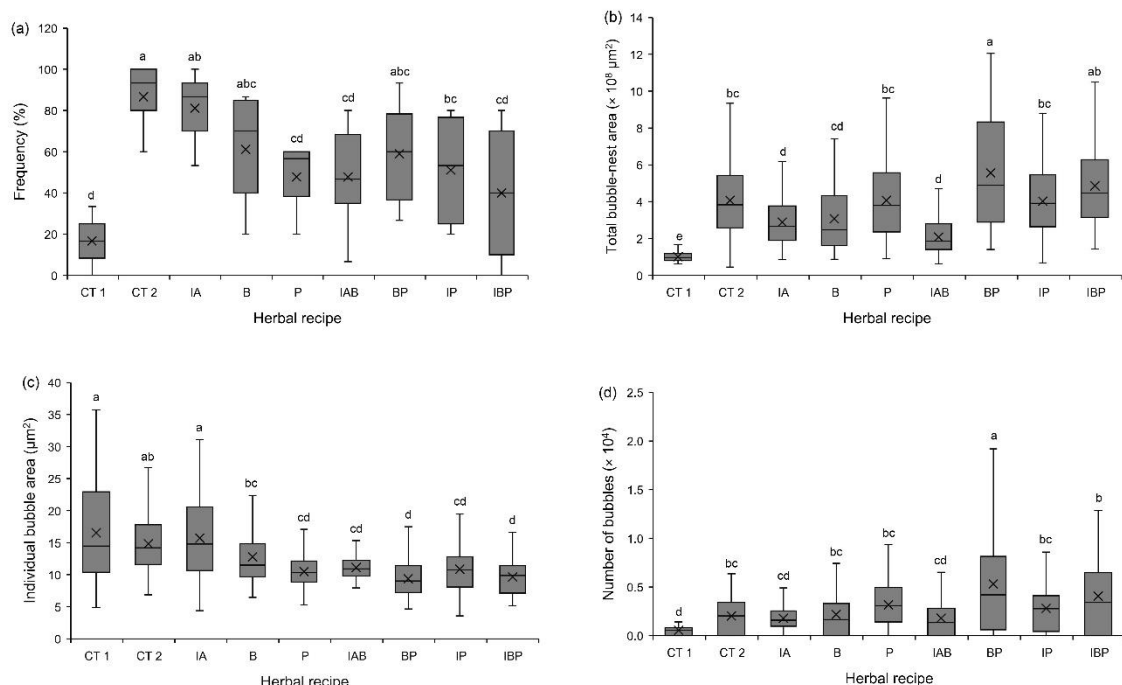


Fig. 13. Frequency (a), total bubble-nest area (b), individual bubble area (c), and number of bubbles (d) of injured male Siamese fighting fish recuperated in fermented water of herbs. Two measurements a week were taken. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Table 10 Hematological parameters of male Siamese fighting fish after caudal fin amputation. The measurements were performed at the end of a five-week trial in which the fish were recuperated in water fermentations of various herbal formulations.

Herbal formulation	Hematological parameter						
	RBC ($\times 10^9$ cells mL ⁻¹)	WBC ($\times 10^7$ cells mL ⁻¹)	Hct (%)	Hb (g dL ⁻¹)	MCHC (g dL ⁻¹)	MCH (pg)	MCV (fL)
CT 1	3.14 $\pm$ 0.12 ^{ab}	3.82 $\pm$ 0.33	46.8 $\pm$ 3.5 ^a	11.2 $\pm$ 1.1 ^{bc}	24.2 $\pm$ 2.4 ^c	35.7 $\pm$ 3.3 ^c	138 $\pm$ 10 ^{bc}
CT 2	2.80 $\pm$ 0.07 ^{cde}	4.15 $\pm$ 0.24	34.3 $\pm$ 3.0 ^c	12.0 $\pm$ 0.4 ^{abc}	35.5 $\pm$ 1.8 ^a	42.8 $\pm$ 1.6 ^{ab}	122 $\pm$ 10 ^{bcd}
IA	3.38 $\pm$ 0.07 ^a	4.68 $\pm$ 0.20	31.9 $\pm$ 6.1 ^c	13.3 $\pm$ 0.7 ^a	37.3 $\pm$ 3.9 ^a	39.5 $\pm$ 2.4 ^{bc}	112 $\pm$ 10 ^{cd}
B	2.99 $\pm$ 0.07 ^{bc}	4.07 $\pm$ 0.16	36.0 $\pm$ 2.1 ^{bc}	12.8 $\pm$ 0.8 ^{ab}	36.0 $\pm$ 2.4 ^a	42.8 $\pm$ 1.6 ^{ab}	120 $\pm$ 6 ^{bcd}
P	3.13 $\pm$ 0.05 ^{ab}	4.34 $\pm$ 0.25	34.8 $\pm$ 4.1 ^c	13.5 $\pm$ 0.4 ^a	34.7 $\pm$ 2.6 ^{ab}	43.2 $\pm$ 1.7 ^{ab}	122 $\pm$ 7 ^{bcd}
IAB	3.07 $\pm$ 0.08 ^b	4.16 $\pm$ 0.36	35.5 $\pm$ 1.5 ^c	12.8 $\pm$ 0.5 ^{ab}	36.4 $\pm$ 2.7 ^a	41.7 $\pm$ 1.8 ^{bc}	116 $\pm$ 6 ^{cd}
BP	2.60 $\pm$ 0.08 ^e	3.46 $\pm$ 0.47	46.3 $\pm$ 4.0 ^{ab}	12.7 $\pm$ 0.3 ^{ab}	28.0 $\pm$ 2.1 ^{bc}	48.6 $\pm$ 2.6 ^a	168 $\pm$ 14 ^a
IP	2.70 $\pm$ 0.11 ^{de}	4.26 $\pm$ 0.14	41.4 $\pm$ 1.8 ^{abc}	10.1 $\pm$ 0.6 ^c	25.6 $\pm$ 2.0 ^c	37.6 $\pm$ 2.3 ^{bc}	144 $\pm$ 7 ^{ab}
IBP	2.88 $\pm$ 0.09 ^{bcd}	3.91 $\pm$ 0.15	30.1 $\pm$ 1.4 ^c	12.6 $\pm$ 0.3 ^{ab}	42.4 $\pm$ 2.1 ^a	44.1 $\pm$ 1.3 ^{ab}	105 $\pm$ 4 ^d
P-value	<0.001	0.170	0.010	0.010	<0.001	0.012	0.001

RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Data are expressed as means $\pm$ SEM ($n = 5$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same column indicate a significant difference ($P < 0.05$).

5. Discussion

5.1 Improvement of water parameters by herbal treatments

The herbs used in our experiment (IAL, BL, PL, and LL) possibly contain phenolic acids, flavonoids, phenols and other phytochemical constituents (Kumar et al., 2021; Muala et al., 2021; Raphaël et al., 2019; Vuong et al., 2013) that could have significantly improve the antioxidant capacity of all the treatments relative to the controls. Free radical quenching by antioxidants in the fermented herbal water treatments was better reflected in the inhibitory activities revealed by the ABTS assay than the DPPH assay. The inconsistency between the results obtained from the same treatments was due to the divergent properties of the reactive media. DPPH[•] is suitable for hydrophobic antioxidants, whereas ABTS⁺⁺ is suitable for hydrophilic and lipophilic antioxidants (Re et al., 1999).

Except for water temperature, the physico-chemical characteristics of the fermented herbal water treatments were also significantly different. The amounts and profiles of the active ingredients in each formulation may have had direct effects on water quality. Water pH levels were significantly higher in CT 2 and all herbal treatments relative to CT 1. The increased pH could be attributed to a higher proportion of phytochemicals with a pH range between 8 and 10, such as flavonoids, than mildly acidic phytochemicals like tannic acid, depending on the nature and position of adjacent groups (Prasad et al., 2023; Yan et al., 2020). However, the observed pH range was in line with water quality recommendations (pH 6.5–8.0) for betta fish aquaculture (Saekhow et al., 2018; Thongprajukaew et al., 2019) and was consistent with some reported usage; for example, IAL extract in betta fish rearing (Malawa et al., 2022; Nugroho et al., 2016). Although total alkalinity and DO fluctuated significantly among treatments, both parameters remained within the most satisfactory range for aquaculture, indicating the capacity of pH-buffering to stabilize water pH (Meade, 1989; Saekhow et al., 2018; Thongprajukaew et al., 2019). DO levels were sufficient for good fish production (Bhatnagar et al., 2004).

The addition of salt to the treatments significantly increased salinity levels due to dissolved ions, compared to CT 1. This finding was consistent with the use by Fabregat et al. (2017) and Puello-Cruz et al. (2010) of saline levels lower than or equal to 5 g L^{-1} that maintained survival and growth but removed ectoparasites in betta larvae. Slightly increased salinity may reduce the metabolic energy required by betta for the osmoregulation process necessary to wound healing (Altinok and Grizzle, 2001). Salt aids wound drying by forcing intracellular fluid out of the body. Therefore, the growth of new tissue is accelerated quicker skin contact (Altinok and Grizzle, 2001; Samidah et al., 2021). Increased water salinity levels, and dissolved minerals from the clay resulted in increased water conductivity, TDS, and hardness (Boyd, 1979; Rusydi, 2018). Although conductivity in our study was slightly above the range generally considered acceptable for betta fish (Thongprajukaew et al., 2019), TDS and hardness levels were within the aquaculture water quality standards (Meade, 1989). In addition, water hardness levels remained between 150 and 320 mg L^{-1} where no adverse effects could be observed on growth, reproductive potential or bubble-nest creation in betta fish (Krishnakumar et al., 2020).

The significant decrement in N-waste levels (NH_3 , NO_2 , and NO_3) observed in all treatments was possibly due to the interaction of tannins (OH groups) with nitrogenous substances (NH_2 , SH, OH, and CO groups) through the formation of hydrogen bridges (Siebert et al., 1996). Braghiroli et al. (2013) described the reaction of condensed tannins with NH_3 in water. The reaction resulted in the amination and cross-linking of phenolic hydroxyl groups and the gelation of tannin/ammonia via the formation of nitrogen bridges between flavonoid units. The improvement of water quality realized with the bio-treatments in the present study is consistent with previous reports of the use of herbs such as IAL (Malawa et al., 2022; Pimolrat et al., 2018; Puttamat and Suwannasarn, 2007) or even BL (El-DougDoug et al., 2020).

Betta fish have relatively slow behavior and are often reared with *ad libitum* feeding in low water volumes that are typically refreshed only every three days. These conditions may lead to low DO levels and elevated NO_2 and NO_3 values in rearing

water, but they can survive in these conditions, and the reported values remained within an acceptable range for betta fish culture (Saekhow et al., 2018). Also, there is a minor effect possibly caused by nitrifying bacteria. However, this effect is minimal because some active ingredients in the fermented herbal extracts may inhibit bacterial growth. Therefore, herbal treatments are recommended for maintaining water quality and reducing the necessity for frequent water changes. Currently, there are some recommended herbal products on the market, including fresh leaves, dried leaves, and concentrated extracts. However, it is possible to develop the treatments proposed here with appropriate usage patterns for commercial applications to improve water quality for fish farming, thereby reducing the need for frequent water changes and maintaining the well-being of the fish. The developed treatments would offer alternatives to consumers and add value to the products used.

5.2 Improvement of bubble-nest creating ability by herbal treatments

At the end of the five-week observation, none of the amputated fish had died. Fin length was completely regenerated, and growth performance and feed utilization were similar across all seven treatments and two controls. These unchanged parameters indicated normal growth without negative effects from the herbal treatments. No external signs, such as non-bleeding wounds, inflammation or mucous covering, were observed in any amputated fish, and positive effects on water quality and fish well-being were clearly observed.

Bubble-nests are composed of glycoprotein-rich mucous-coated bubbles that facilitate oxygenation of the developing fertilized fish eggs and larvae (Kang and Lee, 2010). Behaviorally, the size or height of nests can predict reproductive success in male fish (Jaroensutasinee and Jaroensutasinee, 2001). Bubble-nests of captive betta fish were reported to have an average area of 2.41 to $7.19 \times 10^9 \mu\text{m}^2$ (Srikrishnan et al., 2017). In the present study, significant increments of frequency, total bubble-nest area, number of bubbles, and nest height (gray value) were observed in all herbal treatments, except for CT 1. Individual bubble-nest area was proportionally changed and was the reciprocal of

total bubble-nest area ($r = -0.696$, $P < 0.05$). The injured fish recuperated in the BP treatment showed a higher bubble-nest building capacity regarding total bubble area, number of bubbles, height, and frequency relative to CT 1. A previous study (Malawa et al., 2022) observed healthy betta fish reared in water containing a suitable concentration (0.25 g L^{-1}) of fermented IAL. Total bubble-nest area and frequency were significantly higher than in non-fermented treatments. It is possible that the isoflavones genistein and equol, which are phytoestrogen derivatives, obtained from a combination of plant leaves stimulate bubble-nest building by male betta fish (Clotfelter and Rodriguez, 2006; Vadwala and Kola, 2017), and the amputation of fins reduces the frequency of bubble-nest building. However, among the present treatments, bubble-nests produced by fish recuperated in CT 2, B, BP, and IP showed superior characteristics. Phytoestrogen derivatives or other bioactive compounds in those particular herbal constituents may trigger bubble-nest building. Synergistic effects from the diverse range of compounds in the formulations may also affect this behavioral response. In contrast, since final body weight and total length of all the fish at the end of observation were not significantly different, it was concluded that neither parameter affected nesting ability in this study.

5.3 Hematological responses due to herbal treatments

Hematological analyses can be used as crude indications of health. In the present study, none of the herbal treatments affected the average WBC values of the amputated fish. The observed values were above the range for healthy male betta reported by Motlagh et al. (2012). It is possible that the wound healing process had terminated at the end of five weeks, so that neutrophils, lymphocytes, and monocytes had ceased migrating to damaged tissues (Sonmez and Sonmez, 2017).

The reported ranges of RBC, Hct and Hb in healthy betta erythrocytes are respectively $1.64\text{--}4.20 \times 10^9 \text{ cells mL}^{-1}$, 16.7–44.3%, and $6.22\text{--}11.8 \text{ g dL}^{-1}$ (Kari et al., 2023; Malawa et al., 2022; Motlagh et al., 2012; Nugroho et al., 2016, 2017). In our study, the groups that showed the best bubble-nest building ability (CT 2, B, BP, and IP), exhibited values for these erythrocytes that were similar or within the above ranges.

However, fish reared in the BP treatment were an outlier group in terms of Hct, and might be at risk from a hematological disorder; probably polycythemia (Clauss et al., 2008; Hrubec and Smith, 2000), which reduces the transport of oxygen into tissues. Moreover, based on Pearson correlation analysis, bubble-nesting (total bubble area) was negatively correlated with RBCs ($r = -0.674$, $P < 0.05$). Therefore, the decreased RBCs in the BP treatment may be due to the effect of this herbal recipe on the proliferation of stem cells.

MCV, MCH, and MCHC reflect the size and hemoglobin content of erythrocytes. The amputated fish recuperated in the BP treatment had relatively high MCV and MCH values, which might be linked with macrocytic anemia (Javed et al., 2016). However, the MCHC of fish treated with BP did not change significantly relative to fish in CT 1. Certain negative effects observed in our investigation proscribe recuperating injured fish in the BP formulation.

6. Concluding remarks

Recuperating injured male Siamese fighting fish in suitable formulations of herbs fermented in water significantly supported the well-being of the fish by improving water quality and water antioxidant activity and by accelerating bubble-nest building. At the same time, no undesirable effects on survival, growth, feed utilization, and blood parameters were observed. Phytochemical constituents of the various herbs in these formulations might contribute biological activities that promote these positive responses. Our findings suggest that the well-being of fish suffering damage to their body and fins could be promoted by rearing them in a water fermentation of 1 g L⁻¹ of lemongrass supplemented with 50 g L⁻¹ of dried clay and 5 g L⁻¹ of salt. This formulation was also effective when the water fermentation included 1.5 g L⁻¹ of dried banana leaf, or 0.25 g L⁻¹ of dried Indian-almond leaf, or 2.5 g L⁻¹ of dried papaya leaf. These herbal formulations could also be applied for a short term to enhance the bubble-nest building of mature male betta during courtship and reproduction. Furthermore, these findings support the use of Thai folk wisdom in the management of betta fish farming.

CHAPTER 4

Somatic responses of injured Siamese fighting fish (*Betta splendens*) raised using various recipes of Thai herbal fermented water

1. Introduction

Betta splendens, also known as the Siamese fighting fish, is a small freshwater fish distinguished by its remarkable color variations and captivating fin morphological traits, whose origins can be traced back to Southeast Asia, notably within central Thailand (Goldstein, 2004; Monvises et al., 2009). Furthermore, Thailand's ornamental fish industry, exclusively targeting these male 'betta' fish, has witnessed substantial growth in captive breeding and international exports (DOF, 2021). In the first half of 2021, betta was ranked first among 10 popular ornamental fish in Thailand, with the exported volume surpassing 10 million fish reported at Suvarnabhumi Airport (DOF, 2021). The USA is the primary importer of Siamese fighting fish from Thailand, followed by China, France, Iran and Singapore (Sermwatanakul, 2019).

Inherently, these fish display agonistic behavior in confrontations associated with defending territory, be it between males and females or among individuals of the same gender, resulting in damaged bodies and torn fins (Malawa et al., 2023). Consequently, the breeders raise these fish in solitary captivity, commencing at approximately age 1.5 months (Thongprajukaew et al., 2010a, 2010b), to mitigate the adverse effects of their combative nature. In addition, these injuries can manifest during courtship and reproductive phases (Malawa et al., 2023).

In Thailand, immersion in herbal treatment is the preferred and practical treatment to heal external damage in injured fish rather than using antibiotics or chemicals that cause adverse health effects to the fish and (via the residues) in their surroundings (Malawa et al., 2022, 2023). Typically, some herbs, such as the dried leaves of the Indian-almond (*Terminalia catappa*), banana (*Musa sapientum*), papaya (*Carica papaya*) and

lemongrass (*Cymbopogon citratus*) or dried clay or salts, have been incorporated into the recipes used to treat the fish (Malawa et al., 2023). These ingredients have various phytoconstituents, such as tannin, flavonoid and phenolic compounds, differing in quantity and active ingredient type. For example, the dried leaves of the Indian-almond aid in wound healing and act against harmful monogeneans and *Trichodina* spp. (Chitmanat et al., 2005; Ponpornpisit et al., 2007), while the dried leaves of banana, papaya and lemongrass provide anti-bacterial, anti-oxidant and anti-inflammatory activities that help to heal wounds (Maria and Veronica, 2021; Tanmoy et al., 2021; Wijaya et al., 2020), especially those caused during fish fighting. Dried clay and salt are added to help fish recover more rapidly, to heal wounds, to nourish the skin and to reduce pathogen growth (Chiarachavildham, 2016; Puello-Cruz et al., 2010; Putiyanan, 1999).

Other benefits have been reported regarding the use of certain herbs, specifically the optimal concentration of *T. catappa* leaf extract to enhance the survival rate, growth performance and feed utilization (Nugroho et al., 2016; Nurhidayat et al., 2016; Ramos et al., 2020). Additionally, this herb has been associated with improvements in digestive enzyme activities (Malawa et al., 2022) and skin coloration (Ardi et al., 2020; Malawa et al., 2022), but without affecting muscle quality and the whole-body composition in healthy fish (Malawa et al., 2022). However, an excessive concentration of its extract may adversely affect these parameters (Ikhwanuddin et al., 2014; Mininel et al., 2014).

Although herbal treatment involving Indian-almond leaf has been documented for healthy Siamese fighting fish, there is a noticeable lack of information regarding using a combination of ingredients and their effects on somatic changes in injured fish. Caudal fin amputation was used as a model to simulate a torn fin on the fish resulting from agonistic behavior. The findings from the current study might support using Thai herbal treatment in betta fish farms.

2. Objective

To examine fish survival and growth, feed utilization, skin coloration, digestive enzyme activity, muscle quality, and whole-body composition in injured fish treated with various herbal formulations.

3. Materials and methods

3.1 Ethics statements

The Institutional Animal Care and Use Committee at Prince of Songkla University approved the utilization of the animals in this scientific research (Project Code 2564-01-030). Transportation, acclimatization, surgery, husbandry and sampling were carried out under this agreement.

3.2 Preparation of herbal fermented water

Newly fallen leaves (Indian-almond, banana and papaya), mature leaves (lemongrass) and clay were harvested from a few farms in Songkhla province, Thailand. The samples were cleaned, soaked in 5 mg L⁻¹ of KMnO₄ for 15 min to eliminate attached microorganisms and then washed three times with distilled water. They were dried at 60 °C for 12 h, crushed using a grinder, sieved, homogenously mixed, packed in polyethylene bags, placed inside black plastic bags to exclude light and stored at 4 °C until used.

The components of each herbal formula were detailed in Malawa et al. (2023). The chosen concentration of each ingredient was based on available documents (Banplakatrachong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Amounts of dried clay was estimated from Chiarachavildham (2016). Table salt was directly added to the rearing water to achieve a final concentration of 5 g L⁻¹ (Puello-Cruz et al., 2010). Two control groups were established: one using non-fermented water (CT 1) and the other fermented water (CT 2), to which were added dried lemongrass leaves (1 g L⁻¹), dried clay (50 g L⁻¹), and salt (5 g L⁻¹). Three distinct treatments were created by incorporating dried Indian-almond leaves (0.25 g L⁻¹), dried banana leaves (1.5 g L⁻¹), or dried papaya leaves (2.5 g L⁻¹) into CT 2. These treatments

were denoted as IA, B and P, respectively. Additionally, three more treatments emerged by introducing combinations of dried Indian-almond leaves and dried banana leaves, dried Indian-almond leaves and dried papaya leaves, and dried banana leaves and dried papaya leaves to CT 2. These treatments were labeled as IAB, IP and BP, respectively. The seventh treatment was generated by adding dried Indian-almond leaves, dried banana leaves and dried papaya leaves together to CT 2, and this treatment was designated as IBP. The fermentation was performed by soaking the relevant ingredients in dechlorinated water in 30 L plastic containers (31 cm width $\times$ 30 cm length $\times$ 49.7 cm height) at room temperature for 3 days, stirring twice daily. At the end of 3 days, the mixtures were passed through filter cloth and Whatman No. 4 paper. The obtained filtrate was used for fish rearing.

3.3 Herbal treatment trial

A completely randomized design was conducted using the nine herbal formulations, with 15 individual fish serving as experimental units. The fish preparation and fin amputation were performed as described by Malawa et al. (2023). First, each fish was anesthetized with a 15 mg L⁻¹ solution of clove oil and then the caudal fin lengths were measured using a set of Vernier calipers. Subsequently, one-half of the caudal fin length was amputated in the vertical plane using a sterilized scalpel. The amputated fish ($n = 135$) with their fins excised were carefully carried and raised separately in cylindrical plastic containers measuring 9.3 cm in diameter, 12.5 cm in height and 8 cm in depth. These containers were filled with 350 mL of rearing water, comprised of either freshly fermented water obtained from the eight Thai herbal recipes (CT 2, IA, B, P, IAB, BP IP, and IBP) or dechlorinated tap water (CT 1). All fish were reared under a 12-h light (06.00–18.00 h)/ 12-h dark cycle for 5 weeks until there was complete regeneration of the caudal fin. During the herbal treatment period, all the fish were fed *ad libitum* using a commercial floating feed for fighting fish, which contained 39.4% crude protein (Betta Bio-Gold; Kyorin Food Ind. Ltd.; Himeji, Japan). Feeding occurred twice daily, at 08.00 h. and 17.00 h. After each feeding session, any remaining feed was collected within 30

min and then dried at 60 °C until it reached a constant weight. Obtained data were used to compute the feed conversion ratio (FCR) and the protein efficiency ratio (PER). Every 3 days, 80% of the rearing water was replaced with water containing freshly prepared fermented water. Twice a week, throughout the experimental period, various physio-chemical parameters of the water were measured: temperature (23.9 ± 0.1 °C), pH (6.51 ± 0.03), conductivity (5.41 ± 0.21 mS cm⁻¹), total dissolved solids (3.43 ± 0.13 g L⁻¹), dissolved oxygen (5.71 ± 0.06 mg L⁻¹), salinity (3.00 ± 0.12 g L⁻¹), total alkalinity (129 ± 5 mg L⁻¹), hardness (255 ± 9 mg L⁻¹), total ammonia nitrogen (1.28 ± 0.03 mg L⁻¹), nitrite (0.09 ± 0.01 mg L⁻¹) and nitrate (0.86 ± 0.15 mg L⁻¹).

Fish mortality was monitored daily throughout the experiment. At the end of the trail (5 weeks), all the surviving fish ($n = 15$ per treatment) were starved for 24 h and then sacrificed by chilling in ice before recording body weight (BW) and total length. Samples of viscera ($n = 5$ per treatment), digestive tracts ($n = 5$ per treatment) and white muscle ($n = 5$ per treatment) were gathered from the same group of fish to assess the viscerosomatic index (VSI), digestive enzyme activity and muscle quality, respectively. The whole-body composition analysis used 10 fish chosen from each treatment. Three or four fish were pooled into each lot to obtain one pooled sample (three pooled samples per treatment). The parameters describing survival, growth performance and feed conversion were computed.

$$\text{Survival (\%)} = 100 \times (\text{Final fish number} / \text{Initial fish number})$$

$$\text{Weight gain (g)} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

$$\text{VSI (\%)} = 100 \times [\text{wet weight of viscera (g)} / \text{body weight (g)}]$$

$$\text{FCR (g feed g gain}^{-1}\text{)} = \text{dry feed consumed (g)} / \text{wet weight gain (g)}, \text{ and}$$

$$\text{PER (g gain g protein}^{-1}\text{)} = \text{wet weight gain (g)} / \text{protein intake (g)}$$

3.4 Skin coloration

The fish skin was carefully cleaned using soft blotting paper. Subsequently, a portable spectrophotometer (MiniScan EZ 4500; Hunter Associates Laboratory, Reston, VA, USA) was calibrated using black and white standards before assessing the skin coloration in the mid region of the fish. The color parameters were digitally displayed on the LCD screen as L^* , a^* , h^* and C^* .

3.5 Determination of digestive enzyme activities

3.5.1 Enzyme extraction and protein quantification in crude extracts

The frozen gastrointestinal tract was extracted in cold 0.2 M Na_2HPO_4 - NaH_2PO_4 buffer (1:15 weight per volume) using a tissue homogenizer (THP-220; Omni International, Kennesaw, GA, USA). The homogenate was centrifuged at $15,000 \times g$ and 4°C for 30 min to collect the supernatant from debris and then preserved at -20°C . The total protein concentration in the crude extract was assessed quantitatively based on the method outlined by Lowry et al. (1951) to determine the specific activity of the digestive enzymes (measured in units per milligram of protein).

3.5.2 Digestive enzyme assays

All enzyme assays of the betta fish were performed under optimized conditions of pH and temperature, as reported by Thongprajukaew et al. (2010a, 2010b). Pepsin activity was examined according to the method of Worthington (1993). Trypsin and chymotrypsin activities were assessed based on Rungruangsak-Torrissen et al. (2006). Lipase activity was determined according to the method of Winkler and Stuckmann (1979). The alpha-amylase activity was assayed according to the method of Areekijseree et al. (2004). For these four enzyme tests, one unit (U) was determined as the catalyzed amount to convert $1\ \mu\text{mol}$ of substrate per minute. The amylase-to-trypsin ratio was computed as the amylase activity divided by the trypsin activity from the same fish sample.

3.6 Muscle quality

RNA and the protein concentration were assayed according to the method of Rungruangsak-Torrissen (2007). Myosin, actin and the sarcoplasmic contents were monitored based on weighing defrosted white muscle (approximately 10 mg each), placing in an aluminum pan, sealing with an aluminum lid and then heating at a rate of 5 °C min⁻¹ relative to an empty pan. Muscle thermal characteristics were assessed through differential scanning calorimetry (DSC7; Perkin Elmer; Waltham, MA, USA) within the temperature range 20–100 °C, to identify myosin, actin and sarcoplasmic protein based on the onset (T_o), peak (T_p) and conclusion (T_c) temperatures derived from thermograms as documented in Thongprajukaew et al. (2023) concerning betta fish muscle.

3.7 Whole-body composition

The whole-body of each fish was uniformly minced before undergoing analysis for proximate composition, based on the moisture, ash, crude protein and crude lipid contents, following the standard procedures outlined in AOAC (2005).

3.8 Statistical analysis

The trial used a completely randomized design comprising nine herbal formulations, with 15 individual fish as experimental units. The acquired data were presented as mean $\pm$ standard errors of means (SEM) values. The data were subjected to One-Way analysis of variance and mean comparisons were performed using Duncan's multiple range test at the $P < 0.05$ test level for significance. All statistical analyses were performed using the Statistical Package for Social Sciences version 22 (SPSS Inc.; Chicago, IL, USA).

4. Results

4.1 Survival, growth performance and feed utilization

No mortality was observed in the fish reared in any of the herbal treatments. The herbal treatments did not affect the growth and feed utilization parameters, except for FCR, which significantly increased in the fish raised in the CT 2, P, BP, IP and IBP treatments (Table 11).

4.2 Skin coloration

There were significant differences in skin coloration among the fish in the various herbal treatments, except for L^* (Table 12). Relative to CT 1 and CT 2, there was a notable trend of either unchanged or reduced a^* , h^* and C^* values in all treatment groups ($P < 0.05$).

Table 11 Growth performance, and feed utilization of male Siamese fighting fish reared in various medicinal recipes extract, where parameters were measured at end of experiment (at 5 weeks).

Herbal recipe	Parameter					
	FBW (g)	WG (g)	FTL (cm)	VSI (%)	FCR (g feed g gain ⁻¹)	PER (g gain g protein ⁻¹)
CT 1	1.47 ± 0.05	0.43 ± 0.10	4.88 ± 0.08	6.18 ± 0.84	3.30 ± 0.24 ^c	1.08 ± 0.07
CT 2	1.50 ± 0.04	0.28 ± 0.08	5.02 ± 0.04	5.59 ± 0.33	4.28 ± 0.31 ^{ab}	1.11 ± 0.03
IA	1.56 ± 0.02	0.27 ± 0.01	5.01 ± 0.05	6.73 ± 0.37	3.24 ± 0.12 ^c	1.19 ± 0.02
B	1.52 ± 0.02	0.23 ± 0.02	5.00 ± 0.04	6.50 ± 0.35	4.04 ± 0.38 ^{abc}	1.14 ± 0.03
P	1.50 ± 0.03	0.21 ± 0.01	4.78 ± 0.17	5.65 ± 0.40	4.33 ± 0.18 ^{ab}	1.12 ± 0.02
IAB	1.53 ± 0.02	0.24 ± 0.02	4.97 ± 0.05	7.00 ± 0.43	3.75 ± 0.20 ^{bc}	1.16 ± 0.02
BP	1.42 ± 0.04	0.30 ± 0.09	4.88 ± 0.05	5.05 ± 0.22	4.69 ± 0.33 ^a	1.04 ± 0.03
IP	1.53 ± 0.06	0.31 ± 0.09	5.02 ± 0.06	7.01 ± 0.81	4.32 ± 0.21 ^{ab}	1.10 ± 0.02
IBP	1.47 ± 0.03	0.35 ± 0.09	4.98 ± 0.03	6.54 ± 0.45	4.35 ± 0.35 ^{ab}	1.12 ± 0.02
<i>P</i> -value	0.229	0.449	0.290	0.101	0.002	0.082

FBW, final body weight; WG, weight gain; FTL, final total length; VSI, viscerosomatic index; FCR, feed conversion ratio; PER, protein efficiency ratio; IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Data are expressed as means ± SEM (*n* = 15 per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference (*P* < 0.05).

Table 12 The color parameters of male Siamese fighting fish reared in various fermented water from Thai herbal recipes. The observed parameters were recorded at the end of the 5-week experiment.

Herbal recipe	Color parameter			
	L^*	a^*	h^*	C^*
CT 1	23.0 ± 0.7	13.5 ± 0.3^a	0.51 ± 0.01^a	15.5 ± 0.4^a
CT 2	21.5 ± 0.5	14.2 ± 0.5^a	0.50 ± 0.01^{ab}	16.2 ± 0.6^a
IA	23.5 ± 0.7	12.2 ± 0.5^b	0.47 ± 0.01^{abc}	13.8 ± 0.6^b
B	24.3 ± 0.7	11.4 ± 0.3^{bc}	0.45 ± 0.01^{bc}	12.7 ± 0.4^{bc}
P	23.3 ± 0.7	11.0 ± 0.4^{bc}	0.43 ± 0.02^c	12.2 ± 0.5^c
IAB	23.7 ± 0.7	11.6 ± 0.4^{bc}	0.46 ± 0.02^{abc}	13.0 ± 0.4^{bc}
BP	23.6 ± 0.8	11.2 ± 0.4^{bc}	0.48 ± 0.01^{abc}	12.6 ± 0.5^{bc}
IP	23.0 ± 0.3	11.4 ± 0.4^{bc}	0.49 ± 0.01^{ab}	13.0 ± 0.4^{bc}
IBP	22.8 ± 0.5	10.6 ± 0.4^c	0.46 ± 0.02^{bc}	11.8 ± 0.5^c
<i>P</i> -value	0.208	<0.001	0.012	<0.001

L^* , lightness; a^* , redness; h^* , hue; C^* , chroma. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL. Data are expressed as means $\pm$ SEM ($n = 15$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

4.3 Digestive enzyme activities

The herbal treatments had no significant effects on digestive enzyme-specific activities, except for amylase (Fig. 14), for which there was a significant reduction in the fish reared in treatments CT 2, BP, IP and IBP relative to the control group (dechlorinated water).

4.4 Protein synthesis capacity, and myosin and actin contents

There was no significant difference in the values for the average RNA ($2,704 \pm 224 \mu\text{g g}^{-1}$), protein ($210 \pm 24 \text{ mg g}^{-1}$) or RNA-to-protein ratio ($13.4 \pm 1.5 \mu\text{g mg}^{-1}$) among the fish subjected to any of the herbal treatments. The principal muscle proteins, myosin, actin and sarcoplasmic protein levels exhibited temperature ranges of $41.5\text{--}53.4^\circ\text{C}$, $67.5\text{--}74.2^\circ\text{C}$ and $81.0\text{--}85.5^\circ\text{C}$, respectively, from onset heating to the conclusion. Relative to the control group, there were no significant differences in the values for the average myosin ($0.89 \pm 0.11 \text{ J g}^{-1}$), actin ($0.35 \pm 0.05 \text{ J g}^{-1}$) and sarcoplasmic protein ($0.10 \pm 0.02 \text{ J g}^{-1}$) contents, or their total amounts ($1.34 \pm 0.17 \text{ J g}^{-1}$) or proportions ($0.43 \pm 0.07 \text{ J g}^{-1}$) for all herbal treatments.

4.5 Whole-body composition

Relative to CT 1, the moisture contents remained unchanged in all herbal treatments (Table 13, $P > 0.05$), except for the significant reduction in the fish in the BP treatment. No significant differences were observed in the crude protein, crude lipid and crude ash contents across the nine treatments.

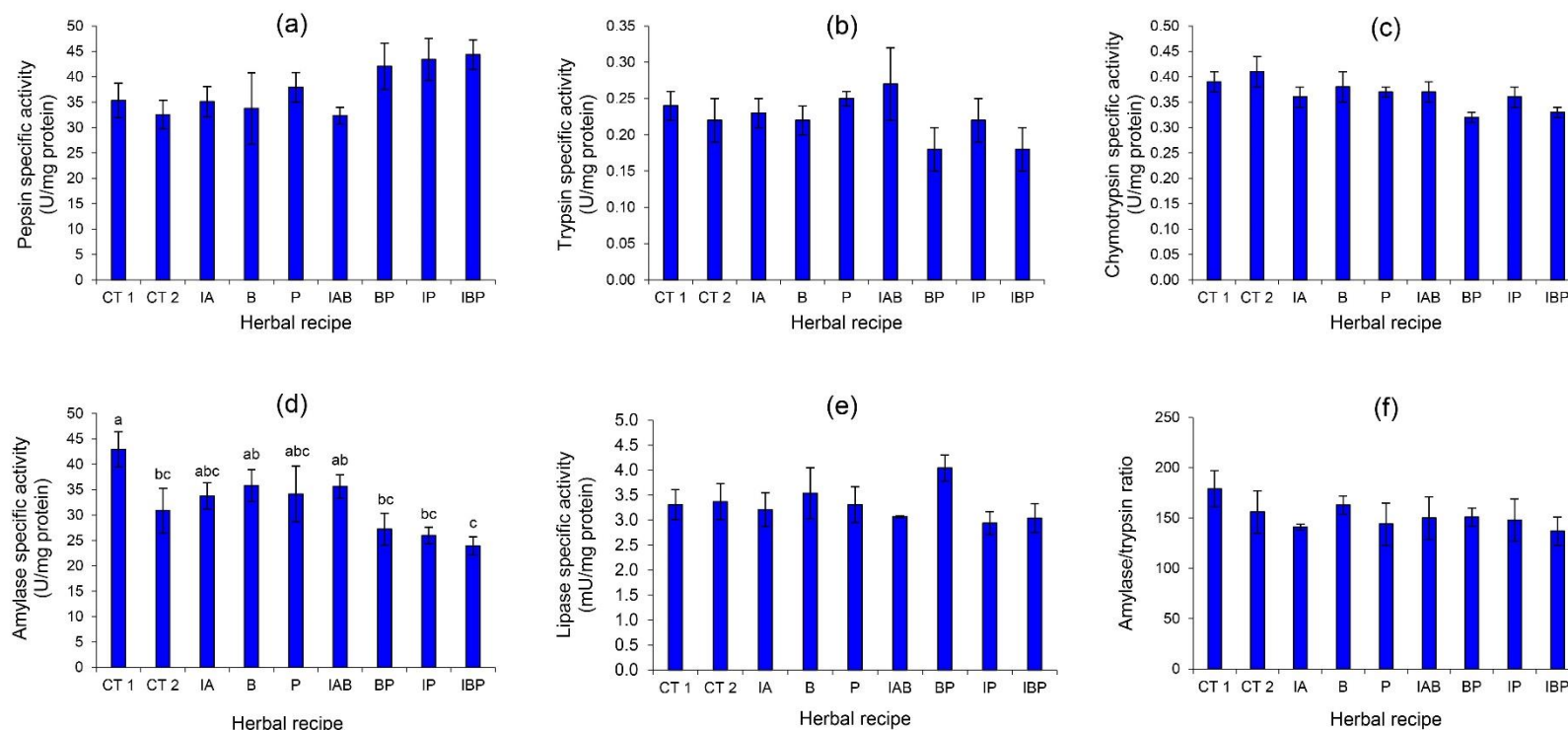


Fig. 14. Specific activities of digestive enzymes in male Siamese fighting fish reared in various treatments using water fermented with Thai herbal recipes: pepsin (a); trypsin (b); chymotrypsin (c); amylase (d); lipase (e); amylase-to-trypsin ratio (f), where parameters measured at end of experiment (at 5 weeks), data = mean + SEM values, different lowercase letters above columns indicate significant ($P < 0.05$) differences between treatments; IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Table 13 Whole-body composition (% of wet weight) of male Siamese fighting fish reared in treatments using water fermented with Thai herbal recipes, where parameters measured at end of experiment (at 5 weeks).

Herbal recipe	Composition			
	Moisture	Crude protein	Crude lipid	Ash
CT 1	70.1 ± 0.8 ^{ab}	17.2 ± 0.7	6.22 ± 0.19	3.82 ± 0.13
CT 2	71.5 ± 1.4 ^a	16.2 ± 0.9	5.52 ± 0.42	4.41 ± 0.29
IA	68.8 ± 0.5 ^{bc}	16.6 ± 0.4	6.59 ± 0.29	5.12 ± 0.56
B	71.2 ± 0.6 ^{ab}	15.6 ± 0.3	5.62 ± 0.58	4.25 ± 0.07
P	70.2 ± 0.3 ^{ab}	16.0 ± 0.6	5.46 ± 0.49	5.12 ± 0.30
IAB	70.6 ± 0.6 ^{ab}	15.5 ± 0.3	6.24 ± 0.43	4.20 ± 0.16
BP	67.4 ± 0.9 ^c	17.1 ± 0.6	6.73 ± 0.57	5.13 ± 0.36
IP	71.9 ± 0.2 ^a	15.6 ± 0.1	4.82 ± 0.17	5.45 ± 0.97
IBP	69.7 ± 1.1 ^{abc}	16.0 ± 0.6	6.12 ± 0.81	4.79 ± 0.31
<i>P</i> -value	0.020	0.210	0.219	0.199

IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

Data are expressed as means ± SEM ($n = 3$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same row indicate a significant difference ($P < 0.05$).

5. Discussion

Another study reported enhancements in growth parameters across several species following rearing in Indian-almond leaf extract at appropriate concentrations (Ikhwanuddin et al., 2014; Nurhidayat et al., 2016; Ramos et al., 2020). Nevertheless, excessive concentrations of these extracts have been proven detrimental to aquatic fauna, such as the neon tetra fish, *Paracheiroidon innesi* (Ardi et al., 2020), the black tiger shrimp, *Penaeus monodon* (Ikhwanuddin et al., 2014), the Siamese fighting fish, *Betta* sp. (Malawa et al., 2022; Nugroho et al., 2016, 2017), the cardinal tetra, *Paracheiroidon axelrodi* (Nurhidayat et al., 2016) and the Amazon leaf fish, *Monocirrhus polyacanthus* (Ramos et al., 2020).

In the current study, there were no fatalities among the injured fish in all herbal treatments throughout the experimental period (5 weeks). Furthermore, the growth-related parameters exhibited no significant variations relative to the control groups (CT 1 and CT 2). Regarding feed utilization, PER remained consistent, aligning with the unchanged specific activities of protein-digesting enzymes (pepsin, trypsin and chymotrypsin). In addition, the lipid-digesting enzyme (lipase) maintained its activity. These results were consistent with the findings reported by Malawa et al. (2022) where 0.25 g L⁻¹ of *T. catappa* leaf extract had no impact on the proteolytic specific activity but led to lipase-specific activity improvement, indicating normal functioning of gastrointestinal digestion for both proteins and lipids.

Notably, there was a significant increase in the FCR among the injured fish reared in the CT 2, P, BP, IP and IBP treatments. This was consistent with a decrease in the amylase-specific activity, except for the P treatment relative to CT 1. However, the amylase-to-trypsin ratio remained constant, suggesting the utilization of available carbohydrates per unit of protein in all herbal remedies, operating in a usual way (Thongprajukaew et al., 2011). These findings aligned with those reported by Malawa et al. (2022) on fish immersed in 0.25 g L⁻¹ of *T. catappa* leaf extract or even for *in vitro* studies with incorporated tannin. Additionally, findings by Maitra and Ray (2003) and Mandal and Ghosh (2010) noted a reduction in the performance of intestinal enzyme

activities, including protease, amylase and lipase. This could be attributed to the herbs selected in the experiment, which contained phytochemical constituents, namely polyphenols categorized into phenolic acids, flavonoids or non-flavonoids (Kumar et al., 2021; Muala et al., 2021; Raphaël et al., 2019; Vuong et al., 2013). These polyphenols, particularly the tannin contents, disrupt protein and dry matter digestibility by creating indigestible complexes (Krogdahl, 1989). However, betta fish are naturally carnivorous and can effectively utilize the protein in their diet. Therefore, herbal treatments are less effective with carnivorous species than with herbivorous fish, specifically in terms of amylase activity, as observed in the study by Mandal and Ghosh (2010). In the current study, an immersion method was used to deliver the herbal extract, resulting in a diminished impact on fish compared to direct dietary intake (Prusty et al., 2007).

Furthermore, the coloration of ornamental fish can serve as a crucial indicator not only of their aesthetic quality but also of their health status (Yi et al., 2014). Nonetheless, the injury stress that occurred during the caudal fin regeneration and color development processes, as documented in the report by Malawa et al. (2023), is a possible factor influencing various physiological processes, including altered shades of body color (Ardi et al., 2020). The current findings identified notable changes, such as significant decreases in some body color parameters (a^* , h^* , C^*) in the injured fish in all herbal treatments, except for CT 2 relative to CT 1. However, the L^* values remained steady. These findings conflicted with the reports by Ardi et al. (2020) and Malawa et al. (2022) that *T. catappa* leaf extracts improved body color quality in healthy fish. The decrease in these values may have been impacted by the color of the fermented herbal water that ranged from deep brown, dark brown and dark gray to dark magenta (Fig. 15). Consequently, the body color of the fish shifted to a shade of brown. In contrast, CT 2 produced a lighter yellow-colored skin than CT 1. This finding was consistent with other research results concerning the utilization of tannin extracted from various leaves as a natural dye, resulting in brown hues, such as those obtained from Indian-almond, papaya or banana leaves (Chitnis, 2013; Rani et al., 2020; Saleh et al., 2013).

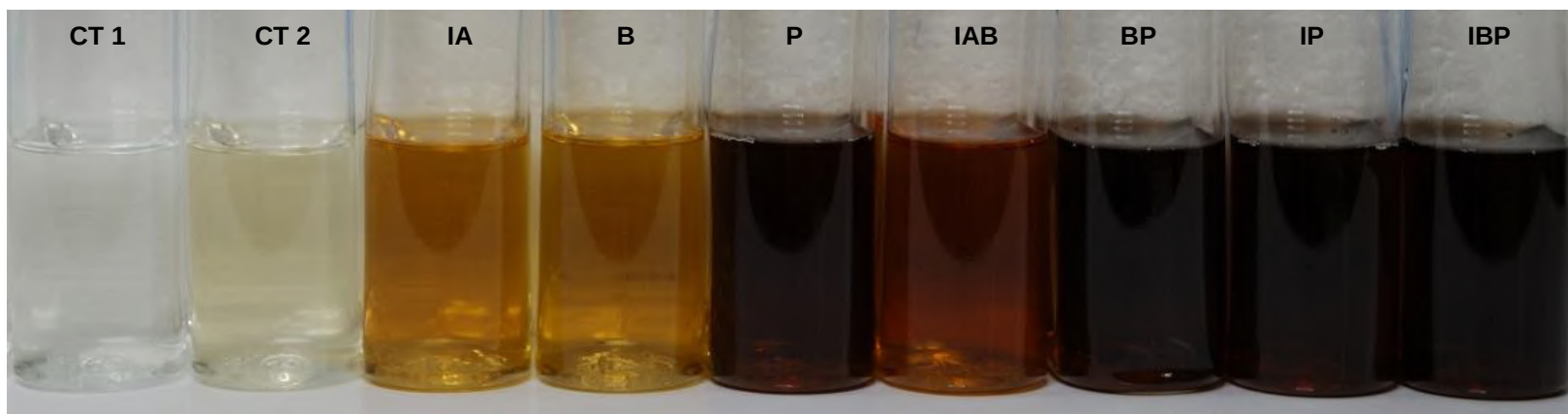


Fig. 15. The color tones of fermented herbal water used for rearing injured male fighting fish. IAL, dried Indian-almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + IAL; B = CT 2 + BL; P = CT 2 + PL; IAB = CT 2 + IAL + BL; BP = CT 2 + BL + PL; IP = CT 2 + IAL + PL; IBP = CT 2 + IAL + BL + PL.

There were no significant differences in the RNA, protein and RNA-to-protein ratio among the fish subjected to any of the herbal treatments compared to CT 1 and CT 2. Additionally, these results corresponded with the stability of the chief muscle components, such as myosin, actin and sarcoplasmic protein and of their total amounts and proportions, suggesting that the herbal treatments had no substantial impact on general physiological movement in the fish. These findings matched well with the unchanged whole-body protein contents. In addition, compensation in the whole-body lipid and ash contents was observed in all the herbal treatments, whereas the moisture content changed significantly only in fish in the BP treatment. Since the biochemical composition would be influenced by the surrounding water in which the fish were reared, the fish immersed in an external solution with a combined concentration (1.5 g L^{-1} BL versus 2.5 g L^{-1} PL) that may have been higher than their body fluids, might have caused water to leave the fish cells through osmosis. In contrast, IAL (0.25 g L^{-1}) alone (Malawa et al., 2022), or perhaps in combination, in a remedy had no impact on the overall whole-body composition.

6. Concluding remarks

Although some herbal recipes have reportedly improved fin regeneration and bubble-nest creation in injured male betta fish (Malawa et al., 2023), some treatments in the current study had negative effects due to lowering feed utilization, as indicated by the FCR, amylase specific activity and skin coloration. Maintained feed utilization without changed growth performance, muscle quality and whole-body composition (albeit with a significant change in whole-body moisture content) were also observed in fish in the water fermentation treatment of 1 g L^{-1} of lemongrass, 50 g L^{-1} of dried clay and 5 g L^{-1} of salt with or without singly 0.25 g L^{-1} of dried Indian-almond leaf, 1.5 g L^{-1} of dried banana leaf, 2.5 g L^{-1} of dried papaya leaf or their combinations, except for the mixture between dried banana and papaya leaves. However, as somatic growth is not a main assessment criterion during fish injury, the current results still endorse using fermented water of Thai herbs for short-term wound healing in betta fish rather than relying on

antibiotics or chemical products that can adversely affect aquatic fauna or their surroundings, or both. Additionally, the active ingredients found in each herb may be affected by the leaf age, extraction solvent, extraction protocol, seasonal variation, geographical location and soil fertility. Differences in these variables could result in differences in the observed outcomes in fish.

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APPENDICES

Appendix A

Manuscript 1

Rearing water containing fermented extracts of Thai herbal formulations improved well-being of injured Siamese fighting fish (*Betta splendens*)

Running title: Herbal treatment improves well-being of injured *B. splendens*

Saowalak Malawa¹, Nutt Nuntapong¹, Naraid Suanyuk¹, Sugree Waeowannajit², Krueawan Yoonram², Karun Thongprajukaew^{2, 3, *}

¹ *Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand*

² *Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand*

³ *Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand*

*** Correspondence:** Karun Thongprajukaew

Emails: karun.t@psu.ac.th (or) karun114@hotmail.com

Abstract

In Thailand, injured Siamese fighting fish (*Betta splendens*) are traditionally treated with herbs rather than antibiotics or chemicals. In this study of traditional herbal treatments, we amputated half the caudal fin of male Siamese fighting fish (1.29 ± 0.01 g) and then divided the fish into nine experimental groups ($n = 15$ per treatment, each fish serving as an experimental unit). The experimental groups comprised a negative control (CT 1) of dechlorinated water and a positive control (CT 2) made up of a water fermentation of dried lemongrass (*Cymbopogon citratus*, 1 g L^{-1}) leaf, dried clay (50 g L^{-1}), and salt (5 g L^{-1}). The seven treatments were based on CT 2 which was supplemented with combinations of dried leaf of one, two or all three of the following: Indian almond (*Terminalia catappa*, 0.25 g L^{-1}), banana (*Musa sapientum*, 1.5 g L^{-1}), and papaya (*Carica papaya*, 2.5 g L^{-1}). Every fish was reared for five weeks in its own small tank. The antioxidant activity of fermented herbal extracts in both controls and all treatments was determined. The water in all treatments showed significantly better antioxidant activity than both controls ($P < 0.05$). Physico-chemical water parameters improved compared with CT 1, especially total ammonia nitrogen, nitrite, and nitrate ($P < 0.05$). Survival, growth, and feed utilization were similar across all treatments ($P > 0.05$). Bubble-nests were superior in height, frequency, area, and number in CT 2 and three treatments that contained either banana leaf, banana leaf and papaya leaf, or Indian almond leaf and papaya leaf ($P < 0.05$). Negative effects in hematological parameters were observed in the treatment that contained banana leaf and papaya leaf together. CT 2 and three herbal treatments improved the antioxidant and physico-chemical properties of the rearing water, and bubble-nest building ability of injured *B. splendens*.

Keywords: *B. splendens*; blood parameter; bubble-nest; fin amputation; herbal treatment

1. Introduction

The Siamese fighting fish (*Betta splendens*), or betta fish, is a freshwater fish with gorgeous coloration and fascinating fin characteristics. The species originated in Southeast Asia, and is especially associated with the central region of Thailand (Goldstein, 2004; Monvises et al., 2009). Decades ago, the ornamental fish industry in Thailand began breeding and exporting Siamese fighting fish (DOF, 2021). The United States is now the biggest importer of Siamese fighting fish from Thailand, followed by China, France, Iran, and Singapore (Sermwatanakul, 2019).

Since the male fish are bigger, more vibrantly colored, and have longer fins than females (Jaroensutasinee and Jaroensutasinee, 2001; Thongprajukaew et al., 2014), only male fish are of interest to keepers of *B. splendens*, and only male fish are exported. The male betta fish is agonistic and displays to defend his territory or upon encountering opponents. These behaviors dictate the practices used in the commercial production and management of this species. At 1.5 months, male fish are placed in solitary captivity to avoid the negative effects of their aggressive behavior (Thongprajukaew et al., 2010). However, damaged bodies and torn fins often result from the matching of male fish for betting, as well as courtship and reproductive behaviors during mating (Malawa et al., 2023). Secondary infections of wounds by *Mycobacterium* result in clamped fins, fin wasting, dulled coloration, lethargy and loss of appetite (Lichak et al., 2022).

Injured and infected betta fish are traditionally treated in Thailand with fermented herbal extracts. The herbal formulations used are well-established combinations that differ in the quantity and type of active ingredients but typically comprise phytoconstituents such as tannin, flavonoids and phenolic compounds. For instance, the dried leaf of the Indian almond (*Terminalia catappa*) aids wound healing and combats harmful monogenean and *Trichodina* spp. (Chitmanat et al., 2005; Ponpornpisit et al., 2007). The dried leaf of banana (*Musa sapientum*), papaya (*Carica papaya*), and lemongrass (*Cymbopogon citratus*) provide anti-bacterial, anti-oxidant, and anti-inflammatory activities that help heal wounds (Maria et al., 2021; Tanmoy et al., 2021; Wijaya et al., 2020), especially those caused by fighting. However, the literature about

fighting fish contains no report that describes the efficacy of treating wounds with herbal fermentations (Banplakatrachong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Some treatments have included dried clay and salt, added to heal wounds, nourish the skin, reduce pathogen growth and help fish recover faster (Chiarachavildham, 2016; Puello-Cruz et al., 2010; Putiyanan, 1999). Certain herbs are used to improve water quality. Extracts of *T. catappa* leaf, for instance, reduce water turbidity, pH, and total ammonia (Malawa et al., 2022; Nozaleda, 2019; Pimolrat et al., 2018; Puttamat and Suwannasarn, 2007) but excessive concentrations can increase acidity and ammonia (Ikhwanuddin et al., 2014).

Apart from the therapeutic effects of herbal treatments, male betta fish behaviors can be influenced by some herbs that contain phytoestrogens. Males construct bubble-nests and betta fish reared in water containing herbs have been observed to construct significantly bigger bubble-nests (Malawa et al., 2022). In general, male fish, juveniles as well as adults, create a bubble-nest by exhaling mucus-coated bubbles rich in glycoproteins. The quality, size and area of the bubble-nest are essential for supplying oxygen to eggs and larvae, which are nursed by males (Jaroensutasinee and Jaroensutasinee, 2003). Moreover, bubble-nest building capacity plays a vital role in health evaluations. Active bubble-nest builders exhibit lower levels of stress-induced cortisol than non-builders (Agues-Barbosa et al., 2022). Furthermore, herbal treatments can also improve hematological parameters (Malawa et al., 2022; Nugroho et al., 2016, 2017) and stimulate skin coloration in aquatic animals (Malawa et al., 2022).

Although the treatment of Siamese fighting fish with Indian almond leaf has been reported (Malawa et al., 2022; Purivirojkul, 2012), information regarding the right combination of ingredients and their effects is still unavailable. Therefore, the objective of this study was to assess the well-being of injured fish treated with a range of herbal formulations. The anti-oxidant activity of fermented herbal water, the physico-chemical parameters of rearing water, bubble-nest construction, and blood parameters were used as assessment criteria. The caudal fins of male fish were amputated to simulate fins torn in male-female courtship and reproductive behaviors or in male-male combative behavior

(Malawa et al., 2023). The most effective herbal formulations used in this study could boost animal welfare and help support betta fish farming.

2. Materials and methods

2.1. Animal protocols

The present protocols conformed to the use of animals for scientific purposes approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Project Code 2564-01-030) under a larger project entitled “Innovations from indigenous knowledge for using Indian-almond leaf (*Terminalia catappa*) and herbal formulations for rearing and wound healing in Siamese fighting fish (*Betta splendens*)”.

2.2. Preparation of fermented herbal extract

All the fermented herbal formulations were based on powdered dried leaf of lemongrass (LL, 1 g L⁻¹), dried clay (50 g L⁻¹), and salt (5 g L⁻¹). Additional constituents were powdered dried leaf of Indian almond (IAL, 0.25 g L⁻¹), powdered dried leaf of banana (BL, 1.5 g L⁻¹), and powdered dried leaf of papaya (PL, 2.5 g L⁻¹). The plant leaves were from farms in Songkhla province in southern Thailand. The leaves were first immersed in 5 mg L⁻¹ of potassium permanganate (KMnO₄) for 15 min (Carpenter et al., 2001; Noga, 1996) and washed three times with distilled water to remove attached impurities. The clean samples were oven-dried at 60°C for 12 h, and then finely powdered using a blender (WF-04, Siam Wealth Food Co., Ltd., Nonthaburi, Thailand), sieved, homogeneously mixed, packed in polyethylene bags, and kept at 4°C until used. Clay was oven-dried after visible impurities were removed, and prepared as described for the components above.

The ingredients and amounts of each ingredient in all the studied herbal formulations are shown in Table 1. Two controls were designed: dechlorinated tap water (CT 1), and a dechlorinated water fermentation of LL, containing clay and salt (CT 2). CT 2 was then used as the basis of seven treatments. Three treatments, respectively labeled IA, B and P, were obtained by adding Indian almond leaf (IAL), banana leaf (BL), or

papaya leaf (PL) to CT 2. Three more treatments, respectively labeled IAB, IP and BP, were obtained by adding IAL and BL, IAL and PL, and BL and PL to CT 2. The seventh treatment, labeled IBP, was obtained by adding IAL, BL and PL to CT 2. To prepare each treatment, the appropriate dried leaf powders were mixed with dechlorinated tap water in 30 L plastic containers (31 cm width $\times$ 30 cm length $\times$ 49.7 cm height). The mixtures were fermented at room temperature for three days, with stirring twice daily, before being passed through filter cloth and filtered through Whatman No. 4 paper (Malawa et al., 2022). Dried clay was wrapped in filter cloth and placed in each small individual fish tank, except in CT 1. The chosen concentration of each herb in the formulations was based on available data (Banplakatrachong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Amounts of dried clay were estimated from Chiarachavildham (2016). Table salt was directly added to the rearing water of CT 2 and treatments to achieve a final concentration of 5 g L⁻¹ (Puello-Cruz et al., 2010).

2.3. Antioxidant characteristics of fermented herbal extracts

2.3.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free DPPH radical scavenging activity was measured based on the method described by Madhujith et al. (2006) with some modifications. In brief, a DPPH working solution was prepared by diluting a stock solution of 0.2 mM DPPH in 95% ethanol until absorbance was 1.0 ± 0.5 when measured at 517 nm. Then, 3 mL of the working solution were mixed with 100 μ L of CT 1, CT 2, and the seven treatments. Scavenging activity (%) was calculated from the equation $[(A_0 - A_i)/A_0] \times 100$ where A_0 and A_i were the absorbance values of the buffer and sample, respectively.

2.3.2. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

Free ABTS^{•+} radical scavenging activity was determined according to the method of Oladipo and Ibukun (2017). Briefly, stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate in phosphate buffer saline (pH 7.4) were mixed in a ratio of 1:1 and left for 16 h in darkness. The working solution was prepared by adding 5 mL of the mixed

stock solutions to 145 mL of distilled water. Scavenging capacity was measured at 734 nm by mixing 900 μ L of working solution with 100 μ L of CT 1, CT 2, and the seven treatments. Scavenging activity (%) was calculated from equation $[(A_0 - A_i)/A_0] \times 100$ where A_0 and A_i were the absorbance values of buffer and sample, respectively.

2.4. Fish preparation and fin amputation

Three-month-old, solid-red male Siamese fighting fish were purchased from a farm in Nakhon Pathom Province, Thailand. They were separately acclimatized in cylindrical plastic containers (9.3 cm diameter $\times$ 12.5 cm height $\times$ 8 cm depth, containing 350 mL of water) for two weeks. The fish were then screened (1.29 ± 0.01 g body weight; 6.67 ± 0.02 cm total length; $n = 135$) and randomly divided into nine treatments of fifteen fish each. The fish were anesthetized with a 15 mg L⁻¹ solution of clove oil (L.B. Science, Songkhla, Thailand) and half the caudal fin was amputated in the vertical plane using a sterilized scalpel (Fig. 1).

2.5. Herbal treatment trial

A diagram showing the herbal treatment of amputated fish is showed in Figure 1. After amputation, each fish was carefully transferred to a cylindrical plastic container (9.3 cm diameter $\times$ 12.5 cm height $\times$ 8 cm depth) holding 350 mL of CT 1, or CT 2, or one of the seven treatments (IA, B, P, IAB, BP IP, and IBP). Each fish was reared in isolation from the other fish for five weeks under a 12-h light (06.00 to 18.00 h)/12-h dark light regime. Every three days, 80% of the water in each container was replaced, including fresh control or treatment. Twice daily, at 08.00 and 17.00 h, the fish received a commercial floating feed (39.4% crude protein) for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). After 30 min, remaining feed was collected and dried at 60°C until a constant weight was reached. The data were used to compute the feeding rate, and the amount of feed given was adjusted weekly according to the recorded feed intake. Fish mortality was monitored daily throughout the experiment. At the end of the

trial, all the fish ($n = 15$ per treatment) were starved for 24 h and sacrificed by chilling in ice. Body weight was then measured and used to calculate specific growth rates. The parameters describing growth and feeding rate were calculated as follows:

$$\text{Specific growth rate (\% body weight day}^{-1}\text{)} = 100 \times [(\ln W_t - \ln W_o)/(t - t_o)]$$

where W_t = mean weight (g) at day t and W_o = mean weight (g) at day t_o ; and

$$\text{Feeding rate (\% body weight day}^{-1}\text{)} = 100 \times C/[(W_o + W_t)/2]/t \text{ where } C = \text{daily feed consumption (g), } W_o = \text{initial body weight (g), } W_t = \text{final body weight (g) and } t = \text{feeding duration (day).}$$

2.6. Water quality

During the experimental period, the quality of rearing water was monitored twice weekly before the water was changed. Physical and chemical water qualities, including pH, temperature, conductivity, salinity, and total dissolved solids (TDS), were measured using a multi-parameter water quality meter (U-52G; Horiba, Kyoto, Japan). Dissolved oxygen (DO) was determined using a modified Winkler method. Total alkalinity and hardness were measured by titration with sulfuric acid and ethylenediaminetetraacetic acid (EDTA), using methyl orange and Eriochrome Black-T as respective indicators. Total ammonia nitrogen was measured by using the phenate method. These four parameters were analyzed based on the procedure of Boyd and Tucker (1992). Nitrite (by colorimetry) and nitrate (by cadmium reduction) were examined based on the methods of APHA, AWWA, and WPCF (1998).

2.7. Bubble-nest building behavior

Each cylinder with fish ($n = 15$ per treatment) was placed in a box (20 cm width $\times$ 20 cm length $\times$ 26 cm height) with a black background illuminated by an off-white LED. Twice a week, a top-view photograph was taken with a smartphone (iPhone 13 Pro, Apple Inc., China) to record bubble-nest formations. The frequency of bubble nest building (%) was calculated by dividing the number of cylinders containing bubble-nests by the number of cylinders in each treatment (15), and multiplying the quotient by 100. The total

area of each bubble-nest (μm^2) and the individual bubble area (μm^2) were quantified by Image J software, as described by Malawa et al. (2022). The number of bubbles was computed by dividing the total area of each bubble-nest by the individual bubble area. The Plot Profile tool in Image J was selected to generate a graph between gray values and bubble-nest distance (μm) from each top-view image. Bubble-nest height was expressed as the calculated mean gray value. A diagram showing how bubble-nest parameters were monitored is presented in Figure 2.

2.8. Hematological parameters

Blood samples were taken from the caudal peduncle and samples from three fish were pooled to obtain five pooled samples per treatment ($n = 5$ per treatment). Each pooled sample was placed in an Eppendorf tube coated with 1% EDTA. Red and white blood cells (RBC and WBC) were counted manually on a hemacytometer under a compound microscope at $40\times$ magnification (cells mL^{-1}). Hemoglobin (Hb) was analyzed by the cyanmethemoglobin method (Blaxhall and Daisley, 1973). To calculate hematocrit (Hct), blood samples were centrifuged in capillary tubes and Hct % was calculated from the length of the RBC component divided by the length of the total blood sample. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\begin{aligned}\text{MCV (fL)} &= 10 \times [\text{Hct (\%)} / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})], \\ \text{MCH (pg)} &= 10 \times [\text{Hb (g dL}^{-1}) / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})], \text{ and} \\ \text{MCHC (g dL}^{-1}) &= 100 \times [\text{Hb (g dL}^{-1}) / \text{Hct (\%)}].\end{aligned}$$

2.9. Statistical analysis

The sample size was computed on the R 3.6.0 program to obtain a test power of 0.8. The trial was a completely randomized design comprising eight herbal formulations and a non-herbal control, each of which contained fifteen fish serving as individual experimental units. The obtained data were expressed as means $\pm$ standard errors (SEM). All statistical values were performed under the Statistical Package for Social Sciences

version 22 (SPSS Inc., Chicago, USA). Differences between treatment groups were assessed by One-Way Analysis of Variance (ANOVA) with Duncan's Multiple Range Test as a *post hoc* test. Rejection of hypothesis was based on a *P*-value equal to or lower than 0.05.

3. Results

3.1. Antioxidant characteristics of fermented herbal extract

Significant improvement in radical scavenging activity was observed in all herbal treatments relative to CT 1 and CT 2 (Fig. 3, $P < 0.05$). Relative to controls, treatment B had the lowest DPPH scavenging activity followed by treatment BP (Fig. 3a). High DPPH scavenging activity was observed in treatments IA, P, and IAB, whereas treatments IB and IBP showed intermediate results. The ABTS assay did not show the same inhibition trend as the DPPH assay, except for the higher scavenging activity of the treatments relative to CT 1 and CT 2 (Fig. 3b). The highest inhibition was found in treatment IBP followed by treatments IP and BP. The remaining treatments (P, IAB, IA, and B) exhibited relatively low scavenging activity.

3.2. Chemical and physical qualities of water

Compared with CT 1, water pH, salinity, total alkalinity, and hardness significantly were higher in all herbal treatments while total ammonia nitrogen (TAN), nitrite, and nitrate were lower (Fig. 4, $P < 0.05$). DO levels were the same or higher in all treatments compared to CT 1, except for treatment IBP (Fig. 4, $P < 0.05$). The water temperature profile remained similar over the five-week trial (Fig. 5, $P > 0.05$) while conductivity and TDS increased significantly in all treatments (Fig. 5, $P < 0.05$).

3.3. Survival, growth performance, and feed utilization of amputated fish

At the end of the trial, the survival of fish was 100% in all treatments. The treatments had no effect on growth (final body weight = 1.50 ± 0.03 g, final total length = 4.95 ± 0.06 cm, specific growth rate = $0.46 \pm 0.03\%$ body weight day⁻¹) or feed utilization (feeding rate = $1.82 \pm 0.03\%$ body weight day⁻¹) ($P > 0.05$).

3.4. Bubble-nest ability of amputated fish

Top-view photographs of bubble-nests are presented in Figure 6a. Relative to CT 1, significant increases in bubble-nest height, indicated by gray values, were observed in all experimental treatments except IAB (Fig. 6b, $P < 0.05$). The frequency of bubble-nest creation was similar in treatments P, IAB, and IBP (Fig. 7a, $P > 0.05$), while the other remaining treatments raised creating ability significantly ($P < 0.05$). Total bubble-nest area was significantly greater in all treatments relative to CT 1 (Fig. 7b, $P < 0.05$) but individual bubble-nest area was lower compared to CT 1 in all treatments except IA, and in CT 2 (Fig. 7c, $P > 0.05$). The number of bubbles produced was similar in CT 1, IA, and IAB (Fig. 7d, $P > 0.05$) but significantly higher in all other treatments relative to CT 1 ($P < 0.05$).

3.5. Hematological parameters of amputated fish

Hematological parameters were significantly different in all treatments (Table 2, $P < 0.05$), except for WBC. Significantly lower RBC counts were observed in BP and IP treatments ($P < 0.05$) but Hct % in these two treatments remained stable compared to CT 1 ($P > 0.05$). Slightly improved ($P < 0.05$) or constant ($P > 0.05$) Hb, MCHC, MCH, and MCV were observed in all treatments except for treatment IBP, where fish showed a significantly lower MCV ($P < 0.05$).

4. Discussion

4.1. Improvement of water parameters by herbal treatments

The herbs used in our experiment (IAL, BL, PL, and LL) possibly contain phenolic acids, flavonoids, phenols and other phytochemical constituents (Kumar et al., 2021; Muala et al., 2021; Raphaël et al., 2019; Vuong et al., 2013) that could have significantly improve the antioxidant capacity of all the treatments relative to the controls. Free radical quenching by antioxidants in the fermented herbal water treatments was better reflected in the inhibitory activities revealed by the ABTS assay than the DPPH assay. The inconsistency between the results obtained from the same treatments was due to the divergent properties of the reactive media. DPPH[•] is suitable for hydrophobic antioxidants, whereas ABTS⁺⁺ is suitable for hydrophilic and lipophilic antioxidants (Re et al., 1999).

Except for water temperature, the physico-chemical characteristics of the fermented herbal water treatments were also significantly different. The amounts and profiles of the active ingredients in each formulation may have had direct effects on water quality. Water pH levels were significantly higher in CT 2 and all herbal treatments relative to CT 1. The increased pH could be attributed to a higher proportion of phytochemicals with a pH range between 8 and 10, such as flavonoids, than mildly acidic phytochemicals like tannic acid, depending on the nature and position of adjacent groups (Prasad et al., 2023; Yan et al., 2020). However, the observed pH range was in line with water quality recommendations (pH 6.5–8.0) for betta fish aquaculture (Saekhow et al., 2018; Thongprajukaew et al., 2019) and was consistent with some reported usage; for example, IAL extract in betta fish rearing (Malawa et al., 2022; Nugroho et al., 2016). Although total alkalinity and DO fluctuated significantly among treatments, both parameters remained within the most satisfactory range for aquaculture, indicating the capacity of pH-buffering to stabilize water pH (Meade, 1989; Saekhow et al., 2008; Thongprajukaew et al., 2019). DO levels were sufficient for good fish production (Bhatnagar et al., 2004).

The addition of salt to the treatments significantly increased salinity levels due to dissolved ions, compared to CT 1. This finding was consistent with the use by Fabregat et al. (2017) and Puello-Cruz et al. (2010) of saline levels lower than or equal to 5 g L⁻¹ that maintained survival and growth but removed ectoparasites in betta larvae. Slightly increased salinity may reduce the metabolic energy required by betta for the osmoregulation process necessary to wound healing (Altinok and Grizzle, 2001). Salt aids wound drying by forcing intracellular fluid out of the body. Therefore, the growth of new tissue is accelerated quicker skin contact (Altinok and Grizzle, 2001; Samidah et al., 2021). Increased water salinity levels, and dissolved minerals from the clay resulted in increased water conductivity, TDS, and hardness (Boyd, 1979; Rusydi, 2018). Although conductivity in our study was slightly above the range generally considered acceptable for betta fish (Thongprajukaew et al., 2019), TDS and hardness levels were within the aquaculture water quality standards (Meade, 1989). In addition, water hardness levels remained between 150 and 320 mg L⁻¹ where no adverse effects could be observed on growth, reproductive potential or bubble-nest creation in betta fish (Krishnakumar et al., 2020).

The significant decrement in N-waste levels (NH₃, NO₂, and NO₃) observed in all treatments was possibly due to the interaction of tannins (OH groups) with nitrogenous substances (NH₂, SH, OH, and CO groups) through the formation of hydrogen bridges (Siebert et al., 1996). Braghiroli et al. (2013) described the reaction of condensed tannins with NH₃ in water. The reaction resulted in the amination and cross-linking of phenolic hydroxyl groups and the gelation of tannin/ammonia via the formation of nitrogen bridges between flavonoid units. The improvement of water quality realized with the bio-treatments in the present study is consistent with previous reports of the use of herbs such as IAL (Malawa et al., 2022; Pimolrat et al., 2018; Puttamat and Suwannasarn, 2007) or even BL (El-DougDoug et al., 2020).

Betta fish have relatively slow behavior and are often reared with *ad libitum* feeding in low water volumes that are typically refreshed only every three days. These conditions may lead to low DO levels and elevated NO₂ and NO₃ values in rearing water,

but they can survive in these conditions, and the reported values remained within an acceptable range for betta fish culture (Saekhow et al., 2018). Also, there is a minor effect possibly caused by nitrifying bacteria. However, this effect is minimal because some active ingredients in the fermented herbal extracts may inhibit bacterial growth. Therefore, herbal treatments are recommended for maintaining water quality and reducing the necessity for frequent water changes. Currently, there are some recommended herbal products on the market, including fresh leaves, dried leaves, and concentrated extracts. However, it is possible to develop the treatments proposed here with appropriate usage patterns for commercial applications to improve water quality for fish farming, thereby reducing the need for frequent water changes and maintaining the well-being of the fish. The developed treatments would offer alternatives to consumers and add value to the products used.

4.2. Improvement of bubble-nest creating ability by herbal treatments

At the end of the five-week observation, none of the amputated fish had died. Fin length was completely regenerated, and growth performance and feed utilization were similar across all seven treatments and two controls. These unchanged parameters indicated normal growth without negative effects from the herbal treatments. No external signs, such as non-bleeding wounds, inflammation or mucous covering, were observed in any amputated fish, and positive effects on water quality and fish well-being were clearly observed.

Bubble-nests are composed of glycoprotein-rich mucous-coated bubbles that facilitate oxygenation of the developing fertilized fish eggs and larvae (Kang and Lee, 2010). Behaviorally, the size or height of nests can predict reproductive success in male fish (Jaroensutasinee and Jaroensutasinee, 2001). Bubble-nests of captive betta fish were reported to have an average area of 2.41 to $7.19 \times 10^9 \mu\text{m}^2$ (Srikrishnan et al., 2017). In the present study, significant increments of frequency, total bubble-nest area, number of bubbles, and nest height (gray value) were observed in all herbal treatments. Individual bubble-nest area was proportionally changed and was the reciprocal of total bubble-nest

area ($r = -0.696$, $P < 0.05$). The injured fish recuperated in the BP treatment showed the highest bubble-nest building capacity in terms of total bubble area, number of bubbles, height and frequency relative to CT 1. A previous study (Malawa et al., 2022) observed healthy betta fish reared in water containing a suitable concentration (0.25 g L^{-1}) of fermented IAL. Total bubble-nest area and frequency were significantly higher than in non-fermented treatments. It is possible that the isoflavones genistein and equol, which are phytoestrogen derivatives, obtained from a combination of plant leaves stimulate bubble-nest building by male betta fish (Clotfelter and Rodriguez, 2006; Vadvalla and Kola, 2017), and the amputation of fins reduces the frequency of bubble-nest building. However, among the present treatments, bubble-nests produced by fish recuperated in CT 2, B, BP, and IP showed superior characteristics. Phytoestrogen derivatives or other bioactive compounds in those particular herbal constituents may trigger bubble-nest building. Synergistic effects from the diverse range of compounds in the formulations may also affect this behavioral response. In contrast, since final body weight and total length of all the fish at the end of observation were not significantly different, it was concluded that neither parameter affected nesting ability in this study.

4.3. Hematological responses due to herbal treatments

Hematological analyses can be used as crude indications of health. In the present study, none of the herbal treatments affected the average WBC values of the amputated fish. The observed values were above the range for healthy male betta reported by Motlagh et al. (2012). It is possible that the wound healing process had terminated at the end of five weeks, so that neutrophils, lymphocytes, and monocytes had ceased migrating to damaged tissues (Sonmez and Sonmez, 2017).

The reported ranges of RBC, Hct and Hb in healthy betta erythrocytes are respectively $1.64\text{--}4.20 \times 10^9 \text{ cells mL}^{-1}$, 16.7–44.3%, and $6.22\text{--}11.8 \text{ g dL}^{-1}$ (Kari et al., 2023; Malawa et al., 2022; Motlagh et al., 2012; Nugroho et al., 2016, 2017). In our study, the groups that showed the best bubble-nest building ability (CT 2, B, BP, and IP), exhibited values for these erythrocytes that were similar or within the above ranges.

However, fish reared in the BP treatment were an outlier group in terms of Hct, and might be at risk from a hematological disorder; probably polycythemia (Clauss et al., 2008; Hrubec and Smith, 2000), which reduces the transport of oxygen into tissues. Moreover, based on Pearson correlation analysis, bubble nesting (total bubble area) was negatively correlated with RBCs ($r = -0.674$, $P < 0.05$). Therefore, the decreased RBCs in the BP treatment may be due to the effect of this herbal recipe on the proliferation of stem cells.

MCV, MCH, and MCHC reflect the size and hemoglobin content of erythrocytes. The amputated fish recuperated in the BP treatment had relatively high MCV and MCH values, which might be linked with macrocytic anemia (Javed et al., 2016). However, the MCHC of fish treated with BP did not change significantly relative to fish in CT 1. Certain negative effects observed in our investigation proscribe recuperating injured fish in the BP formulation.

5. Conclusions

Recuperating injured male Siamese fighting fish in suitable formulations of herbs fermented in water significantly supported the well-being of the fish by improving water quality and water antioxidant activity and by accelerating bubble-nest building. At the same time, no undesirable effects on survival, growth, feed utilization, and blood parameters were observed. Phytochemical constituents of the various herbs in these formulations might contribute biological activities that promote these positive responses. Our findings suggest that the well-being of fish suffering damage to their body and fins could be promoted by rearing them in a water fermentation of 1 g L⁻¹ of lemongrass supplemented with 50 g L⁻¹ of dried clay and 5 g L⁻¹ of salt. This formulation was also effective when the water fermentation included 1.5 g L⁻¹ of dried banana leaf, or 0.25 g L⁻¹ of dried Indian almond leaf, or 2.5 g L⁻¹ of dried papaya leaf. These herbal formulations could also be applied for a short term to enhance the bubble-nest building of mature male betta during courtship and reproduction. Furthermore, these findings support the use of Thai folk wisdom in the management of betta fish farming.

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Table 1 The composition of traditional Thai herbal formulations used to treat male Siamese fighting fish after caudal fin amputation. The symbols × and ✓ indicate the absence and presence of ingredients, respectively. Permission to reuse this table was granted by Elsevier (License No. 5617450078926).

Herbal formulation	Ingredient					
	IAL (0.25 g L ⁻¹) ¹	BL (1.5 g L ⁻¹) ²	PL (2.5 g L ⁻¹) ³	LL (1 g L ⁻¹) ⁴	Clay (50 g L ⁻¹) ⁵	Salt (5 g L ⁻¹) ⁶
CT 1	×	×	×	×	×	×
CT 2	×	×	×	✓	✓	✓
IA	✓	×	×	✓	✓	✓
B	×	✓	×	✓	✓	✓
P	×	×	✓	✓	✓	✓
IAB	✓	✓	×	✓	✓	✓
BP	×	✓	✓	✓	✓	✓
IP	✓	×	✓	✓	✓	✓
IBP	✓	✓	✓	✓	✓	✓

IAL, dried Indian almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT, control; IA, treatment containing IAL as key ingredient; B, treatment containing BL as key ingredient; P, treatment containing PL as key ingredient; IAB, treatment containing IAL and BL as key ingredients; BP, treatment containing BL and PL as key ingredients; IP, treatment containing IAL and PL as key ingredients; IBP, treatment containing IAL, BL, and PL as key ingredients.

¹⁻⁶ The herbal formulation concentrations are as recommended by Malawa et al. (2022)¹, Rucksapram (2007)², Banplakatraying (2022)³, Institute for Innovative Learning (2022)⁴, Chiarachavildham (2016)⁵, and Puello-Cruz et al. (2010)⁶.

Table 2 Hematological parameters of male Siamese fighting fish after caudal fin amputation. The measurements were performed at the end of a five-week trial in which the fish were recuperated in water fermentations of various herbal formulations.

Herbal formulation	Hematological parameter						
	RBC ($\times 10^9$ cells mL ⁻¹)	WBC ($\times 10^7$ cells mL ⁻¹)	Hct (%)	Hb (g dL ⁻¹)	MCHC (g dL ⁻¹)	MCH (pg)	MCV (fL)
CT 1	3.14 $\pm$ 0.12 ^{ab}	3.82 $\pm$ 0.33	46.8 $\pm$ 3.5 ^a	11.2 $\pm$ 1.1 ^{bc}	24.2 $\pm$ 2.4 ^c	35.7 $\pm$ 3.3 ^c	138 $\pm$ 10 ^{bc}
CT 2	2.80 $\pm$ 0.07 ^{cde}	4.15 $\pm$ 0.24	34.3 $\pm$ 3.0 ^c	12.0 $\pm$ 0.4 ^{abc}	35.5 $\pm$ 1.8 ^a	42.8 $\pm$ 1.6 ^{ab}	122 $\pm$ 10 ^{bcd}
IA	3.38 $\pm$ 0.07 ^a	4.68 $\pm$ 0.20	31.9 $\pm$ 6.1 ^c	13.3 $\pm$ 0.7 ^a	37.3 $\pm$ 3.9 ^a	39.5 $\pm$ 2.4 ^{bc}	112 $\pm$ 10 ^{cd}
B	2.99 $\pm$ 0.07 ^{bc}	4.07 $\pm$ 0.16	36.0 $\pm$ 2.1 ^{bc}	12.8 $\pm$ 0.8 ^{ab}	36.0 $\pm$ 2.4 ^a	42.8 $\pm$ 1.6 ^{ab}	120 $\pm$ 6 ^{bcd}
P	3.13 $\pm$ 0.05 ^{ab}	4.34 $\pm$ 0.25	34.8 $\pm$ 4.1 ^c	13.5 $\pm$ 0.4 ^a	34.7 $\pm$ 2.6 ^{ab}	43.2 $\pm$ 1.7 ^{ab}	122 $\pm$ 7 ^{bcd}
IAB	3.07 $\pm$ 0.08 ^b	4.16 $\pm$ 0.36	35.5 $\pm$ 1.5 ^c	12.8 $\pm$ 0.5 ^{ab}	36.4 $\pm$ 2.7 ^a	41.7 $\pm$ 1.8 ^{bc}	116 $\pm$ 6 ^{cd}
BP	2.60 $\pm$ 0.08 ^e	3.46 $\pm$ 0.47	46.3 $\pm$ 4.0 ^{ab}	12.7 $\pm$ 0.3 ^{ab}	28.0 $\pm$ 2.1 ^{bc}	48.6 $\pm$ 2.6 ^a	168 $\pm$ 14 ^a
IP	2.70 $\pm$ 0.11 ^{de}	4.26 $\pm$ 0.14	41.4 $\pm$ 1.8 ^{abc}	10.1 $\pm$ 0.6 ^c	25.6 $\pm$ 2.0 ^c	37.6 $\pm$ 2.3 ^{bc}	144 $\pm$ 7 ^{ab}
IBP	2.88 $\pm$ 0.09 ^{bcd}	3.91 $\pm$ 0.15	30.1 $\pm$ 1.4 ^c	12.6 $\pm$ 0.3 ^{ab}	42.4 $\pm$ 2.1 ^a	44.1 $\pm$ 1.3 ^{ab}	105 $\pm$ 4 ^d
P-value	<0.001	0.170	0.010	0.010	<0.001	0.012	0.001

RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; CT, control; IA, treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient; B, treatment containing dried banana (*Musa sapientum*) leaf as key ingredient; P, treatment containing dried papaya (*Carica papaya*) leaf as key ingredient; IAB, treatment containing dried Indian almond and banana leaves as key ingredients; BP, treatment containing dried banana and papaya leaves as key ingredients; IP, treatment containing dried Indian almond and papaya leaves as key ingredients; IBP, treatment containing dried Indian almond, banana, and papaya leaves as key ingredients.

Data are expressed as means $\pm$ SEM ($n = 5$ per treatment).

Differences between means were tested with Duncan's multiple range test.

Different superscripts in the same column indicate a significant difference ($P < 0.05$).

Figure legends

Fig. 1 The diagram shows herbal treatment of amputated fish.

Fig. 2 The diagram shows the monitoring of bubble-nest parameters through Image J software.

Fig. 3 Column charts show the percent inhibition of free radicals due to water fermentations of various herbal formulations used for rearing male Siamese fighting fish: (a) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and (b) 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity. The assays were performed in triplicate. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. CT = control, IA = treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient, B = treatment containing dried banana (*Musa sapientum*) leaf as key ingredient, P = treatment containing dried papaya (*Carica papaya*) leaf as key ingredient, IAB = treatment containing dried Indian almond leaf and banana leaf as key ingredients, BP = treatment containing dried banana leaf and papaya leaf as key ingredients, IP = treatment containing dried Indian almond leaf and papaya leaf as key ingredients, IBP = treatment containing dried Indian almond leaf, banana leaf, and papaya leaf as key ingredients.

Fig. 4 Boxplots show the chemical characteristics of water fermentations of various herbal formulations over the five-week trial: pH (a), salinity (b), total alkalinity (c), hardness (d), dissolved oxygen (e), TAN (f), nitrite (g), and nitrate (h). Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. TAN = total ammonia nitrogen. CT = control, IA = treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient, B = treatment containing dried banana (*Musa sapientum*) leaf as key ingredient, P = treatment containing dried papaya (*Carica papaya*) leaf

as key ingredient, IAB = treatment containing dried Indian almond leaf and banana leaf as key ingredients, BP = treatment containing dried banana leaf and papaya leaf as key ingredients, IP = treatment containing dried Indian almond leaf and papaya leaf as key ingredients, IBP = treatment containing dried Indian almond leaf, banana leaf, and papaya leaf as key ingredients.

Fig. 5 Boxplots show physical characteristics of water fermentations of various herbal formulations over the five-week trial: temperature (a), conductivity (b), and TDS (c). Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. TDS = total dissolved solid. CT = control, IA = treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient, B = treatment containing dried banana (*Musa sapientum*) leaf as key ingredient, P = treatment containing dried papaya (*Carica papaya*) leaf as key ingredient, IAB = treatment containing dried Indian almond leaf and banana leaf as key ingredients, BP = treatment containing dried banana leaf and papaya leaf as key ingredients, IP = treatment containing dried Indian almond leaf and papaya leaf as key ingredients, IBP = treatment containing dried Indian almond leaf, banana leaf, and papaya leaf as key ingredients.

Fig. 6 Representative photographs show the morphological appearance (a) and gray values (b) of bubble-nests of injured male Siamese fighting fish recuperated in water fermentations of various herbal formulations. Measurements were taken twice a week. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. CT = control, IA = treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient, B = treatment containing dried banana (*Musa sapientum*) leaf as key ingredient, P = treatment containing dried papaya (*Carica papaya*) leaf as key ingredient, IAB = treatment containing dried Indian almond leaf and banana leaf as key ingredients, BP =

treatment containing dried banana leaf and papaya leaf as key ingredients, IP = treatment containing dried Indian almond leaf and papaya leaf as key ingredients, IBP = treatment containing dried Indian almond leaf, banana leaf, and papaya leaf as key ingredients.

Fig. 7 Frequency (a), total bubble-nest area (b), individual bubble area (c), and number of bubbles (d) of injured male Siamese fighting fish recuperated in fermented water of herbs. Two measurements a week were taken. Significant differences are indicated by different superscripts ($P < 0.05$). Means were tested by One-Way ANOVA using Duncan's Multiple Range Test for *post hoc* analysis. CT = control, IA = treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient, B = treatment containing dried banana (*Musa sapientum*) leaf as key ingredient, P = treatment containing dried papaya (*Carica papaya*) leaf as key ingredient, IAB = treatment containing dried Indian almond leaf and banana leaf as key ingredients, BP = treatment containing dried banana leaf and papaya leaf as key ingredients, IP = treatment containing dried Indian almond leaf and papaya leaf as key ingredients, IBP = treatment containing dried Indian almond leaf, banana leaf, and papaya leaf as key ingredients.

Fig.1

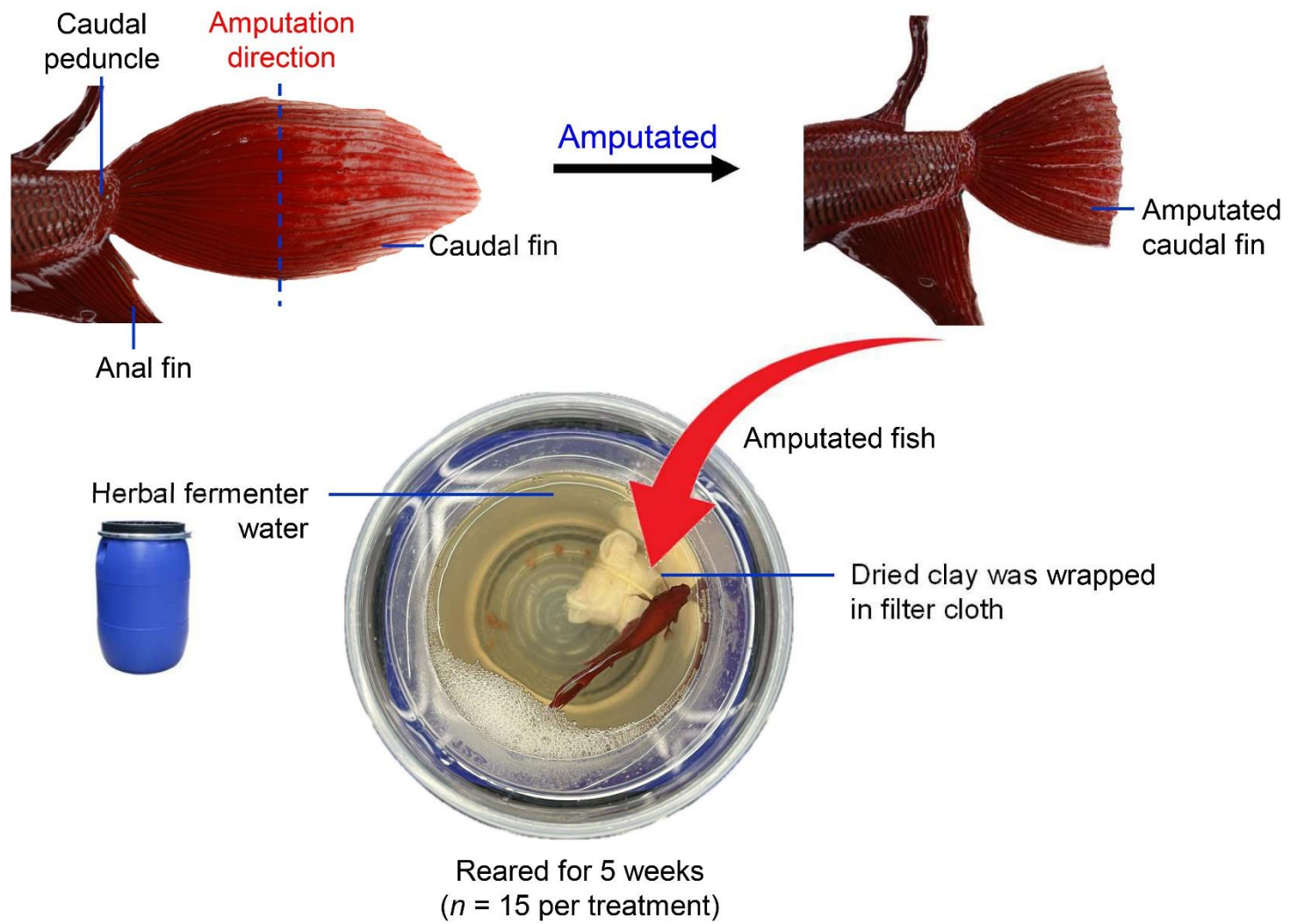


Fig. 2

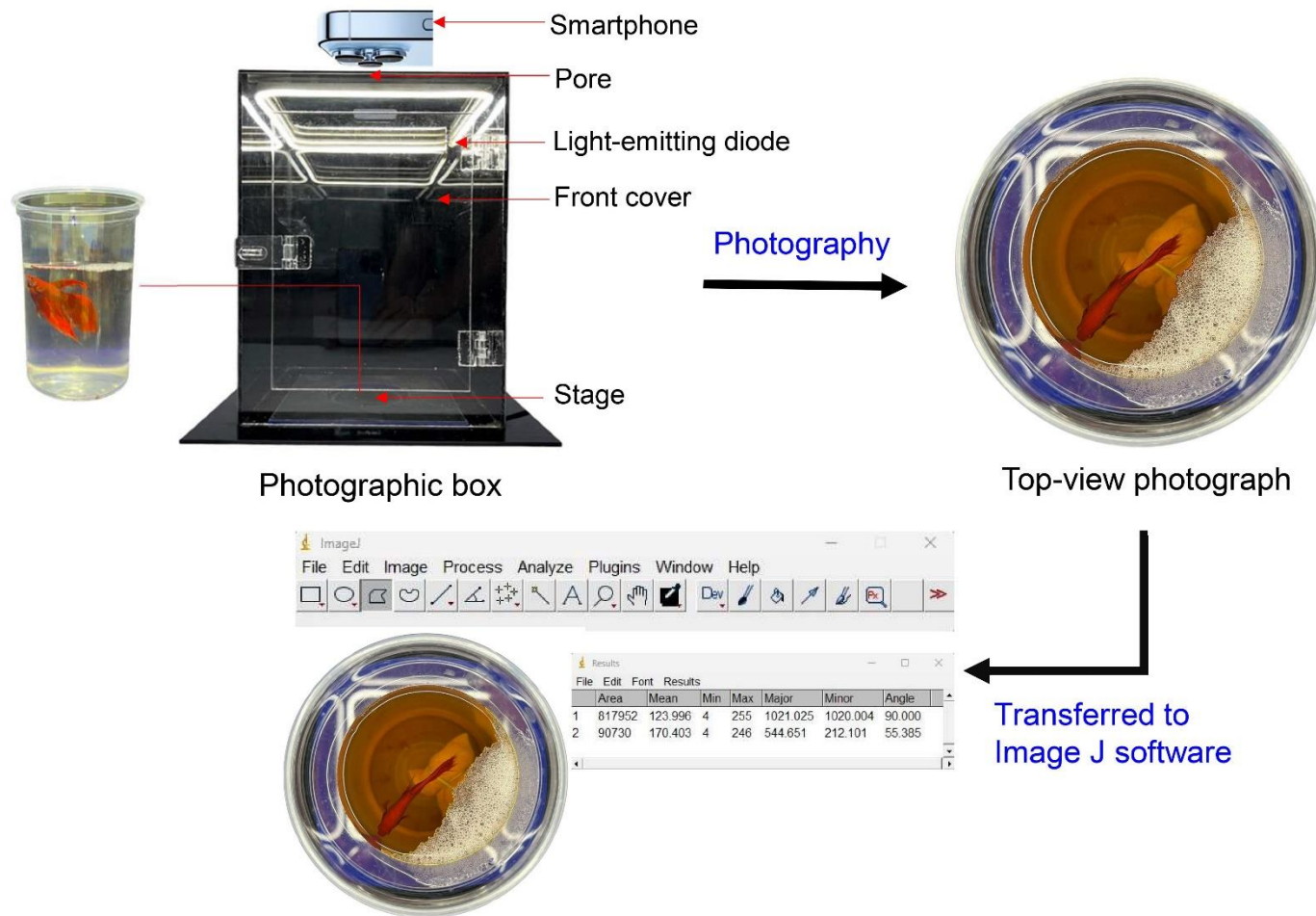


Fig. 3

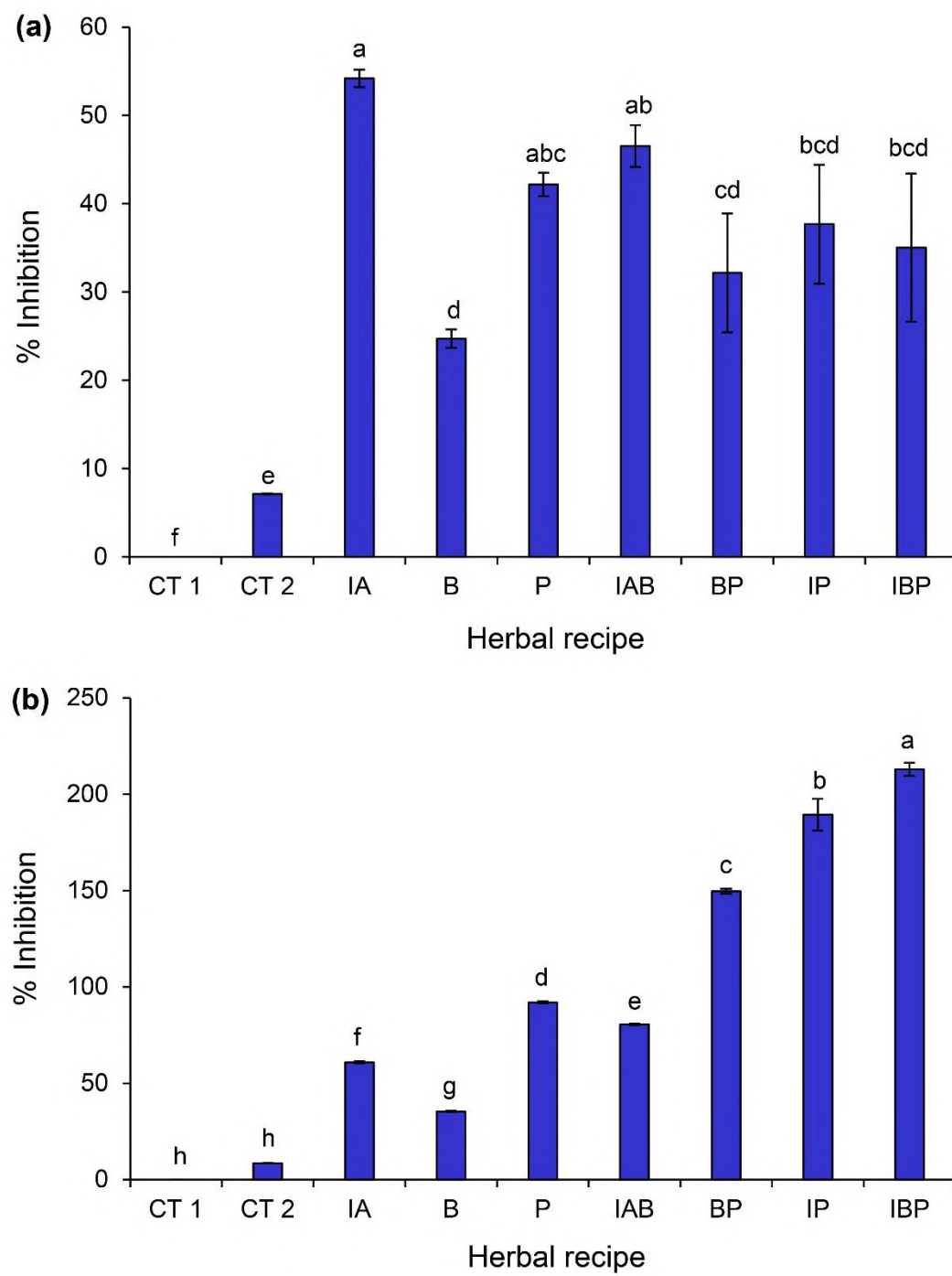


Fig. 4

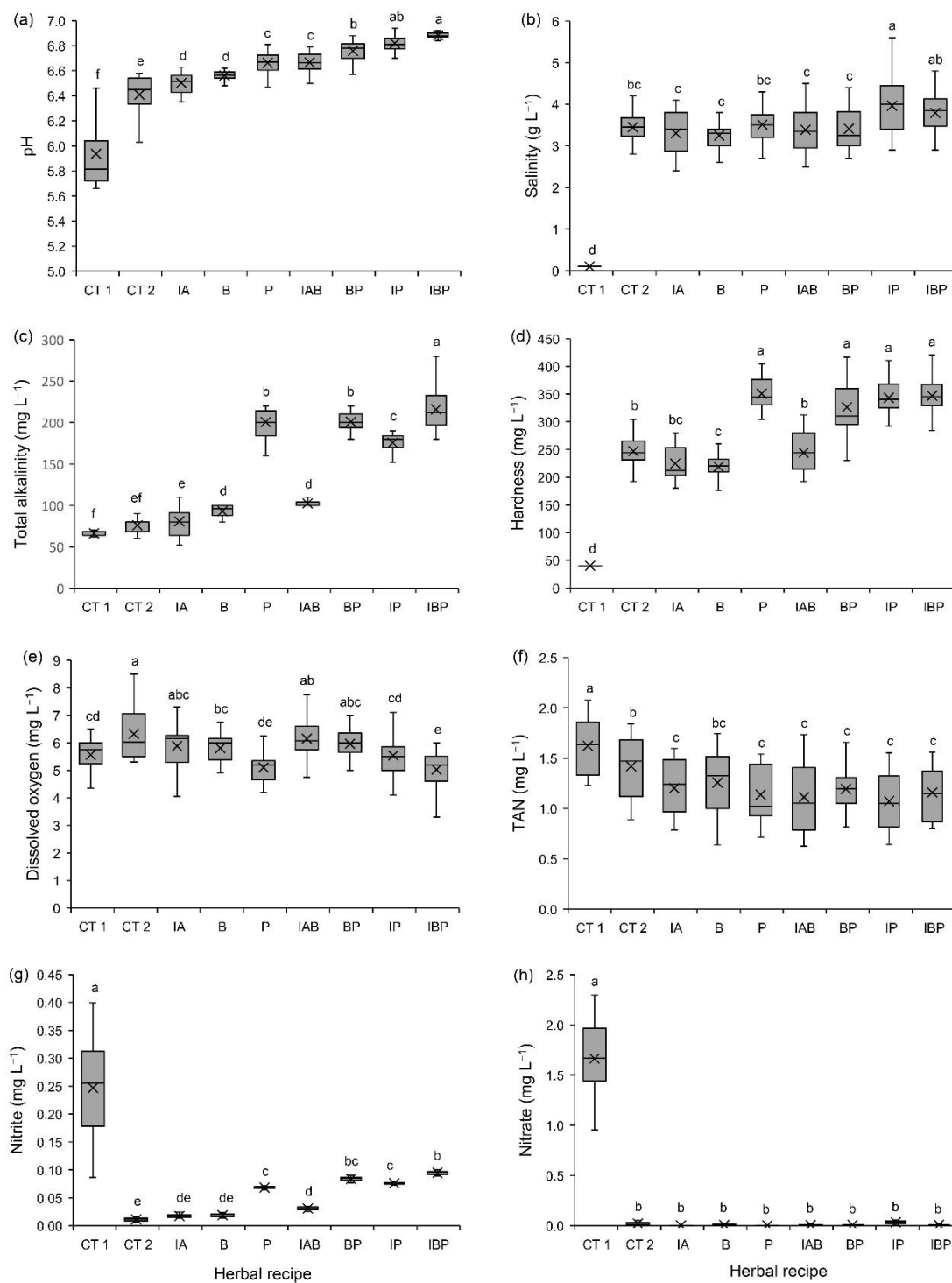


Fig. 5

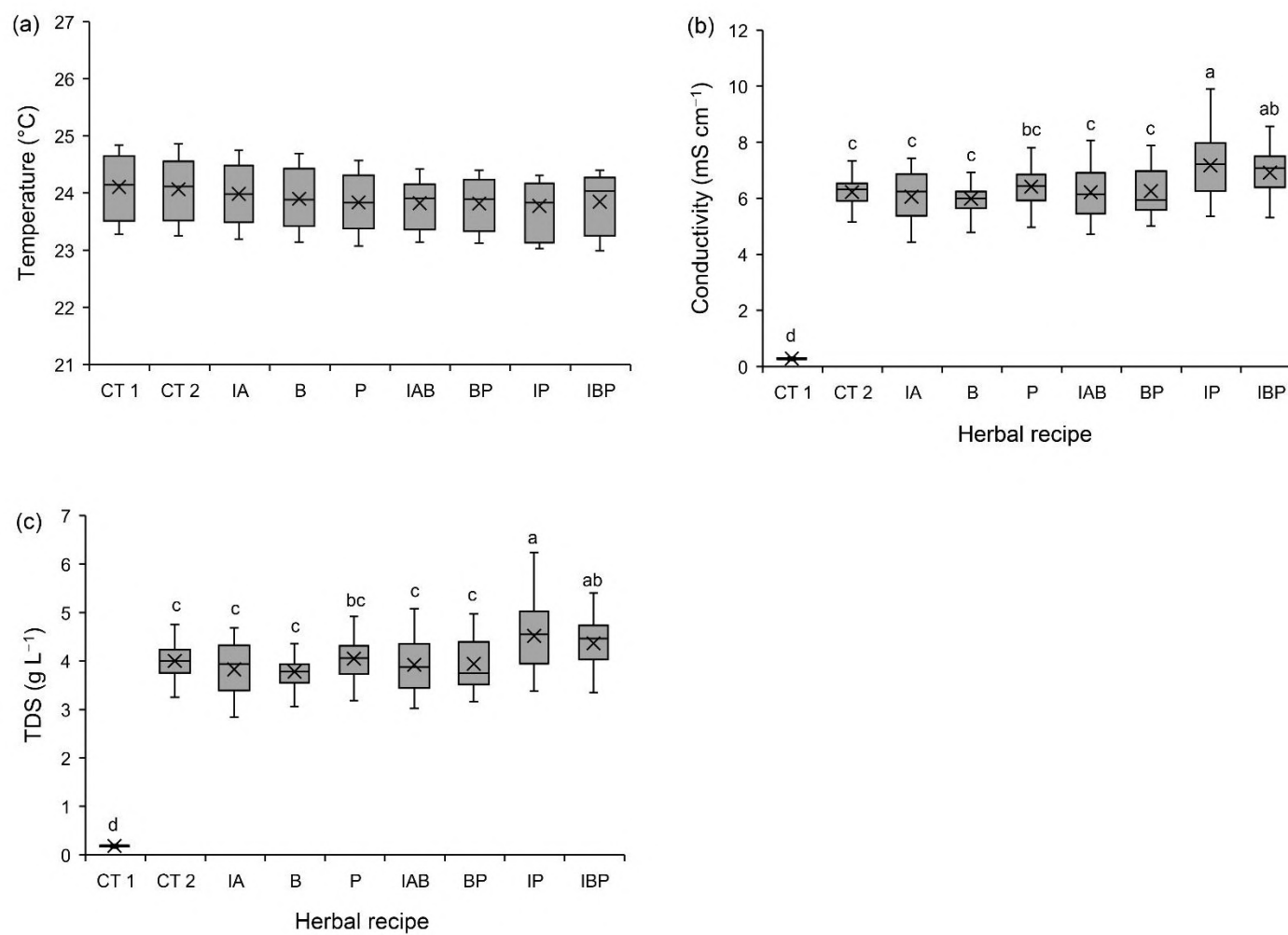


Fig. 6

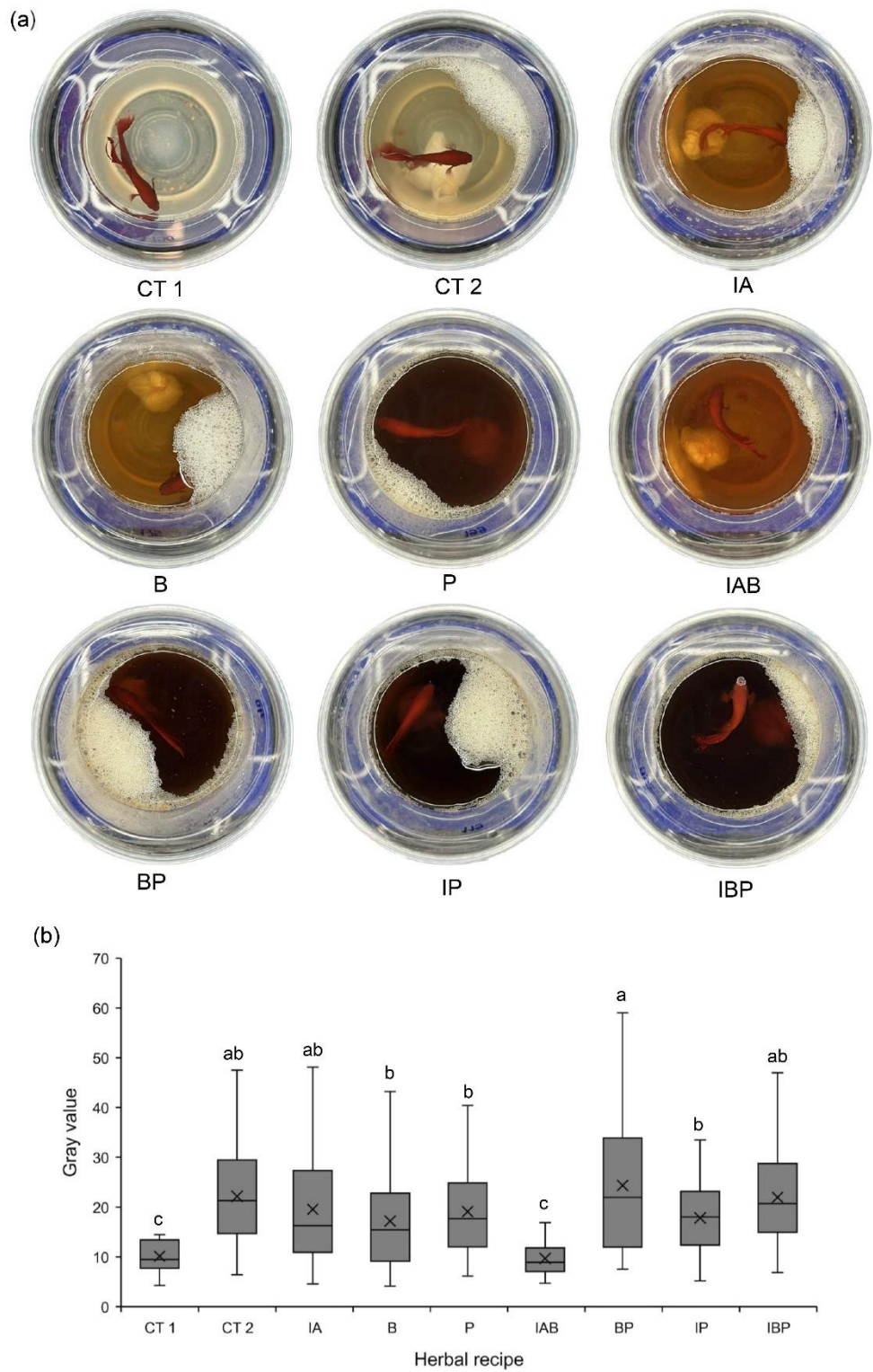
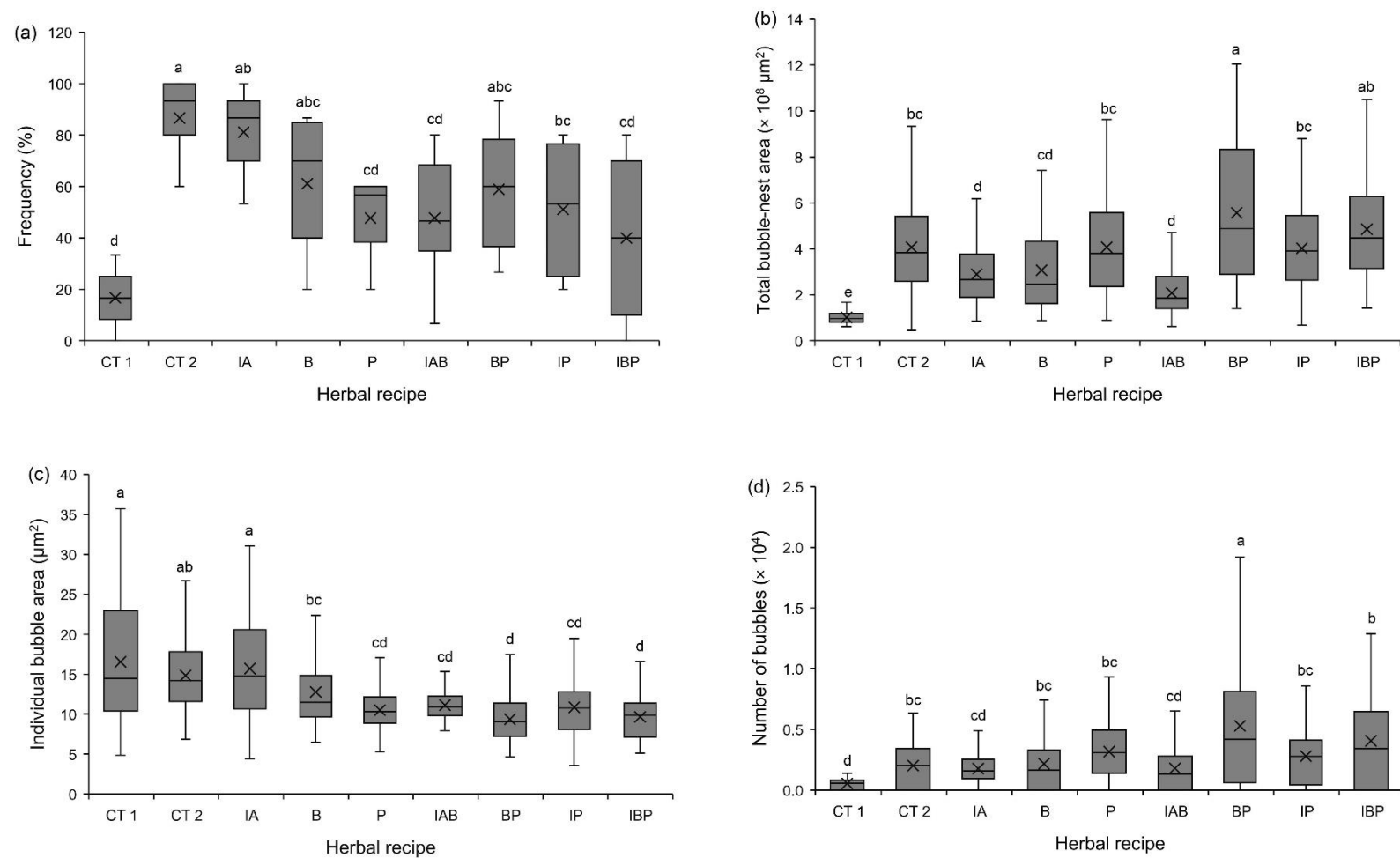


Fig. 7



Appendix B

Paper 1



Addition of different concentrations of Indian almond (*Terminalia catappa*) leaf extract to aquarium water resulted in improved water quality and increased bubble nest formation by male Siamese fighting fish (*Betta splendens*) without having any consistent negative effects on growth metrics and blood chemistry

Saowalak Malawa¹ · Nutt Nuntapong¹ · Naraid Suanyuk¹ · Karun Thongprajukaew^{2,3}

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Abstract

Breeders and keepers of Siamese fighting fish (*Betta splendens*) often add Indian almond (*Terminalia catappa*) leaf extract to the water in the aquarium, but there is little information available about the effects of extract concentration on water quality and fish performance. In the current study, the effects of *T. catappa* extract on water quality, growth performance, feed utilization, skin coloration, bubble nest formation, digestive enzyme activity, muscle quality, whole-body composition, and hematological parameters of *B. splendens* were investigated. Seventy-five juvenile male fish (2-month-old, 0.80–0.95 g initial weight) were individually reared in 350-mL cylindrical plastic containers (8.3 cm diameter × 12.5 cm height). The fish were reared for 8 weeks in four concentrations of *T. catappa* leaf extract (0.125, 0.25, 0.5, and 1 g L⁻¹) and a control group was reared in water without the extract. The fish received an excess of a commercial floating feed (39.4% crude protein) for fighting fish twice daily (08.00 and 17.00 h). A leaf extract concentration of 0.25 g L⁻¹ showed no significant effect on growth (specific growth rate = 1.62% body weight day⁻¹), feed utilization (feed conversion ratio = 1.21 g feed g gain⁻¹), viscerosomatic index, muscle quality, whole-body composition, and hematological parameters of reared fish ($P > 0.05$), but did show effects on some of the other metrics that were monitored. Water quality significantly improved during the experiment ($P < 0.05$). Increased skin lightness (L^*) was observed in all experimental groups, but only fish reared in 0.125 g L⁻¹ leaf extract increased skin redness/greenness (a^*), hue (h^*), and chroma (C^*) ($P < 0.05$). Relative to the control, increments in bubble nest formation frequency (1.70-fold on average) and bubble nest size (1.52-fold on average) were correlated with the concentration of *T. catappa* leaf extract ($P < 0.05$). Specific activities of protein- and lipid-digesting enzymes were not negatively affected but some modulations were observed in treatments containing leaf extract at 0.125, 0.25, and 1 g L⁻¹. Fish reared in the

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0.25 g L⁻¹ treatment exhibited a sparing effect of carbohydrate utilization indicated by decreased amylase specific activity, increased lipase specific activity, and stable amylase/trypsin ratios. The results suggest that 0.25 g L⁻¹ of *T. catappa* leaf extract is a suitable concentration of the additive during the growing phase of male Siamese fighting fish.

Keywords Coloration · Growth metrics · Hematological parameter · Muscle quality · Ornamental fish farming · Water treatment

Introduction

The Siamese fighting fish (*Betta splendens*) is a globally important species in the ornamental aquarium fish trade. The species is native to Thailand and is found in neighboring Cambodia, Laos, Malaysia, and Vietnam (Monvises et al. 2009; Panijpan et al. 2017). Outside of its native area, it has spread to southeast Australia, Brazil, Colombia, the Dominican Republic, southeast United States, and Singapore (Brammah 2015). The USA is the largest market for Siamese fighting fish from Thailand (DOF 2018). Since spectacular coloring, fin characteristics, aggressive behavior, and sexual dimorphism are attractive to keepers of ornamental fish, breeders only market male fish (Goldstein 2004; Thongprajukaew et al. 2014), which are individually reared in small containers since they exhibit aggressive behavior toward other males (Goldstein 2004; Monvises et al. 2009).

It is common practice to add fallen, reddish-brown Indian almond (*Terminalia catappa*) leaves to the aquarium water (Chansue and Assawawongkasem 2008; Nugroho et al. 2016). The leaves contain a number of active ingredients, including tannins, flavonoids, and saponins (Nurhidayat et al. 2016). This practice mimics the natural habitat conditions to provide a more suitable captive environment during rearing and before export. This practice has also been reported in the rearing of cardinal tetra, *Paracheirodon axelrodi* (Walker 2004), and Amazon leaf fish, *Monocirrhus polyacanthus* (Ramos et al. 2020).

Besides the leaves themselves, extracts of *T. catappa* leaf have been used to reduce water turbidity, pH, and total ammonia (Abe et al. 2019; Nozaleda 2019; Ramos et al. 2020). Positive effects of *T. catappa* leaf extracts have also been reported on survival, growth performance, and feed utilization of reared fish (Nugroho et al. 2016, 2017; Nurhidayat et al. 2016; Ramos et al. 2020). On the other hand, excessive concentrations of *T. catappa* leaf extracts adversely affected some water quality parameters (Ikhwanuddin et al. 2014; Mininel et al. 2014).

Although *T. catappa* leaf and extracts of the leaf are commercially available and widely used by fighting fish culturists, an assessment of the effects of *T. catappa* on overall fish performance has never been reported. This study investigated the optimal concentration of dried *T. catappa* leaf extract that should be used in rearing male Siamese fighting fish. The assessment was based on water quality, fish survival and growth, feed utilization, skin coloration, bubble nest formation, digestive enzyme activity, muscle quality, whole-body composition, and hematological parameters. Findings from the present study can help determine the most suitable concentration of *T. catappa* leaf extracts for use in Siamese fighting fish production.

Materials and methods

Collection and preparation of leaf extract

Newly fallen *T. catappa* leaves were collected within the Prince of Songkla University campus, Hat Yai, Thailand. Attached dirt was removed and the leaves were immersed in 5 mg L⁻¹ of KMnO₄ for 15 min to eliminate microorganisms. The leaves were then rinsed three times with distilled water. The clean leaves were dried at 60 °C for 12 h, chopped in a blender, sieved, pooled, packed in polyethylene bags, covered with black plastic bags to exclude light, and then stored at 4 °C until used. The same pooled samples of leaves were used to prepare stocks of extract throughout the experiment.

To prepare the stock, 1 g of leaf sample was macerated in 1 L of distilled water at room temperature for 3 days. The mixture was stirred twice a day. The macerated mixture was passed through a filter cloth and the extract was obtained by passing the filtrate through Whatman paper No. 4 (Chansue and Assawawongkasem 2008).

The tannin contents of the extract were determined by adding 1 mL of extract to a volumetric flask containing 75 mL distilled water, 5 mL of Folin and Ciocalteu's phenol reagent, and 10 mL of 35% Na₂CO₃. The final volume was adjusted to 100 mL. The mixture was shaken and kept at room temperature for 30 min, and absorbance was spectrophotometrically measured at 760 nm against the linear range of tannic acid standard solution (AOAC 1990). The average concentration of tannins in the stock (1 g L⁻¹) of leaf extract was 0.14 g L⁻¹. This initial concentration was serially diluted to obtain leaf extract concentrations of 0.125, 0.25, and 0.5 g L⁻¹.

Fish trial

Solid-red, 1.5-month-old, male Siamese fighting fish were purchased from a local farm in Nakhon Pathom Province, Thailand. They were individually acclimatized for 2 weeks in cylindrical plastic containers measuring 8.3 cm in diameter and 12.5 cm in height. The containers were filled with 350 mL of water, giving a water depth of 8 cm. After acclimatization, the fish were individually weighed immediately prior to the start of the trial to confirm the suitability of their initial body weight (0.80–0.95 g). The fish were then randomly divided into four treatments and a control group, each containing fifteen fish. The fish were reared for 8 weeks in 350 mL of water containing the prepared *T. catappa* leaf extract at concentrations of 0, 0.125, 0.25, 0.5, and 1 g L⁻¹. These concentrations were chosen based on a preliminary study that covered the reported concentrations of *T. catappa* leaf extract used in rearing various fish species (Nugroho et al. 2016; Nurhidayat et al. 2016; Ardi et al. 2020; Ramos et al. 2020). During the present study, the photoperiod was a 12-h light (06.00 to 18.00 h)/12-h dark cycle.

Twice a day at 08.00 and 17.00 h, the fish were given an excess of a commercial floating feed for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). The amount of feed given was adjusted weekly based upon the record of amounts of feed consumed. The proximate chemical composition of the diet on a dry matter basis included 39.4% crude protein, 3.23% crude lipid, 1.97% crude fiber, and 7.72% ash. The procedures for chemical analysis (moisture, crude protein, crude lipid, and ash) are detailed in the whole-body composition data, while crude fiber content was determined based on acid and alkali digestion (AOAC 2005), using a fiber analyzer (F800; Hanon Advanced Technology Group, Jinan, China). Uneaten food was collected 30 min after

feeding, and dried at 60 °C until constant weight was reached. The obtained data were used to calculate the feeding rate (FR), feed conversion ratio (FCR), and protein efficiency ratio (PER).

Water management and quality determination

Every other day, 80% of the rearing water was changed, and water quality was monitored twice weekly. pH, temperature, dissolved oxygen (DO), conductivity, and turbidity were measured using a multiparameter water quality meter (U-52G; Horiba, Kyoto, Japan). Total alkalinity (by methyl orange indicator), hardness (by ethylenediaminetetraacetic acid (EDTA) titration), and total ammonia nitrogen (by the phenate method) were analyzed based on the procedure of Boyd and Tucker (1992). Nitrite (by colorimetry) and nitrate (by cadmium reduction) were analyzed according to the method of APHA, AWWA, and WPCF (1998).

Fish sampling and biometrics

At the end of the 8-week trial, the fish were fasted for 12 h and then euthanized with clove oil (75 mg L⁻¹). Body weight and length (total and standard lengths) were recorded for all the surviving fish ($n=15$ per treatment) before skin coloration was measured. The skin was carefully cleaned with soft blotting paper, and a rapid color measurement was taken from the middle part of the fish body using a MiniScan EZ (Hunter Associates Laboratory, Reston, VA, USA) calibrated with white and black standards. The color coordinates were automatically recorded in terms of lightness (L^*), redness/greenness (a^*), hue (h^*), and chroma (C^*). The value L^* indicates darkness (0) to brightness (100), a^* indicates the degree of redness (+ a) to greenness (− a), h^* indicates color differences due to wavelength, from red (0°) to blue (270°), and C^* indicates color purity from vividness (+ C) to dullness (− C). Blood samples were taken from the excision of the caudal fin and transferred to a 1% EDTA-coated Eppendorf tube. Samples were collected from all the fish and then pooled from lots of three fish to obtain five pooled samples per treatment ($n=5$ per treatment). Viscera ($n=5$ per treatment), digestive tracts ($n=5$ per treatment), and white muscle ($n=5$ per treatment) were collected from the same lots of fish to determine the viscerosomatic index (VSI), digestive enzyme activity, and muscle quality, respectively. The whole-body composition analysis used ten fish selected from each treatment after blood collection. The fish were pooled into lots, with the first and second lots comprising three fish and the third lot four fish ($n=3$ per treatment).

The parameters describing growth performances and feed conversion were calculated as follows:

$$\text{Condition factor (CF)} = 100 \times [\text{body weight (g)/standard length (cm)}^3]$$

$$\text{VSI (\%)} = 100 \times [\text{wet weight of viscera (g)/body weight (g)}]$$

$$\text{Specific growth rate (SGR, \%body weight day}^{-1}\text{)} = 100 \times [(\ln W_t - \ln W_o)/(t - t_o)],$$

where W_t = mean weight (g) at day t , and W_o = mean weight (g) at day t_o ;

$$\text{FR (\%body weight day}^{-1}\text{)} = 100 \times C/[W_0 + W_t]/2/t,$$

where C = daily feed consumption (g), W_0 = initial body weight (g), W_t = final body weight (g), and t = feeding duration (day);

$$\text{FCR (g feed g gain}^{-1}\text{)} = \text{dry feed consumed (g)/wet weight gain (g)}$$

and

$$\text{PER (g gain g protein}^{-1}\text{)} = \text{wet weight gain (g)/protein intake (g)}.$$

Bubble nest behavior

The bubble nest production of each fish was photographed from above with a smartphone (iPhone 11 Pro Max, Apple Inc., China). The photographs were taken three times weekly during weeks 6 to 8 of the experiment. The images were transferred to file and each nest area was analyzed (cm^2) by ImageJ software, as described by Bautista et al. (2019). The frequency of bubble nesting (%) was calculated by dividing the number of aquaria containing bubble nests by the total number of aquaria in each treatment and then multiplying the quotient by 100.

Determination of digestive enzyme activities

Enzyme extraction and protein quantification in crude extracts

Frozen digestive tract was extracted in cold 0.2 M Na_2HPO_4 - NaH_2PO_4 buffer (pH 8) at a ratio of 1:15 w/v, using a tissue micro-homogenizer (THP-220; Omni International, Kennewick, WA, USA). The homogenates were centrifuged at 15,000 g for 30 min at 4 °C. Supernatants were collected and kept in small portions at −20 °C. The protein concentration of crude extracts (mg mL^{-1}) was assayed using the method described by Lowry et al. (1951) with a protein standard of bovine serum albumin. These protein concentration values were used to quantify the specific activity of digestive enzymes (U mg protein^{-1}).

Digestive enzyme assays

The digestive enzymes were assayed according to the optimal conditions recommended for Siamese fighting fish by Thongprajukaew et al. (2010a, b). These conditions were pH 2 and 40 °C for pepsin (EC 3.4.23.1); pH 8 and 50 °C for trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and α -amylase (EC 3.2.1.1); and pH 8 and 40 °C for lipase (EC 3.1.1.3). All assays were performed within 1 month of enzyme extraction. Pepsin activity was assayed using hemoglobin as the substrate according to the method of Worthington (1993). One enzyme unit (U) of pepsin was defined as an increase of 1.0 in absorbance at 280 nm. The activities of trypsin and chymotrypsin were assayed according to the method of Rungruangsak-Torrissen et al. (2006), using *N*-benzoyl-*L*-Arg-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the respective specific substrates. The liberated *p*-nitroanilide from both enzymatic hydrolyses was spectrophotometrically measured at 410 nm against the linear range of its standard. Alpha-amylase activity was determined using soluble starch as the substrate according to the method of Areekijseree

et al. (2004). The liberated product was measured at 540 nm and compared against the linear range of standard maltose. Lipase activity was determined using *p*-nitrophenyl palmitate as the substrate according to the method of Winkler and Stuckmann (1979). The liberated *p*-nitrophenol was spectrophotometrically measured at 410 nm. One U of the last four enzymes above was defined as the amount that catalyzed the conversion of 1 μmol of substrate per minute. To obtain the amylase/trypsin ratio (A/T ratio), the activity of amylase was divided by the activity of trypsin from the same fish sample.

Muscle protein synthesis capacity

Concentrations of RNA and protein in white muscle were determined based on the method of Rungruangsak-Torrissen (2007). Briefly, using an ultrasonic processor (VCX; Sonic and Materials Inc., Newtown, CT, USA), 50 mg of frozen muscle was sonicated with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). After sonication, chloroform was added to obtain an upper aqueous phase (RNA) and a lower organic phase (protein). The aqueous phase was precipitated with isopropanol, washed with ethanol, oven-dried, dissolved in sodium acetate, and its absorbance was measured at 260 nm. Similar protocols were applied to the organic phase, except that protein was dissolved with sodium dodecyl sulfate and absorbance was measured at 280 nm. The extinction coefficients for calculating RNA and protein concentrations were $E_{260}=40 \mu\text{g RNA mL}^{-1}$ and $E_{280}=2.1 \text{ mg protein mL}^{-1}$, respectively.

Muscle myosin and actin contents

Myosin, actin, and sarcoplasmic protein in white muscle were detected using differential scanning calorimetry (DSC7; Perkin Elmer, Waltham, MA, USA) within a range of 20 to 100 °C. Approximately 10 mg of muscle was placed in an aluminum pan, sealed with an aluminum lid, and then heated at a rate of 5 °C min⁻¹ against an empty pan. Identification of muscle proteins was based on onset (T_o), peak (T_p), and conclusion (T_c) temperatures from previous reports of muscle of Nile tilapia, *Oreochromis niloticus* (Thongprajukaew et al. 2015). Amounts of native protein were interpreted from the amounts of energy required to completely denature each protein within a specific range of temperature, and the values were automatically converted from the areas under the curves of the thermograms.

Whole-body composition

Whole bodies of fish were minced to homogeneity and their proximate compositions, including moisture, crude protein, crude lipid, and ash, were determined as described by the AOAC (2005). The samples were first dried for 24 h in a hot air oven (WOF-155; Wisd Laboratory Instruments, Wertheim, Germany) at 105 °C for moisture analysis. Crude protein was analyzed using the Kjeldahl analyzer (Kjeltec™ 8200; Foss, Hillerød, Denmark). Crude lipid was extracted by petroleum ether using a Soxhlet extraction unit (Soxtec™ 8000; Foss, Hillerød, Denmark). Ash was gravimetrically determined after burning at 600 °C for 2 h in a muffle furnace (E30-HT; Thai Furnaces Engineering, Lampang, Thailand). These procedures were also applied for diet proximate composition.

Hematological analysis

Red (RBC) and white (WBC) blood cells were manually counted with a hemocytometer under a compound microscope. Hematocrit (Hct) was determined based on the procedure of Blaxhall and Daisley (1973). Hemoglobin (Hb) was determined by measuring the formation of cyanmethemoglobin according to the method of Larsen and Snieszko (1961). Plasma protein was determined according to the method of Lowry et al. (1951). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\text{MCV (fL)} = 10 \times [\text{Hct (\%)} / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})],$$

$$\text{MCH (pg)} = 10 \times [\text{Hb (g dL}^{-1}) / \text{RBC} (\times 10^6 \text{ cells } \mu\text{L}^{-1})] \text{ and}$$

$$\text{MCHC (g dL}^{-1}) = 100 \times [\text{Hb (g dL}^{-1}) / \text{Hct (\%)}].$$

Statistical analysis

The suitable sample size per treatment was estimated through Program R 3.6.0, requiring the power of test at 0.8. Since the fish were individually reared in isolation, each fish served as an experimental unit. A completely randomized design (CRD) was formulated, comprising five alternative treatments with fifteen fish in each. All data were reported as means and standard errors of means (SEM). The Statistical Package for Social Sciences Version 22 (SPSS Inc., Chicago, USA) was used for all statistical evaluations. Significant differences between means were analyzed by one-way analysis of variance (one-way ANOVA) and Duncan's multiple range test (DMRT) at 95% confidence level was used as the post hoc test.

Results

Water quality

All the water quality parameters, except temperature (data not shown), were significantly changed depending on the concentration of *T. catappa* leaf extract (Fig. 1, $P < 0.05$). Rearing water containing leaf extract at 0.125 g L^{-1} showed reduced pH and nitrite and increased DO and hardness, while conductivity, turbidity, alkalinity, ammonia, and nitrate were unchanged compared to the control. Rearing water that contained leaf extract at 0.25 g L^{-1} showed the same trends in all parameters except for DO, which was unchanged, and ammonia, which was reduced. Rearing water containing leaf extract at 0.5 g L^{-1} showed decreased pH, ammonia, and nitrite while DO and turbidity increased. pH, conductivity, and nitrogen wastes were significantly reduced in the treatment containing the leaf extract at 1 g L^{-1} while alkalinity increased relative to control.

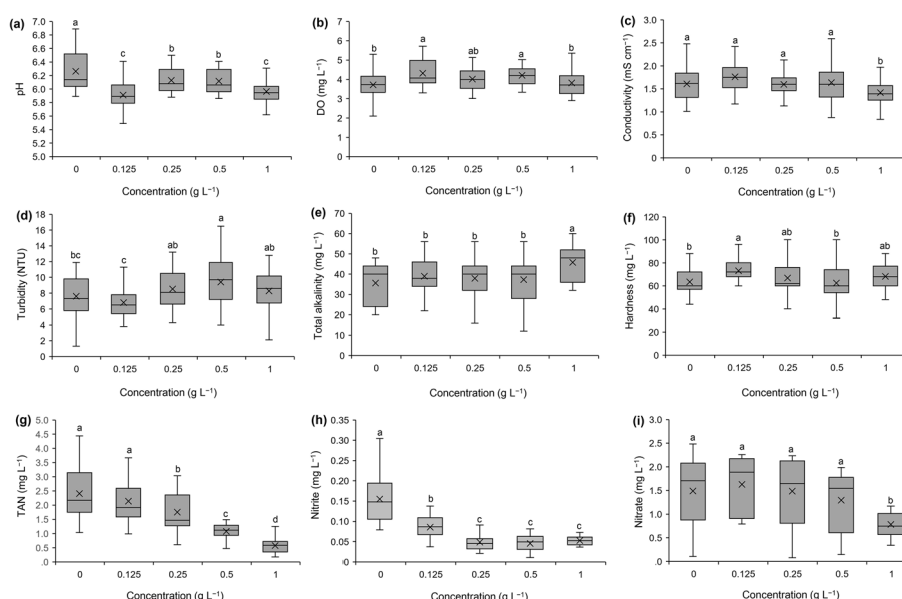


Fig. 1 Boxplots represent water quality parameters during the 8-week experiment: pH (a), DO (b), conductivity (c), turbidity (d), total alkalinity (e), hardness (f), TAN (g), nitrite (h), and nitrate (i). The fish were reared in various concentrations of *T. catappa* leaf extract, and measurements were taken two times a week. DO, dissolved oxygen; NTU, nephelometric turbidity units; TAN, total ammonia nitrogen

Survival, growth, and feed utilization of reared fish

No mortality occurred over the 8-week trial. Final body weight, SGR, and VSI were not affected by any concentration of *T. catappa* leaf extracts (Table 1, $P > 0.05$). Compared to control, standard length (SL) and total length (TL) were significantly smaller in fish reared in all treatments, except the treatment containing the extract at 0.25 g L⁻¹. As a result, CF significantly increased in the other three treatments relative to control. Significant reductions in FR were observed in fish reared in the treatment containing 0.5 g L⁻¹ while FCR and PER did not differ between groups.

Skin coloration

Improved skin coloration (L^* , a^* , h^* , and C^*) was most apparent in fish reared in the 0.125 g L⁻¹ treatment (Table 2, $P < 0.05$). Only partial improvements in color coordinates were observed in the other three treatments, where fish reared in *T. catappa* leaf extract at 0.25 and 0.5 g L⁻¹ showed improvements in the L^* parameter, and fish reared in the extract at 1 g L⁻¹ showed improvements in L^* and a^* parameters.

Bubble nest behavior during experimental duration

The characteristics of bubble nest formation are shown in Fig. 2a. The frequency of bubble nest formation and bubble nest size were influenced by the concentration of *T.*

Table 1 Survival, growth performance, and feed utilization of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment

Parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
IBW (g)	0.88 ± 0.01	0.88 ± 0.03	0.87 ± 0.02	0.86 ± 0.02	0.87 ± 0.01	1.000
FBW (g)	2.08 ± 0.12	2.29 ± 0.10	2.24 ± 0.07	2.34 ± 0.05	2.40 ± 0.04	0.091
SGR (% BW day ⁻¹)	1.47 ± 0.11	1.65 ± 0.07	1.62 ± 0.06	1.61 ± 0.07	1.74 ± 0.02	0.158
SL (cm)	4.19 ± 0.04 ^a	3.56 ± 0.07 ^c	4.14 ± 0.03 ^{ab}	4.04 ± 0.02 ^b	4.06 ± 0.03 ^b	< 0.001
TL (cm)	8.69 ± 0.08 ^a	7.94 ± 0.07 ^c	8.49 ± 0.06 ^{ab}	8.46 ± 0.08 ^{ab}	8.37 ± 0.12 ^b	< 0.001
CF	1.65 ± 0.16 ^c	3.22 ± 0.27 ^a	1.92 ± 0.10 ^{bc}	2.30 ± 0.06 ^b	2.19 ± 0.08 ^b	< 0.001
VSI (%)	7.49 ± 1.01	5.68 ± 0.36	5.51 ± 0.69	6.13 ± 1.12	5.60 ± 0.31	0.390
FR (% BW day ⁻¹)	1.92 ± 0.09 ^a	1.79 ± 0.06 ^a	1.77 ± 0.05 ^a	1.35 ± 0.18 ^b	1.64 ± 0.12 ^{ab}	0.008
FCR (g feed g gain ⁻¹)	1.28 ± 0.10	1.10 ± 0.06	1.21 ± 0.08	1.10 ± 0.04	1.07 ± 0.02	0.187
PER (g gain g protein ⁻¹)	1.77 ± 0.17	2.05 ± 0.14	2.03 ± 0.11	2.09 ± 0.09	2.14 ± 0.02	0.292

IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; BW, body weight; SL, standard length; TL, total length; CF, condition factor; VSI, viscerosomatic index; FR, feeding rate; FCR, feed conversion ratio; PER, protein efficiency ratio

Data are expressed as means ± SEM (*n* = 15 per treatment)

Differences between means were tested with Duncan's multiple range test

Different superscripts in the same row indicate a significant difference (*P* < 0.05)

catappa leaf extract (Fig. 2b and c, *P* < 0.05). Relative to the control, the frequency of bubble nest formation was significantly higher in all experimental treatments (Fig. 2b). The areas of bubble nest were similar between fish reared in 0.125 and 0.25 g L⁻¹ treatments and then increased progressively at concentrations of 0.5 and 1 g L⁻¹ (Fig. 2c).

Digestive enzyme activities

The concentration of the leaf extract had no effect on pepsin specific activity (Fig. 3a, *P* > 0.05). Significantly increased trypsin specific activity relative to control was only

Table 2 Color parameters of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment

Color parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
<i>L</i> [*]	4.00 ± 0.54 ^b	7.45 ± 0.75 ^a	9.13 ± 0.93 ^a	9.79 ± 1.31 ^a	10.6 ± 1.4 ^a	< 0.001
<i>a</i> [*]	18.4 ± 1.8 ^b	23.8 ± 0.8 ^a	21.5 ± 0.7 ^{ab}	20.7 ± 1.1 ^{ab}	22.0 ± 1.0 ^a	0.025
<i>h</i> [*]	19.2 ± 1.1 ^{bc}	25.0 ± 1.7 ^a	23.6 ± 1.7 ^{ab}	19.8 ± 2.1 ^{bc}	18.3 ± 1.8 ^c	0.021
<i>C</i> [*]	19.6 ± 1.9 ^b	25.8 ± 1.0 ^a	23.3 ± 0.7 ^{ab}	22.3 ± 1.3 ^{ab}	23.4 ± 1.2 ^{ab}	0.021

Data are expressed as means ± SEM (*n* = 15 per treatment)

Differences between means were tested with Duncan's multiple range test

Different superscripts in the same row indicate a significant difference (*P* < 0.05)

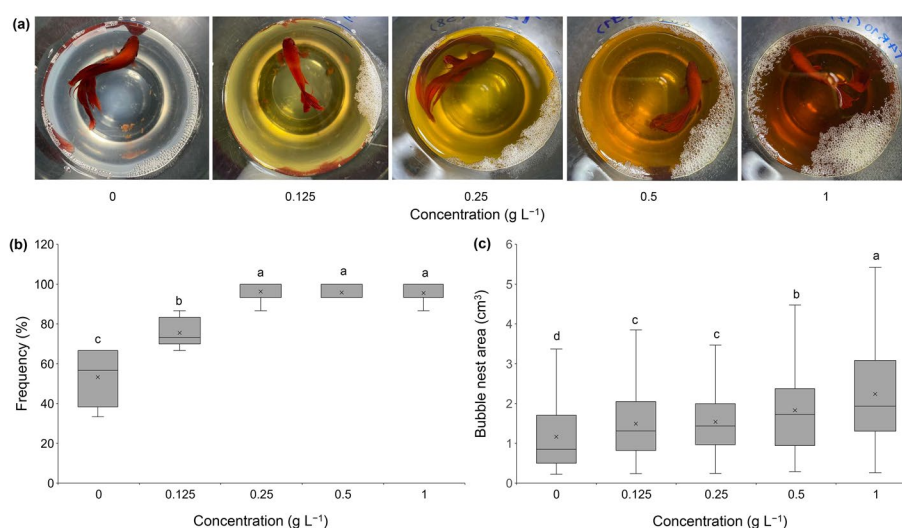


Fig. 2 Characteristics (a), frequency (b), and area (c) of bubble nest formation by male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The data on frequency and area were recorded from all fish three times weekly from week 6 to 8. Differences between means were tested with Duncan's multiple range test. Different superscripts indicate a significant difference ($P < 0.05$) between treatments

observed in fish reared in the lowest concentration (Fig. 3b, $P < 0.05$). The highest chymotrypsin-specific activity was observed in fish reared in the 0.125 and 1 g L⁻¹ treatments (Fig. 3c). Increased lipase specific activity was found only in fish reared in the 0.25 g L⁻¹ treatment (Fig. 3d). Amylase specific activity was significantly reduced compared to control in all treatments except 0.125 g L⁻¹ (Fig. 3e). The A/T ratio remained unchanged compared to the control only in fish reared in the 0.25 g L⁻¹ treatment. Significant reductions were found in the other three treatments (Fig. 3f).

Protein synthesis capacity, and myosin and actin muscle contents

RNA, protein, and the RNA/protein ratio did not differ between treatments (Table 3, $P > 0.05$). The main muscle proteins, myosin and actin, were found within onset to conclusion temperature ranges of 28.5 to 56.9 °C and 65.4 to 76.4 °C, respectively. Sarcoplasmic protein was found within a range of 79.3 to 91.3 °C (data not shown). According to the thermal responses, no significant differences were present in myosin, actin, and sarcoplasmic protein contents, or their summations or ratios, between the control and all four treatments (Table 3, $P > 0.05$).

Whole-body composition

No significant differences were found in moisture, crude protein, crude lipid, and ash contents between the control and all four treatments (Table 4, $P > 0.05$). On a wet weight basis, the respective average values were 70.4, 16.9, 5.84, and 4.07%.

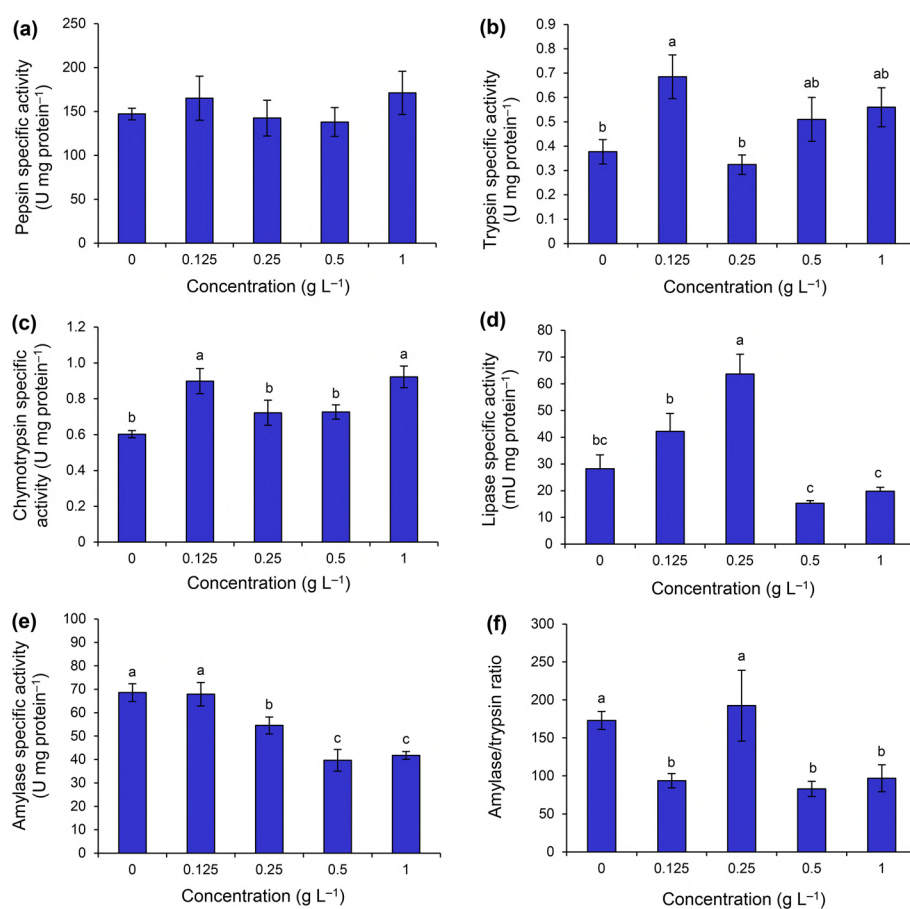


Fig. 3 Specific activities of digestive enzymes were analyzed in male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract: pepsin (a), trypsin (b), chymotrypsin (c), lipase (d), amylase (e), and amylase to trypsin ratio (f). The observed parameters were recorded at the end of the 8-week experiment. Data are expressed as tank means $\pm$ SEM ($n=5$ per treatment). Differences between means were tested with Duncan's multiple range test. Different superscripts indicate a significant difference ($P < 0.05$) between treatments

Hematological parameters

RBC, Hct, Hb, MCV, MCH, and MCHC were similar across all groups (Table 5, $P > 0.05$). WBC was significantly higher in fish reared in water containing the leaf extract at 0.25 and 0.5 g L⁻¹, and plasma protein was statistically higher compared to control in fish reared in the treatment containing leaf extract at 0.5 g L⁻¹ (Table 5, $P < 0.05$).

Table 3 The muscle quality of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment

Parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
Protein synthesis capacity						
RNA (μg g ⁻¹)	6,200±619	7,676±435	7,818±623	7,405±794	6,619±304	0.243
Protein (mg g ⁻¹)	314±23	267±7	292±15	246±15	292±40	0.294
RNA/protein ratio (μg mg ⁻¹)	20.4±3.0	28.8±1.7	27.1±2.6	30.2±2.8	24.7±2.9	0.101
Muscle protein						
ΔH Myosin (J g ⁻¹)	1.11±0.09	1.08±0.16	1.29±0.13	1.20±0.06	1.26±0.05	0.553
ΔH Actin (J g ⁻¹)	0.38±0.11	0.32±0.03	0.35±0.08	0.39±0.07	0.42±0.02	0.866
ΔH Sarcoplasmic protein (J g ⁻¹)	0.11±0.03	0.15±0.03	0.12±0.02	0.22±0.09	0.12±0.02	0.492
ΣΔH (J g ⁻¹)	1.60±0.21	1.54±0.15	1.77±0.21	1.81±0.13	1.96±0.16	0.483
ΣΔH Myosin and actin (J g ⁻¹)	1.49±0.19	1.40±0.15	1.65±0.20	1.59±0.05	1.68±0.07	0.630
ΔH Actin/myosin	0.33±0.08	0.33±0.08	0.27±0.06	0.34±0.08	0.34±0.00	0.943

Data are expressed as means ± SEM (*n* = 5 per treatment)

Differences between means were tested with Duncan's multiple range test

Different superscripts in the same row indicate a significant difference (*P* < 0.05)

Discussion

Water quality

A number of water qualities were significantly improved by the various concentrations of *T. catappa* leaf extract. The low water pH produced by all concentrations of *T. catappa* leaf extract represents a significant improvement in water quality since Siamese fighting fish prefer a slightly acidic environment (Nugroho et al. 2016). In studies of Amazon leaf fish larvae, Abe et al. (2019) and (Ramos et al. 2020) reported that aqueous *T. catappa*

Table 4 Whole-body compositions (% of weight weight) of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment

Composition	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
Moisture	70.9 ± 0.1	69.6 ± 0.5	70.0 ± 0.2	70.5 ± 0.6	70.8 ± 0.9	0.441
Crude protein	16.5 ± 0.1	16.7 ± 0.3	17.3 ± 0.3	17.1 ± 0.3	16.9 ± 0.5	0.525
Crude lipid	5.75 ± 0.31	6.54 ± 0.24	5.71 ± 0.24	5.45 ± 0.06	5.73 ± 0.30	0.086
Ash	4.01 ± 0.08	4.12 ± 0.10	4.13 ± 0.03	4.15 ± 0.19	3.94 ± 0.06	0.804

Data are expressed as means ± SEM (*n* = 3 per treatment)

Differences between means were tested with Duncan's multiple range test

Different superscripts in the same row indicate a significant difference (*P* < 0.05)

Table 5 Hematological parameters of male Siamese fighting fish reared in various concentrations of *T. catappa* leaf extract. The observed parameters were recorded at the end of the 8-week experiment

Hematological parameter	Concentration (g L ⁻¹)					P-value
	0	0.125	0.25	0.5	1	
RBC (× 10 ⁹ cells mL ⁻¹)	2.66 ± 0.09	3.09 ± 0.05	2.78 ± 0.16	2.85 ± 0.10	2.99 ± 0.10	0.145
WBC (× 10 ⁷ cells mL ⁻¹)	3.65 ± 0.43 ^b	5.43 ± 0.28 ^{ab}	7.27 ± 1.04 ^a	7.29 ± 0.84 ^a	5.82 ± 0.48 ^{ab}	0.047
Hct (%)	44.3 ± 3.0	31.9 ± 6.7	39.4 ± 3.0	46.0 ± 6.1	40.2 ± 3.0	0.355
Hb (g dL ⁻¹)	11.8 ± 1.7	13.5 ± 0.9	12.3 ± 0.8	12.1 ± 0.5	15.0 ± 0.9	0.210
Plasma protein (g L ⁻¹)	56.2 ± 2.2 ^b	54.3 ± 2.2 ^b	60.2 ± 2.2 ^b	88.0 ± 2.2 ^a	60.5 ± 1.7 ^b	0.021
MCV (fL)	167 ± 12	104 ± 22	143 ± 10	162 ± 20	109 ± 26	0.122
MCH (pg)	44.9 ± 7.6	43.5 ± 2.3	44.8 ± 2.8	48.9 ± 6.0	56.3 ± 6.3	0.434
MCHC (g dL ⁻¹)	26.8 ± 4.1	48.3 ± 10.0	31.6 ± 1.9	31.4 ± 3.4	37.5 ± 2.1	0.095

RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

Data are expressed as means ± SEM (*n* = 5 per treatment)

Differences between means were tested with Duncan's multiple range test

Different superscripts in the same row indicate a significant difference (*P* < 0.05)

leaf extract at 0.75 g L⁻¹ improved pH and total ammonia levels of rearing water and subsequent survival and growth performances. In a study of cardinal tetra (Nurhidayat et al. 2016), similar results were reported for water treatment with a dose of *T. catappa* leaf at 0.5 g L⁻¹. In general, *T. catappa* leaves contain a lot of tannins, so water pH should gradually decline as the concentration of the extract increases (Ramos et al. 2020).

We found that DO levels remained within an acceptable range of 3 to 5 mg L⁻¹ (Bhatnagar and Devi 2013) but improved in some treatments. Possibly, tannins reduce the breakdown of organic matter by precipitating proteins and inhibit the secretion of bacterial digestive enzymes (Smith et al. 2005). Water conductivity was also within the desirable range, at 0.1 to 2 mS cm⁻¹ for fish ponds (Stone and Thomforde 2004), and at 0.5 to 3 mS cm⁻¹ for natural courses (Hem 1985).

The increased water turbidity we observed conflicts with the report by Nozaleda (2019). The results of our study are possibly due to the use of whole *T. catappa* leaves. *T. catappa* leaves contain pectic substances which can interact with water, so it is possibly more appropriate to use only the leaf blade, without the midrib. Although some significant changes in alkalinity and hardness were observed in the 1 and 0.125 g L⁻¹ treatments, these water qualities would not be toxic to fish and are similar to or lower than those recorded in previous reports (Chansue and Assawawongkasem 2008; Purivirojkul 2012; Saekhow et al. 2018).

In the present study, significantly decreased N-wastes were observed in all treatments. Reduced N-wastes were facilitated by hydrogen bridges between OH functional groups of tannins and N-waste (Siebert et al. 1996), which help precipitate the products. Similar reductions in N-wastes due to *T. catappa* leaf extract water additives have been found (Putamat and Suwannasarn 2007; Pimolrat et al. 2018).

Growth, weight-length morphometrics, and feed utilization

Several species have shown significantly improved growth parameters that were dependent on the concentration of *T. catappa* leaf extract used in the treatment. Growth parameters

of post-larvae of the black tiger shrimp (*Penaeus monodon*) improved in the presence of *T. catappa* leaf extract at 1 to 4 g L⁻¹ (Ikhwanuddin et al. 2014); growth parameters of juvenile cardinal tetra improved at concentrations of 0.5 and 1.5 g L⁻¹ (Nurhidayat et al. 2016); and growth parameters of Amazon leaf fish larvae improved at a concentration of 0.75 g L⁻¹ (Ramos et al. 2020). In the present study, water treatment with *T. catappa* leaf extract did not significantly affect weight-related parameters (final body weight and SGR) of the Siamese fighting fish at any concentration. Length-related parameters (SL, TL, and CF), however, were significantly influenced by the extract, except in fish reared in a concentration of 0.25 g L⁻¹. Similar changes in weight- and length-related growth parameters were observed in neon tetra fish (*Paracheirodon innesi*) reared in *T. catappa* leaf extract at 0.75 g L⁻¹ (Ardi et al. 2020). It is possible, as reported by Nurhidayat et al. (2016), that the leaf extract might disturb bone development, and if so, the application of *T. catappa* leaf extract during the growing phase of Siamese fighting fish may well be unsuitable. However, the amount of ash in the whole fish body at the end of the present trial showed insignificant differences across all groups. Therefore, the effect of *T. catappa* leaf extract on bone development and mineral availability requires further investigation.

Regarding feed utilization, treatment with *T. catappa* leaf extract at 0.5 and 1.0 g L⁻¹ produced an undesirable FR while FCR and PER were still acceptable. Since these fish take a long time to consume their feed, these results show that growth performances in the present study were in line with the known behavior of the fish. Theoretically, tannins in *T. catappa* leaf bind to proteins and this protein-binding reduces the digestion of food by inhibiting the activities of digestive enzymes, such as proteases, amylase, and lipase (Maitra and Ray 2003). Since Siamese fighting fish are carnivorous, they require large amounts of protein in their diet but, although a tannin-rich diet can reduce appetite and digestive efficiency (Maitra and Ray 2003), the effects of *T. catappa* leaf extract water additive on the secretion of fish digestive enzymes have never been reported. In the present study, the absence of negative effects on the specific activities of protein-digesting enzymes (pepsin, trypsin, and chymotrypsin) indicates normal gastrointestinal functionality in protein digestion.

Although some treatments produced positive results, the adverse effects on growth performance and feed utilization negated them. Fish reared in water containing 0.25 g L⁻¹ of leaf extract exhibited the highest lipase specific activity. This change indicates improved digestion of dietary lipids, and would support the use of *T. catappa* leaf extract in fighting fish farms since live diets comprise high amounts of lipid (Thongprajukaew et al. 2014, 2019). In the 0.25 g L⁻¹ treatment, the A/T ratio was maintained while amylase specific activity decreased. These results indicate normal use of available carbohydrate per unit of protein (Thongprajukaew et al. 2011). Since no negative effects on growth and feed utilization were found in this treatment, it is possible that some flexibility might exist between lipid- and carbohydrate-digesting enzyme activities that contributed a carbohydrate-sparing effect in the studied fish. In other words, the increased digestion of fat reduced the need to digest carbohydrate.

Bubble nest formation

T. catappa contains isoflavones, a functional group of phytoestrogens that are structurally similar to animal estrogens (Vadwala and Kola 2017). Exposing male *B. splendens* to isoflavones (genistein and equol) in a previous study was shown to promote bubble nest building (Clotfelter and Rodriguez 2006). In the present study, the increased frequency and area of bubble nest formation were closely associated with the concentration of leaf extract in the

treatment. Increased gonadosomatic indices have been reported after the dietary incorporation of isoflavones (Pastore et al. 2018; Rzepkowska et al. 2020), and a hydroalcoholic extract containing phytoestrogenic compounds (Enayat Gholampour et al. 2020). On the other hand, phytoestrogens can inhibit steroidogenesis, which could interfere in the transition from courtship to parental behavior in fish (Sisneros et al. 2004; Desmawati and Sulastri 2019), and significant changes in hormones and gonads in male fish are known to reduce aggression (Ofir et al. 2003; Perreault et al. 2003; Stevenson et al. 2011), a chief attraction of *B. splendens* to keepers. The conflicting results reported above were dependent on the concentration of leaf extract and fish species, and we concluded that only the optimal concentration used in the present study is suitable for male Siamese fighting fish.

Skin coloration, body homeostasis, and general health status

Although improved skin color coordinates were observed in all experimental treatments, the negative effects of the treatments on body length have to be taken into account. Body length was impacted the least in fish reared in water containing 0.25 g L^{-1} of leaf extract and only this treatment represents a suitable choice. However, information on the mechanisms involved in improving color parameters is limited. It is possible that some active ingredients from *T. catappa*, such as flavonoids, serve as potent tyrosinase inhibitors that inhibit melanogenesis without provoking melanocytotoxicity (Zhu and Gao 2008). Some elicitors from active ingredients may trigger coloration and since water soluble extracts from *T. catappa* leaf can be used as natural dyes in the presence of mordants (Chitnis 2013), direct immersion of fish in the extract might contribute some skin pigmentation.

The absence of significant changes in RNA, protein, and the RNA/protein ratio among all treatments in the present study indicates that normal muscle protein growth occurred even in the presence of the higher concentrations of *T. catappa* leaf extract. Also, no significant changes were observed in the amount of major muscle proteins (mainly myosin and actin) and their proportional ratios, and whole-body compositions. These results indicate that no negative effect on the health status of Siamese fighting fish resulted from the use of *T. catappa* leaf extract during this study.

The health status of aquatic animals can also be monitored through hematological parameters. In our study, fish reared in the 0.25 g L^{-1} treatment showed significantly increased WBC and those reared in the 0.5 g L^{-1} treatment showed significantly increased WBC and plasma protein levels. Improved results for WBC have been reported for fighting fish treated with *T. catappa* extract at concentrations above 0.375 g L^{-1} (Nugroho et al. 2016); and treatment of the same species with *T. catappa* extract at 0.5 g L^{-1} increased the percentage of monocytes and granulocytes against *Aeromonas hydrophila* (Nugroho et al. 2017). Exposure to 0.5 g L^{-1} *T. catappa* leaf extract was associated with increased plasma protein concentrations in Tambaqui, *Colossoma macropomum* (Meneses et al. 2020).

Some of the positive effects observed in the current study might be attributed to active ingredients such as tannins, flavonoids, and saponins in *T. catappa* leaves (Nurhidayat et al. 2016). In a previous study of fish (Pandey 2013), these components increased both the non-specific defense and the specific immune response. Elsewhere, flavonoids were shown to modulate the production of cytokines such as interferon and interleukin, and also enhance T-cell activities (Lyu and Park 2005; Helal and Melzig 2011; Xiong et al. 2012; Nugroho et al. 2016). Flavonoids also have antioxidant, antiparasitic, antifungal, and antibacterial activities that prevent and cure disease, and are applied in aquaculture as an alternative to antibiotics (Chitmanat et al. 2005; Chansue and Assawawongkasem 2008; Purivirojkul 2012).

Conclusions

Terminalia catappa leaf and its products are widely used by fighting fish culturists but reports of some negative effects on reared fish have been published. Therefore, we set out to clarify the overall effects of *T. catappa* leaf extract on fish performance. Based on observations of water quality, fish growth, feed utilization, skin coloration, bubble nest creation, digesting enzyme activities, muscle protein, whole-body composition, and hematological parameters, the findings of this work indicated that an aquarium additive of *T. catappa* leaf extract at a concentration of 0.25 g L⁻¹ of water is suitable for rearing male Siamese fighting fish. On the other hand, observations of skeletal growth based on body length and condition factor indicated undesirable effects on fish reared in less appropriate concentrations. Practically, this environmentally friendly leaf extract appears to be most suitable if used occasionally for specific purposes such as improving water quality, prophylactic treatment, or courtship issues. Despite not being appropriate for the whole duration of fish rearing, the correct use of *T. catappa* leaf should yield not only the desirable results mentioned, but also reductions in the use of antibiotics or chemicals that cause side effects and residue problems in the commercial production of aquatic animals. However, the active ingredients, mainly tannins, found in *T. catappa* leaf may be affected by seasonal variation, geographical location, soil fertility, leaf age, extraction solvent, and extraction protocol. Differences in these parameters can lead to differences in observed outcomes in fish.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval The transportation, acclimatization, rearing, sampling, and euthanasia of the fish conformed to the “Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes,” National Research Council, Thailand (Application No. U1-06514–2560). This research project was approved by the Institutional Animal Care and Use Committees, Prince of Songkla University (Project Code 2564–01-030).

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Conflict of interest The authors declare no competing interests.

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Authors and Affiliations

Saowalak Malawa¹ · Nutt Nuntapong¹ · Naraid Suanyuk¹ · Karun Thongprajukaew^{2,3}

✉ Karun Thongprajukaew
karun.t@psu.ac.th; karun114@hotmail.com

¹ Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

² Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

³ Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

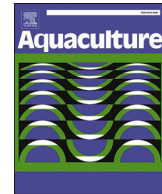
Appendix C

Paper 2



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Fermented water of Thai herbs effectively enhanced amputated fin regeneration in Siamese fighting fish (*Betta splendens*)

Saowalak Malawa^a, Nutt Nuntapong^a, Sugree Waeowannajit^b, Karun Thongprajukaew^{b,c,*}

^a Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

^b Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

^c Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

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ABSTRACT

The aggression of Siamese fighting fish (*Betta splendens*) during courtship and reproductive behaviors can result in torn fins. However, the natural regeneration of torn fins has been significantly improved by treating fish with fermented water of plants. We amputated half of the caudal fin of male Siamese fighting fish (1.29 ± 0.01 g body weight) in the vertical plane. The amputated fish were individually reared in fermented water containing eight combinations of herbs that included dried Indian almond (*Terminalia catappa*) leaf (IAL, 0.25 g L^{-1}), dried banana (*Musa sapientum*) leaf (BL, 1.5 g L^{-1}), and dried papaya (*Carica papaya*) leaf (PL, 2.5 g L^{-1}) as major ingredients. Other ingredients included dried lemongrass (*Cymbopogon citratus*) leaf (LL, 1 g L^{-1}), dried clay (50 g L^{-1}), and salt (5 g L^{-1}). For comparison, other fish were reared in non-fermented water. The experiment was conducted in a completely randomized design for five weeks, comprising fifteen fish per treatment. Individual fish served as experimental units. Fin regeneration was monitored weekly while growth performance was recorded at the end of the trial. The herbal treatments had no effects on growth, body morphometrics, and feed utilization ($P > 0.05$). However, fin regeneration was statistically improved for the fish reared in combinations of IAL, BL, and PL ($P < 0.05$). Among these alternative treatments, red color intensity was fully redeveloped in fish reared in three herbal treatments ($P < 0.05$) that all contained dried papaya leaf (BL + PL, IAL + PL, and IAL + BL + PL). Therefore, those three treatments effectively enhanced fin regeneration.

1. Introduction

The Siamese fighting fish (*Betta splendens*) is a globally important ornamental fish prized for its vibrant colors and the pugnacious nature and beauty of the male fish (Monvises et al., 2009; Rahmati-Holasoo et al., 2019, 2020). From 2010 to 2019, the species was one of the two most commercially profitable ornamental fish in Thailand. The United States is the largest market and the biggest importer of Siamese fighting fish from Thailand, followed by China, France, Iran, and Singapore (Sermwatanakul, 2019). The declaration of this fish as a national aquatic animal was not only due to the economic importance of the species, but also to its place in the cultural heritage of Thailand (Sermwatanakul, 2019).

Behaviorally, *B. splendens* are bubble-nest builders in which males are responsible for raising eggs and larvae until they can support

themselves (Monvises et al., 2009). The courtship and reproductive behaviors (male-female) include combative behavior (male-male or female-female) when fish encounter each other. Threatening poses are struck towards opponents. Body color darkens, gill covers flare, fins are erected, and tails beat. These displays can lead to chasing, biting, butting, and locking jaws. These actions result in damaged bodies and torn fins, especially pelvic and pectoral fins, and sometimes dorsal, anal, or caudal fins (Braddock and Braddock, 1955; Rainwater, 1967; Ramos and Gonçalves, 2019; Simpson, 1968). Therefore, male fish are reared individually in small containers until they reach 1.5 months of age (Thongprajukaew et al., 2010a, 2010b).

In general, fish can completely regenerate injured or lost fins naturally. Previous studies have investigated fin regeneration amputating fins, especially the caudal fin (Pfefferli and Jazwińska, 2015). Different caudal fin excision sites resulted in divergent reconstruction times/rates.

* Corresponding author at: Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand.
E-mail address: karun.t@psu.ac.th (K. Thongprajukaew).

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Proximal amputations took longer time than distal amputations to be regenerated and for the fin to regain its original size (length) (Uemoto et al., 2020). Previous studies have reported species-related differences in complete regeneration time: fourteen days for zebrafish, *Danio rerio* (Geraudie et al., 1995; Poleo et al., 2001); twenty-four days for mosquito fish, *Gambusia affinis* (Johnson and Denton, 1977) and guppy, *Poecilia reticulata* (Kolluru et al., 2006); and twenty-eight days for Nile tilapia, *Oreochromis niloticus* (Ngamsiri, 2015). However, the capacity to regenerate fin length has been significantly improved by rearing fish in water containing herbs (Chansue, 2006; Ngamsiri and Laumsomboon, 2021).

B. splendens culturists have always soaked dried Indian almond (*Terminalia catappa*) leaves in rearing water (Malawa et al., 2022). The properties of this plant for wound healing and eliminating harmful monogenean and *Trichodina* spp. have been demonstrated (Chitmanat et al., 2005; Ponpornpisit et al., 2007). Apart from dried Indian almond leaves, other herbs commonly used by *B. splendens* culturists in Thailand are dried banana leaves (*Musa sapientum*), papaya leaves (*Carica papaya*), and lemongrass (*Cymbopogon citratus*) leaves. These herbs contain active ingredients with various biological activities that especially help heal wounds caused by fighting (Maria and Veronica, 2021; Tanmoy et al., 2021; Wijaya et al., 2020). As well as these herbs, some *B. splendens* culturists also add clay and salt to rearing water. The first helps the fish recover faster (Chiarachavildham, 2016) while the second helps heal wounds, nourishes the skin, and reduces pathogen growth (Puello-Cruz et al., 2010; Putiyanan, 1999).

Although indigenous knowledge of the above ingredients is widely applied by bettas fish farmers in Thailand, there is still a lack of research to support local knowledge for further commercialization. Therefore, this study aims to investigate the ability of Thai herbal recipes to improve the healing of wounds of Siamese fighting fish. To mimic the type of damage caused during mating or fighting contests, the caudal fin was amputated. The results of this study will help develop simple herbal recipes for commercial application in betta fish farming.

2. Materials and methods

2.1. Animal protocols

The transportation, acclimatization, fin amputation, rearing, sampling, and euthanasia of the fish conformed to the “Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes,” National Research Council, Thailand (Application No. U1–06514-2560). This research project was conducted as part of a larger project entitled “Innovations from indigenous knowledge for using Indian-almond leaf (*Terminalia catappa*) and herbal recipes for rearing and wound healing in Siamese fighting fish (*Betta splendens*)”, which was approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Project Code 2564–01-030).

2.2. Preparation of ingredients and fermented water from Thai herbal recipes

The principal herbs of interest included dried Indian almond (*T. catappa*) leaves (IAL), dried banana (*M. sapientum*) leaves (BL), and dried papaya (*C. papaya*) leaves (PL). While dried lemongrass (*C. citratus*) leaves (LL), dried clay, and salt were secondary ingredients, they were included in every formulation except one of two controls. The leaves were harvested from a few farms within Songkhla Province in southern Thailand. Each leaf type was cleaned, separately soaked in 5 mg L⁻¹ of KMnO₄ for 15 min, and washed three times with distilled water to remove any attached microorganisms and impurities. The cleaned leaves were dried at 60 °C for 12 h, crushed using a grinder (WF-04, Siam Wealth Food Co., Ltd., Nonthaburi, Thailand), sieved, homogeneously mixed, packed in polyethylene bags, placed inside black plastic bags to exclude light, and stored at 4 °C until used. The formulations of

Table 1
The composition of traditional Thai herbal recipes used to treat male Siamese fighting fish after caudal fin amputation. The symbols × and ✓ indicate absence and presence of ingredients, respectively.

Treatment	Recipe ingredients					
	IAL (0.25 g L ⁻¹) ¹	BL (1.5 g L ⁻¹) ²	PL (2.5 g L ⁻¹) ³	LL (1 g L ⁻¹) ⁴	Clay (50 g L ⁻¹) ⁵	Salt (5 g L ⁻¹) ⁶
CT 1	×	×	×	×	×	×
CT 2	×	×	×	✓	✓	✓
IA	✓	×	×	✓	✓	✓
B	×	✓	×	✓	✓	✓
P	×	×	✓	✓	✓	✓
IAB	✓	✓	×	✓	✓	✓
BP	×	✓	✓	✓	✓	✓
IP	✓	×	✓	✓	✓	✓
IBP	✓	✓	✓	✓	✓	✓

IAL, dried Indian almond (*Terminalia catappa*) leaf; BL, dried banana (*Musa sapientum*) leaf; PL, dried papaya (*Carica papaya*) leaf; LL, dried lemongrass (*Cymbopogon citratus*) leaf; CT, control; IA, treatment containing IAL as key ingredient; B, treatment containing BL as key ingredient; P, treatment containing PL as key ingredient; IAB, treatment containing IAL and BL as key ingredients; BP, treatment containing BL and PL as key ingredients; IP, treatment containing IAL and PL as key ingredients; IBP, treatment containing IAL, BL, and PL as key ingredients.

1–6 The herbal recipe concentrations as described by Malawa et al. (2022)¹, Rucksapram (2007)², Banplakatrayong (2022)³, Institute for Innovative Learning (2022)⁴, Chiarachavildham (2016)⁵, and Puello-Cruz et al. (2010)⁶.

the Thai herbal recipes are summarized in Table 1. Raw tap water was dechlorinated using sodium thiosulfate coupled with aeration for three days. Fermentation was performed by soaking ingredients in dechlorinated water in 30 L plastic containers (31 cm width × 30 cm length × 49.7 cm height) at room temperature for three days, stirring twice daily. At the end of three days, the mixtures were passed through filter cloth and Whatman No. 4 paper, as described by Malawa et al. (2022).

2.3. Fish acclimatization and fin amputation

Three-month-old, solid-red, male Siamese fighting fish were purchased from a local farm in Nakhon Pathom Province, Thailand. During a two-week acclimatization period, specimens were separately reared in cylindrical plastic containers (9.3 cm diameter × 12.5 cm height × 8 cm depth), containing 350 mL of water. At the end of the acclimatization period, each fish was immediately weighed to obtain initial body weight (1.29 ± 0.01 g, n = 135). After weighing, the fish were randomly divided into nine treatments of fifteen fish. The acclimatized fish were anesthetized with a 15 mg L⁻¹ solution of clove oil and caudal fin lengths were measured with a Vernier caliper. The fin amputation procedure was conducted as described by Kolluru et al. (2006). Half of the caudal fin length was amputated in the vertical plane using a sterilized scalpel (see Fig. 1).

2.4. Fish treatments

After amputation, all the fish were carefully transferred to 350-mL cylindrical plastic containers (9.3 cm diameter × 12.5 cm height × 8 cm depth) containing seven different treatments (n = 15 per treatment) and reared for 35 days under a 12-h light (06.00 to 18.00 h)/12-h dark cycle. Two controls (n = 15 per treatment) were designed: non-fermented water (CT 1), and fermented water containing LL, clay, and salt (CT 2). All of fifteen fish, each kept apart from all others in an individual container. Each fish, therefore, served as an experimental unit. Three treatments were obtained by adding IAL (treatment IA), BL (treatment B), or PL (treatment P) to CT 2. Three more treatments were obtained by adding IAL and BL (treatment IAB), IAL and PL (treatment IP), and BL and PL (treatment BP) to CT 2. The seventh treatment was

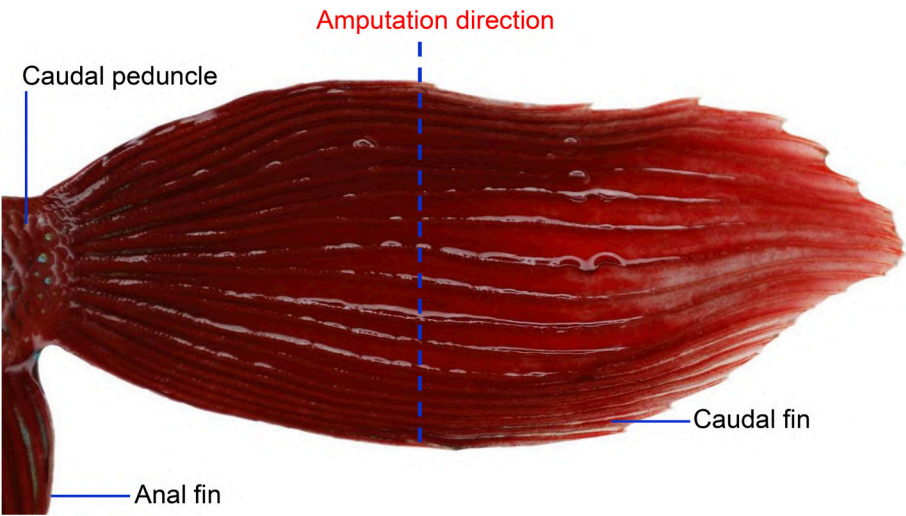


Fig. 1. The amputation of caudal fins for studying regeneration in male Siamese fighting fish.

obtained by adding IAL, BL and PL (treatment IBP) to CT 2. The formulations of these treatments are summarized in Table 1.

All fish were fed *ad libitum* twice daily at 08.00 and 17.00 h with a

commercial floating feed for fighting fish (Betta Bio-Gold; Kyorin Food Ind. Ltd., Himeji, Japan). The proximate chemical composition of the feed was analyzed according to AOAC (2005), and the results on a dry

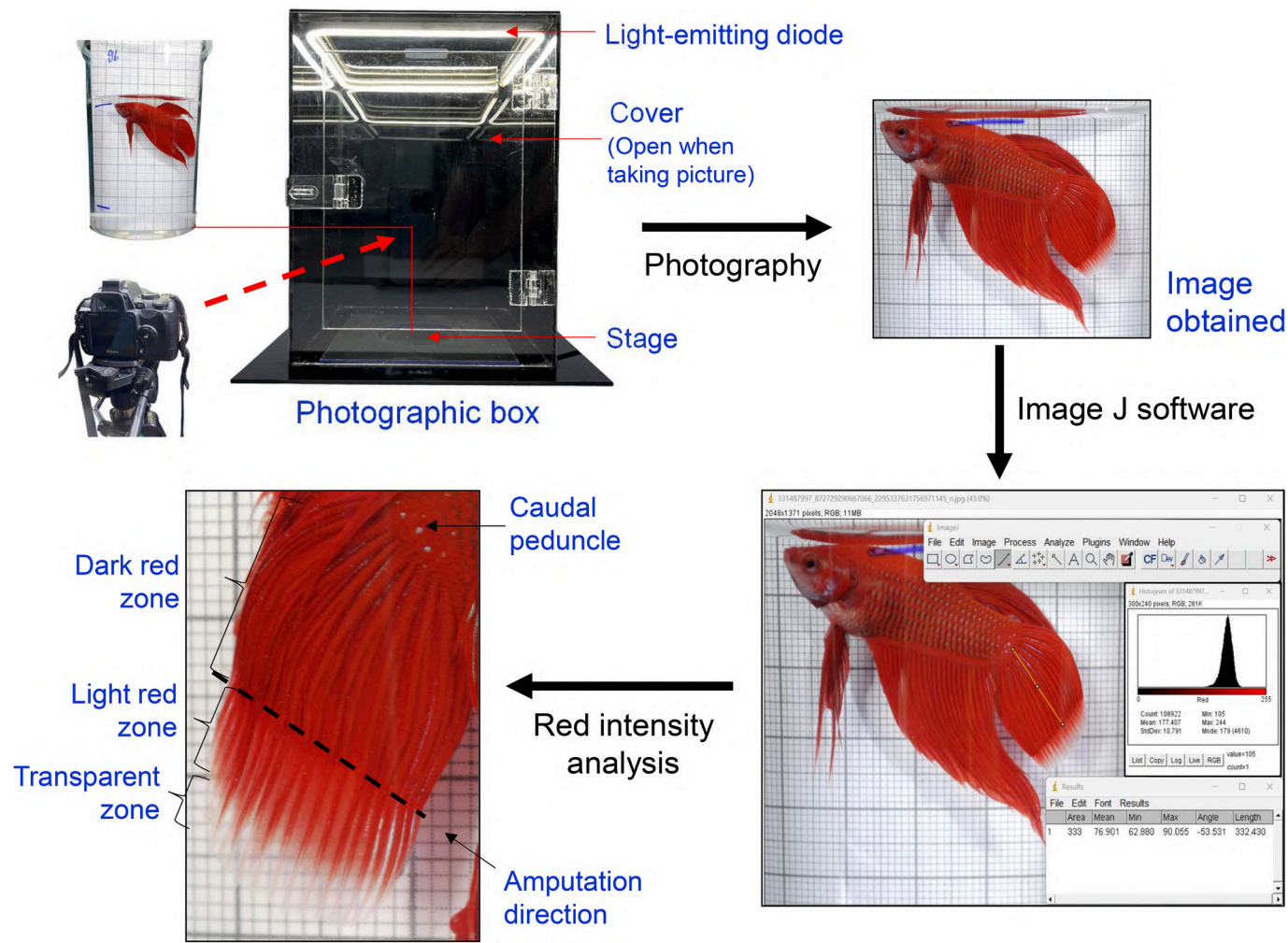


Fig. 2. Weekly monitoring of fin length and color development after caudal fin amputation. The fins were photographed in a closed box. The recorded images were transferred to Image J software to calculate regenerated fin lengths and regenerated color zones. The regenerated fins were divided for color development monitoring into dark red, light red, and transparent zones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

matter basis were: $39.4 \pm 0.2\%$ crude protein, $3.23 \pm 0.12\%$ crude lipid, $1.97 \pm 0.18\%$ crude fiber, and $7.72 \pm 0.24\%$ crude ash. Any feed uneaten after 30 min was recovered and dried at $60\text{ }^{\circ}\text{C}$ until a constant weight was achieved. Obtained data were used to calculate the feeding rate, and the amounts of feed given were adjusted weekly according to the rate of consumption. Static system without aeration was conducted over five-week experiment. Every three days, 80% of the rearing water was replaced with water containing fresh fermented water. During the experimental period, the quality of rearing water was monitored twice weekly. The parameters of interest were water temperature ($23.9 \pm 0.1\text{ }^{\circ}\text{C}$), pH (6.51 ± 0.03), conductivity ($5.41 \pm 0.21\text{ mS cm}^{-1}$), total dissolved solids ($3.43 \pm 0.13\text{ g L}^{-1}$), dissolved oxygen ($5.71 \pm 0.06\text{ mg L}^{-1}$), salinity ($3.00 \pm 0.12\text{ g L}^{-1}$), total alkalinity ($129 \pm 5\text{ mg L}^{-1}$), hardness ($255 \pm 9\text{ mg L}^{-1}$), total ammonia nitrogen ($1.28 \pm 0.03\text{ mg L}^{-1}$), nitrite ($0.09 \pm 0.01\text{ mg L}^{-1}$), and nitrate ($0.86 \pm 0.15\text{ mg L}^{-1}$).

Mortality and morbidity were monitored daily over the duration of the experiment. At the end of the trial, all the fish ($n = 135$) were starved for 24 h and then sacrificed by chilling in ice before body weight and total length were measured. Both values were then used to calculate specific growth rates and condition factors. The parameters describing survival, growth performance and feed consumption were calculated as follows:

Survival (%) = $100 \times (\text{final fish number} / \text{initial fish number})$,
Specific growth rate (% body weight day⁻¹) = $100 \times [(\ln W_t - \ln W_0) / (t - t_0)]$
where W_t = mean weight (g) at day t , W_0 = mean weight (g) at day t_0 ,
Condition factor = $100 \times [\text{body weight (g)} / \text{standard length (cm)}^3]$,
and
Feeding rate (% body weight day⁻¹) = $100 \times C / [(W_0 + W_t) / 2] / t$
where C = daily feed consumption (g), W_0 = initial body weight (g), W_t = final body weight (g), t = feeding duration (day).

2.5. Fin regeneration monitoring

Caudal fins ($n = 15$ per treatment) were photographed every seven days (Nikon D60, Nikon Inc., Thailand). An off-white light-emitting diode (LED) was used as a light source inside a closed box (20 cm width $\times$ 20 cm length $\times$ 26 cm height) with a black background. Each fish in its cylindrical container was placed in the box, and a plastic millimeter grid was placed behind the fish as a reference for measurements. All images were produced with the flash off and automatic white balance turned on. The recorded images were processed by Image J software to calculate caudal fin length. Regeneration (% in length) was calculated from $100 \times [(\text{length on day } N \text{ (cm)} - \text{length on day } 0 \text{ (cm)}) / \text{fin length amputated (cm)}]$.

Based on fin regeneration ability, the suitable treatments (IA, IAB, BP, IP, and IBP) were chosen for evaluating red pigmentation development. The same set of images from five alternative treatments were processed with the Image J software, and automatically analyzed for red intensity values. The original images were decomposed and returned numerical values ranging from 0 to 255. All parts of regenerated fins, and the parts of fins that remained after amputation, were individually classified into three zones of red intensity: dark red zone (DRZ), light red zone (LRZ), and transparent zone (TZ). A total red zone (TRZ) and the ratio TRZ: TZ were also calculated. After measuring the length of each zone, the calculation was performed as described for percent regeneration in length. The monitoring of fin regeneration is illustrated in Fig. 2.

2.6. Statistical analysis

Sample size per treatment was calculated using Program R 3.6.0, requiring the power of test at 0.8. The present study was formulated as a complete randomized design with seven treatments and two controls. The obtained data were expressed as means $\pm$ standard errors (SE). All statistical comparisons were made using the Statistical Package for Social Sciences Version 22 (SPSS Inc., Chicago, USA). Differences among

Table 2

Survival, growth, and feed consumption of male Siamese fighting fish after caudal fin amputation. The measurements were performed at the end of a five-week trial.

Treatment	Parameter					
	Survival (%)	FBW (g)	SGR (% BW day ⁻¹)	FTL (cm)	CF	FR (% BW day ⁻¹)
CT 1	100	1.47 $\pm$ 0.05	0.48 $\pm$ 0.04	4.88 $\pm$ 0.08	1.27 $\pm$ 0.04	1.75 $\pm$ 0.04
CT 2	100	1.50 $\pm$ 0.04	0.43 $\pm$ 0.04	5.02 $\pm$ 0.04	1.19 $\pm$ 0.04	1.83 $\pm$ 0.03
IA	100	1.56 $\pm$ 0.02	0.53 $\pm$ 0.03	5.01 $\pm$ 0.05	1.25 $\pm$ 0.04	1.81 $\pm$ 0.02
B	100	1.52 $\pm$ 0.02	0.50 $\pm$ 0.04	5.00 $\pm$ 0.04	1.22 $\pm$ 0.03	1.84 $\pm$ 0.02
P	100	1.50 $\pm$ 0.03	0.43 $\pm$ 0.02	4.78 $\pm$ 0.17	1.25 $\pm$ 0.02	1.82 $\pm$ 0.03
IAB	100	1.53 $\pm$ 0.02	0.46 $\pm$ 0.03	4.97 $\pm$ 0.05	1.26 $\pm$ 0.03	1.84 $\pm$ 0.03
BP	100	1.42 $\pm$ 0.04	0.42 $\pm$ 0.03	4.88 $\pm$ 0.05	1.23 $\pm$ 0.03	1.87 $\pm$ 0.02
IP	100	1.53 $\pm$ 0.06	0.43 $\pm$ 0.03	5.02 $\pm$ 0.06	1.23 $\pm$ 0.07	1.80 $\pm$ 0.04
IBP	100	1.47 $\pm$ 0.03	0.46 $\pm$ 0.04	4.98 $\pm$ 0.03	1.20 $\pm$ 0.04	1.84 $\pm$ 0.02
P-value	–	0.229	0.250	0.290	0.882	0.205

FBW, final body weight; SGR, specific growth rate; BW, body weight; FTL, final total length; CF, condition factor; FR, feeding rate; CT, control; IA, treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient; B, treatment containing dried banana (*Musa sapientum*) leaf as key ingredient; P, treatment containing dried papaya (*Carica papaya*) leaf as key ingredient; IAB, treatment containing dried Indian almond and banana leaves as key ingredients; BP, treatment containing dried banana and papaya leaves as key ingredients; IP, treatment containing dried Indian almond and papaya leaves as key ingredients; IBP, treatment containing dried Indian almond, banana, and papaya leaves as key ingredients.

Data are expressed as means $\pm$ SE ($n = 15$ per treatment). Differences between means were tested with Duncan’s multiple range test. Different superscripts in the same row indicate a significant difference ($P < 0.05$).

means between treatment groups were subjected to one-way analysis of variance using Duncan’s multiple range test as a post hoc test. The hypothesized condition was rejected if the *P-value* was 0.05 or below.

3. Results

3.1. Growth and feed utilization characteristics

No mortality was observed over five weeks after caudal fin amputation ($P > 0.05$, Table 2). The herbal treatments had no effect on growth (specific growth rate), body morphometrics (condition factor), or feed utilization (feeding rate) ($P > 0.05$). The respective average values were 0.46 body weight day⁻¹, 1.23, and 1.82% body weight day⁻¹.

3.2. Fin length regeneration

Among the seven treatments and two controls, approximately half of the original caudal fin length was regenerated within the first week. On day 14, fin length had increased 1.55-fold. Thereafter, regeneration was slower (1.06 to 1.14-fold increases) until day 35. At the end of observation, the length of regenerated fins was statistically higher for fish reared in IA, IAB, BP, IP, and IBP treatments, relative to treatments CT 1 and B ($P < 0.05$, Table 3). However, intermediate results were observed for fish reared in treatments CT 2 and P ($P > 0.05$). Based on third-order polynomial regression equations, the fish in the IA treatment completely regenerated the caudal fin within 30.8 days. The coefficient variation (CV) of 9.78% was relatively low at the end of day 35 (Fig. 3a). Caudal

Table 3
Percent regeneration (in length) of caudal fin after amputation, followed by treatment in fermented water from Thai herbal recipes. The data were recorded on days 7, 14, 21, 28, and 35.

Treatment	Percent regeneration				
	Day 7	Day 14	Day 21	Day 28	Day 35
CT 1	42.4 ± 1.6 ^d	72.3 ± 2.5 ^d	75.9 ± 2.1 ^c	90.0 ± 3.5 ^{bc}	95.7 ± 4.0 ^b
CT 2	43.5 ± 1.4 ^d	74.6 ± 1.9 ^{bcd}	78.2 ± 2.0 ^c	90.1 ± 2.3 ^{bc}	99.6 ± 3.0 ^{ab}
IA	50.9 ± 1.4 ^{bc}	80.8 ± 2.3 ^{ab}	85.9 ± 2.1 ^a	98.3 ± 2.5 ^a	108 ± 3 ^a
B	46.4 ± 1.4 ^{cd}	73.5 ± 2.0 ^{cd}	78.4 ± 2.0 ^c	86.8 ± 2.5 ^c	96.2 ± 3.2 ^b
P	48.9 ± 1.4 ^c	75.4 ± 1.6 ^{bcd}	79.2 ± 1.8 ^{bc}	89.7 ± 1.8 ^{bc}	100 ± 3 ^{ab}
IAB	54.0 ± 1.4 ^b	83.3 ± 2.2 ^a	87.3 ± 2.1 ^a	97.2 ± 2.6 ^{ab}	108 ± 4 ^a
BP	50.6 ± 1.2 ^{bc}	79.8 ± 1.7 ^{abc}	84.6 ± 1.7 ^{ab}	94.3 ± 2.0 ^{abc}	108 ± 2 ^a
IP	50.8 ± 1.9 ^{bc}	76.1 ± 2.3 ^{bcd}	81.6 ± 2.3 ^{abc}	92.7 ± 2.5 ^{abc}	107 ± 3 ^a
IBP	58.6 ± 2.1 ^a	77.4 ± 2.0 ^{abcd}	85.0 ± 2.2 ^{ab}	97.5 ± 2.6 ^{ab}	107 ± 3 ^a
P-value	< 0.001	0.003	< 0.001	0.008	0.005

CT, control; IA, treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient; B, treatment containing dried banana (*Musa sapientum*) leaf as key ingredient; P, treatment containing dried papaya (*Carica papaya*) leaf as key ingredient; IAB, treatment containing dried Indian almond and banana leaves as key ingredients; BP, treatment containing dried banana and papaya leaves as key ingredients; IP, treatment containing dried Indian almond and papaya leaves as key ingredients; IBP, treatment containing dried Indian almond, banana, and papaya leaves as key ingredients. Data are expressed as means ± SE (n = 15 per treatment). Differences between means were tested with Duncan's multiple range test. Different superscripts in the same row indicate a significant difference (P < 0.05).

fin regeneration times for fish in other treatments were as follows: IAB 32.3 days (CV = 11.6%, Fig. 3b), BP 32.7 days (CV = 10.2%, Fig. 3c), IP 32.4 days (CV = 14.2%, Fig. 3d), and IBP 31.3 days (CV = 8.56%, Fig. 3e).

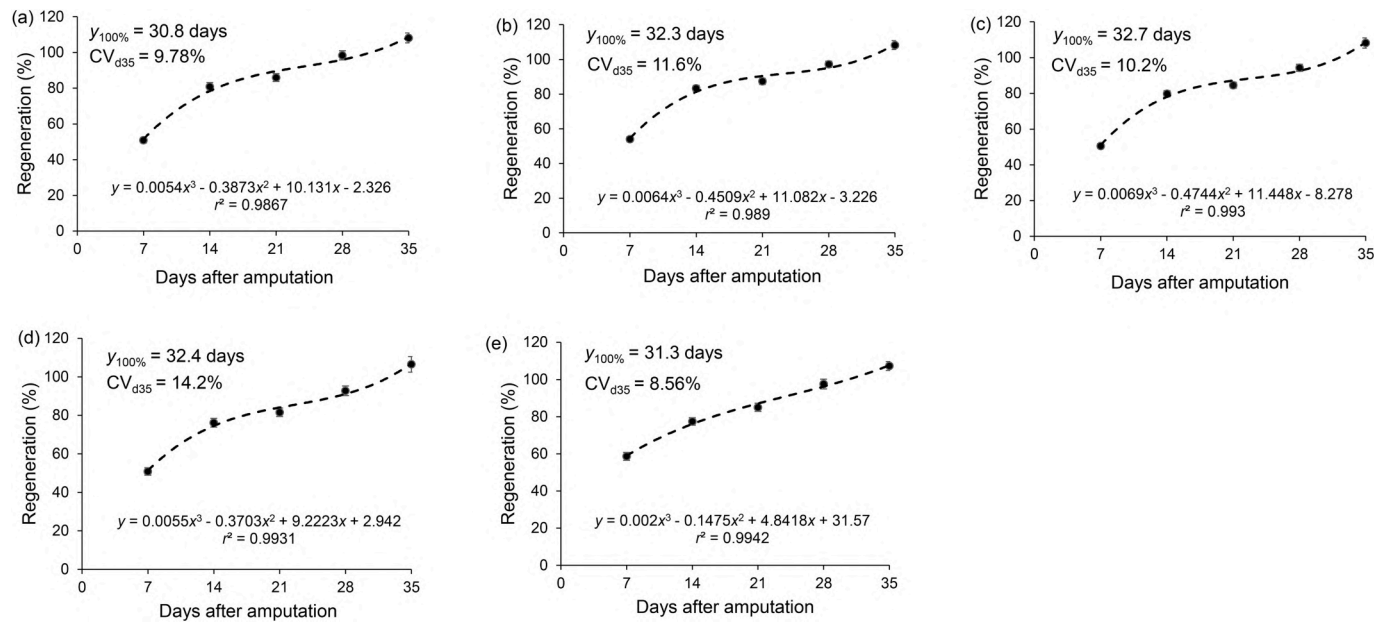


Fig. 3. Caudal fin regeneration in male Siamese fighting fish after amputation. The fish were reared in water fermented with herbs. The water contained dried lemongrass leaf, clay and salt. Added ingredients were dried Indian almond leaf (a), dried Indian almond and banana leaf (b), dried banana and papaya leaf (c), dried Indian almond and papaya leaf (d), and dried Indian almond, banana, and papaya leaf (e). Each data point was calculated from all individual fish (n = 15).

3.3. Color development in regenerated fins

Herbal recipes and rearing times had significant effects on the development of red pigmentation in the regenerated caudal fins (P < 0.05, Table 4). As the fins grew in length, color development was most pronounced in the TRZ followed by the LRZ. Color intensity in the TRZ ranged from 87.9 to 100% and the TRZ: TZ ratio ranged from 88:12 to 100:0. TRZ and TRZ: TZ developed faster in the fish reared in BP, IP, and IBP than in IA and IAB (P < 0.05). At the end of day 35, the development of red pigmentation was complete in those three treatments (P < 0.05).

4. Discussion

In this study, the amputated caudal fins of male Siamese fighting fish were treated over 35 days with water-based fermentations of various herbs. At the end of observation, no mortality was found, and growth performance (final body weight, specific growth rate, and final total length), body morphometrics (condition factor), and feed utilization (feeding rate) parameters did not differ across seven treatments and two controls (P > 0.05). Unchanged these parameters indicate normal growth and no negative effects due to herbal treatments. No effects of survival due to herbal treatments in amputated fish has also been reported (Ngamsiri and Laumsomboon, 2021), without existing records on growth biometrics (Geraudie et al., 1995; Johnson and Denton, 1977; Kolluru et al., 2006; Ngamsiri, 2015; Poleo et al., 2001). High growth obtained in this study was higher than those previously reported in male Siamese fighting fish (James and Sampath, 2006; Saekhow et al., 2018), suggesting that the amputated fish recovered well under the treatment conditions. In addition, concurrent observations in bubble-nest creating ability and hematological parameters (data not shown) of the same fish set indicate well-being of the fish reared in herbal recipes.

Phytoconstituents contained in the herbs' fermented water, such as tannin, can inhibit bacterial infection (Chansue and Assawawongkasem, 2008; Chitmanat et al., 2005; Purivirojkul, 2012). During the experiment, external signs such as non-bleeding wounds, inflammation, or mucous covering were not observed in all amputated fish. The length of damaged fins was rapidly regenerated in the first week (~50%), and then increased more gradually until the end of the five-week study. Preliminary studies showed that caudal fins of fighting fish regenerated

Table 4
Color development (in length) on regenerated caudal fin of male Siamese fighting fish. The values were recorded on day 7, 14, 21, 28, and 35 of the experiment.

Day	Treatment	DRZ (%)	LRZ (%)	TZ (%)	TRZ (%)	TRZ: TZ
7	IA	77.2 ± 0.8 ^a	15.1 ± 0.6 ^b	7.70 ± 0.41 ^a	92.3 ± 0.4 ^b	92:8
	IAB	74.7 ± 0.7 ^b	17.1 ± 0.4 ^b	8.22 ± 0.59 ^a	91.8 ± 0.6 ^b	92:8
	BP	77.8 ± 0.6 ^a	16.3 ± 0.7 ^b	5.91 ± 0.70 ^{ab}	94.1 ± 0.7 ^{ab}	94:6
	IP	78.0 ± 0.8 ^a	17.5 ± 1.1 ^b	4.58 ± 1.01 ^b	95.4 ± 1.0 ^a	95:5
	IBP	75.1 ± 0.7 ^b	20.8 ± 1.1 ^a	4.12 ± 1.21 ^b	95.9 ± 1.2 ^a	96:4
	<i>P</i> -value	0.002	< 0.001	0.002	0.002	
14	IA	66.2 ± 2.3 ^b	21.7 ± 2.5 ^{ab}	12.2 ± 1.0 ^a	87.9 ± 1.0 ^b	88:12
	IAB	72.5 ± 2.5 ^b	17.5 ± 2.9 ^b	10.0 ± 1.8 ^a	90.0 ± 1.8 ^b	90:10
	BP	71.4 ± 1.9 ^b	27.9 ± 1.8 ^a	0.73 ± 0.51 ^b	99.3 ± 0.5 ^a	99:1
	IP	68.3 ± 2.2 ^b	29.0 ± 2.2 ^a	2.70 ± 1.29 ^b	97.3 ± 1.3 ^a	97:3
	IBP	82.3 ± 3.8 ^a	14.3 ± 2.9 ^b	3.47 ± 1.41 ^b	96.5 ± 1.4 ^a	97:3
	<i>P</i> -value	0.001	< 0.001	< 0.001	< 0.001	
21	IA	70.8 ± 1.7 ^b	18.1 ± 2.1 ^{ab}	11.2 ± 1.3 ^a	88.8 ± 1.3 ^b	89:11
	IAB	76.1 ± 2.5 ^{ab}	13.8 ± 3.0 ^b	10.1 ± 2.1 ^a	89.9 ± 2.1 ^b	90:10
	BP	74.8 ± 3.1 ^b	24.1 ± 2.8 ^a	1.04 ± 0.61 ^b	99.0 ± 0.6 ^a	99:1
	IP	71.7 ± 2.9 ^b	25.3 ± 3.1 ^a	3.05 ± 1.40 ^b	97.0 ± 1.4 ^a	97:3
	IBP	84.0 ± 3.8 ^a	13.8 ± 3.3 ^b	2.24 ± 0.95 ^b	97.8 ± 1.0 ^a	98:2
	<i>P</i> -value	0.014	0.010	< 0.001	< 0.001	
28	IA	75.3 ± 2.4	14.5 ± 3.3	10.2 ± 1.9 ^a	89.8 ± 1.9 ^{bc}	90:10
	IAB	79.2 ± 3.3	10.2 ± 3.8	10.1 ± 2.5 ^a	89.9 ± 2.5 ^c	90:10
	BP	78.3 ± 4.7	20.4 ± 4.4	1.34 ± 1.12 ^b	98.7 ± 1.1 ^a	99:1
	IP	75.1 ± 4.3	21.5 ± 4.5	3.40 ± 1.71 ^b	96.6 ± 1.7 ^{abc}	97:3
	IBP	85.8 ± 4.9	13.2 ± 4.8	1.01 ± 0.70 ^b	99.0 ± 0.7 ^{ab}	99:1
	<i>P</i> -value	0.338	0.267	< 0.001	< 0.001	
35	IA	84.0 ± 2.4	8.3 ± 2.6	7.78 ± 1.76 ^a	92.2 ± 1.8 ^b	92:8
	IAB	90.2 ± 2.8	2.4 ± 1.5	7.41 ± 2.23 ^a	92.6 ± 2.2 ^b	93:7
	BP	87.5 ± 4.2	12.5 ± 4.2	nd	100 ± 0 ^a	100:0
	IP	83.2 ± 5.2	16.9 ± 5.2	nd	100 ± 0 ^a	100:0
	IBP	86.5 ± 5.3	13.5 ± 5.3	nd	100 ± 0 ^a	100:0
	<i>P</i> -value	0.753	0.117	< 0.001	< 0.001	

DRZ, dark red zone; LRZ, light red zone; TZ, transparent zone; TRZ, total red zone; IA, treatment containing dried Indian almond (*Terminalia catappa*) leaf as key ingredient; IAB, treatment containing dried Indian almond and banana leaves as key ingredients; BP, treatment containing dried banana and papaya leaves as key ingredients; IP, treatment containing dried Indian almond and papaya leaves as key ingredients; IBP, treatment containing dried Indian almond, banana, and papaya leaves as key ingredients; nd, not detected. Data are expressed as means ± SE (*n* = 15 per treatment). Differences between means were tested with Duncan's multiple range test. Different superscripts in the same row indicate a significant difference (*P* < 0.05).

faster when ~50% was amputated rather than ~25%. Divergent reconstruction times/rates were observed among different fin excision sites and fish species. More proximal amputations took longer to regain their original length than distal amputations (Uemoto et al., 2020). Observations of a number of fish species have reported varying regeneration times: 24 days for mosquito fish (Johnson and Denton, 1977) and guppy (Kolluru et al., 2006); 14 days for zebrafish (Geraudie et al., 1995; Poleo et al., 2001) and fancy carp, *Cyprinus carpio* (Chansue, 2006); 42 days for goldfish, *Carassius auratus* (Ngamsiri and Laumsomboon, 2021); and 28 days for Nile tilapia (Ngamsiri, 2015).

In the current study, fish reared in the formulations containing Indian almond leaf (IA), Indian almond leaf and banana leaf (IB), banana leaf and papaya leaf (BP), Indian almond leaf and papaya leaf (IP), and Indian almond leaf, banana leaf, and papaya leaf (IBP) completely regenerated fin length within a duration of 31–33 days. Fish reared in CT 1 were calculated to have regenerated their fins in 35.8 days. Fish can completely regenerate injured or lost organs naturally (Pfefferli and Jazwińska, 2015), but the herbal treatments improved regeneration rates.

Several studies have improved wound healing and fin regeneration processes using natural products at optimal levels: 0.2 g L⁻¹ of dried Indian almond leaves for goldfish (Ngamsiri and Laumsomboon, 2021); and 0.03 g L⁻¹ of creat (*Andrographis paniculata*) and dried Indian almond leaves for fancy carp (Chansue, 2006). For Siamese fighting fish, since scientific information is limited, all ingredients (including leaves of Indian almond, banana, papaya, and lemongrass) were applied based on the limited available documents (Banplakatrayong, 2022; Chiarachavildham, 2016; Institute for Innovative Learning, 2022; Malawa et al., 2022; Puello-Cruz et al., 2010; Rucksapram, 2007). These Thai herbs contain phytoconstituents such as tannins, flavonoids, and phenolic compounds that provide anti-oxidant, anti-parasitic, anti-fungal, anti-bacterial, and anti-inflammatory activities (Chitmanat et al., 2005; Maria and Veronica, 2021; Ponpornpisit et al., 2007; Tanmoy et al., 2021; Wijaya et al., 2020). The addition of clay to the water helps fish recover faster (Chiarachavildham, 2016) whereas salt helps heal wounds, nourishes the skin, and reduces pathogenic growth (Puello-Cruz et al., 2010; Putiyanan, 1999). These ingredients promote three main stages of wound healing: they shorten the inflammation phase and accelerate the transition from proliferation to remodeling (Maria and Veronica, 2021; Pastore et al., 2012; Tanmoy et al., 2021; Wijaya et al., 2020).

Caudal fin coloration is consistent with body color. Primarily, regenerated fins will have a light-colored region, or non-pigmented regenerated tissue that requires a pigment progenitor to transfer chromatophores (Huang et al., 2017; Yi et al., 2014). Chromatophores containing pteridine-based pigment cells are color-associated cells derived from the neural crest during embryonic development and determine the differential expression of color in individual fish species. These pigments emerge from the proximal end of the base of the excision and spread distally with fin regrowth (Goodrich and Nichols, 1931; Huang et al., 2017). In the present study, solid-red male Siamese fighting fish were the model, and therefore the focus was on the deposition of erythrophores (Khoo et al., 2012).

The herbal treatments and treatment times affected the development of red pigmentation in the caudal fin, from light to dark red. The regenerated fin was classified into three color zones (dark red, light red, and transparent), and the red coloration developed faster in fish reared in BP, IP, or IBP than in IA and IAB treatments. Some studies have reported that herb extracts added to rearing water stimulated pigmentation. Examples include fermented water from Indian almond leaf for fighting fish (Malawa et al., 2022) and neon tetra fish, *Paracheirodon innesi* (Ardi et al., 2020). Based on our observations, the three most effective treatments were BP, IP and IBP. The ingredient common to all three was dried papaya leaf. Papaya leaf contains flavonoids that reduce oxidative stress by inhibiting free radicals. They also contain dietary β-carotene that improves skin coloration (Chavarria and Flores, 2013;

Das and Biswas, 2020; Van der Salm et al., 2006). Papaya leaf extracts are also used to dye textiles (Rani et al., 2020). Therefore, it is possible that dried papaya leaf might be a mordant that contributes some skin pigmentation to fish immersed in its extract.

5. Conclusions

Male Siamese fighting fish automatically regenerate damaged fins in normal rearing water. Fin length develops primarily followed by the migration of pigmentation. However, findings from the current study demonstrate the ability of fermented water from Thai herbal formulations to significantly enhance fin regeneration, in terms of both length and color development. All the formulations included dried lemongrass leaf, clay and salt. Fully developed fins were observed in fish reared in fermented water containing the above components with the addition of dried papaya leaf and papaya leaf with dried leaf of either banana or Indian almond or both. Findings from the current study could be applied to enhance fin regeneration in betta fish farming.

CRedit authorship contribution statement

Saowalak Malawa: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nutt Nuntapong:** Investigation, Resources, Writing – review & editing. **Sugree Waeowannajit:** Investigation, Methodology, Writing – review & editing. **Karun Thongprajukaew:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix D

Paper 3

Research article

Somatic responses of injured Siamese fighting fish (*Betta splendens*) raised using various recipes of Thai herbal fermented water

Saowalak Malawa^a, Nutt Nuntapong^a, Karun Thongprajukaew^{b,c,*}

^a Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

^b Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

^c Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

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Abstract

Importance of the work: Thai herbal remedies offer an alternative to chemicals to help in the recuperation of injured Siamese fighting fish (*Betta splendens*).

Objective: To evaluate fish survival and growth, feed utilization, skin coloration, digestive enzyme activity, muscle quality and whole-body composition at the end of herbal treatment for 5 wk.

Materials & Methods: Each male fighting fish had one-half of its caudal fin amputated and then was allocated to one of nine experimental groups. Each fish was reared separately for 5 wk in its own small tank. Treatments consisted of two controls, with one using non-fermented water (CT 1), while the other control consisted of fermented water (CT 2) and was mixed with various products—dried lemongrass leaves (1 g/L), dried clay (50 g/L) and salt (5 g/L). Subsequently, CT 2 was added to different combinations of dried Indian almond leaves (0.25 g/L), dried banana leaves (1.5 g/L) or dried papaya leaves (2.5 g/L) to make seven other treatments.

Results: No mortality occurred and fish growth performance was not influenced by the herbal treatments. The feed conversion ratio significantly ($p < 0.05$) increased in association with decreasing amylase-specific activity, whereas protein- and lipid-digesting enzymes remained consistent. Skin coloration parameters were either reduced or preserved due to the herbal treatments. Muscle quality was unchanged, whereas the crude protein, crude lipid and ash contents in whole-body were similar across the nine treatments.

Main findings: Whereas entire fish can compensate for growth normally, these findings indicated minor effects of long-term herbal treatments on the somatic changes in the injured fish. The results endorsed the therapeutic approach of fermented water of Thai herbs in Siamese fighting fish.

* Corresponding author.
E-mail address: karun.t@psu.ac.th (K. Thongprajukaew)

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Introduction

Betta splendens, also known as the Siamese fighting fish, is a small freshwater fish distinguished by its remarkable color variations and captivating fin morphological traits, whose origins can be traced back to Southeast Asia, notably within central Thailand (Goldstein, 2004; Monvises et al., 2009). Furthermore, Thailand's ornamental fish industry, exclusively targeting these male 'betta' fish, has witnessed substantial growth in captive breeding and international exports (Department of Fisheries, 2021). In the first half of 2021, betta was ranked first among 10 popular ornamental fish in Thailand, with the exported volume surpassing 10 million fish reported at Suvarnabhumi Airport (Department of Fisheries, 2021). The USA is the primary importer of Siamese fighting fish from Thailand, followed by China, France, Iran and Singapore (Sermwatanakul, 2019).

Inherently, these fish display agonistic behavior in confrontations associated with defending territory, be it between males and females or among individuals of the same gender, resulting in damaged bodies and torn fins (Malawa et al., 2023). Consequently, the breeders raise these fish in solitary captivity, commencing at approximately age 1.5 mth (Thongprajukaew et al., 2010a, b), to mitigate the adverse effects of their combative nature. In addition, these injuries can manifest during courtship and reproductive phases (Malawa et al., 2023).

In Thailand, immersion in herbal treatment is the preferred and practical treatment to heal external damage in injured fish rather than using antibiotics or chemicals that cause adverse health effects to the fish and (via the residues) in their surroundings (Malawa et al., 2022, 2023). Typically, some herbs, such as the dried leaves of the Indian almond (*Terminalia catappa*), banana (*Musa sapientum*), papaya (*Carica papaya*) and lemongrass (*Cymbopogon citratus*) or dried clay or salts, have been incorporated into the recipes used to treat the fish (Malawa et al., 2023). These ingredients have various phytoconstituents, such as tannin, flavonoid and phenolic compounds, differing in quantity and active ingredient type. For example, the dried leaves of the Indian almond aid in wound healing and act against harmful monogeneans and *Trichodina* spp. (Chitmanat et al., 2005; Ponpornpisit et al., 2007), while the dried leaves of banana, papaya and lemongrass provide anti-bacterial, anti-oxidant and anti-inflammatory activities that help to heal wounds (Wijaya et al., 2020; Maria and Veronica, 2021; Tanmoy et al., 2021), especially

those caused during fish fighting. Dried clay and salt are added to help fish recover more rapidly, to heal wounds, to nourish the skin and to reduce pathogen growth (Putiyanan, 1999; Puello-Cruz et al., 2010; Chiarachavildham, 2016).

Other benefits have been reported regarding the use of certain herbs, specifically the optimal concentration of *T. catappa* leaf extract to enhance the survival rate, growth performance and feed utilization (Nugroho et al., 2016; Nurhidayat et al., 2016; Ramos et al., 2020). Additionally, this herb has been associated with improvements in digestive enzyme activities (Malawa et al., 2022) and skin coloration (Ardi et al., 2020; Malawa et al., 2022), but without affecting muscle quality and the whole-body composition in healthy fish (Malawa et al., 2022). However, an excessive concentration of its extract may adversely affect these parameters (Ikhwanuddin et al., 2014; Mininel et al., 2014).

Although herbal treatment involving Indian almond leaf has been documented for healthy Siamese fighting fish, there is a noticeable lack of information regarding using a combination of ingredients and their effects on somatic changes in injured fish. Therefore, the current study examined fish survival and growth, feed utilization, skin coloration, digestive enzyme activity, muscle quality and whole-body composition. Caudal fin amputation was used as a model to simulate a torn fin on the fish resulting from agonistic behavior. The findings from the current study might support using Thai herbal treatment in betta fish farms.

Materials and Methods

Ethics statements

The Institutional Animal Care and Use Committee at Prince of Songkla University approved the utilization of the animals in this scientific research (Project Code 2564-01-030). Transportation, acclimatization, surgery, husbandry and sampling were carried out under this agreement.

Preparation of herbal fermented water

Newly fallen leaves (Indian almond, banana and papaya), mature leaves (lemongrass) and clay were harvested from a few farms in Songkhla province, Thailand. The samples were cleaned, soaked in 5 mg/L of KMnO_4 for 15 min to eliminate attached microorganisms and then washed three times with distilled water. They were dried at 60°C for 12 hr,

crushed using a grinder, sieved, homogenously mixed, packed in polyethylene bags, placed inside black plastic bags to exclude light and stored at 4°C until used.

The components of each herbal formula were detailed in Malawa et al. (2023). The chosen concentration of each ingredient was based on available documents (Banplakatravyong, 2022; Institute for Innovative Learning, 2022; Malawa et al., 2022; Rucksapram, 2007). Amounts of dried clay was estimated from Chiarachavildham (2016). Table salt was directly added to the rearing water to achieve a final concentration of 5 g/L (Puello-Cruz et al., 2010). Two control groups were established: one using non-fermented water (CT 1) and the other fermented water (CT 2), to which were added dried lemongrass leaves (1 g/L), dried clay (50 g/L), and salt (5 g/L). Three distinct treatments were created by incorporating dried Indian almond leaves (0.25 g/L), dried banana leaves (1.5 g/L), or dried papaya leaves (2.5 g/L) into CT 2. These treatments were denoted as IA, B and P, respectively. Additionally, three more treatments emerged by introducing combinations of dried Indian almond leaves and dried banana leaves, dried Indian almond leaves and dried papaya leaves, and dried banana leaves and dried papaya leaves to CT 2. These treatments were labeled as IAB, IP and BP, respectively. The seventh treatment was generated by adding dried Indian almond leaves, dried banana leaves and dried papaya leaves together to CT 2, and this treatment was designated as IBP. The fermentation was performed by soaking the relevant ingredients in dechlorinated water in 30 L plastic containers (31 cm width × 30 cm length × 49.7 cm height) at room temperature for 3 d, stirring twice daily. At the end of 3 d, the mixtures were passed through filter cloth and Whatman No. 4 paper. The obtained filtrate was used for fish rearing.

Herbal treatment trial

A completely randomized design was conducted using the nine herbal formulations, with 15 individual fish serving as experimental units. The fish preparation and fin amputation were performed as described by Malawa et al. (2023). First, each fish was anesthetized with a 15 mg/L solution of clove oil and then the caudal fin lengths were measured using a set of Vernier calipers. Subsequently, one-half of the caudal fin length was amputated in the vertical plane using a sterilized scalpel. The amputated fish ($n = 135$) with their fins excised were carefully carried and raised separately in cylindrical plastic containers measuring 9.3 cm in diameter, 12.5 cm in

height and 8 cm in depth. These containers were filled with 350 mL of rearing water, comprised of either freshly fermented water obtained from the eight Thai herbal recipes (CT 2, IA, B, P, IAB, BP IP, and IBP) or dechlorinated tap water (CT 1). All fish were reared under a 12 hr light (0600–1800 hours)/ 12 hr dark cycle for 5 wk until there was complete regeneration of the caudal fin. During the herbal treatment period, all the fish were fed *ad libitum* using a commercial floating feed for fighting fish, which contained 39.4% crude protein (Betta Bio-Gold; Kyorin Food Ind. Ltd.; Himeji, Japan). Feeding occurred twice daily, at 0800 hours and 1700 hours. After each feeding session, any remaining feed was collected within 30 min and then dried at 60°C until it reached a constant weight. Obtained data were used to compute the feed conversion ratio (FCR) and the protein efficiency ratio (PER). Every 3 d, 80% of the rearing water was replaced with water containing freshly prepared fermented water. Twice a week, throughout the experimental period, various physio-chemical parameters of the water were measured: temperature ($23.9 \pm 0.1^\circ\text{C}$), pH (6.51 ± 0.03), conductivity (5.41 ± 0.21 mS/cm), total dissolved solids (3.43 ± 0.13 g/L), dissolved oxygen (5.71 ± 0.06 mg/L), salinity (3.00 ± 0.12 g/L), total alkalinity (129 ± 5 mg/L), hardness (255 ± 9 mg/L), total ammonia nitrogen (1.28 ± 0.03 mg/L), nitrite (0.09 ± 0.01 mg/L) and nitrate (0.86 ± 0.15 mg/L).

Fish mortality was monitored daily throughout the experiment. At the end of the trial (5 wk), all the surviving fish ($n = 15$ per treatment) were starved for 24 hr and then sacrificed by chilling in ice before recording body weight (BW) and total length. Samples of viscera ($n = 5$ per treatment), digestive tracts ($n = 5$ per treatment) and white muscle ($n = 5$ per treatment) were gathered from the same group of fish to assess the viscerosomatic index (VSI), digestive enzyme activity and muscle quality, respectively. The whole-body composition analysis used 10 fish chosen from each treatment. Three or four fish were pooled into each lot to obtain one pooled sample (three pooled samples per treatment). The parameters describing survival, growth performance and feed conversion were computed using Equations 1–5:

$$\text{Survival (\%)} = 100 \times (\text{Final fish number} / \text{Initial fish number}) \quad (1)$$

$$\text{Weight gain} = \text{Final BW} - \text{Initial BW} \quad (2)$$

$$\text{VSI} = 100 \times [\text{Wet weight of viscera} / \text{BW}] \quad (3)$$

$$\text{FCR} = \text{Dry feed consumed} / \text{Wet weight gain} \quad (4)$$

$$\text{PER} = \text{Wet weight gain} / \text{Protein intake} \quad (5)$$

Where all parameters are measured in grams and the VSI is determined as a percentage.

Skin coloration

The fish skin was carefully cleaned using soft blotting paper. Subsequently, a portable spectrophotometer (MiniScan EZ 4500; Hunter Associates Laboratory; Reston, VA, USA) was calibrated using black and white standards before assessing the skin coloration in the mid region of the fish. The color parameters were digitally displayed on the LCD screen as L^* , a^* , h^* and C^* .

Determination of digestive enzyme activities

Enzyme extraction and protein quantification in crude extracts

The frozen gastrointestinal tract was extracted in cold 0.2 M Na_2HPO_4 - NaH_2PO_4 buffer (1:15 weight per volume) using a tissue homogenizer (THP-220; Omni International; Kennesaw, GA, USA). The homogenate was centrifuged at $15,000\times g$ and 4°C for 30 min to collect the supernatant from debris and then preserved at -20°C . The total protein concentration in the crude extract was assessed quantitatively based on the method outlined by Lowry et al. (1951) to determine the specific activity of the digestive enzymes (measured in units per milligram of protein).

Digestive enzyme assays

All enzyme assays of the betta fish were performed under optimized conditions of pH and temperature, as reported by Thongprajukaew et al. (2010a, b). Pepsin activity was examined according to the method of Worthington (1993). Trypsin and chymotrypsin activities were assessed based on Rungruangsak-Torrissen et al. (2006). Lipase activity was determined according to the method of Winkler and Stuckmann (1979). The alpha-amylase activity was assayed according to the method of Areekijserree et al. (2004). For these four enzyme tests, one unit (U) was determined as the catalyzed amount to convert $1\ \mu\text{mol}$ of substrate per minute. The amylase-to-trypsin ratio was computed as the amylase activity divided by the trypsin activity from the same fish sample.

Muscle quality

RNA and the protein concentration were assayed according to the method of Rungruangsak-Torrissen (2007). Myosin,

actin and the sarcoplasmic contents were monitored based on weighing defrosted white muscle (approximately 10 mg each), placing in an aluminum pan, sealing with an aluminum lid and then heating at a rate of $5^\circ\text{C}/\text{min}$ relative to an empty pan. Muscle thermal characteristics were assessed through differential scanning calorimetry (DSC7; Perkin Elmer; Waltham, MA, USA) within the temperature range 20 – 100°C , to identify myosin, actin and sarcoplasmic protein based on the onset (T_o), peak (T_p) and conclusion (T_c) temperatures derived from thermograms as documented in Thongprajukaew et al. (2023) concerning betta fish muscle.

Whole-body composition

The whole body of each fish was uniformly minced before undergoing analysis for proximate composition, based on the moisture, ash, crude protein and crude lipid contents, following the standard procedures outlined in Association of Official Analytical Chemists (2005).

Statistical analysis

The trial used a completely randomized design comprising nine herbal formulations, with 15 individual fish as experimental units. The acquired data were presented as mean $\pm$ SD values. The data were subjected to one-way analysis of variance and mean comparisons were performed using Duncan's multiple range test at the $p < 0.05$ test level for significance. All statistical analyses were performed using the Statistical Package for Social Sciences version 22 (SPSS Inc.; Chicago, IL, USA).

Results

Survival, growth performance and feed utilization

No mortality was observed in the fish reared in any of the herbal treatments. The herbal treatments did not affect the growth and feed utilization parameters, except for FCR, which significantly increased in the fish raised in the CT 2, P, BP, IP and IBP treatments (Table 1).

Table 1 Growth performance and feed utilization of male Siamese fighting fish reared in various medicinal recipes extract, where parameters were measured at end of experiment (at 5 wk)

Herbal recipe	Parameter					
	FBW (g)	WG (g)	FTL (cm)	VSI (%)	FCR	PER
CT 1	1.47±0.19	0.43±0.39	4.88±0.28	6.18±2.06	3.30±0.75 ^c	1.08±0.26
CT 2	1.50±0.14	0.28±0.32	5.02±0.14	5.59±0.80	4.28±0.97 ^{ab}	1.11±0.09
IA	1.56±0.07	0.27±0.06	5.01±0.18	6.73±0.82	3.24±0.44 ^c	1.19±0.06
B	1.52±0.08	0.23±0.09	5.00±0.17	6.50±0.86	4.04±1.42 ^{abc}	1.14±0.12
P	1.50±0.10	0.21±0.05	4.78±0.65	5.65±0.97	4.33±0.69 ^{ab}	1.12±0.08
IAB	1.53±0.09	0.24±0.07	4.97±0.21	7.00±1.05	3.75±0.73 ^{bc}	1.16±0.08
BP	1.42±0.14	0.30±0.34	4.88±0.19	5.05±0.55	4.69±1.11 ^a	1.04±0.11
IP	1.53±0.23	0.31±0.33	5.02±0.24	7.01±1.97	4.32±0.70 ^{ab}	1.10±0.06
IBP	1.47±0.12	0.35±0.35	4.98±0.14	6.54±1.10	4.35±1.28 ^{ab}	1.12±0.08
<i>p</i> -Value	0.229	0.449	0.290	0.101	0.002	0.082

FBW = final body weight; WG = weight gain; FTL = final total length; VSI = viscerosomatic index; FCR = feed conversion ratio; PER = protein efficiency ratio; CT 1 = negative control (dechlorinated water); CT 2 = positive control (dried lemongrass leaves + dried clay + salt); IA = CT 2 + dried Indian almond (*Terminalia catappa*) leaf; B = CT 2 + dried banana (*Musa sapientum*) leaf; P = CT 2 + dried papaya (*Carica papaya*) leaf; IAB = CT 2 + dried Indian almond and banana leaves; BP = CT 2 + dried banana and papaya leaves; IP = CT 2 + dried Indian almond and papaya leaves; IBP = CT 2 + dried Indian almond, banana and papaya leaves.

Different lowercase superscripts in the same row indicate significant ($p < 0.05$) differences.

Skin coloration

There were significant differences in skin coloration among the fish in the various herbal treatments, except for L^* (Table 2). Relative to CT 1 and CT 2, there was a notable trend of either unchanged or reduced a^* , h^* and C^* values in all treatment groups ($p < 0.05$).

Table 2 Color parameters of male Siamese fighting fish reared in various treatments involving fermented water based on Thai herbal recipes, where parameters measured at end of experiment (at 5 wk)

Herbal recipe	Color parameter			
	L^*	a^*	h^*	C^*
CT 1	23.0±2.9	13.5±1.2 ^a	0.51±0.06 ^a	15.5±1.5 ^a
CT 2	21.5±2.0	14.2±2.0 ^a	0.50±0.05 ^{ab}	16.2±2.1 ^a
IA	23.5±2.8	12.2±2.0 ^b	0.47±0.05 ^{abc}	13.8±2.3 ^b
B	24.3±2.6	11.4±1.3 ^{bc}	0.45±0.04 ^{bc}	12.7±1.6 ^{bc}
P	23.3±2.6	11.0±1.4 ^{bc}	0.43±0.09 ^c	12.2±1.8 ^c
IAB	23.7±2.6	11.6±1.4 ^{bc}	0.46±0.07 ^{abc}	13.0±1.7 ^{bc}
BP	23.6±3.2	11.2±1.6 ^{bc}	0.48±0.05 ^{abc}	12.6±1.9 ^{bc}
IP	23.0±1.1	11.4±1.3 ^{bc}	0.49±0.04 ^{ab}	13.0±1.6 ^{bc}
IBP	22.8±2.1	10.6±1.5 ^c	0.46±0.07 ^{bc}	11.8±1.9 ^c
<i>p</i> -value	0.208	< 0.001	0.012	< 0.001

L^* = lightness; a^* = redness; h^* = hue; C^* = chroma.

Details for each treatment are shown in Table 1 footnote.

Different lowercase superscripts in the same row indicate significant ($p < 0.05$) differences.

Digestive enzyme activities

The herbal treatments had no significant effects on digestive enzyme-specific activities, except for amylase (Fig. 1), for which there was a significant reduction in the fish reared in treatments CT 2, BP, IP and IBP relative to the control group (dechlorinated water).

Protein synthesis capacity, and myosin and actin contents

There was no significant difference in the values for the average RNA (2,704±520 µg/g), protein (210±46 mg/g) or RNA-to-protein ratio (13.4±3.1 µg/mg) among the fish subjected to any of the herbal treatments. The principal muscle proteins, myosin, actin and sarcoplasmic protein levels exhibited temperature ranges of 41.5–53.4°C, 67.5–74.2°C and 81.0–85.5°C, respectively, from onset heating to the conclusion. Relative to the control group, there were no significant differences in the values for the average myosin (0.89±0.30 J/g), actin (0.35±0.12 J/g) and sarcoplasmic protein (0.10±0.04 J/g) contents, or their total amounts (1.34±0.36 J/g) or proportions (0.43±0.19 J/g) for all herbal treatments.

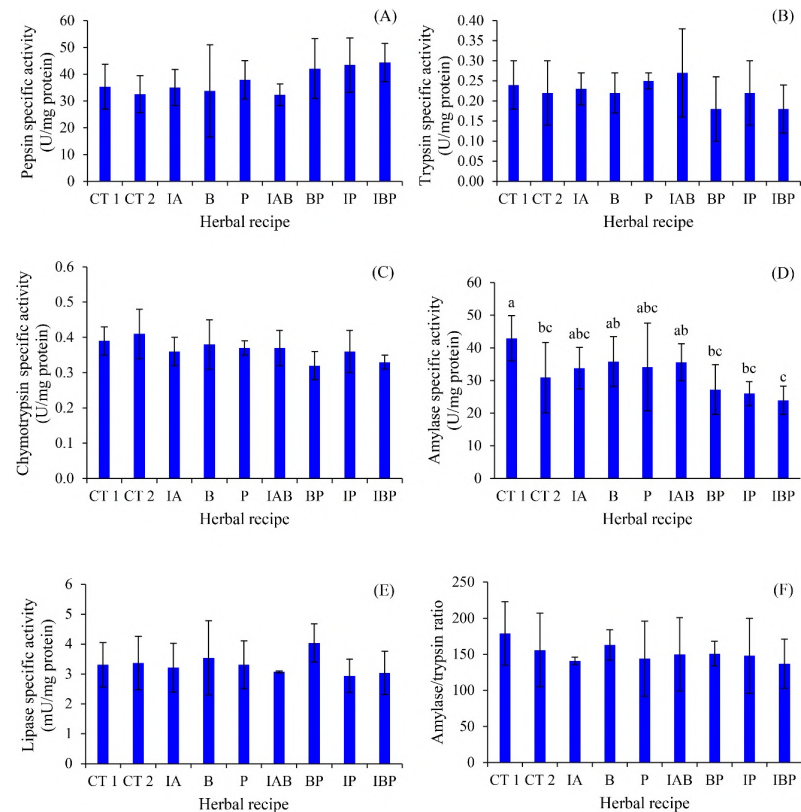


Fig. 1 Specific activities of digestive enzymes in male Siamese fighting fish reared in various treatments using water fermented with Thai herbal recipes: (A) pepsin; (B) trypsin; (C) chymotrypsin; (D) amylase; (E) lipase; (F) amylase-to-trypsin ratio, where parameters measured at end of experiment (at 5 wk), data =mean + SD values, different lowercase letters above columns indicate significant ($p < 0.05$) differences between treatments; details for each treatment are shown in Table 1 footnote.

Whole-body composition

Relative to CT 1, the moisture contents remained unchanged in all herbal treatments (Table 3, $p > 0.05$),

Table 3 Whole-body composition of male Siamese fighting fish reared in treatments using water fermented with Thai herbal recipes, where parameters measured at end of experiment (at 5 wk)

Herbal recipe	Composition (% of wet weight)			
	Moisture	Crude protein	Crude lipid	Ash
CT 1	70.1±1.4 ^{ab}	17.2±1.2	6.22±0.27	3.82±0.23
CT 2	71.5±2.4 ^a	16.2±1.2	5.52±0.73	4.41±0.51
IA	68.8±0.9 ^{bc}	16.6±0.6	6.59±0.50	5.12±0.98
B	71.2±1.0 ^{ab}	15.6±0.6	5.62±1.01	4.25±0.13
P	70.2±0.5 ^{ab}	16.0±1.0	5.46±0.69	5.12±0.53
IAB	70.6±1.0 ^{ab}	15.5±0.6	6.24±0.74	4.20±0.28
BP	67.4±1.6 ^c	17.1±1.0	6.73±1.00	5.13±0.63
IP	71.9±0.3 ^a	15.6±0.1	4.82±0.29	5.45±1.68
IBP	69.7±1.9 ^{abc}	16.0±1.1	6.12±1.41	4.79±0.53
p-value	0.020	0.210	0.219	0.199

Details for each treatment are shown in Table 1 footnote. Different lowercase superscripts in the same row indicate significant ($p < 0.05$) differences.

except for the significant reduction in the fish in the BP treatment. No significant differences were observed in the crude protein, crude lipid and crude ash contents across the nine treatments.

Discussion

Another study reported enhancements in growth parameters across several species following rearing in Indian almond leaf extract at appropriate concentrations (Ikhwanuddin et al., 2014; Nurhidayat et al., 2016; Ramos et al., 2020). Nevertheless, excessive concentrations of these extracts have been proven detrimental to aquatic fauna, such as the neon tetra fish, *Paracheiroduon innesi* (Ardi et al., 2020), the black tiger shrimp, *Penaeus monodon* (Ikhwanuddin et al., 2014), the Siamese fighting fish, *Betta* sp. (Nugroho et al., 2016, 2017; Malawa et al., 2022), the cardinal tetra, *Paracheiroduon axelrodi* (Nurhidayat et al., 2016) and the Amazon leaf fish, *Monocirrhys polyacanthus* (Ramos et al., 2020).

In the current study, there were no fatalities among the injured fish in all herbal treatments throughout the experimental period (5 wk). Furthermore, the growth-related parameters exhibited no significant variations relative to the control groups (CT 1 and CT 2). Regarding feed utilization, PER remained consistent, aligning with the unchanged specific activities of protein-digesting enzymes (pepsin, trypsin and chymotrypsin). In addition, the lipid-digesting enzyme (lipase) maintained its activity. These results were consistent with the findings reported by Malawa et al. (2022) where 0.25 g/L of *T. catappa* leaf extract had no impact on the proteolytic-specific activity but led to lipase-specific activity improvement, indicating normal functioning of gastrointestinal digestion for both proteins and lipids.

Notably, there was a significant increase in the FCR among the injured fish reared in the CT 2, P, BP, IP and IBP treatments. This was consistent with a decrease in the amylase-specific activity, except for the P treatment relative to CT 1. However, the amylase-to-trypsin ratio remained constant, suggesting the utilization of available carbohydrates per unit of protein in all herbal remedies, operating in a usual way (Thongprajukaew et al., 2011). These findings aligned with those reported by Malawa et al. (2022) on fish immersed in 0.25 g/L of *T. catappa* leaf extract or even for *in vitro* studies with incorporated tannin. Additionally, findings by Maitra and Ray (2003) and Mandal and Ghosh (2010) noted a reduction in the performance of intestinal enzyme activities, including protease, amylase and lipase. This could be attributed to the herbs selected in the experiment, which contained phytochemical constituents, namely polyphenols categorized into phenolic acids, flavonoids or non-flavonoids (Vuong et al., 2013; Raphaël et al., 2019; Kumar et al., 2021; Muala et al., 2021). These polyphenols, particularly the tannin contents, disrupt protein and dry matter digestibility by creating indigestible complexes (Krogdahl, 1989). However, betta fish are naturally carnivorous and can effectively utilize the protein in their diet. Therefore, herbal treatments are less effective with carnivorous species than with herbivorous fish, specifically in terms of amylase activity, as observed in the study by Mandal and Ghosh (2010). In the current study, an immersion method was used to deliver the herbal extract, resulting in a diminished impact on fish compared to direct dietary intake (Prusty et al., 2007).

Furthermore, the coloration of ornamental fish can serve as a crucial indicator not only of their aesthetic quality but also of their health status (Yi et al., 2021). Nonetheless, the injury stress that occurred during the caudal fin regeneration and color development processes, as documented in the report

by Malawa et al. (2023), is a possible factor influencing various physiological processes, including altered shades of body color (Ardi et al., 2020). The current findings identified notable changes, such as significant decreases in some body color parameters (a^* , h^* , C^*) in the injured fish in all herbal treatments, except for CT 2 relative to CT 1. However, the L^* values remained steady. These findings conflicted with the reports by Ardi et al. (2020) and Malawa et al. (2022) that *T. catappa* leaf extracts improved body color quality in healthy fish. The decrease in these values may have been impacted by the color of the fermented herbal water that ranged from deep brown, dark brown and dark gray to dark magenta (Fig. S1). Consequently, the body color of the fish shifted to a shade of brown. In contrast, CT 2 produced a lighter yellow-colored skin than CT 1. This finding was consistent with other research results concerning the utilization of tannin extracted from various leaves as a natural dye, resulting in brown hues, such as those obtained from Indian almond, papaya or banana leaves (Chitnis, 2013; Saleh et al., 2013; Rani et al., 2020).

There were no significant differences in the RNA, protein and RNA-to-protein ratio among the fish subjected to any of the herbal treatments compared to CT 1 and CT 2. Additionally, these results corresponded with the stability of the chief muscle components, such as myosin, actin and sarcoplasmic protein and of their total amounts and proportions, suggesting that the herbal treatments had no substantial impact on general physiological movement in the fish. These findings matched well with the unchanged whole-body protein contents. In addition, compensation in the whole-body lipid and ash contents was observed in all the herbal treatments, whereas the moisture content changed significantly only in fish in the BP treatment. Since the biochemical composition would be influenced by the surrounding water in which the fish were reared, the fish immersed in an external solution with a combined concentration (1.5 g/L BL versus 2.5 g/L PL) that may have been higher than their body fluids, might have caused water to leave the fish cells through osmosis. In contrast, IAL (0.25 g/L) alone (Malawa et al., 2022), or perhaps in combination, in a remedy had no impact on the overall whole-body composition.

Although some herbal recipes have reportedly improved fin regeneration and bubble-nest creation in injured male betta fish (Malawa et al., 2023), some treatments in the current study had negative effects due to lowering feed utilization, as indicated by the FCR, amylase specific activity and skin coloration. Maintained feed utilization without changed growth performance, muscle quality and whole-body composition

(albeit with a significant change in whole-body moisture content) were also observed in fish in the water fermentation treatment of 1 g/L of lemongrass, 50 g/L of dried clay and 5 g/L of salt with or without singly 0.25 g/L of dried Indian almond leaf, 1.5 g/L of dried banana leaf, 2.5 g/L of dried papaya leaf or their combinations, except for the mixture between dried banana and papaya leaves. However, as somatic growth is not a main assessment criterion during fish injury, the current results still endorse using fermented water of Thai herbs for short-term wound healing in betta fish rather than relying on antibiotics or chemical products that can adversely affect aquatic fauna or their surroundings, or both. Additionally, the active ingredients found in each herb may be affected by the leaf age, extraction solvent, extraction protocol, seasonal variation, geographical location and soil fertility. Differences in these variables could result in differences in the observed outcomes in fish.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix E

Conference Abstract 1



IBSC

International Bioscience Conference and the
8th International PSU – UNS Bioscience Conference

Towards the SDG Challenges

ONLINE

25–26 November 2021, Novi Sad, Serbia

BOOK OF ABSTRACTS



IBCS2021 is organized jointly by:



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University Prince of Songkla,
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University of Novi Sad,
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TRACK 1 - Participants 1

RESULTS:

Viability of EA.hy926 cells was not significantly affected after 24 h and 48 h of exposure to all concentrations of DBP. Decreased adhesion to gelatin was observed after 24 h of exposure to 10^{-5} M DBP, followed by diminished expression of five genes encoding the integrin family of proteins: *ITGA2*, *ITGA5*, *ITGAV*, *ITGB1*, and *ITGB5*. Cell migration was increased after 24h of exposure to 10^{-6} M and 10^{-5} M DBP. Observed increase in cell migration was not due to altered expression of *MMP1*, *MMP2*, and *MMP9*, but rather increased gelatinolytic activity of *MMP-2* in response to a 24 h-long exposure to DBP. After 24h of DBP pre-treatment, angiogenesis was increased after additional 20 h-long exposure of EA.hy926 cells to 10^{-4} M DBP. To investigate signaling pathways involved in DBP-induced migration of EA.hy926 cells, we used several pharmacological inhibitors: ICI 182 780, a specific estrogen receptor (ER) down-regulator, G15, a specific G-protein-coupled ER (GPER) antagonist, wortmannin, a specific inhibitor of the phosphoinositide-3-kinase – protein kinase B/Akt pathway, and U0126, a potent and selective inhibitor of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. G15 and U0126 inhibited DBP-induced migration, indicating that DBP acts through the GPER and ERK1/2 pathway. Western blotting analysis confirmed activation of ERK1/2 in DBP-exposed EA.hy926 cells: 10^{-5} M and 10^{-4} M DBP activated ERK1/2 after 15 min, whereas only 10^{-5} M DBP activated ERK1/2 after 30 and 60 min of exposure. ERK1/2 activation was GPER dependent, which was confirmed on Western blots by using G15. DBP-induced increase in angiogenesis was ERK1/2 dependent, as shown by U0126-inhibited tube formation.

CONCLUSIONS:

Obtained results suggest that DBP acts through GPER in EA.hy926 cells to increase ERK1/2 activity, cell migration, and angiogenesis, thereby providing novel information regarding the molecular mechanism of DBP's action in human vascular ECs. Understanding the molecular mechanisms behind different diseases with vascular etiology is critical for formulating effective prevention strategies and identifying novel therapeutic interventions. Additional studies are needed in order to provide a more detailed insight into the exact mechanism of DBP's action in the vasculature.

T1-P-40-ORAL Efficiency of Indian-almond leaf (*Terminalia catappa* Linnaeus, 1767) extracts in rearing Siamese fighting fish (*Betta Splendens* Regan, 1910)

Saowalak Malawa^{112/113}, Karun Thongprajukaew^{113/114}, Nutt Nuntapong, Naraid Suanyuk¹¹²

KEYWORDS: *T. catappa*; *B. splendens*; Growth; Water quality; Hematological parameter

INTRODUCTION:

The lack of adequate management in aquaculture causes stress to aquatic animals, lowering immunity that leads to pathogenic infection and outbreak of diseases. Whereas antibiotic and chemical treatments can cause undesirable side effects and residue problems in the production of aquatic animals and their surrounding environments, herbal treatment based on indigenous knowledge is an alternative approach in aquaculture. Indian-almond (*Terminalia catappa* Linnaeus, 1767) leaf has been widely used in rearing of economically important Siamese fighting fish (*Betta splendens* Regan, 1910). Generally, this plant contains active ingredients include tannins, flavonoids, phenols, alkaloids, triterpenoids, steroids, saponins and quinones. Therefore, some improvements in water quality, and better growth performance of a few aquatic animals have

¹¹² Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90112, Thailand.

Corresponding author: 6310630001@psu.ac.th

¹¹³ Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90112, Thailand

¹¹⁴ Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand

TRACK 1 - Participants 1

been reported after addition of the aqueous *T. catappa* leaves extracts. However, perceptive information is unavailable on the efficiency of *T. catappa* leaves extracts in rearing the growing phase of Siamese fighting fish.

OBJECTIVE:

This study was performed to determine the optimal concentration of Indian-almond leaf extracts for rearing growing phase of male Siamese fighting fish.

METHOD/DESIGN:

Seventy-five solid-red male fish (2-month-old, 0.80-0.95 g initial body weight) were individually distributed into aquaria (8.3 cm diameter × 12.5 cm height, with 8 cm water depth). Aquaria were divided into five groups, containing fifteen fish each. Each group was treated with a different concentration of Indian-almond leaf extracts (0, 0.125, 0.25, 0.5 and 1 g/L of water). The water was 80% exchanged every other day and the qualities were monitored two times weekly. At the end of the 8-week trial, growth performance, feed utilization, skin coloration and hematological parameters of reared fish were investigated.

RESULTS:

No mortality was observed over the eight weeks of trial. Only the fish reared in the highest concentration of leaf extracts exhibited the highest growth (specific growth rate 1.74% body weight/day) relative to control treatment ($P < 0.05$). No significant differences in feed conversion ratio (1.16 g feed/g gain on averages) and protein efficiency ratio (2.28 g gain/g protein on averages) were observed across all groups. The last treatment also improved skin coloration, in terms of lightness and redness, as compared to control group. White blood cells, red blood cells, plasma protein concentration and hematocrit did not differ between the fish reared in the highest concentration of leaf extracts and in control treatment ($P > 0.05$), while minor improvement was observed in fish subjected to 0.125, 0.25 and 0.5 g/L of leaf extracts. Also, rearing the fish in the highest concentration of leaf extracts significantly improved key water quality parameters (pH, ammonia, nitrite and nitrate) as compared to control ($P < 0.05$).

CONCLUSIONS:

These findings suggest that the highest concentration of Indian-almond leaf extracts (1 g/L of water) is suitable in rearing growing phase of male Siamese fighting fish. Application of this herbal extract is environmentally-friendly and can reduce chemical or antibiotic usages in aquaculture, inhibiting residue problems in the production of aquatic animals and the surrounding environments.

T1-P-41 Effects of chronic dietary cadmium on midgut superoxide dismutase (SOD) and catalase (CAT) in larvae from two *Lymantria dispar* populations

Dragana Matić, Milena Vlahović, Larisa Ilijin, Anja Grčić, Aleksandra Filipović, Marija Mrdaković, Vesna Perić-Mataruga¹¹⁵

KEYWORDS: superoxide dismutase; catalase; *Lymantria dispar*; cadmium; biomarker

INTRODUCTION:

Cadmium (Cd) levels in the environment have increased during decades of intensive industrial development and urbani-

¹¹⁵ Department of Insect Physiology and Biochemistry, Institute for Biological Research "Siniša Stanković", National Institute of Republic of Serbia, University of Belgrade, Despot Stefan Blv. 142, 11060 Belgrade, Serbia. Corresponding author: dragana.matic@ibiss.bg.ac.rs

Appendix F

Conference Abstract 2

1st AINR

AQO048

**ผลของการใช้สารสกัดจากใบหูกวาง (*Terminalia catappa*) เป็นสารเติมแต่งในน้ำเลี้ยง
ปลากัด (*Betta splendens*) เพศผู้ระยะวัยรุ่น**
**Effect of Aquarium Additive from Indian Almond (*Terminalia catappa*) Leaf Extract for
Juvenile Male Siamese Fighting Fish (*Betta splendens*)**

เสาวลักษณ์ มาลาเว^{1, 2} นันทน์ นันทพงศ์¹ นเรศ ชวนยุก¹ และ การุณ ทองประจุแก้ว^{2, 3*}
Malawa, S.^{1, 2}, Nuntapong, N.¹, Suanyuk, N.¹ and Thongprajukaew, K.^{2, 3*}

บทคัดย่อ

การใช้ยาปฏิชีวนะและสารเคมีทำให้เกิดผลข้างเคียงและสารตกค้างที่ไม่พึงประสงค์ในการผลิตสัตว์น้ำ สารเติมแต่งจากพืชจึงเป็นอีกทางเลือกหนึ่งที่ปลอดภัยกว่า การศึกษาครั้งนี้ได้นำสารสกัดจากใบหูกวาง (*Terminalia catappa*) มาเติมในน้ำที่ใช้เลี้ยงปลากัด (*Betta splendens*) โดยนำปลากัดเพศผู้สีแดงทั้งตัว (น้ำหนัก 0.87 ± 0.01 กรัม) จำนวน 75 ตัว มาเลี้ยงเดี่ยวในน้ำที่มีการเติมสารสกัดจากใบหูกวางความเข้มข้นแตกต่างกัน 4 ระดับ (0.125, 0.25, 0.5 และ 1 กรัม/ลิตร) และกลุ่มควบคุมที่ไม่เติมสารสกัดจากใบหูกวางเป็นเวลา 8 สัปดาห์ ผลการทดลองพบว่าคุณภาพน้ำ ได้แก่ ความเป็นกรด-ด่าง ออกซิเจนละลายน้ำ แอมโมเนียรวม ไนไตรท์ และไนเตรท ดีขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) ปลาที่เลี้ยงในน้ำที่มีสารสกัด 0.25 กรัม/ลิตร มีการเจริญเติบโต (อัตราการเจริญเติบโตเฉพาะเท่ากับ 1.62 เปอร์เซ็นต์/วัน) และการใช้ประโยชน์จากอาหาร (อัตราการเปลี่ยนอาหารเป็นเนื้อเท่ากับ 1.21) ไม่ต่างจากชุดควบคุม ($P > 0.05$) กิจกรรมจำเพาะของเอนไซม์ย่อยโปรตีน ได้แก่ เปปซิน ทริปซิน และไคโมทริปซิน มีค่าไม่แตกต่างจากปลาในชุดควบคุม แต่การใช้ประโยชน์จากคาร์โบไฮเดรตผ่านกิจกรรมของอะไมเลสลดลง เนื่องจากกิจกรรมของไลเปสเพิ่มขึ้น และมีอัตราส่วนของอะไมเลส/ทริปซิน คงที่ ขณะที่ความถี่ (1.7 เท่า) และพื้นที่หูด (1.52 เท่า) เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับปลาในชุดควบคุม ผลการศึกษาทั้งหมดแสดงให้เห็นว่าความเข้มข้นของสารสกัดจากใบหูกวางที่เหมาะสมสำหรับเลี้ยงปลากัด คือ 0.25 กรัม/ลิตร

คำสำคัญ: ปลากัด การเจริญเติบโต ใบหูกวาง คุณภาพน้ำ

Abstract

In the production of aquatic animals, the use of antibiotics and chemicals causes undesirable side effects and residues. Phyto-genic additives can be safer alternatives. Here, Indian almond (*Terminalia catappa*) leaf extract was added to the rearing water of male Siamese fighting fish (*Betta splendens*). Seventy-five solid-red male fish (0.87 ± 0.01 g body weight) were individually reared in water containing four different treatments of *T. catappa* leaf extract (0.125, 0.25, 0.5, and 1 g/L) and a control group reared in water without the extract. The water quality parameters including pH, dissolved oxygen, total ammonia, nitrite, and nitrate were significantly improved ($P < 0.05$). The fish reared in water containing 0.25 g of leaf extract/L maintained growth (specific growth rate = 1.62%/day) and feed utilization (feed conversion ratio = 1.21) in comparison with the fish in the control group ($P > 0.05$). In the preferred treatment, specific activities of protein-digesting enzymes (pepsin, trypsin, and chymotrypsin) did not differ as compared to control treatment. The sparing effects of carbohydrate utilization through decreased amylase specific activity were observed but increased lipase specific activity and maintained amylase/trypsin ratios. The frequency of bubble-nest formation (1.7 folds) and bubble-nest size (1.52 folds) were significantly increased as compared to control fish. The results suggest that 0.25 g/L of *T. catappa* leaf extract is a suitable concentration of the additive for male Siamese fighting fish.

Keywords: Betta, Growth, Tropical almond, Water quality

¹ ศูนย์วิจัยสุขภาพสัตว์น้ำ กิจกรรม ศูนย์มาตย์ สาขาวิชาวิทยาศาสตร์และนวัตกรรมการจัดการ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์

¹ Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

² ศูนย์วิจัยความเป็นเลิศเทคโนโลยีชีวภาพเกษตรและทรัพยากรธรรมชาติ ระยะที่ 3 คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์

² Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

³ สาขาวิทยาศาสตร์สุขภาพและวิทยาศาสตร์ประยุกต์ คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์

³ Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

* Corresponding author: karun.t@psu.ac.th (or) karun114@hotmail.com

Appendix G

Conference Abstract 3

การประชุมวิชาการระดับชาติ
และนานาชาติ ครั้งที่ 1 มหาวิทยาลัยทักษิณ
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มุ่งสู่เป้าหมายของการพัฒนาที่ยั่งยืน

Abstract book
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ตำรับสมุนไพรไทยมีประสิทธิภาพส่งเสริมสุขภาพและสวัสดิภาพของปลากัด (*Betta splendens*)

เสาวลักษณ์ มาลาวัชะ^{1*} นันทน์ นันทพงษ์² นเรศ ช้วนยุค³ และ การุณ ทองประจุแก้ว^{4, 5}

บทคัดย่อ

การแช่ปลาด้วยสารสกัดสมุนไพรจากภูมิปัญญาชาวบ้านเป็นวิธีการทางธรรมชาติเพื่อรักษาและฟื้นฟูปลากัด (*Betta splendens*) ที่มีบาดแผล ทดแทนการใช้ยาปฏิชีวนะหรือสารเคมีที่ก่อให้เกิดผลกระทบในเชิงลบต่อสุขภาพหรือการเกิดสารตกค้างในปลาและสิ่งแวดล้อม การศึกษาครั้งนี้ได้วางแผนการทดลองแบบสุ่มสมบูรณ์ โดยนำปลากัดเพศผู้สีแดงทั้งตัว (น้ำหนัก 1.11–1.44 กรัม) มาตัดครีบหางออกครึ่งหนึ่งในระนาบแนวตั้ง มาเลี้ยงเดี่ยวในน้ำหมักสมุนไพรไทย 8 สูตร ($n = 15$ ต่อสูตร) ประกอบด้วย วัตถุดิบหลัก ได้แก่ ใบหูกวางแห้ง (*Terminalia catappa*, TC, 0.25 กรัม/ลิตร) ใบกล้วยแห้ง (*Musa sapientum*, MS, 1.5 กรัม/ลิตร) และใบมะละกอแห้ง (*Carica papaya*, CP, 2.5 กรัม/ลิตร) โดยเลือกใช้เพียง 1, 2 หรือ 3 ชนิดร่วมกัน ขณะที่ใบตะไคร้แห้ง (*Cymbopogon citratus*, CC, 1 กรัม/ลิตร) ดินเหนียวแห้ง (50 กรัม/ลิตร) และเกลือแกง (5 กรัม/ลิตร) คงที่ในทุกสูตร เปรียบเทียบกับกลุ่มควบคุมที่ไม่เติมน้ำหมักเป็นเวลา 5 สัปดาห์ ผลการทดลองพบว่าคุณภาพน้ำ ได้แก่ แอมโมเนียรวม ไนไตรท์ และไนเตรท) และค่าทางโลหิตวิทยาของปลาดีขึ้นอย่างมีนัยสำคัญในปลาที่เลี้ยงด้วยน้ำหมักทุกสูตร ($P < 0.05$) แต่ความถี่ของการสร้างหวอดและพื้นที่หวอดมีค่าสูงในปลาที่เลี้ยงในสูตร CC อย่างเดียวหรือผสมระหว่าง MS และ CP ($P < 0.05$) ดังนั้น การรักษาด้วยสมุนไพรจึงสามารถประยุกต์ใช้เพื่อส่งเสริมสุขภาพและสวัสดิภาพของปลากัดได้

คำสำคัญ: การก่อหวอด, ครีบหาง, ค่าโลหิตวิทยา, ปลาสวยงาม, สมุนไพร

¹ นักศึกษาปริญญาเอก, สาขาวิชาวาริชศาสตร์และนวัตกรรมการจัดการ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ จ.สงขลา 90110

² ดร., ศูนย์วิจัยสุขภาพสัตว์น้ำ กิจการ ศุภมาตย์ สาขาวิชาวาริชศาสตร์และนวัตกรรมการจัดการ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ จ.สงขลา 90110

³ ผู้ช่วยศาสตราจารย์, ศูนย์วิจัยสุขภาพสัตว์น้ำ กิจการ ศุภมาตย์ สาขาวิชาวาริชศาสตร์และนวัตกรรมการจัดการ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ จ.สงขลา 90110

⁴ รองศาสตราจารย์, สาขาวิทยาศาสตร์สุขภาพและวิทยาศาสตร์ประยุกต์ คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ จ.สงขลา 90110

⁵ รองศาสตราจารย์, ศูนย์วิจัยความเป็นเลิศเทคโนโลยีชีวภาพเกษตรและทรัพยากรธรรมชาติ ระยะที่ 3 คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ จ.สงขลา 90110

¹ Ph.D. Student, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

² Dr., Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

³ Assistant Professor, Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

⁴ Associate Professor, Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110

⁵ Associate Professor, Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

* Corresponding Author: E-mail: 6310630001@psu.ac.th (or) saowalak4866@gmail.com

Thai herbal recipes effectively promoted health and welfare of Siamese fighting fish (*Betta splendens*)

Saowalak Malawa^{1*}, Nutt Nuntapong², Naraid Suanyuk³, and Karun Thongprajukaew^{4,5}

Abstract

Immersing fish with herbal extracts from indigenous knowledge is a natural way to heal and recover injured Siamese fighting fish (*Betta splendens*) instead of using antibiotics or chemicals that cause adversely affect health or residue in fish and the environment. In the current study, half of the caudal fin of solid-red male Siamese fighting fish (1.11–1.44 g body weight) was amputated in the vertical plane, and they were assigned to trial based completely randomized design. The amputated fish ($n = 15$ per treatment) were divided to individually rear in fermented water from eight combinations of Thai herbal recipes (1, 2, or 3 items of chief ingredients); Indian almond (*Terminalia catappa*) dried leaves (TC, 0.25 g/L), banana (*Musa sapientum*) dried leaves (MS, 1.5 g/L), and papaya (*Carica papaya*) dried leaves (CP, 2.5 g/L) as chief ingredients, while lemon grass (*Cymbopogon citratus*) dried leaves (CC, 1 g/L), dried clay (50 g /L), and salt (5 g/L) were constant in all herbal treatments, in comparison to fish rear in non-fermented water for five weeks. The results showed that the water quality (including total ammonia nitrogen, nitrite, and nitrate) and hematological parameters of reared fish were statistically improved in all herbal treatments ($P < 0.05$). However, the frequency and bubble-nesting area for the fish raised in CC singly or a combination between MS and CP were significantly increased compared to control fish ($P < 0.05$). Therefore, herbal treatments can be applied to promote betta fish's health and welfare.

Keywords: Bubble-nesting creation, Caudal fin, Hematological parameter, Ornamental fish, Herb

Appendix H

Conference Abstract 4

บทคัดย่อ

Book of Abstract



The 2nd Academic Conference
on Agricultural Innovation and Natural Resources 2023

งานประชุมวิชาการนวัตกรรมการเกษตร
และทรัพยากรธรรมชาติ ครั้งที่ 2

ระหว่างวันที่ 3 - 4 และ 18 สิงหาคม 2566

ณ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่

Efficacy of Thai herbal Recipes to Accelerate Fin Regeneration in Siamese Fighting Fish (*Betta splendens*)

Malawa, S.^{1,2*}, Nuntapong, N.¹, Waeowannajit, S.³ and Thongprajukaew, K.^{2,3}

¹ Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

² Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110

³ Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110

* Corresponding author: 6310630001@psu.ac.th

Abstract

The courtship and reproductive behaviors or combating behavior encounter each other of Siamese fighting fish (*Betta splendens*) resulted in body damage and tear fins. In the current study, a half of the caudal fin of solid-red male Siamese fighting fish (1.11–1.44 g body weight) were amputated in the vertical plane. The amputated fish ($n = 15$ per treatment) were divided to individually rear in fermented water containing eight combinations of Thai herbal recipes containing dried Indian almond (*Terminalia catappa*) leaf (IAL, 0.25 g L⁻¹), dried banana (*Musa sapientum*) leaf (BL, 1.5 g L⁻¹), and dried papaya (*Carica papaya*) leaf (PL, 2.5 g L⁻¹) as major ingredients, while dried lemongrass (*Cymbopogon citratus*) leaf (LL, 1 g L⁻¹), dried clay (50 g L⁻¹), and salt (5 g L⁻¹) were constant, in comparison to fish rear in non-fermented water for five weeks. The fin regeneration rate was monitored weekly, while growth performance was recorded at the end of the trial. The herbal treatments had no effect on growth (specific growth rate = 0.46% day⁻¹ on average) and feed utilization (feeding rate = 1.82% day⁻¹ on average) ($P > 0.05$). The amputated fin completely regenerated fins within 35 days. However, the fin regeneration rate was statistically improved for the fish reared in combinations of IAL, BL, and PL (31–33 days) than for fish kept in non-fermented water (35.8 days) ($P < 0.05$). Among these alternative treatments, red color intensity was fully redeveloped in fish reared in three herbal treatments ($P < 0.05$) that all contained dried papaya leaf (BL + PL, IAL + PL, and IAL + BL + PL). Therefore, those three treatments could be applied effectively to enhance fin regeneration in betta fish.

Keywords: *B. splendens*, caudal fin, color development, fin amputation

Appendix I

Conference Abstract 5



SAKURA Science Exchange Program

Abstract Book

Prince of Songkla University, Thailand
Rajamangala University of Technology Srivijaya,
Thailand

Vellore Institute of Technology, India

Feb. 15-16, 2024
(The Research Institute of Marine Bioresources,
Miraisouzou-kan)

Fukuyama University, JAPAN

(要旨の日本語版は Deep L の自動翻訳を使用した)

Fermented herbal extracts from Thai recipes successfully improved fin regeneration and color development in amputated Siamese fighting fish (*Betta splendens*)

Saowalak Malawa^{1,2*}, Karun Thongprajukaew^{2,3}, Nutt Nuntapong¹ and Sugree Waeowannajit³

¹ Kidchakan Supamattaya Aquatic Animal Health Research Center, Aquatic Science and Innovative Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

² Center of Excellence in Agricultural and Natural Resources Biotechnology Phase 3, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

³ Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand

* Corresponding author: 6310630001@psu.ac.th

Abstract

Courtship and reproductive behavior, or confrontations between Siamese fighting fish (*Betta splendens*), often lead to physical injuries such as torn fins and body damage. In the current study, a half of the caudal fin of solid-red male Siamese fighting fish (1.11–1.44 g body weight) were amputated in the vertical plane. The amputated fish ($n = 15$ per treatment) were divided to individually rear in fermented water containing eight combinations of Thai herbal recipes containing dried Indian almond (*Terminalia catappa*) leaf (IAL, 0.25 g L⁻¹), dried banana (*Musa sapientum*) leaf (BL, 1.5 g L⁻¹), and dried papaya (*Carica papaya*) leaf (PL, 2.5 g L⁻¹) as major ingredients, while dried lemongrass (*Cymbopogon citratus*) leaf (LL, 1 g L⁻¹), dried clay (50 g L⁻¹), and salt (5 g L⁻¹) were constant, in comparison to fish rear in non-fermented water for five weeks. The fin regeneration rate was monitored weekly, while growth performance was recorded at the end of the trial. The herbal treatments had no effect on growth (specific growth rate = 0.46% day⁻¹ on average) and feed utilization (feeding rate = 1.82% day⁻¹ on average) ($P > 0.05$). The amputated fin completely regenerated fins within 35 days. However, the fin regeneration rate was statistically improved for the fish reared in combinations of IAL, BL, and PL (31–33 days) than for fish kept in non-fermented water (35.8 days) ($P < 0.05$). Among these alternative treatments, red color intensity was fully redeveloped in fish reared in three herbal treatments ($P < 0.05$) that all contained dried papaya leaf (BL + PL, IAL + PL, and IAL + BL + PL). Therefore, those three treatments could be applied effectively to enhance fin regeneration in betta fish.

Keywords: *B. splendens*, caudal fin, color development, fin amputation

タイのレシピから抽出した発酵ハーブエキ스는、切断されたシャム闘魚 (*Betta splendens*) の鰭の再生と発色を改善することに成功した。

概要

シャム闘魚 (*Betta splendens*) 同士の求愛行動や繁殖行動、あるいは対決は、しばしばヒレの断裂や体の損傷などの身体的傷害につながる。本研究では、赤色の雄シャム闘魚 (体重1.11~1.44g) の尾鰭の半分を垂直面で切断した。切断した魚 (各処理につきn = 15) を、乾燥インドアーモンド (*Terminalia catappa*) の葉 (IAL、0.25 g L⁻¹)、乾燥バナナ (*Musa sapientum*) の葉 (BL、1.5 g L⁻¹)、乾燥パパイヤ (*Carica papaya*) の葉 (PL、2.5 g L⁻¹) を主成分とし、乾燥レモングラス (*Cymbopogon citratus*) の葉 (LL、1 g L⁻¹)、乾燥粘土 (50 g L⁻¹)、食塩 (5 g L⁻¹) は一定とし、非発酵水で5週間飼育した魚と比較した。ヒレの再生率は毎週モニターし、成長成績は試験終了時に記録した。ハーブ処理は成長 (比成長率 = 平均0.46% day⁻¹) と飼料利用率 (給餌率 = 平均1.82% day⁻¹) に影響を与えなかった (P > 0.05)。切断されたヒレは35日以内に完全に再生した。しかし、IAL、BL、PLの組み合わせで飼育した魚 (31-33日) では、非発酵水で飼育した魚 (35.8日) よりもヒレの再生率が統計的に改善した (P < 0.05)。これらの代替処理のうち、乾燥パパイヤ葉を含む3つのハーブ処理 (BL+PL, IAL+PL, IAL+BL+PL) で飼育した魚では、赤色強度が完全に回復した (P < 0.05)。したがって、これら3つの処理法はベタ魚のヒレの再生を促進するために効果的に適用できる可能性がある。

キーワード *B. splendens*, 尾鰭, 発色, 鰭切断

DeepL.com で翻訳しました。

Appendix J

Petty Patent 1

สำหรับเจ้าหน้าที่			
จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร ☐ กลุ่มวิศวกรรม สิทธิบัตรการประดิษฐ์ (วิศวกรรม) สิทธิบัตรการประดิษฐ์ (ไฟฟ้า) สิทธิบัตรการประดิษฐ์ (ฟิลิกส์)	☐ กลุ่มเคมี สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค) สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี) สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ) สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)	สิทธิบัตรการออกแบบ ☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1) ☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2) ☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)	อนุสิทธิบัตร ☐ อนุสิทธิบัตร (วิศวกรรม) ☐ อนุสิทธิบัตร (เคมี)

8. การยื่นคำขออนุญาตนำเข้า ☐ PCT ☐ เพิ่มเติม (ดังแนบ)				
วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				
8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย ☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้				
9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด วันแสดง _____ วันเปิดงานแสดง _____ ผู้จัด _____				
10. การประดิษฐ์เกี่ยวกับจุลชีพ				
10.1 เลขทะเบียนฝากเก็บ		10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ	
11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา ☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ _____				
12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ _____ ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข _____ ในการประกาศโฆษณา				
13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย ก. แบบพิมพ์คำขอ _____ 3 _____ หน้า ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์ _____ 5 _____ หน้า ค. ข้อถ้อยสิทธิ _____ 1 _____ หน้า ง. รูปเขียน _____ รูป _____ หน้า จ. ภาพแสดงแบบผลิตภัณฑ์ ☐ รูปเขียน _____ รูป _____ หน้า ☐ ภาพถ่าย _____ รูป _____ หน้า ฉ. บทสรุปการประดิษฐ์ _____ 1 _____ หน้า			14. เอกสารประกอบคำขอ ☒ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์ ☐ หนังสือมอบอำนาจ ☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ ☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย ☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ ☐ เอกสารอื่นๆ	
15. ข้าพเจ้าขอรับรองว่า ☒ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน ☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก _____				
16. ลายมือชื่อ ☒ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ ตัวแทน (_____ มหาวิทยาลัยสงขลานครินทร์ _____)				

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

ใบแนบต่อท้าย สป/สผ/001-ก

5. ตัวแทน (ถ้ามี)

2. ชื่อ นางสาวขวัญชนก ขวัญปลอด

ที่อยู่ 15

ต.คองหงส์ อ.หาดใหญ่ จ.สงขลา 90110

อีเมล kwanchanok.kwa@gmail.com

เลขประจำตัวประชาชน 1930400044536

ตัวแทนเลขที่ 2618 โทรศัพท์ 074-289335 โทรสาร

3. ชื่อ นางสาวเพชรดา สัตยากุล

ที่อยู่ อาคารอำนวยการอุทยานวิทยาศาสตร์ภาคใต้ (จ.สงขลา) มหาวิทยาลัยสงขลานครินทร์ส่วนขยาย

ต.ทุ่งใหญ่ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

อีเมล phetrada.sk@gmail.com

เลขประจำตัวประชาชน 1920600104818

ตัวแทนเลขที่ 2440 โทรศัพท์ 0-7485-9516 โทรสาร 0-7485-9511

4. ชื่อ นายจิตติยุทธ เยี่ยมยกกุล

ที่อยู่ ศูนย์ทรัพยากรเส้นทางปัญญา อาคารอำนวยการอุทยานวิทยาศาสตร์ภาคใต้ (จ.สงขลา) มหาวิทยาลัยสงขลานครินทร์ส่วนขยาย

ต.ทุ่งใหญ่ อ.หาดใหญ่ จ.สงขลา 90110

อีเมล jittiyut.y@gmail.com

เลขประจำตัวประชาชน 3959900384187

ตัวแทนเลขที่ 2266 โทรศัพท์ 0-7485-9516 โทรสาร 0-7485-9511

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์

2. ชื่อ นางสาวเสาวลักษณ์ มัลลวระ

ที่อยู่ 15 ถ.กาญจนวนิชย์ ต.คองหงส์ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

สัญชาติ ไทย

เลขประจำตัวประชาชน 1800300082143

Appendix K

Petty Patent 2

หมายเหตุ ในกรณีที่ไม้อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย			
สำหรับเจ้าหน้าที่			
จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร			
☐ กลุ่มวิศวกรรม	☐ กลุ่มเคมี	สิทธิบัตรการออกแบบ	อนุสิทธิบัตร
สิทธิบัตรการประดิษฐ์ (วิศวกรรม)	สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)	☐ อนุสิทธิบัตร (วิศวกรรม)
สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)	สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)	☐ อนุสิทธิบัตร (เคมี)
สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)	สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)	
	สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)		

8. การยื่นคำขออนุญาตนำเข้า ☐ PCT ☐ เพิ่มเติม (ดังแนบ)				
วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				
8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย ☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้				
9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด วันแสดง 29 มิ.ย. 2566 วันเปิดงานแสดง 29 มิ.ย. 2566 ผู้จัด อุทยานวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์				
10. การประดิษฐ์เกี่ยวกับจุลชีพ				
10.1 เลขทะเบียนฝากเก็บ		10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ	
11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา ☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ				
12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา				
13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย ก. แบบพิมพ์คำขอ 3 หน้า ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์ 6 หน้า ค. ข้อถ้อยสิทธิ 1 หน้า ง. รูปเขียน รูป หน้า จ. ภาพแสดงแบบผลิตภัณฑ์ ☐ รูปเขียน รูป หน้า ☐ ภาพถ่าย รูป หน้า ฉ. บทสรุปการประดิษฐ์ 1 หน้า			14. เอกสารประกอบคำขอ ☒ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร ☒ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์ ☐ หนังสือมอบอำนาจ ☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ ☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย ☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ ☐ เอกสารอื่นๆ	
15. ข้าพเจ้าขอรับรองว่า ☒ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน ☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก				
16. ลายมือชื่อ ☒ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ ตัวแทน (มหาวิทยาลัยสงขลานครินทร์)				

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษ จำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

ใบแนบต่อท้าย สป/สผ/001-ก

5. ตัวแทน (ถ้ามี)

2. ชื่อ นางสาวเพชรดา สัตยากุล

ที่อยู่ อุทยานวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ชั้น 12 อาคารLRC

ต.คอหงส์ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

อีเมล phetrada.sk@gmail.com

เลขประจำตัวประชาชน 1920600104818

ตัวแทนเลขที่ 2440 โทรศัพท์ 083-5329596, 074-289335 โทรสาร 074-289333
3. ชื่อ นายธิปไตย ศรีนวลขาว

ที่อยู่

ต.ทุ่งใหญ่ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

อีเมล tippatai.s@gmail.com

เลขประจำตัวประชาชน 3900200121282

ตัวแทนเลขที่ 2674 โทรศัพท์ 084-9688988 โทรสาร 074-859511
4. ชื่อ นายจิตติยุทธ เยี่ยมยกกุล

ที่อยู่ อาคารอำนวยการอุทยานวิทยาศาสตร์ภาคใต้ (จ.สงขลา) มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตส่วนขยาย 15

ต.ทุ่งใหญ่ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

อีเมล jittiyut.y@gmail.com

เลขประจำตัวประชาชน 3959900384187

ตัวแทนเลขที่ 2266 โทรศัพท์ 089-1345190 โทรสาร 074859511

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์

2. ชื่อ นางสาวเสาวลักษณ์ มาลาวะ

ที่อยู่ 15 ถ.กาญจนวนิชย์ ต.คอหงส์ อ.หาดใหญ่ จ.สงขลา 90110 ประเทศไทย

สัญชาติ ไทย

เลขประจำตัวประชาชน 1800300082143

VITAE

Name Miss Saowalak Malawa

Student ID 6310630001

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Chemistry-Biology)	Prince of Songkla University	2020

Scholarship Awards during Enrolment:

1. Research and Development Office (Contract No. SCI6402049S), Prince of Songkla University
2. Discipline of Excellence in Sustainable Aquaculture (Contract No. TAM-DOE60040), Prince of Songkla University
3. Center of Excellence in Agricultural and Natural Resources Biotechnology (CoE-ANRB) Phase 3, Prince of Songkla University

List of Publication and Proceedings:

1. **Malawa, S.**, Nuntapong, N., Suanyuk, N., Thongprajukaew, K., 2022. Addition of different concentrations of Indian almond (*Terminalia catappa*) leaf extract to aquarium water resulted in improved water quality and increased bubble nest formation by male Siamese fighting fish (*Betta splendens*) without having any consistent negative effects on growth metrics and blood chemistry. *Aquac. Int.* 30, 3269–3288.
2. **Malawa, S.**, Nuntapong, N., Waeowannajit, S., Thongprajukaew, K., 2023. Fermented water of Thai herbs effectively enhanced amputated fin regeneration in Siamese fighting fish (*Betta splendens*). *Aquaculture* 577, 739980.
3. **Malawa, S.**, Nuntapong, N., Thongprajukaew, K., 2024. Somatic responses of injured Siamese fighting fish (*Betta splendens*) raised using various recipes of Thai herbal fermented water. *Agric. Nat. Resour.* 58, 101–11.

4. **Malawa, S.**, Nuntapong, N., Suanyuk, N., Waeowannajit, S., Thongprajukaew, K.
Rearing water containing fermented extracts of Thai herbal formulations improved well-being of injured Siamese fighting fish (*Betta splendens*). Aquac. Rep. (Under review).
5. Rodjaroen, S., Thongprajukaew, K., Khongmuang, P., **Malawa, S.**, Tuntikawinwong, K., Saekhow, S., 2020. Ontogenic development of digestive enzymes in mealworm larvae (*Tenebrio molitor*) and their suitable harvesting time for use as fish feed. Insects 11, 393.
6. Thongprajukaew, K., Takaeh, S., Esor, N., Saekhow, S., **Malawa, S.**, Nuntapong, N., Hahor, W., Choodum, A., 2023. Optimal water volume for transportation of male Siamese fighting fish (*Betta splendens*). Aquac. Rep. 28, 101430.
7. **Malawa, S.**, Thongprajukaew, K., Nuntapong, N., Suanyuk, N., 2021. Efficiency of Indian-almond leaf (*Terminalia catappa* Linnaeus, 1767) extracts in rearing Siamese fighting fish (*Betta splendens* Regan, 1910). In: Book of Abstract of the International BioScience Conference and the 8th International PSU–UNS BioScience Conference, pp. 76–77, 25–26 November 2021, Novi Sad (Oral presentation).
8. **Malawa, S.**, Nuntapong, N., Suanyuk, N., Thongprajukaew, K., 2022. Effect of aquarium additive from Indian almond (*Terminalia catappa*) leaf extract for juvenile male Siamese fighting fish (*Betta splendens*). In: Book of Abstract of the 1st Conference on Agricultural Innovation and Natural Resources 2022, p. 94, 18–19 August 2022, Songkhla (Oral presentation).
9. **Malawa, S.**, Nuntapong, N., Suanyuk, N., Thongprajukaew, K., 2023. Thai herbal recipes effectively promoted health and welfare of Siamese fighting fish (*Betta splendens*). In: Book of Abstract of the 34th National Conference and The 1st International Conference on ASEAN Sustainable Development (ICASD 2023), pp. 27–28, 20–21 July 2023, Songkhla (Oral presentation).
10. **Malawa, S.**, Nuntapong, N., Waeowannajit, S., Thongprajukaew, K., 2023. Efficacy of Thai herbal recipes to accelerate fin regeneration in Siamese fighting fish (*Betta splendens*). In: Book of Abstract of the 2nd Academic Conference on Agricultural Innovation and Natural Resources 2023, p. 89, 3–4 and 18 August 2023, Songkhla (Oral presentation).

11. **Malawa, S.**, Thongprajukaew, K., Nuntapong, N., Waeowannajit, S., 2024.
Fermented herbal extracts from Thai recipes successfully improved fin regeneration and color development in amputated Siamese fighting fish (*Betta splendens*). In: Book of Abstract of SAKURA Science Exchange Program, pp. 9–10, 15–16 February 2024, Fukuyama (Oral presentation).
12. Hahor, W., Thongprajukaew, K., Takaeh, S., Esor, N., Saekhow, S., **Malawa, S.**, Nuntapong, N., Choodum, A., 2023. Study of water volume for domestic transportation of packed male Siamese fighting fish (*Betta splendens* Regan, 1910). In: Book of Abstract of the 34th National Conference and The 1st International Conference on ASEAN Sustainable Development (ICASD 2023), pp. 27–28, 20–21 July 2023, Songkhla (Oral presentation).

List of Petty Patents:

1. **Malawa, S.**, Thongprajukaew, K., 2022. Pelleted Indian almond leaf extract formulation and its manufacturing process. (Application date August 30, 2022)
2. **Malawa, S.** and Thongprajukaew, K., 2023. Herb recipes for enhancing fin regeneration of Siamese fighting fish. (Application date June 29, 2023)