รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลของอาหารเสริมและการฉีดสารสกัดใบฝรั่ง, ทับทิม และรางจืด
ต่อปลานิล และปลาตะเพียน

Effect of dietary supplement and intraperitoneal injection of
Psidium guajava, Punica granatum* and *Thunbergia laurifolia
leaves extract on *Oreochromis niloticus* and *Puntius altus*

โดย ดร.วรรณีย์ จิรอังกูรสกุล

14 พฤษภาคม 2554

สัญญาเลขที่…..RMU5180001……

รายงานวิจัยฉบับสมบูรณ์

โครงการ: ผลของอาหารเสริมและการฉีดสารสกัดใบฝรั่ง, ทับทิม และรางจืด ต่อปลานิล และปลาตะเพียน

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(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และสกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

CONTENTS

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Wannee Jiraungkoorskul

ABSTRACT

Project Code : RMU5180001

Project Title : Effect of dietary supplement and intraperitoneal injection of *Psidium guajava, Punica granatum* and *Thunbergia laurifolia* leaves extract on *Oreochromis niloticus* and *Puntius altus*

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Objective: To investigate the effect of intraperitoneal injection of various doses of plant leaves extract at 48 hours and 7 days; and to investigate the effect of dietary supplement of various doses of dry plant leaves at 1 and 2 months.

Method: Fish in each group was evaluated in term growth rate; macrophage studies; nitroblue tetra zolium test; lysozyme activity test; micronucleus and nuclear abnormality tests; hematology studies; liver function test; histopathological and ultrastructural studies.

Result: The results had shown the time-dose dependent, the higher dose the more effect and the longer time the higher response. The administration route via the food supplement was the greater than the intraperitonial injection. The highest efficiency of leaf extract was *T. laurifolia* follow by *P. granatum* and *P. guajava.*

Conclusion: In conclusion, the results presented in this study show no harmful of these leaf extracts.

Keywords : *Psidium guajava; Punica granatum; Thunbergia laurifolia; Oreochromis niloticus; Puntius altus*

บทคัดย่อ

รหัสโครงการ : RMU5180001

ชื่อโครงการ : ผลของอาหารเสริมและการฉีดสารสกัดใบฝรั่ง, ทับทิม และรางจืด ต่อปลานิล และปลาตะเพียน

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<u>วัตถุประสงค์ :</u> การศึกษาประสิทธิผลของการฉีดสารสกัดจากใบฝรั่ง, ทับทิม และรางจืด เข้าช่องท้องปลาในระยะเฉียบพลัน48 ชั่วโมงและ 7 วัน และศึกษาประสิทธิผลของอาหารเสริมจากใบพืชอบแห้งในระยะยาว 1 และ 2 เดือน

<u>วิธีทดลอง :</u> ทำการตรวจวัด อัตราการเจริญเติบโต การเพิ่มจำนวนเซลล์มาคโครฟาส์กและนิวโตรฟิวส์ในน้ำล้างช่องท้องและเนื้อเยื่อไต การเปลี่ยนแปลงระดับไลโซไซม์ การเปลี่ยนแปลงรูปร่างของนิวเคลียสเม็ดเลือดแดง การเปลี่ยนแปลงทางโลหิตวิทยา การเปลี่ยนแปลงทางเคมีของการทำงานของตับ และการเกิดพยาธิสภาพของเนื้อเยื่อ ได้แก่ เหงือก ตับและไต ในระดับกล้องจุลทรรศน์ธรรมดาและกล้องจุลทรรศน์อิเลคตรอน

<u>ผลการทดลอง :</u> พบว่าใบพืชที่ระดับความเข้มข้นสูงและให้ในระยะเวลานานจะมีประสิทธิภาพดีกว่าความเข้มข้นต่ำและให้ในระยะเวลาสั้น การให้ในรูปแบบอาหารเสริมจะมีประสิทธิภาพดีกว่าการฉีดเข้าช่องท้อง นอกจากนั้น ใบรางจืดจะแสดงประสิทธิภาพดีกว่าใบทับทิม ดีกว่าใบฝรั่งตามลำดับ

<u>สรุป :</u> การศึกษาพบว่าการให้อาหารเสริมใบพืชมีประสิทธิภาพในการกระตุ้นภูมิคุ้มกันในปลา

คำหลัก : *Psidium guajava; Punica granatum; Thunbergia laurifolia; Oreochromis niloticus; Puntius altus*

LIST OF TABLES

LIST OF TABLES (cont.)

Table **Page**

LIST OF TABLES (cont.)

LIST OF TABLES (cont.)

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

EXECUTIVE SUMMARY

1. ความสำคัญและที่มาของปัญหา

มีการศึกษามากมายที่มุ่งพัฒนาการป้องกันโรค ในการเพาะเลี้ยงสัตว์น้ำให้ได้อย่างมีประสิทธิภาพพอๆกับการใช้สารเคมีเพื่อการรักษา ได้แก่ การใช้วัคซิน การใช้สารกระตุ้นภูมิคุ้มกัน แต่ก็ยังคงมีข้อเสียบางประการ เช่น ราคาของวัคซินค่อนข้างสูง และก่อให้ความเครียดต่อสัตว์น้ำ

สารกระตุ้นภูมิคุ้มกันที่มีการทดสอบในการเพิ่มประสิทธิผลในการเพาะเลี้ยงสัตว์น้ำ เช่น ปลา ได้แก่ glucan (Chen and Ainsworth, 1992), lactoferrin (Sakai et al., 1993), levamisole (Findlay et al., 2000), chitosan (Siwicki et al., 1994) และพืชบางชนิด เช่น Solanum trilobatum (Divyagnaneswari et al., 2007), *Azadirachta indica* (Harikrishnan et al., 2003), *Radix astragalin* และ *Angelicae Sinensis* (Jian and Wu., 2004) โดยพบว่ามีการเพิ่มจำนวนเซลล์มาคโครฟาส์ก เพิ่มระดับของเอนไซม์ไลโซโซม, ความต้านทานต่อโรค, phagocytic activity และ complement activities

พืชถูกนำมาใช้ในทางการแพทย์นานพอๆกับประวัติศาสตร์ของมนุษย์ มีพืชมากกว่า 150,000 ชนิดที่นำมาศึกษาและหลายชนิดในนั้นมีสรรพคุณในการรักษาโรค และกระตุ้นการสร้างภูมิคุ้มกันทั้งชนิดจำเพาะ และ ไม่จำเพาะ ประเทศไทยเป็นประเทศหนึ่งที่มีพืชสมุนไพรมากมาย เช่นเดียวกับอินเดียและจีน มีการทดลองและศึกษาถึงประโยชน์และโทษของพืชสมุนไพรต่อมนุษย์และสัตว์ แต่ยังมีข้อมูลการวิจัยอยู่น้อย ในการพัฒนาการใช้พืชเพื่อกระต้นภูมิคุ้มกันในเพาะเลี้ยงปลา ในการทดลองนี้ได้เลือกพืชสมุนไพรที่มีการบริโภคมาก หาได้ง่าย ราคาถูก มาทำการศึกษา ได้แก่ ฝรั่ง, ทับทิม และรางจืด

ฝรั่งมีชื่อวิทยาศาสตร์ว่า *Psidium guajava* อยู่ในตระกูล Myrtaceae เป็นไม้ยืนต้นขนาดกลาง เปลือกลำต้นมีสีเขียวปนน้ำตาล เรียบเป็นมัน ใบรูปไข่ ผลรูปร่างกลม ผิวอาจเรียบหรือขรุขระ ผลอ่อนมีสีเขียวแก่เนื้อแข็ง เมื่อผลแก่จะมีสีเขียวอ่อนเนื้อกรอบ เนื้อในเป็นสีขาว เมล็ดมีลักษณะกลม แข็ง มีสีเหลืองขนาดเล็ก ฝรั่งมีสรรพคุณในการลดไข้ แก้ท้องเสีย รักษาโรคบิด กระเพาะและลำไส้อักเสบ (Lozoya et al., 2002) การทดลองศึกษาสารสกัดจากใบพบว่าสามารถใช้ลดการอักเสบ, ลดระดับน้ำตาลในหนูได้ (Oh et al., 2005) มีความสามารถขจัดอนุมูลอิสระ (Chen and Yen, 2007) มีฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรีย (Akinpelu and Onakoya, 2006) องค์ประกอบทางเคมีที่พบ ได้แก่ fatty acid (Opute, 1978), ascorbic acid และ carotenoids (Nogueira et al., 1978), saponin (Cuellar et al., 1984), tannin (Tanaka et al., 1992), lactins (Coutino-Rodriguez et al., 2001) tripenoids (Begum el al.,2002), และ flavonoids (Lozoya et al., 2002),

ทับทิมมีชื่อวิทยาศาสตร์ว่า *Punica granatum* อยู่ในตระกูล Punicaceae เป็นไม้พุ่มขนาดกลาง แตกกิ่งก้าน โคนต้นมีกิ่งที่เปลี่ยนไปเป็นหนามยาวแข็ง ใบเดี่ยวแผ่นใบแคบขอบใบเป็นรูปขอบขนาน ยอดอ่อนเป็นสีแดง ใบออกเป็นคู่ๆตรงข้ามกัน ดอกเดี่ยว กลีบเลี้ยงหนาสีแดงจะคงทนอยู่จนเป็นผล กลีบดอกสีแดงอมส้ม ผลกลมโต เปลือกนอกของผลหนาค่อนข้างเหนียว เปลือกด้านในสีเหลือง ภายในมีเมล็ดเป็นจำนวนมาก อัดกันแน่นเต็มเปลือก แต่ละเมล็ดมีเนื้อสีชมพูอมแดงใส รสหวานอมเปรี้ยว ทับทิมมีสรรพคุณในการลดการอักเสบ ป้องกันมะเร็ง (Lansky and Newman, 2007) แก้ท้องเสีย มีฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรีย (Mathabe el al., 2006) การทดลองศึกษาสารสกัดจากดอกพบว่าสามารถใช้ลดระดับน้ำตาลในหนูได้ (Huang et al., 2005) มีความสามารถขจัดอนุมูลอิสระ (Sestili et al., 2007) องค์ประกอบทางเคมีที่พบ ได้แก่ punicalagin (Cerda et al., 2003), ellagitannins และ gallotannins (Seeram et al., 2005) flavonoids ชนิด quercetin, kaempferol และ luteolin glycosides (Lansky and Newman, 2007)

รางจืดมีชื่อวิทยาศาสตร์ว่า *Thunbergia laurifolia* อยู่ในตระกูล Acanthaceae เป็นไม้เถาขนาดกลาง ลำต้นจะเลื้อยพันกับต้นไม้อื่นไม่มีมือจับ ใบเป็นใบเดี่ยวแยกออกจากลำต้นเป็นคู่ตรงบริเวณข้อ สีเขียวเข้ม รูปยาวรีขอบขนาน ปลายเรียวแหลมโดยเว้าขอบเรียบหรือหยักตื้น ดอกสีม่วงอมฟ้า ออกเป็นช่อตามซอกใบ ห้อยลงมีใบประดับสีเขียว กลีบดอกมีลักษณะเป็นถ้วยแผ่ออกเป็นรูปแตร ผลรูปทรงกลม ส่วนปลายสอบแหลมเป็นจงอย รางจืดมีสรรพคุณ ใช้น้ำคั้นใบสดลดการอักเสบ (Charumanee et al., 1998) แก้ไข้ ถอนพิษของยาพิษ, พิษจากพืชและสัตว์, มีการศึกษาทดลองในหนูพบว่าน้ำสกัดใบรางจืดมีฤทธิ์ถอนพิษแอลกอฮอล์ในหนูได้ (Pramyothin et al., 2005) ป้องกันการเปลี่ยนแปลงยีนส์ (Saenphet et al., 2005) มีความสามารถขจัดอนุมูลอิสระ (Chan and Lim, 2006) ช่วยรักษาอาการติดยา (Thongsaard et al., 2005) องค์ประกอบทางเคมีที่พบ ได้แก่ apigenin, cosmosiin, glucosides (Kanchanapoom et al., 2002)

2. วัตถุประสงค์

การทดลองส่วนใหญ่ของสารสกัดจากพืชทั้ง 3 ชนิด มักเป็นรายงานใน หนู หมูและกระต่าย แต่ยังไม่มีงานวิจัยที่ศึกษาเกี่ยวกับประสิทธิผลของสารสกัดต่อสัตว์น้ำเช่น ปลานิล และปลาตะเพียน ดังนั้นการวิจัยในครั้งนี้จึงมีจุดประสงค์ ดังต่อไปนี้

1. **วัตถุประสงค์ที่ 1 : การศึกษาประสิทธิผลของการฉีดสารสกัดจากใบพืชเข้าช่องท้องปลาในระยะเฉียบพลัน (Efficacy of plant leaves extract in acute treatment)**

การศึกษาถึงประสิทธิผลแบบเฉียบพลัน ในช่วงเวลา 48 ชั่วโมง และ 7 วัน ของสารสกัดจากใบพืชที่ระดับความเข้มข้นต่างๆ โดยการฉีดเข้าช่องท้องปลา ต่อการกระตุ้นระบบภูมิคุ้มกัน โดยใช้ตัววัด ได้แก่

1.1 การเปลี่ยนแปลงจำนวนเซลล์มาคโครฟาส์ก (Macrophage studies)

1.2 การเปลี่ยนแปลงจำนวนเซลล์นิวโตรฟิวส์ (Nitroblue tetra zolium test)

1.3 การเปลี่ยนแปลงระดับไลโซไซม์ (Lysozyme activity test)

1.4 การเปลี่ยนแปลงรูปร่างนิวเคลียสเม็ดเลือดแดง (Micronucleus and nuclear abnormality tests)

1.5 การเปลี่ยนแปลงทางโลหิตวิทยา (Hematology)

1.6 การเปลี่ยนแปลงทางเคมีของการทำงานของตับ (Liver function test) ได้แก่ Alanine และ Aspartate aminotransferase และ Alkaline Phosphatase

1.7 การเปลี่ยนแปลงทางพยาธิสภาพของเนื้อเยื่อ ได้แก่ เหงือก ตับ ไต ทั้งในระดับกล้องจุลทรรศน์ธรรมดาและกล้องจุลทรรศน์อิเลคตรอน (Histopathological and Ultrastructural studies)

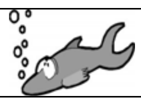
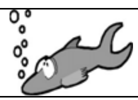
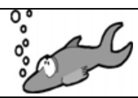
2. วัตถุประสงค์ที่ 2 : การศึกษาประสิทธิผลของอาหารเสริมจากใบพืชอบแห้งในระยะยาว (Efficacy of dietary supplement of dry plant leaves in chronic treatment)

ศึกษาถึงประสิทธิผลของอาหารเสริมของใบพืชอบแห้งในระดับความเข้มข้นต่างๆ ต่อการกระตุ้นระบบภูมิคุ้มกัน ในปลาตะเพียนและปลานิล โดยใช้ตัววัด ได้แก่ อัตราการเจริญเติบโต การเพิ่มจำนวนเซลล์มาคโครฟาส์กและนิวโตรฟิวส์ในน้ำล้างช่องท้องและเนื้อเยื่อไต การเปลี่ยนแปลงระดับไลโซไซม์ การเปลี่ยนแปลงรูปร่างของนิวเคลียสเม็ดเลือดแดง การเปลี่ยนแปลงทางโลหิตวิทยา การเปลี่ยนแปลงทางเคมีของการทำงานของตับ และการเกิดพยาธิสภาพของเนื้อเยื่อ ได้แก่ เหงือก ตับ และไต ในระดับกล้องจุลทรรศน์ธรรมดาและกล้องจุลทรรศน์อิเลคตรอน ในช่วงเวลา 2 เดือน

3. ระเบียบวิธีวิจัย : การทดลองแบ่งออกเป็น 3 ส่วน ดังนี้คือ

การทดลองเลือกใช้ ปลานิลและปลาตะเพียน ขนาดน้ำหนัก 35-45 กรัม ความยาว 12-15 ซม นำมาเลี้ยงในห้องปฏิบัติการ ภาควิชาพยาธิชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ก่อนการทดลองให้ เตรียมใบพืชอบแห้ง สารสกัดจากใบพืชอบแห้งและผสมอาหารปลากับใบพืชอบแห้ง

การจัดกลุ่มทดลอง (Experimental design) ตามวัตถุประสงค์ที่ 1:

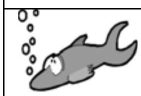
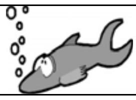
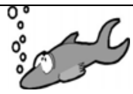
	กลุ่มที่ 1	**กลุ่มที่ 2**	**กลุ่มที่ 3**
			
จำนวนปลาที่เริ่มทดลอง	10 ตัว	10 ตัว	10 ตัว
ความเข้มข้นของสารสกัดจากใบพืชอบแห้ง	------	200 มก ต่อ กก น้ำหนักตัวปลา	600 มก ต่อ กก น้ำหนักตัวปลา
48 ชม เก็บตัวอย่างเพื่อวิเคราะห์	5 ตัว	5 ตัว	5 ตัว
7 วัน เก็บตัวอย่างเพื่อวิเคราะห์	5 ตัว	5 ตัว	5 ตัว

ปลาจำนวน 30 ตัว แบ่งเป็น 3 กลุ่ม กลุ่มละ 10 ตัว ฉีดสารสกัดจากใบพืชอบแห้งเข้าช่องท้องปลา ด้วยขนาดความเข้มข้นที่ระบุตามกลุ่มได้แก่

1. กลุ่มที่ 1 กลุ่มควบคุม ไม่ได้ฉีดสารสกัดจากใบพืชอบแห้งเข้าช่องท้องปลา
2. กลุ่มที่ 2 ฉีดสารสกัดจากใบพืชอบแห้งเข้าช่องท้องปลา ปริมาณ 0.5 มล. ในระดับความเข้มข้นต่ำ 200 มิลลิกรัม ต่อ กิโลกรัมน้ำหนักตัวปลา
3. กลุ่มที่ 3 ฉีดสารสกัดจากใบพืชอบแห้งเข้าช่องท้องปลา ปริมาณ 0.5 มล. ในระดับความเข้มข้นสูง 600 มิลลิกรัม ต่อ กิโลกรัมน้ำหนักตัวปลา

หลังจากฉีด 48 ชั่วโมง และ 7 วัน จะสุ่มเลือกปลาจำนวน 5 ตัวจากแต่ละกลุ่ม มาทำให้สลบ โดยใช้ MS-222 ปริมาณ 0.2 กรัม/ลิตร ตัวอย่างที่ต้องทำการเก็บเพื่อวิเคราะห์ต่อไป ได้แก่ น้ำล้างช่องท้อง เลือด และอวัยวะ เหงือก ตับและ ไต

การจัดกลุ่มทดลอง (Experimental design) ตามวัตถุประสงค์ที่ 2:

	กลุ่มที่ 1	**กลุ่มที่ 2**	**กลุ่มที่ 3**
			
จำนวนปลาที่เริ่มทดลอง	10 ตัว	10 ตัว	10 ตัว
ความเข้มข้นของอาหารเสริม	อาหารปลาปกติ	20 มิลลิกรัม ต่อกรัม อาหารปลา ต่อ กิโลกรัม น้ำหนักตัวปลา	60 มิลลิกรัม ต่อกรัม อาหารปลา ต่อ กิโลกรัมน้ำหนักตัวปลา
1 เดือน เก็บตัวอย่างเพื่อวิเคราะห์	5 ตัว	5 ตัว	5 ตัว
2 เดือน เก็บตัวอย่างเพื่อวิเคราะห์	5 ตัว	5 ตัว	5 ตัว

ปลาจำนวน 30 ตัว แบ่งเป็น 3 กลุ่ม กลุ่มละ 10 ตัว ได้รับอาหารที่ระบุตามกลุ่ม วันละครั้ง ขนาดประมาณ 2 เปอร์เซ็นต์ของน้ำหนักตัว ได้แก่

1. กลุ่มที่ 1 กลุ่มควบคุมได้รับอาหารปลาปกติ
2. กลุ่มที่ 2 ได้รับอาหารผสมใบพืชในระดับความเข้มข้นต่ำ 20 มิลลิกรัม ต่อ กรัมอาหารปลา ต่อ กิโลกรัมน้ำหนักตัวปลา
3. กลุ่มที่ 3 ได้รับอาหารผสมใบพืชในระดับความเข้มข้นสูง 60 มิลลิกรัม ต่อ กรัมอาหารปลา ต่อ กิโลกรัมน้ำหนักตัวปลา

เดือนที่ 1 และ 2 หลังจากให้อาหารปลาตามที่กำหนด จะสุ่มเลือกปลาจำนวน 5 ตัวจากแต่ละกลุ่ม มาทำให้สลบ ตัวอย่างที่ต้องทำการเก็บเพื่อวิเคราะห์ต่อไป ได้แก่ น้ำล้างช่องท้อง เลือด และอวัยวะ เหงือก ตับและ ไต

4. แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วง 6 เดือน

	ปีที่ 1		ปีที่ 2		ปีที่ 3	
	เดือน 1-6	เดือน 7-12	เดือน 1-6	เดือน 7-12	เดือน 1-6	เดือน 7-12
การศึกษาประสิทธิผลของการฉีดสารสกัดจากใบฝรั่งเข้าช่องท้องปลาในระยะเฉียบพลัน	✓					
การศึกษาประสิทธิผลของการฉีดสารสกัดจากใบทับทิมเข้าช่องท้องปลาในระยะเฉียบพลัน		✓				
การศึกษาประสิทธิผลของการฉีดสารสกัดจากใบรางจืดเข้าช่องท้องปลาในระยะเฉียบพลัน			✓			
การศึกษาประสิทธิผลของอาหารเสริมจากใบฝรั่งในระยะยาว				✓		
การศึกษาประสิทธิผลของอาหารเสริมจากใบทับทิมในระยะยาว					✓	
การศึกษาประสิทธิผลของอาหารเสริมจากใบรางจืดในระยะยาว						✓

5. ผลการวิจัยในครั้งนี้คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติในหัวข้อเรื่อง

1. Effect of dietary supplement and intraperitoneal injection of *Psidium guajava, Punica granatum* and *Thunbergia laurifolia* leaves extract on *Oreochromis niloticus*

ชื่อวารสาร Environmental Toxicology มี Impact factor =1.582

2. Effect of dietary supplement and intraperitoneal injection of *Psidium guajava, Punica granatum* and *Thunbergia laurifolia* leaves extract on *Puntius altus*

ชื่อวารสาร Journal of Fish Biology มี Impact factor = 1.198

3. Efficacy of plant leaves aqueous extract enhance immune response in fish

ชื่อวารสาร Acta Tropica มีImpact Factor = 1.952

6. งบประมาณโครงการ (ตามระยะเวลาโครงการที่ได้เสนอรับทุน)

	ปีที่ 1	**ปีที่ 2**	**ปีที่ 3**	**รวม**
1.หมวดค่าตอบแทน ได้แก่ ค่าตอบแทนหัวหน้าโครงการ	180,000	180,000	180,000	540,000
2.หมวดค่าวัสดุวิทยาศาสตร์	173,000	173,000	173,000	519,000
3.หมวดค่าใช้สอย	35,000	35,000	35,000	105,000
4. หมวดค่าจ้าง	12,000	12,000	12,000	36,000
รวมงบประมาณโครงการ	**400,000**	**400,000**	**400,000**	**1,200,000**

CHAPTER I
INTRODUCTION

Immunostimulants have been playing an important role in stimulatory effects on the non-specific immune system of both humans and animals. However, immunostimulants are used quite often for intensive fish and animal farming, in order to reduce the risk of disease outbreak thereby improving the health, eventually, antibiotics use can be reduced (Sintef, 2005). The immunostimulants that have been bought into trials in aquaculturing may include glucan (Chen and Ainsworth, 1992), lactoferrin (Sakai et al., 1993), levamisole (Anderson and Jeney, 1992), chitosan (Siwicki et al., 1994), growth hormone (Sakai et al., 1996), vitamin C or ascorbic acid (Qin et al., 2000; Sahoo et al., 1999), aloe (Kim et al., 1999) and extracellular products of *Mycobacterium* spp. (Chen et al., 1998).

The effect of these immunostimulants has shown certain influence to augment the non-specific immune system in aspects like the innate immunity which includes the phagocytes, lysozyme and the complement levels which includes the immunoglobulin levels. One of the researches in the past have shown that rainbow trouts can be protected against furunculosis, boils or lesions on the skin caused by *Staphylococcus aureus*, whereby its mortality rate also decreases with the use of immunostimulants (Siwicki et al., 1994; Wahli et al., 1998).

Immunostimulants are brought into use due to its easier availability and its cheap price with excellent influence on improving health and decreasing diseases of organisms. Furthermore, the adverse effects are less when compared to antibiotics or other forms of therapies. China is considered one of the main countries which bring immunostimulants into use and sometimes they refer to it as “Traditional Chinese Medicine (TCM)”, which can be used to treat humans and animals diseases. Examples of TCM can include Astragalus Root (*Radix astragalin seu Hedysari*), Chinese Angelica Root (*R. Angelicae Sinensis*) (Jian and Wu, 2004). India is also endowed with high availability of natural medicinal plants, for example the *Solanum trilobatum* (Divyaganansewari et al., 2007). Other examples of wide-reaching immunostimulants can include Laurel clock vine (*Thunbergia laurifolia*), Pomegranate (*Punica granatum*) and Guava (*Psidium guajava*). These immunostimulants have been used in various human medicines in countries like China and India, but however research on fishes is being progressed.

1.1 Fish model

1.1.1 *Puntius altus*

Barb name for some freshwater fish species of the family Cyprinidae, order Cypriniformes. Ten species of barbs are within two genera *Puntius* (9 species) and *Oreichthys* (1 species). The moderate to deeply compressed body of this fish is silvery to greenish silvery or reddish brown in color. Spots, blotches, bands on the body, and 4, 2 or no barbells are important identifying characters. Body length varies from 5 cm to about 20 cm.

The body shape is elongated to high-backed and slightly compressed laterally. The mouth has two pairs of barbells. The body is silvery with a bluish to yellow iridescence. The scales have a metallic look. Broad red distal margin with no black sub-marginal stripe along each lobe of the caudal fin; red pelvic and anal fins; a black distal blotch on the dorsal fin; the body depth 1.8-2.2 times in standard length. The iris of the eye is amber-gold (Fig. 1).

Tinfoil barbs are a peaceful fish that can reach a length greater than 40 cm, and like other, larger barbs, can often live more than 10 years in captivity. In nature, tinfoil barbs are primarily macrophages; that is, they feed on vascular aquatic plants. However, they will eat most commercially prepared diets consisting of vegetable and fishmeal proteins. Water quality,parameters of the tinfoil barb's natural habitat ranges from soft to moderately hard water (100-200 mg/L calcium carbonate), pH of 6.5 to 7.0, and a temperature of 22^{o} to 25^{o}C. Tinfoil barbs are a hardy fish that can do well in conditions exceeding the water quality parameters of their natural habitat.

Taxonomy

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Neopterygii
Class	Teleostei

Subclass	Euteleostei
Order	Cypriniformes
Suborder	Cyprinoidei
Family	Cyprinidae
Genus	Puntius
Species	altus

Figure 1. Red-tailed tinfoil barb *(Puntius altus)*

1.1.2 *Oreochromis niloticus*

Nile tilapia has one nostril on each side of the snout. The body is oblong, moderately deep, compressed. The dorsal and ventral profiles are about equally convex. The caudal peduncle is broadly short. The mouth is slightly oblique and protractile. The dorsal fin is with a long base; spinout dorsal fin with 16-17 spines, followed by 11-15 soft fin rays. The anal fin base is relatively short, consisting of 3 spines and 8-11 soft fin rays. The posterior part of both fins, especially in adult male fishes, is usually and considerably extended. The pectoral fins are moderately large and pointed, with 15 soft fin rays. The caudal fin is broadly round in adult, but almost truncate in young. The scales are fairly large and cycloid. There are two lateral lines. The upper one extends from the upper corner of operculum vertically to below the origin of soft dorsal fin, with 19-25 scales. The lower portion begins perpendicular and transposes to the middle of caudal peduncle, with 11-18 scales, totally consisting of 31-35 scales (Fig. 2, Nelson, 1984).

In all *Oreochromis* species the male excavates a nest in the pond bottom and mates with several females. After a short mating ritual the female spawns in the nest, the male fertilizes the eggs, and holds and incubates the eggs in mouth or buccal cavity until they hatch. Fry remain in the female mouth through yolk sac absorption and often seek refuge in her mouth for several days after they begin to feed.

Sexual maturity in tilapia is a function of age, size and environmental conditions. When growth is slow, sexual maturity in Nile tilapia is delayed a month or two but stunted fish may spawn at a weight of less than 20 grams. Under good growing conditions in ponds, the Nile tilapia may reach sexual maturity in as little as 3 months of age, when they seldom weigh more than 60 to 100 grams.

The sex of a 25-gram tilapia fingerling can be determined by examining the genital papilla located immediately behind the anus. In males the genital papilla has only one opening, the urinary pore of the ureter, through which both milt and urine pass. In females the eggs exit through a separate oviduct and only urine passes through the urinary pore. Placing a drop of dye, methylene blue or food coloring, on the genital region helps to highlight the papilla and its openings (Popma and Masser, 1999).

Taxonomy

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Order	Perciformes
Suborder	Labroidei
Family	Cichlidae
Genus	Oreochromis
Species	niloticus

Figure 2. Nile tilapia (*Oreochromis niloticus*)

1.2 Plant model

1.2.1 *Psidium guajava*

Psidium guajava L. of the myrtle family (Myrtaceae) is considered one of the extroverted fruit trees known (Morton, 1987). It is commonly called guave, goyave or goyavier in French; guave, guavenbaum, guayave in German; banjiro in Japanese; goiaba, goiabeiro in Portugal; araçá-goiaba, araçá-guaçú, guaiaba in Brazil; guayaba, guayabo in Español; guava in English and farang in Thai (Killion, 2000).

Taxonomy

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Myrtales
Family	Myrtaceae
Genus	Psidium
Species	guajava

It is a small tree to 10 m high with thin, smooth, patchy, peeling bark.The leaves are opposite, elliptic to ovate about 2-5 cm long. The flowers are white, with five petals up to 2 cm long and numerous stamens. This plant produces edible round or pear shaped fruit about 5 cm in diameter which contains high amounts of calcium and containing numerous small hard white seeds (USDA, 2007). Fig.3-6 has shown the overall picture of guava plant.

These readily available type of plants yield edible fruits which can be eaten with prune powder or salt like most people in Asia. Furthermore Guava juice, and candies or jellies made from guava are also quite popular worldwide. Guava is rich in tannins, phenols, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit). The leaves of guava are rich in flavonoids (Tropical plant database, 1996). They are also containing essential oil with the main components being α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, cineol, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene and curcumene (Zakaria et al., 1994, Li et al., 1999). Flavonoids, and saponins combined with oleanolic acid have been isolated from the leaves (Arima and Danno, 2002). Nerolidiol, β-sitosterol, ursolic, crategolic, and guayavolic acids have also been identified (Iwu, 1993). In addition, the leaves contain triterpenic acids as well as flavonoids; avicularin and its 3-L-4-pyranoside with strong antibacterial action (Oliver, 1986), fixed oil 6%, 3.15% resin, and 8.5% tannin, and a number of other fixed substances, fat, cellulose, tannin, chlorophyll and mineral salts (Nadkarni and Nadkarni, 1999). Also have been isolated from the leaves of *P. guajava* guavanoic acid, guavacoumaric acid, 2α-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol D and β-sitosterol-3-*O*-β-D-glucopyranoside (Begum et al., 2002). In mature leaves, the greatest concentrations of flavonoids were found in July: Myricetin (208.44 mg/kg), quercetin (2883.08 mg kg^{-1}), luteolin (51.22 mg/kg and kaempferol (97.25 mg/kg) (Vargas et al., 2006). Two triterpenoids, 20β-acetoxy-2α,3β-dihydroxyurs-12-en-28-oic acid (guavanoic acid), and 2α,3β-dihydroxy-24-*p*-*z*-coumaroyloxyurs-12-en-28-oic acid (guavacoumaric acid), along with six known compounds 2α-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol D and β-sitosterol-3-*O*-β-D-glucopyranoside, have been isolated from the leaves of *P. guajava*. Guajavolide (2a-,3β-6β-,23-tetrahydroxyurs-12-en-28,20β-olide, and guavenoic acid, were isolated from fresh leaves of *P. guajava* (Guti´errez and Solis 2007).

However our main interest is not about the fruit itself but the leaf extract which is going to be used in my research. Guava leaves are used for medicinal purposes, as a remedy to diarrhea. Furthermore they also have antimicrobial properties, which make them strong immunostimulants. Recent studies prove that guava has sugar lowering properties to help diabetics lower their sugar count (USDA, 2007). In addition, crushed leaves from guava plants can be applied on wounds, ulcers and rheumatic places and toothache can be relieved by chewing the leaves. Lastly, leaves extracts can also be gargled to relieve oral ulcers and can also be remedy for coughs (Morton, 1987). For this research guava leaves extract will be mixed with fish food under different concentrations. Much of guava's therapeutic activity is due to the flavonoids present in it. The flavonoids have antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava as it is able to relax intestinal smooth muscle and inhibit bowel contractions. Guava also has antioxidant properties which are primarily due to the polyphenols found in the leaves (Tropical plant database, 1996).

Figure 3. The tree of *Psidium guajava*

Figure 4. The leaves of *Psidium guajava*

Figure 5. The flower of *Psidium guajava*

Figure 6. The fruit of *Psidium guajava*

1.2.2 *Punica granatum*

Punica granatum, commonly known as pomegranate, is a deciduous shrub or small tree of around 5-8 m tall. Pomegranate is a native fruit of all regions from Iran to the Himalayas in northern India and has been cultivated and naturalized over the whole Mediterranean region. It is widely cultivated throughout Armenia, Iran, India, the drier parts of Southeast Asia, Malaya, the East Indies, and tropical Africa. Introduced into Latin America and California by Spanish settlers in 1769, pomegranate is now cultivated mainly in the drier parts of California and Arizona for its fruits exploited commercially as juice products gaining in popularity since 2001. Pomegranate is best grown in mild temperature where there is little humidity (Figs. 7-12).

Taxonomy

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Myrtales
Family	Lythraceae
Genus	Punica
Species	granatum

Figure 7. The tree of *Punica granatum*

Figure 8. The leaves of *Punica granatum*

Figure 9. The flowers of *Punica granatum*

Figure 10. The fruit of *Punica granatum*

Figure 11. The fruit of *Punica granatum*

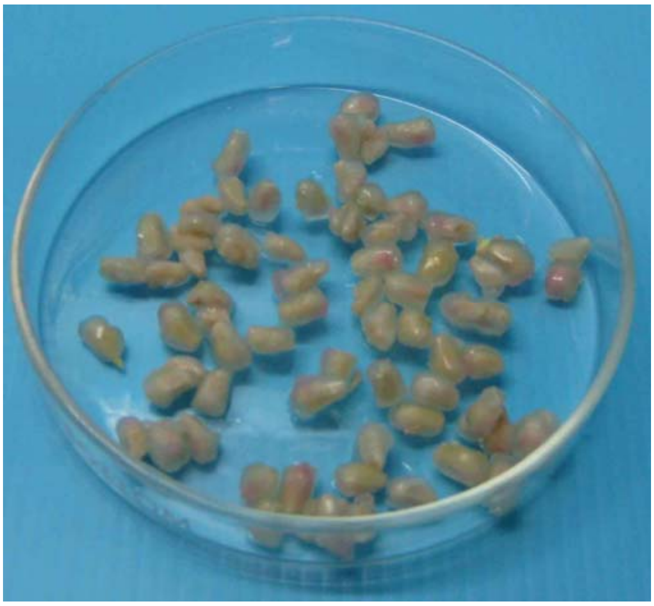


Figure 12 The seeds of *Punica granatum*

Pomegranate has many health benefits that have been proven scientifically. Pomegranate contains high level of antioxidant that prevents the oxidization of low density lipoprotein (LDL, bad cholesterol), prevents the onset of atherosclerosis, high blood pressure and also the risk oh heart disease (Aviram et al., 2000).

According to one of the studies in China, extracts from pomegranate leaves may help to control weight gained as a result of consuming a high fat diet (Lei et al., 2007). In addition, pomegranate juice, like aspirin, can help keep blood platelets from clumping together to form unwanted clots. A new study, published in the *International Journal of Obesity*, suggests that the leaves of the pomegranate may also offer significant health benefits, particularly for weight management supplements market (Lei et al., 2007).

1.2.3 *Thunbergia laurifolia*

Thunbergia laurifolia or Laurel clock vine can be found in most tropical rain forest. It is a large woody vine that can grow from 2-6 m tall. The leaf oval-shaped appearance about 8-15 cm wide and 10-20 cm long (THD, 2008). The leaves grow in the opposite pairs along the stalks. Its flower has the shape of a trumpet, and its white outside with a yellow neck flower. Its flower opens out into give pale purple-blue petals and it has the length of around 6-8 cm long. Its seed is cover up by brown capsule and has the oval shape where it is pinched at one end. Its seed has the size of 1 cm. long and

0.4 cm. wide. An important characteristic of this plant is that it has tuberous root system that can resprout when cut. Some countries like Australia consider this plant as an invasive threat weed (Figs. 13-14, WMG, 2003).

Taxonomy

Kingdom	Plantae
Phylum	Tracheobionta
Subphylum	Spermatophyta
Superclass	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Scrophualariales
Family	Acanthaceae
Genus	Thunbergia
Species	laurifolia

Figure 13. The leaves of *Thunbergia laurifolia*

Figure 14. The flowers of *Thunbergia laurifolia*

CHAPTER II
OBJECTIVES

The objectives of this study, first to investigate the effect of intraperitoneal injection of various doses of plant leaves extract at 48 hours and 7 days; second to investigate the effect of dietary supplement of various doses of dry plant leaves at 1 and 2 months in the term of:

1.1 Growth rate

1.2 Macrophage studies

1.3 Nitroblue tetra zolium test

1.4 Lysozyme activity test

1.5 Micronucleus and nuclear abnormality tests

1.6 Hematology studies i.e., Hematocrit, Hemoglobin, White blood cell count, Red cell indices

1.7 Liver function test i.e., Alanine and Aspartate aminotransferase, Alkaline Phosphatase

1.8 Histopathological and Ultrastructural studies in gills, liver and kidney

CHAPTER III
LITERATURE REVIEWS

3.1 *Psidium guajava*

Guti´errez and Solis (2007) have reviewed the phytochemical and pharmacological of *P. guajava*. A survey of the literature shows *P. guajava* is mainly known for its antispasmodic and antimicrobial properties in the treatment of diarrhea and dysentery. It has also been used extensively as a hypoglycemic agent. Many pharmacological studies have demonstrated the ability of this species to exhibit antioxidant, hepatoprotection, anti-allergy, antimicrobial, antigenotoxic, antiplasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, antiinflammatory and antinociceptive activities, supporting its traditional uses.

3.1.1 Cardiovascular and hypotensive effects

Garcia et al. (2003) evaluated the effectiveness of guava extract on the mammalian myocardium by measuring isometric force. This study showed that the chemicals quercetin (flavonoid) found in leaves decreased the contraction of the heart muscle but it was concentration-dependent. From the interaction between quercetin and calcium, it decreased the smooth muscle contractile force which reduced the release of acetylcholine in neuromuscular junction. The cardio-inhibitory actions in rats and guinea pigs of the aqueous leaf extract of *P. guajava* also appeared to be due to cholinergic involvement in the mechanism of action. Ojewole (2005) showed that the aqueous leaf extract caused hypotension in the experimental animal model used via cholinergic mechanisms. Moreover, acute intravenous administrations of the leaf extract (50-800 mg/kg i.v.) produced dose-dependent, significant reductions in systemic arterial blood pressures and heart rates of hypertensive, Dahl salt-sensitive rats. Although the exact mechanisms of action of the extract remain speculative at present, it is unlikely that the extract causes hypotension in the mammalian experimental animal model used via cholinergic mechanisms since its cardiodepressant effects are resistant to atropine pre-treatment (Ojewole, 2005).

3.1.2 Anti-inflammatory and anti-analgesic effects

Many parts of Africa use *P. guajava* leaf, stem, bark, and roots to use as traditional treatment for illnesses. In effort to prove the usefulness and safety of this plant, a study was been made to examine the anti-inflammatory and analgesic effects in rats and mice (Ojewole, 2006). An aqueous extract was obtained from guava leaves. To observe the anti-inflammatory property of the extract, fresh egg albumin was used to induce pedal edema, while to observe the analgesic property, a hot-plate and acetic acid test was performed. Diclofenac and morphine were used as a comparison for anti-inflammatory and pain relieving response. *P. guajava* leaf aqueous extract (50-800 mg/kg, i.p.) showed a significant inhibition of fresh egg albumin induced acute inflammation in rats and also a significant analgesic effect against heat and chemical induced pain in mice. The two experiments used to determine the anti-inflammatory and analgesic property of the leaf was concentration dependent. The numerous chemical compounds found in the leaf such as tannins, polyphenolic compound, flavonoids, and quercetin possesses the analgesic and anti-inflammatory properties. These findings may allow pharmacological credence to manage pain control and inflammation conditions in rural areas elsewhere as well.

3.1.3 Anticestodal effects

Temgenmogla and Arun (2006) investigated the anticestodal efficacy of *P. guajava*. The leaf extract was administered orally to different groups of rat that has been experimentally infected by *Hymenolepis diminuta,* an intestinal parasite in rats. The result was compared to a standard anticestodal drug, praziquantel. After treatment from the guava extract, there was a significant decrease of egg count in the feces and a low recovery number of scolices in the small intestine. This experiment is dose-dependent but it has a significant lower parasite count than the number from praziquantel treatment. The use of guava extract used as anticestodal drug is more efficient and has been used as folk medicine in north India.

3.1.4 Hepatoprotective effects

The hepatoprotective effect of an aqueous leaf extract of *P. guajava* was studied on rat liver damage induced by carbon tetrachloride by monitoring serum aspartate and alanine transaminase, alkaline posphatase, serum cholesterol, serum total lipids and histopathological alterations. The leaf extract at doses of 500 mg kg^{-1} produced significant hepatoprotection (Roy et al., 2006). Pretreatment with asiatic acid (triterpenoid) at doses of 25, 50 or 100 mg/kg significantly blocked the lipopolysaccharide and D-galactosamine-induced increases in both serum aspartate and alanine aminotransferase levels, showing improved nuclear condensation, ameliorated proliferation and less lipid deposition (Gao et al., 2006). Several studies have indicated the ability of guava to reduce several parameters associated with liver injury.

3.1.5 Antioxidant and free radical scavenger effects

Dried leaves of *P. guajava* were extracted with hot water. The total phenolic content in the extract was determined spectrophotometrically according to Folin-Ciocalteu's phenol method and calculated as gallic acid equivalent. A remarkably high total phenolic content 575.3$\pm$15.5 was obtained. The antioxidant activity of lyophilized leaf extracts was determined using free radical DPPH (2,2-diphenyl-1-picrylhydryzyl) scavenging (Qian and Nihorimbere 2004). These antioxidant properties are associated with its phenolic compounds such as protocatechuic acid, ferulic acid, quercetin and guavin B (Thaipong et al., 2005), quercetin, ascorbic acid, gallic acid and caffeic acid (Jimenez et al., 2001). Guava leaf extracts are a potential source of natural antioxidants.

3.2 *Punica granatum*

Furthermore, the uses of pomegranate are so diverse that many studies have been conducted on it. Pomegranate has been revered through the ages for its medicinal properties. Studies in mice and humans indicate that it may also have a potential therapeutic and chemopreventive adjuvant effect in cardiovascular disorders. Anthocyanins were shown to be effective inhibitors of lipid peroxidation, the production of nitric oxide (NO) and inducible nitric oxide synthase activity in different model systems (Ahmed et al., 2005).

Another study focused on the anti-diarrhea activity of *P. granatum* extracts in rats. Methanol extract of *P. granatum* seed was evaluated for anti-diarrhea activity against different experimental models of diarrhea in rats. *P. granatum* seed extract treated animals showed significant inhibitory activity against castrol-oil induced diarrhea and PGE_2 induced enteropooling in rats. The extract also showed a significant reduction in gastrointestinal motility in charcoal meal test in rats. The results obtained established the efficacy of *P. granatum* seed extract as an anti-diarrhea agent (Das et al., 1999).

3.2.1 Antibacterial effects

Mathabe et al. (2006) had used methanol, ethanol, acetone and hot water extract the different plant parts (leaves, root, bark and stem rhizome) of *P. granatum* and the other traditional medical plants. These extracts were screened for antibacterial activity against *Vibro cholera, Escherichia coli* and *Staphylococcus aureus, Shigella* spp., *Salmonella typhi*. The antibacterial activity was determined by agar-well diffusion and expressed as the average diameter of the zone of inhibition of bacterial growth around the wells. The minimum inhibitory concentration of active extracts was determined by using the micro-plate dilution assay. Most of the extracts showed relatively high antibacterial activity against most of the tested microorganisms with the diameter of inhibition zones ranging between 10 and 31 mm. Of the plants studied, the most active extracts were those obtained from *P. granatum* and *Indigofera daleoides*. Water extract of *P. granatum* were equally active as organic extracts against bacteria such as *S. aureus*, *S. sonnei* and *S. flexneri*. The minimum inhibitory concentration values for active extracts ranged between 0.039 and 0.6 mg/ml. The results obtained appeared to confirm the antibacterial potential of the plants investigated, and their usefulness in the treatment of diarrhea.

3.2.2 Antidiabetic effects

Bagri et al. (2009) had investigated the effects of *P. granatum* aqueous extract on 60 mg/kg streptozotocin induced diabetic albino Wistar rats by measuring fasting blood glucose, lipid profiles (atherogenic index), lipid peroxidation and activities of both non-enzymatic and enzymatic antioxidants. The increase in blood glucose level, total cholesterol, triglycerides, low-density lipoprotein cholesterol, very low density lipoprotein, lipid peroxidation level with decrease in high density lipoprotein cholesterol, reduced glutathione content and antioxidant enzymes namely, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase and catalase were the salient features observed in diabetic rats. On the other hand, oral administration of *P. granatum* aqueous extract at doses of 250 mg/kg and 500 mg/kg for 21 days resulted in a significant reduction in fasting blood glucose, total cholesterol, triglycerides, low-density lipoprotein cholesterol, very low density lipoprotein and tissue lipid peroxidation levels coupled with elevation of high density lipoprotein cholesterol, reduced glutathione content and antioxidant enzymes in comparison with diabetic control group. The results suggest that PG could be used, as a dietary supplement, in the treatment of chronic diseases characterized by atherogenous lipoprotein profile, aggravated antioxidant status and impaired glucose metabolism and also in their prevention.

3.2.3 Antioxidant and free radical scavenger effects

Kaur et al. (2006) had evaluated antioxidant and hepatoprotective activity of pomegranate flowers. Ethanolic extract of flowers was found to contain a large amount of polyphenols and exhibit enormous reducing ability, both indicative of potent antioxidant ability. The extract showed 81.6% antioxidant activity in DPPH model system. The ability of extract to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) was tested and it was found to significantly scavenge superoxide ($O_2^{\bullet -}$) (by up to 53.3%), hydrogen peroxide (H_2O_2) (by up to 30%), hydroxyl radicals ($^{\bullet}OH$) (by up to 37%) and nitric oxide (NO) (by up to 74.5%). The extract also inhibited $^{\bullet}OH$ induced oxidation of lipids and proteins in vitro. These results indicated pomegranate flower extract to exert a significant antioxidant activity in vitro. The efficacy of extract was tested in vivo and it was found to exhibit a potent protective activity in acute oxidative tissue injury animal model: ferric nitrilotriacetate (Fe-NTA) induced hepatotoxicity in mice. Intraperitoneal administration of 9 mg/kg body wt. Fe-NTA to mice induced oxidative stress and liver injury. Pretreatment with pomegranate flower extract at a dose regimen of 50–150 mg/kg body wt. for a week significantly and dose dependently protected against Fe-NTA induced oxidative stress as well as hepatic injury. The extract afforded up to 60% protection against hepatic lipid peroxidation and preserved glutathione (GSH) levels and activities of antioxidant enzymes viz., catalase (CAT), glutathione peroxidase (GPX) glutathione reductase (GR) and glutathione-S-transferase (GST) by up to 36%, 28.5%, 28.7%, 40.2% and 42.5% respectively. A protection against Fe-NTA induced liver injury was apparent as inhibition in the modulation of liver markers viz., aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin and albumin in serum. The histopathological changes produced by Fe-NTA, such as ballooning degeneration, fatty changes, necrosis were also alleviated by the extract. These results indicate pomegranate flowers to possess potent antioxidant and hepatoprotective property, the former being probably responsible for the latter.

3.3 *Thunbergia laurifolia*

3.3.1 Antidiabetic effects

Aritajat and co-workers (2004) had studies on the effect of *T. laurifolia* on the reproductive system and blood glucose level of diabetic rats. *T. laurifolia* leaf extract were given to the rats at the concentration of 60 mg/mL orally for 15 days. The results show that both diabetic rats and control rats show a decrease in blood glucose level. Thus, there was no affect on both the rats' reproductive system.

3.3.2 Antidrug-addiction effects

Thongsaard et al. (2005) had done an experiment to treat alcohol and drug addiction from *T. laurifolia* leaf extract in rat. The purpose was to test function of *T. laurifolia* extract on alcohol and drug in rat brain region activity. In *vivo* functional nuclear magnetic resonance imaging to observe effects in vitro on dopamine release. Investigations were made on different part of rat's brain that was induced by 200 mg/kg of *T. laurifolia* extract. Results showed that *T. laurifolia* had increase the single intensity in different brain areas like nucleus, accumenbens, amygldala, globus pallidus, frontrol cortex, hippocampus and caudate putamen. *T. laurifolia* increases neuronal activity in regions that are responsible for locomotors behavior and reward.

3.3.3 Hepatoprotective effects

Pramyothin et al. (2005) had evaluate the hepatoprotective activity of aqueous extract from *T. laurifolia* by using primary cultures of rat hepatocyte and rats as the in vitro and in vivo models. Ethanol was selected as hepatotoxin. Silymarin was the reference hepatoprotective agent. In the in vitro study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and release of transaminases (ALT and AST) were the criteria for cell viability. Primary cultures of rat hepatocyte (24 h culturing) were treated with ethanol (96 μl/ml) and various concentrations of *T. laurifolia* (2.5, 5.0, 7.5 and 10.0 mg/ml) or silymarin (1, 2 and 3 mg/ml) for 2 h. Ethanol decreased MTT (%) nearly by half. Both *T. laurifolia* and silymarin increased MTT reduction and brought MTT (%) back to normal. Ethanol induced release of ALT and AST was also reduced by *T. laurifolia* (2.5 and 5.0 mg/ml) and silymarin (1 mg/ml). In the in vivo study, serum transaminases, serum triglyceride together with hepatic triglyceride and histopathological examination were the criteria for evidences of liver injury. Ethanol (4 g/kg/day, po for 14 days) caused the increase in ALT, AST, hepatic triglyceride and centrilobular hydropic degeneration of hepatocytes. *T. laurifolia* at 25 mg/kg/day, po, or silymarin at 5 mg/kg/day, po, for 7 days after ethanol enhanced liver cell recovery by bringing hepatic triglyceride, ALT and/or AST back to normal. These results suggest that *T. laurifolia* and silymarin possess the hepatoprotective activity against ethanol induced liver injury in both primary cultures of rat hepatocyte and rats.

CHAPTER IV
MATERIALS AND METHODS

4.1 Instruments

- Digital weighing machine (Sartorius BP 3100 S, Minneapolis, U.S.A)
- Hot plate and magnetic stirrer (Lab-Line Pyro Magnestir, Melrose Park, ILL 1267, USA)
- Vortex Genie 2 G560F (Scientific Industries, Bohemia, NY, USA)
- Hematocrit centrifuge machine (BOECO, Germany)
- Micro-Capillary Reader (IEC 2201, Damon, USA)
- Eppendoff Centrifuge (Spectrafuge C0160-B-230, Labnet International, Edison, NJ, USA)
- Nikon E600 light microscope and Nikon DXM 1200 digital camera (Tokyo, Japan)
- Tissue grinder (Sigma CD-1, Germany)
- Magnetic stirrer (Lab-Line Pyro Magnestir, Melrose Park, ILL # 1267)
- Embedding machine (Axel Johnson Lab system, U.S.A)
- Microtome (Histo STAT, Reichert, U.S.A).

4.2 Materials

- Syringe and hypodermic needle (Nipro, Thailand)
- Transfermicropette (TreffLab, Switzerland)
- Hematocrit tubes (Vitrex 0705427, Herlev, Denmark)
- Neubauer hemacytometer (Precicolor, HBG,Germany)
- Red and White cell pipettes
- 0.45 µm Syringe filter 25 mm GD/X (Whatman, NJ, USA)
- Screen 100 mesh (Sigma S3895, Germany)

4.3 Chemicals

- 0.85% NaCl (Merck, Germany).

- Ethyl 3-aminobenzoate methanesulfonate salt (MS 222, Anesthetizing drug, Sigma 886-86-2, Germany)
- Phosphate Buffer Saline (PBS, Sigma 1000-3, Germany)
- Fetal calf serum (Gibco 2011-09, USA)
- Nitroblue tetrazolium (NBT, Sigma N6876, Germay)
- *Micrococcus lysodeikticus* (Sigma M3770, Germany)
- Leibovitz15 medium (L-15, Sigma L4386, Germany)
- Heparin 5,000 IU/mL (Leo, Denmark)
- Wright Instant Stain Set (BML lab, Thailand)
- Natt and Herrick diluting solution
- Drabkin's cyanmethemoglobin (Hemasol) solution
- Distilled Water

4.4 Animal model

This study was performed at the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand. The fish, *O. niloticus and P. altus* were bought from the commercial fish farm in Rachaburi Province, Thailand. Fishes with similar size were chosen for the experiment to enhance accuracy. The average body weight of the fishes was 40-45 g and the average length was 10-15 cm. All the fishes were acclimatized under laboratory conditions for 30 days before performing the experiment. The fish were watched carefully for signs of diseases, stress, physical damage or mortality. If the mortality rate of the fish exceeded 10%, the entire stock was discarded. Fish were fed twice a day with 28%-protein, 3%-fat, and 4%-fibre commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was given based upon the initial weight of the fish that was 2% of the initial body weight per day.

4.5 Water conditions

The fishes were kept in the glass flow through aquaria (50x50x120 cm) with continuous aeration filled with 200L of dechlorinated tap water. According to the experimental procedures described in *Standard Methods for the Examination of Water and Wastewater,* the physicochemical characteristics of water are measured daily

(APHA, 2005). The pH was measured with a Hanna microprocessor pH/mV/^{o} C meter model 8417 and the temperature was measured with a glass mercury thermometer. Dissolved oxygen was supplied by a diffused air system. Furthermore, a 16:8 hour light-dark cycle was maintained throughout.

4.6Preparation of the aqueous leaves extract

Fresh leaves (*P. guajava, P. granatum* and *T. laurifolia)* were and washed several times in running water, air dried for 1 h and cut into small fragments. The extraction was done by following the method of Winkaler et al. (2007) with slight modifications. The leaves were dried in an incubator at 45^{o}C for 72 hours. The dried leaves were then be reduced to coarse powder with mortar and pestle and stored in an airtight desiccator prior to the experiment. The powdered leaves were then dissolved in distilled water. Heat can be added to catalyze the action. The solution was then filtered through a 0.45 μm membrane filter.

4.7 Preparation of dry leaf with fish food

Fresh leaves were washed several times in water, dried in incubator at 45^{o}C for 72 hours and made semi powder by crushing using a mortar and pestle same as the above following.

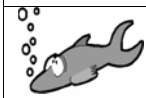
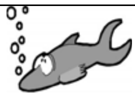
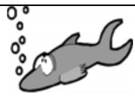
Fish food was grounded in a blender and hydrated with 0.7 mL/g distilled water, mixed with the leaf semi powder extract and extruded through a pasta maker or minced-meat processing machine. Later, the mixture was broken into small pellets by hands and air dried at 60^{o}C in hot air oven for 48 hours. The fishes were fed twice a day (2% body weight), with the prepared dry leaf food supplementation within 2 months.

4.8 Experimental design I

The fish (n=30) were separated into 3 groups (Table 1), control group, low concentration group, and high concentration group. The fish were then injected at the intraperitoneal area with 0.5 ml of the aqueous leaf extraction with the concentration of either 200 or 600 mg/kg body weight using a 1-ml syringe with 26-gauge needle, respectively according to their experimental group. The selection of test concentrations is based on the working immunostimulants concentration to determine the effect on fish with a broad margin of safety.

Forty-eight hours and seven days after intraperitoneal injection of aqueous leaf extraction, the fish were not fed for 24 hours prior to dissection. Five fish from each group were anesthetized with 0.2 g/L MS-222, the total length, body length and body weight were measured.

Table 1. Summarize in experimental design I.

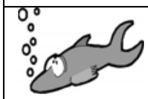
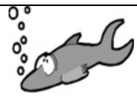
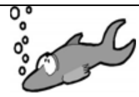
	Group 1	**Group 2**	**Group 3**
			
Number of fish	Fish n= 10	Fish n= 10	Fish n= 10
Concentration of aqueous leaves extract	0 mg/kg BW	200 mg/kg BW	600 mg/kg BW
48 hours after IP	Fish n= 5	Fish n= 5	Fish n= 5
	Specimen collection		
7 days after IP	Fish n= 5	Fish n= 5	Fish n= 5
	Specimen collection		

4.9 Experimental design II

Thirty fish were randomly assigned to three equally sized groups containing 10 fish each (Table 2). Group I; the control group obtains food, which was not mixed with dry leaf. Group II; the low concentration exposure obtains leaf extract 20 mg/g fish food. Group III; the high concentration exposure obtains leaf extract 60 mg/g fish food.

After 1 and 2 months, 5 fish from each group was taken and anesthetized with 0.2 g/L MS-222, length and weight were measured.

Table 2. Summarize in experimental design II.

	Group 1	Group 2	Group 3
			
Number of fish	Fish n= 10	Fish n= 10	Fish n= 10
Concentration of dry leaf with fish food	0 mg/g fish food	20 mg/g fish food	60 mg/g fish food
1 month after feeding	Fish n= 5	Fish n= 5	Fish n= 5
	Specimen collection		
2 month after feeding	Fish n= 5	Fish n= 5	Fish n= 5
	Specimen collection		

4.10 Peritoneal lavage collection

A 23 gauge (G) needle with 3 mL phosphate buffer saline (PBS) and supplemented with 20 U/mL heparin was injected intra-peritoneal in the ventral midline midway between the pelvic and pectoral fins. After gently massaging the ventral surface of the fish for 10 min with a finger, the injected PBS is recovered by a syringe with 23 G needle.

4.11 Blood collection

Peripheral blood was collected by injecting a needle on the mid-ventral line behind the anal fin. The needle was pushed into the musculature until the spinal column is reached, and kept the steady vacuum on the syringe. The needle was slowly withdrawn until blood entered the syringe. The needle and syringe were slowly and carefully withdrawn completely from the fish. The syringe was gently rotated and the needle was removed. The contents of in the syringe were then emptied into an eppendoff tube.

4.12 Tissue collection

The operculum was opened and the gills were collected and rinsed in 0.85% NaCl. The body cavity was opened and the kidney was collected and weighted. The kidney index (KI) calculated and compared with those obtained for unexposed fish at the same time intervals. Same procedure was done with the liver after it was collected in order to calculate the hepato-somatic index (HSI).

$$\text{Kidney Index (KI)} = \frac{\text{Weight of Kidney}}{\text{Weight of Body Fish}} \times 100$$

$$\text{Hepato-Somatic Index (HSI)} = \frac{\text{Weight of liver}}{\text{Weight of Body Fish}} \times 100$$

4.13 Isolation of kidney macrophage

The methods for isolation of kidney macrophage were described by Secombes (1990) with modification by Crumlish et al. (2000). Suspensions of macrophages were prepared by teasing the kidney tissue through a sterile 100 μm wire mesh into a eppendoff tube containing 1 mL of Leibovitz-15 medium (L-15), 10 Units/mL of heparin and 0.1% (v/v) of fetal calf serum. Two slides were prepared for each fish with 100 μL of cell suspension from kidney or peritoneal lavage and incubated for 30 minutes in a Petri dish, after which the unattached cells like the erythrocytes and the lymphocytes were removed by washing the slides three times with sterile saline. Excess moisture was removed by tapping the slides onto a tissue. Adhering macrophages were fixed by incubating the slides for 30 seconds in the alcohol fixative from a Wright Instant Staining Set. The slides were immediately stained for 1 minute in the eosin-based and then the Giemsa-based dyes of Wright Instant Staining Set. The slides were washed twice with tap water to remove excess stain, and examined by Nikon E600 light microscope and photographed by a Nikon DXM 1200 digital camera.

4.14 Respiratory burst activity

Nitroblue tetrazolium was used, to determine the respiratory burst activity using the technique by Serezlü et al (2005). Briefly, 100 μL of cell suspension from kidney or peritoneal lavage of each fish was dropped on a glass cover slip that was placed on a moist paper towel in 60 mm diameter petri dishes and incubated for 30 min 25^{o}C. Thus neutrophils (including some monocytes and macrophages) are adhered to the glass. The excess cells are washed (including the RBC) with 0.067 mM PBS pH 6.4 and excess solution is drained off. The glass cover slip was turned upside down onto a drop (40 μL) of 0.2% NBT in PBS solution on a glass microscope slide. The slide was incubated for

30 minutes at 25^{o}C and was then observed under light microscope under 400 X magnification. The cells that took up the dark blue dye were counted in 10 microscope areas and are compared with untreated control samples (Serezlü et al., 2005; Jian and Wu, 2004).

4.15 Lysozyme activity test

The lysozyme activity assay was followed by the method described by Parry et al. (1965) with some modification by Jian and Wu (2004). In this turbidimetric assay, a suspension of lyophilized *Micrococcus lysodeikticus* was prepared by suspending 20 mg of *M. lysodeikticus* in 100 mL 0.067 mM PBS pH 6.4. Forty microlitres of fish serum was added to 3 mL suspension of *M. lysodeikticus* at 25$\pm$1^{o}C, and the absorbance at 540 nm was measured after 0.5 and 4.5 min. One unit of lysozyme activity is defined as the amount of lysozyme producing a decrease in absorbance of 0.001/min.

4.16. Micronuclei (MN) and nuclear abnormality (NA) studies: Giemsa staining

Peripheral blood samples is smeared immediately on clean grease free microscope slides, air dried for 12 h, and then fixed in absolute ethanol for 20 min. Each slide is stained with 5% Giemsa solution for 30 min. From each slide 1000 cells are scored under 1000× magnification using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera. The slides are scored by a single observer using blind review. Frequencies of micronucleated (MN) and nuclear abnormality (NA) cells are expressed per 1000 cells (%).

Only the cells clearly isolated from the surrounding cells are scored. The criteria for the identification of MN are earlier described: (a) MN must be smaller than one-third of the main nuclei, (b) MN must be clearly separated from the main nuclei, (c) MN must be on the same plane of focus, and have the same color. Cells having two nuclei with approximately equal sizes are considered as binucleates (Fenech et al., 2003). Nuclear abnormality shapes are scored into one of the following categories: blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT), and binuclei (BN) (Carrasco et al., 1990). The result is expressed as the mean value (%) of the sum for all the individual abnormality observed.

4.17 Hematological studies

This study basically deals with the study of the alteration of blood cells and this is further divided into 5 possible tests for checking the blood cell alterations (Dacie and Lewis, 1991).

4.17.1 Hematocrit

Hematocrit was determined by spinning the blood sample contained in heparinized capillary tubes in a microhematocrit centrifuge.

- A capillary hematocrit tube about 7 cm long that was coated on the inside with anticoagulant was used (Na-Heparin).
- The microhematocrit tube was filled by capillary action. The tube should be filled to at least 5 cm. The empty end was sealed with modeling clay.
- The tube was centrifuged in microhematocrit centrifuge machine for 5-10 minutes at 10000 to 12000 g.
- Hematocrit level was measured with a hematocrit reader or calculated by the hematocrit equation which is:

$$\text{Hematocrit (\%)} = \frac{\text{Packed red cell X 100}}{\text{Whole blood}}$$

4.17.2 Hemoglobin

Hemoglobin was estimated by Drabkin's cyanmethemoglobin method. Drabkin's solution containing potassium ferricyanide, potassium cyanide and sodium bicarbonate was prepared. Twenty µL of blood was added to 5 mL of Drabkin's solution. Optical density is measured at 540 nm in a spectrophotometer. Hemoglobin values were calculated from a hemoglobin curve prepared using hemoglobin standard.

4.17.3 Red and white blood cell count

Unlike mammalian cells fish erythrocytes and thrombocytes are nucleated. Therefore, we cannot use the technique of counting blood cells like we used for mammalian blood cells. Furthermore, fish’s blood clots very quickly and the number of erythrocytes and leukocytes varies in different species of fish. Two mL of Natt and Herrick diluting solution (Natt and Herrick, 1952) and 10 µL of blood were taken and dropped onto the hemocytometer at the edge of the coverslip. The suspension was allowed to flow under the cover slip by capillary action into the counting chamber (Henry, 2001).

4.17.4 Red cell indices

The blood indexes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from hematocrit, haemoglobin and RBC.

4.17.4.1 Mean corpuscular volume (MCV): The mean volume of the red cell in terms of cubic microns may be calculated from the formula:

$$MCV = \frac{\text{Hematocrit (volume of packed red cells \%) X 10}}{\text{RBC in millions/cu.mm.}}$$

4.17.4.2 Mean corpuscular hemoglobin (MCH): The average quantity of hemoglobin in each RBC expressed in micrograms may be calculated from the formula:

$$MCH = \frac{\text{gm. Hemoglobin/100 ml. X 10}}{\text{RBC in millions/cu. mm.}}$$

4.17.4.3 Mean corpuscular hemoglobin concentration (MCHC): A more commonly used formula is that of the calculation of the concentration of hemoglobin in the average red cell. This value is derived from the following formula and is expressed in percent.

$$MCHC = \frac{\text{gm. Hemoglobin/100 ml. X 100}}{\text{Hematocrit (\%)}}$$

4.18. Biochemical studies

Serum was determined for the enzyme activity as follows:

4.18.1 Transaminase (Reitman & Frankel, 1957)

A. Aspartate aminotransferase (AST): the enzymatic activity of AST was measured by the oxaloacetate formed in the reaction:

$$\text{alpha-ketoglutarate + L-aspartate} \xrightarrow{\text{AST}} \text{glutamate + oxaloacetate}$$

Oxaloacetate was measured colorimetrically as its hydrazone after the reaction with 2,4 – dinitrophenylhydrazine at 505nm.The procedures for the analysis of glutamic oxaloacetic transaminase activity were as follows:

1. Aspartate transaminase substrate 0.25mL were pipetted into a 12 x 75mm cuvette and warmed for 5min in a water bath at 37°C.

2. Serum 0.05mL was added, mixed and covered with parafilm.

3. The mixture was incubated at 37°C for 60min.

4. Color reagent 0.25mL was added and shaken gently.

5. The tube was allowed to stand at room temperature for 20min.

6. The 2.5mL of 0.4mol/L NaOH was added and mixed.

7. OD of the unknown was read with water as a blank at 505nm.

8. The optical density for the value of oxaloacetic transaminase was referred to the calibration curve.

B. Alanine aminotransferase (ALT): the enzymatic activity of ALT was measured by the pyruvate produced in the reaction:

$$\text{alpha-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{glutamate} + \text{pyruvate}$$

Pyruvate formed was measured colorimetrically as its hydrazone after the reaction with 2, 4-dinitrophenylhydrazine at 505nm. The procedures for the analysis of glutamic pyruvic transaminase activity were performed similar to those of oxaloacetic transaminase, substituting with the alanine transaminase substrate, and incubated at 37°C for 30min.

The procedures for calibration curve were as follows:

1. The solution was pipetted into cuvettes as indicated in Table 1:

Table 3. The calibration curve of AST and ALT.

Tube No.	Calibration standard	Aspartate transaminase substrate (mL)	Water (mL)	Sigma-Frankel units	
				Aspartate substrate	Alanine substrate
1	0.0	1.0	0.2	0	0
2	0.1	0.9	0.2	20	23
3	0.2	0.8	0.2	55	50
4	0.3	0.7	0.2	95	83
5	0.4	0.6	0.2	148	125
6	0.5	0.5	0.2	216	-

2. Color reagent 1mL was added to all cuvettes and mixed. The tube was allowed to stand for 20min at room temperature. Ten mL of 0.4mol/L NaOH was added to all cuvettes and mixed.

3. Exactly 5min after adding NaOH, the optical density of all samples in 12 x 75mm cuvettes was read and recorded, using water as a blank at 505nm.
4. A calibration curve of optical density versus the corresponding units of transminase was plotted.

4.18.2 Alkaline phosphatase (McComb & Bowers, 1972)

It is the enzyme that hydrolyzes organic phosphates such as phenolphthalein monophosphate to phenolphthalein with yellow color under an alkaline condition. The procedures for the analysis of this enzyme activity are indicated in Table 2.

Table 4.The procedures for alkaline phosphatase analysis.

	Sample (mL)	Blank (mL)
Substrate-Phenolphthaline monophosphate Dist. water	1 drop 1.0	1 drop 1.0
Incubate at 37°C 5 min		
Serum	0.1	-
Incubate at 37°C 20 min		
Buffer solution (Na_3PO_4 + NaH_2PO_4)	5.0	5.0

The optical density was read at 550nm, and the value was read from calibration curve. The procedures for calibration curve: standard phenolphthalein concentrations of 25, 50, 75, and 100IU were used (by using standard 1.0mL + buffer 5.14 mL). The optical density was read at 550nm with water as a blank. A calibration curve of optical density and concentration was plotted. The curve was a straight line.

4.19 Light microscopic study

Paraffin technique was follow by Humason (1972) with modification as shown in Table 5. Fish organs were preserved in 10% formalin. To begin the process the organs were placed in small metal caskets and stirred by a magnetic stirrer. The organs were then dehydrated using alcohol and embedded in paraffin so that the original tissue morphology was preserved and tissue is strengthened for sectioning. Following this the organs were sectioned using a rotary ultra microtome in order to make slides. The slides were then stained with the use of different concentrations of xylene and alcohol as well as hematoxylin and eosin (Table 6). These slides were observed under the light microscope for analysis, provided with a digital camera.

Histopathological alteration was assessed using a score ranging from – to + + + depending on the degree and extent of the alteration: (-) none, (+) mild occurrence, (+ +) moderate occurrence, (+ + +) severe occurrence. Ten slides were observed from each organ and treatment.

Table 5. Schedule for histopathological process.

No	Method	Duration
1	Dissection	
2	10% buffered formaldehyde	24 h
3	70% alcohol	24 h
4	80% alcohol	1 h
5	95% alcohol	1 h
6	95% alcohol	1 h
7	Absolute alcohol	1 h
8	Absolute alcohol	1 h
9	Xylene	1 h
10	Xylene	1 h
11	Paraffin	1 h
12	Paraffin	1 h
13	Embedding	
14	Sectioning	24 h
15	Staining	
16	Examination	

Table 6. Schedule for staining section.

No	Method	Duration
1	Xylene (I)	5 min
2	Xylene (II)	5 min
3	Xylene (III)	5 min
4	Absolute alcohol (I)	3 min
5	Absolute alcohol (II)	3 min
6	Absolute alcohol (III)	3 min
7	95% alcohol	3 min
8	80% alcohol	3 min
9	70% alcohol	3 min
10	Running water	7 min
11	Hematoxylin	7 min
12	Running water	7 min
13	Eosin	3 min
14	95% alcohol (I)	3 min
15	95% alcohol (II)	3 min
16	95% alcohol (III)	3 min
17	Absolute alcohol (I)	3 min
18	Absolute alcohol (II)	3 min
19	Absolute alcohol (III)	3 min
20	Xylene (I)	5 min
21	Xylene (II)	5 min
22	Xylene (III)	5 min
23	Mounting	
24	Examination	

4.20 Scanning electron microscopic study

The procedures for scanning electron microscopy are performed following by the protocol routinely practice in the Center of Nanoimaging, Faculty of Science, Mahidol University. Small pieces of tissue were fixed in 4% glutaraldehyde-phosphate buffer (0.1mol/L, pH 7.4) at 4°C for 24h and postfixed in 1% osmium tetroxide for 1h. They were dehydrated through a graded series of alcohol, dried in a Hitachi HCP-2 critical point dryer machine using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminium stubs and coated with platinum and paladium in an ion-sputtering apparatus, Hitachi E-102, at 10-15mA for 6min. They were examined and photographed in a Hitachi scanning electron microscope S-2500, operating at 15kV (Humason, 1972).

4.21 Statistical analysis

All data were expressed as mean values $\pm$ S.D. An analysis of variance was performed separately for each time, and separately tested in each group. Tukey post hoc test for multiple comparisons was used for determination of significant differences between the control and treated groups. The level of statistical significance is set at the probability level of 0.05.

CHAPTER V
RESULTS AND DISCUSSION

5.1 Characteristics of fish

No mortality was detected during the acclimatization. The characteristics of each fish in the experiment are presented in Table 7.

Table 7. The characteristics of each fish used in the experiment (n=45, mean $\pm$ S.D.).

Fish	Body weight (g)	Total length (cm)
O. niloticus	18.89 $\pm$ 0.81	8.98 $\pm$ 0.40
P. altus	18.46 $\pm$ 0.92	8.54 $\pm$ 0.48

5.2 Characteristics of water

The quality of water in the experiment was constant and within a normal requirement of freshwater species. All water quality parameters among treatments were in the same range throughout the tests. They are presented in Table 8.

Table 8. The physical and chemical characteristics of water used in the experiment.

Parameters	**Range**
pH	6.8-7.3
Total hardness	50-70 mg/L as $CaCO_3$
Alkalinity	62-65 mg/L
Conductivity	185-210 µmhos/cm
Temperature	25-27 ^{o}C

5.4 Total length and body weight

The growth parameter i.e., total length and body weight after 1 and 2 month treatment as shown in Table 9-12.

Table 9. Total length (cm) of *O. niloticus* after 1m and 2m in each treatment (n=5, mean ± S.D.).

Total Length (cm)									
Concentration	*O. niloticus*								
	P. guajava			*P. granatum*			*T. laurifolia*		
	0 m	1 m	2 m	0 m	1 m	2 m	0 m	1 m	2 m
0 mg/g	8.94 ±0.49	10.82 ±0.48	11.04 ±0.34	8.94 ±0.51	10.66 ±0.51	11.32 ±0.77	8.86 ±0.27	11.22 ±0.37	11.38 ±0.97
20 mg/g	8.86 ±0.42	10.68 ±0.42	11.26 ±0.50	9.06 ±0.38	11.16 ±0.68	11.32 ±0.77	9.16 ±0.57	11.26 ±0.82	11.74 ±0.96
60 mg/g	8.82 ±0.34	10.94 ±0.49	11.78 ±0.40	9.10 ±0.35	11.48 ±0.83	11.14 ±0.42	9.08 ±0.41	11.56 ±0.76	12.18 ±0.47

Table 10. Total length (cm) of *P.altus* after 1m and 2m in each treatment (n=5, mean ± S.D.).

Total Length (cm)									
Concentration	*P. altus*								
	P. guajava			*P. granatum*			*T. laurifolia*		
	0 m	1 m	2 m	0 m	1 m	2 m	0 m	1 m	2 m
0 mg/g	8.62 ±0.42	10.44 ±0.57	10.82 ±0.53	8.82 ±0.29	10.40 ±0.60	10.94 ±0.27	8.64 ±0.18	10.98 ±0.22	11.18 ±1.10
20 mg/g	8.46 ±0.39	10.12 ±0.53	10.92 ±0.33	8.34 ±0.61	10.72 ±0.72	10.92 ±0.75	8.50 ±0.70	10.56 ±0.50	11.36 ±0.97
60 mg/g	8.60 ±0.39	10.12 ±0.53	10.92 ±0.33	8.34 ±0.61	10.72 ±0.72	10.92 ±0.75	8.50 ±0.70	10.56 ±0.50	11.36 ±0.97

Table 11. Body weight (g) of *O. niloticus* after 1m and 2m in each treatment (n=5, mean ± S.D.).

Body Weight (g)									
Concentration	*O. niloticus*								
	P. guajava			*P. granatum*			*T. laurifolia*		
	0 m	1 m	2 m	0 m	1 m	2 m	0 m	1 m	2 m
0 mg/g	19.06 ±1.17	24.23 ±0.53	31.26 ±2.43	18.67 ±0.77	24.44 ±0.76	30.59 ±1.91	18.77 ±0.82	25.34 ±0.99	31.03 ±2.33
20 mg/g	18.96 ±0.38	25.21 ±2.27	31.54 ±2.04	19.19 ±1.18	26.52 ±2.58	31.09 ±1.95	19.00 ±0.41	27.01 ±1.01	32.40 ±1.71
60 mg/g	18.86 ±0.79	24.51 ±1.37	31.74 ±1.93	19.19 ±0.94	24.38 ±1.35	28.40 ±1.75	18.29 ±0.78	26.77 ±0.73	34.42 ±0.88

Table 12. Body weight (g) of *P.altus* after 1m and 2m in each treatment (n=5, mean ± S.D.).

Body Weight (g)									
Concentration	*P. altus*								
	P. guajava			*P. granatum*			*T. laurifolia*		
	0 m	1 m	2 m	0 m	1 m	2 m	0 m	1 m	2 m
0 mg/g	18.61 ±1.53	23.69 ±0.76	30.53 ±2.15	18.84 ±0.47	23.00 ±0.40	28.99 ±2.12	18.23 ±0.64	24.34 ±1.69	30.21 ±3.62
20 mg/g	17.93 ±1.16	24.61 ±1.73	31.14 ±1.67	18.46 ±1.05	24.83 ±0.45	31.30 ±2.37	18.67 ±0.85	26.04 ±1.32	32.25 ±2.16
60 mg/g	18.23 ±0.77	24.22 ±1.50	30.44 ±1.80	18.46 ±1.05	23.42 ±1.44	29.00 ±2.37	18.67 ±0.85	26.81 ±0.77	33.38 ±2.16

5.5 Isolation of kidney macrophage

The result of macrophage in experiment I and II are shown in Table 13-18. Macrophages are phagocytic cells that play an important role in both specific and non-specific immune responses. These cells are capable of killing a wide range of pathogens by engulfing them and producing reactive superoxide species. These functions are indicators of the non-specific immune response and the general health of the organism, and can be measured by the visible presence of engulfed bacteria and respiratory burst activity. Assays for the measurement of these macrophage functions are widely used for evaluating immune responses in many vertebrates as well as in fish (Secombes, 1990). In such studies macrophage cells in fish are obtained and isolated from the head kidney, maintained in cell culture, and then checked for the amount of reactive superoxide species produced during the respiratory burst activity and the number of phagocytized bacteria (Mustafa et al., 2008).

Table 13. Macrophage (cells/HPF) after 48h and 7d in each treatment (n=5, mean ± S.D.).

Macrophage (cells/HPF)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	2.80 ±0.84	3.00 ±1.22	4.20 ±2.28	3.60 ±1.95	3.00 ±1.22	4.40 ±1.95	4.20 ±2.17	3.00 ±1.22	2.80 ±1.64	2.60 ±1.52	2.40 ±1.14	2.80 ±1.48
200 mg/kg BW	3.00 ±2.35	2.80 ±1.48	3.40 ±2.07	3.60 ±2.70	3.00 ±2.35	3.40 ±2.07	2.60 ±1.34	2.60 ±1.14	3.20 ±2.28	3.80 ±2.17	2.80 ±2.49	2.80 ±1.79
600 mg/kg BW	3.20 ±1.79	3.00 ±1.87	3.20 ±1.79	3.80 ±2.39	2.20 ±1.64	2.80 ±2.95	3.40 ±2.41	3.80 ±2.95	3.40 ±2.61	3.20 ±2.68	3.80 ±3.56	3.40 ±2.88

Table 14. Macrophage (cells/HPF) after 1m and 2m in each treatment (n=5, mean ± S.D.).

Macrophage (cells/HPF)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	2.20 ±1.64	3.00 ±1.58	2.60 ±1.34	2.20 ±0.84	3.00 ±1.87	2.80 ±1.30	3.00 ±1.00	3.40 ±1.52	3.40 ±1.52	3.60 ±1.67	2.40 ±1.34	2.80 ±1.30
20 mg/g	4.00 ±2.55	4.20 ±2.59	4.40 ±2.88	3.80 ±3.83	3.20 ±2.49	3.60 ±1.14	3.40 ±1.52	2.60 ±1.52	3.20 ±2.28	4.40 ±2.07	3.80 ±2.77	3.40 ±2.30
60 mg/g	3.00 ±2.35	2.20 ±1.64	2.00 ±0.71	2.80 ±1.30	3.20 ±2.77	3.80 ±3.11	4.40 ±1.14	3.00 ±1.58	2.80 ±1.64	3.80 ±2.59	3.00 ±2.55	2.00 ±1.22

Table 15. Multiple comparisons in macrophage between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MACROPHA
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	.1500	.38394	.919	-.7592	1.0592
	600 mg/kg	-.0333	.38394	.996	-.9426	.8759
200 mg/kg	0 mg/kg	-.1500	.38394	.919	-1.0592	.7592
	600 mg/kg	-.1833	.38394	.882	-1.0926	.7259
600 mg/kg	0 mg/kg	.0333	.38394	.996	-.8759	.9426
	200 mg/kg	.1833	.38394	.882	-.7259	1.0926

Based on observed means.

Table 16. Multiple comparisons in macrophage between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MACROPHA
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-.2833	.38394	.741	-1.1926	.6259
	T. laurifolia	.0500	.38394	.991	-.8592	.9592
P. granatum	P. guajava	.2833	.38394	.741	-.6259	1.1926
	T. laurifolia	.3333	.38394	.661	-.5759	1.2426
T. laurifolia	P. guajava	-.0500	.38394	.991	-.9592	.8592
	P. granatum	-.3333	.38394	.661	-1.2426	.5759

Based on observed means.

Table 17. Multiple comparisons in macrophage between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MACROPHA
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/g	20 mg/g	-.8000	.36591	.077	-1.6665	.0665
	60 mg/g	-.1333	.36591	.929	-.9999	.7332
20 mg/g	0 mg/g	.8000	.36591	.077	-.0665	1.6665
	60 mg/g	.6667	.36591	.166	-.1999	1.5332
60 mg/g	0 mg/g	.1333	.36591	.929	-.7332	.9999
	20 mg/g	-.6667	.36591	.166	-1.5332	.1999

Based on observed means.

Table 18. Multiple comparisons in macrophage between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MACROPHA
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-.0500	.36591	.990	-.9165	.8165
	T. laurifolia	.1167	.36591	.946	-.7499	.9832
P. granatum	P. guajava	.0500	.36591	.990	-.8165	.9165
	T. laurifolia	.1667	.36591	.892	-.6999	1.0332
T. laurifolia	P. guajava	-.1167	.36591	.946	-.9832	.7499
	P. granatum	-.1667	.36591	.892	-1.0332	.6999

Based on observed means.

Crumlish et al (2000) had developed the sampling method for the isolation of head kidney macrophages from fish at the pond-side. Cells from this modified method were clearly visible after staining, with little cell lysis occurring.

5.6 Respiratory burst activity (NBT)

The result of NBT in experiment I and II are shown in Table 19-24. As emphasized recently by Lunden et al. (2002) there are 2 main phagocytes, namely mononuclear phagocytes (tissue macrophages and circulating monocytes) and polymorph nuclear phagocytes (mainly neutrophils). These are multifunctional non-specific cells in the immune system of fish and other vertebrates. Macrophages are considered the main phagocytic cells in fish and they are also the dominant phagocytes in the head kidney. The polymorph nuclear lecocytes, mainly neutrophils, are the main phagocytic cells in blood (Serezli et al., 2005). Phagocytosis is defined as the ingestion of particles by cells, and this process involves the binding of particles to the surface of phagocytic cells followed by the internalization and destruction of these particles. The nitroblue tetrazolium assay is the most useful test for the rapid determination of active neutrophils. Staining the neutrophils with the NBT dye helps to confirm their activity. The soluble NBT dye, taken in by pinocytosis into the neutrophils, is reduced to dark blue formazan granules that are distinctive on microscopic examination. NBT (+)

neutrophils were observed. These cells were round and multilobar and the granules were stained blue-green (Anderson, 1992; Anderson et al., 1992).

Table 19. NBT (cells/HPF) after 48h and 7d in each treatment (n=5, mean ± S.D.).

NBT (cells/HPF)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	4.20 ±1.92	6.20 ±2.77	4.80 ±1.92	5.60 ±2.61	5.80 ±3.56	5.80 ±2.95	6.60 ±2.30	6.60 ±3.05	5.20 ±2.59	6.60 ±2.51	3.60 ±2.07	5.40 ±2.61
200 mg/kg BW	6.80 ±1.64	7.80 ±2.28	7.00 ±1.41	7.80 ±1.30	9.60 ±2.70	8.60 ±3.21	6.20 ±2.05	6.40 ±2.70	7.20 ±2.17	6.60 ±1.82	7.60 ±1.95*	6.00 ±2.83
600 mg/kg BW	7.40 ±2.30	6.80 ±1.79	7.20 ±2.05	8.60 ±3.36	10.40 ±3.85	10.80 ±2.39*	7.80 ±2.49	8.00 ±1.00	8.60 ±2.97	7.40 ±2.70	8.60 ±1.82*	7.80 ±2.17

Table 20. NBT (cells/HPF) after 1m and 2m in each treatment (n=5, mean ± S.D.).

NBT (cells/HPF)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	4.80 ±0.84	4.60 ±1.14	3.40 ±1.34	2.60 ±1.82	4.60 ±0.55	3.60 ±1.14	4.40 ±1.14	4.20 ±0.84	4.40 ±1.14	4.80 ±1.48	4.00 ±1.00	3.60 ±1.14
20 mg/g	6.80 ±1.92	6.40 ±1.14	7.60 ±2.07*	9.60 ±1.82*	7.40 ±2.30*	9.20 ±1.79*	5.20 ±0.84	5.20 ±2.17	5.20 ±1.30	5.60 ±1.14	7.20 ±2.95	6.40 ±2.07
60 mg/g	6.80 ±1.64	7.60 ±2.07*	8.40 ±1.67*	8.00 ±2.35*	10.00 ±1.58*	9.80 ±0.84*	6.80 ±1.92	6.40 ±1.82	6.40 ±2.41	6.40 ±1.52	6.80 ±1.79	6.80 ±1.64*

Table 21. Multiple comparisons in NBT between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: NBT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	-1.7667*	.44959	.000	-2.8314	-.7020
	600 mg/kg	-2.7500*	.44959	.000	-3.8147	-1.6853
200 mg/kg	0 mg/kg	1.7667*	.44959	.000	.7020	2.8314
	600 mg/kg	-.9833	.44959	.077	-2.0480	.0814
600 mg/kg	0 mg/kg	2.7500*	.44959	.000	1.6853	3.8147
	200 mg/kg	.9833	.44959	.077	-.0814	2.0480

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 22. Multiple comparisons in NBT between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: NBT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-.1500	.44959	.941	-1.2147	.9147
	T. laurifolia	-.7667	.44959	.207	-1.8314	.2980
P. granatum	P. guajava	.1500	.44959	.941	-.9147	1.2147
	T. laurifolia	-.6167	.44959	.358	-1.6814	.4480
T. laurifolia	P. guajava	.7667	.44959	.207	-.2980	1.8314
	P. granatum	.6167	.44959	.358	-.4480	1.6814

Based on observed means.

Table 23. Multiple comparisons in NBT between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: NBT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	-2.7167*	.30031	.000	-3.4279	-2.0055
	60 mg/g	-3.4167*	.30031	.000	-4.1279	-2.7055
20 mg/g	0 mg/g	2.7167*	.30031	.000	2.0055	3.4279
	60 mg/g	-.7000	.30031	.055	-1.4112	.0112
60 mg/g	0 mg/g	3.4167*	.30031	.000	2.7055	4.1279
	20 mg/g	.7000	.30031	.055	-.0112	1.4112

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 24. Multiple comparisons in NBT between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: NBT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-.2833	.30031	.614	-.9945	.4279
	T. laurifolia	-.8500*	.30031	.015	-1.5612	-.1388
P. granatum	P. guajava	.2833	.30031	.614	-.4279	.9945
	T. laurifolia	-.5667	.30031	.146	-1.2779	.1445
T. laurifolia	P. guajava	.8500*	.30031	.015	.1388	1.5612
	P. granatum	.5667	.30031	.146	-.1445	1.2779

Based on observed means.

*. The mean difference is significant at the .05 level.

The respiratory burst activity can be quantified by the Nitroblue Tetazolium assay, which measures by leukocytes. Herbal based immunostimulants can enhance the repiratory burst activity of fish phagocytes. For instance, Rao et al. (2006) reported that superoxide anion production by the blood leucocytes was enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed. Ardo et al. (2008) also reported that feeding Nile tilapia (*O. niloticus*) with two herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocitic cells. Similarly, the plant extracts we used in this study could enhance respiratory burst activity in treatment groups compared to control group. On the other hand, Anderson et al. (1992) reported that the number of NBT (+) cells in rainbow trout injected with levamisole maximize on day 2 after injection, being significant higher than that in the control group. Chen et al (1998) reported that the number of NBT (+) cells observed in the swim bladder of Nile tilapia injected intraperitoneally with extracellular products of *Mycobacterium* spp. had markedly increased on day 4 after injection. These data indicate that the efficacious time of immunostimulant being quickly absorbed and functional in fish, while the oral administration enables the immunostimulant to be slowly absorbed. However, oral administration is a more practical method for fish farming (Jian and Wu, 2004). Additional, the recommended administration time of the traditional Chinese medicine as immunostimulant is considered to be 20 days as a course of treatment (Jian and Wu, 2004). Yoshida et al. (1995) demonstrated that the number of NBT (+) cells in African catfish increased following oral administration of glucan or oligosaccharide over 30 days, but not over 45 days. Although the reasons for these decreases in immune responses in fish by long-term oral administration of immunostimulants are still unknown, negative feedback systems against immunostimulation may function in fish, and the immune response may revert to a previous state. Thus, the effective administration period should be investigated for each immunostimulant (Sakai, 1999).

5.7 Lysozyme activity test

The result of lysozyme activity in experiment I and II are shown in Table 25-30. Lysozyme is an important component in the immune system of fish. It is bactericidal by hydrolyzing β (1 $\rightarrow$ 4) linkages of bacterial cell wall peptidoglycans resulting in bacteriolysis. It is also known to act as opsonin and activate the complement system and phagocytes (Magnadottir, 2006).

Table 25. Lysozyme activity (U/mL) after 48h and 7d in each treatment (n=5, mean ± S.D.).

Lysozyme activity (U/mL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	82.58 ±10.14	79.64 ±12.83	86.06 ±12.43	84.18 ±10.22	94.98 ±9.50	88.38 ±8.76	81.76 ±8.06	85.18 ±11.93	92.44 ±13.93	86.82 ±14.56	91.52 ±13.08	90.32 ±11.69
200 mg/kg BW	104.12 ±10.82*	95.00 ±6.40*	82.76 ±7.89	93.92 ±11.38	88.38 ±10.26	89.88 ±6.85	98.46 ±8.73*	93.84 ±8.68	99.20 ±10.51	96.12 ±7.21	93.98 ±8.99	89.66 ±9.82
600 mg/kg BW	82.68 ±6.37#	88.94 ±5.06	86.80 ±4.18	85.28 9.22	91.28 ±4.60	91.52 ±9.44	83.92 ±10.51	81.66 ±9.21	81.20 ±5.28*	81.70 ±5.88	75.60 ±4.37*	80.54 ±10.60

Table 26. Lysozyme activity (U/mL) after 1m and 2m in each treatment (n=5, mean ± S.D.).

Lysozyme activity (U/mL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	80.30 ±5.98	82.74 ±10.70	82.18 ±11.94	84.80 ±13.84	79.20 ±5.05	77.68 ±9.22	71.44 ±5.51	76.54 ±17.71	70.92 ±7.59	79.90 ±17.50	75.90 ±17.15	77.68 ±16.62
20 mg/g	86.70 ±11.31	88.28 ±11.17	89.42 ±10.92	88.66 ±9.53	89.66 ±9.68	90.18 ±12.88	82.24 ±7.66	80.32 ±10.42	82.46 ±6.61	80.78 ±11.06	84.68 ±16.27	79.60 ±10.31
60 mg/g	89.08 ±8.39	91.24 ±8.43	88.96 ±8.48	91.36 ±7.01	97.82 ±8.43*	95.98 ±10.51	83.06 ±9.67	79.06 ±10.43	83.02 ±7.89	81.04 ±7.64	84.68 ±13.16	82.48 ±10.27

Table 27. Multiple comparisons in lysozyme activity between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: LYSOZYME
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	-6.7883*	1.74169	.000	-10.9130	-2.6637
	600 mg/kg	2.7283	1.74169	.264	-1.3963	6.8530
200 mg/kg	0 mg/kg	6.7883*	1.74169	.000	2.6637	10.9130
	600 mg/kg	9.5167*	1.74169	.000	5.3920	13.6413
600 mg/kg	0 mg/kg	-2.7283	1.74169	.264	-6.8530	1.3963
	200 mg/kg	-9.5167*	1.74169	.000	-13.6413	-5.3920

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 28. Multiple comparisons in lysozyme activity between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: LYSOZYME
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.1083	1.74169	.998	-4.0163	4.2330
	T. laurifolia	-.6883	1.74169	.918	-4.8130	3.4363
P. granatum	P. guajava	-.1083	1.74169	.998	-4.2330	4.0163
	T. laurifolia	-.7967	1.74169	.891	-4.9213	3.3280
T. laurifolia	P. guajava	.6883	1.74169	.918	-3.4363	4.8130
	P. granatum	.7967	1.74169	.891	-3.3280	4.9213

Based on observed means.

Table 29. Multiple comparisons in lysozyme activity between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: LYSOZYME
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	-6.9750*	2.00583	.002	-11.7252	-2.2248
	60 mg/g	-9.0417*	2.00583	.000	-13.7919	-4.2915
20 mg/g	0 mg/g	6.9750*	2.00583	.002	2.2248	11.7252
	60 mg/g	-2.0667	2.00583	.559	-6.8169	2.6835
60 mg/g	0 mg/g	9.0417*	2.00583	.000	4.2915	13.7919
	20 mg/g	2.0667	2.00583	.559	-2.6835	6.8169

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 30. Multiple comparisons in lysozyme activity between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: LYSOZYME
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-1.0417	2.00583	.862	-5.7919	3.7085
	T. laurifolia	-2.0450	2.00583	.566	-6.7952	2.7052
P. granatum	P. guajava	1.0417	2.00583	.862	-3.7085	5.7919
	T. laurifolia	-1.0033	2.00583	.871	-5.7535	3.7469
T. laurifolia	P. guajava	2.0450	2.00583	.566	-2.7052	6.7952
	P. granatum	1.0033	2.00583	.871	-3.7469	5.7535

Based on observed means.

In the present study, it was observed that the lysozyme activity was substantially enhanced on treatment with *T. laurifolia*. Similar results of elevated lysozyme activity was observed on Jian carp (Jian and Wu, 2004) after feeding with traditional Chinese medicine formulated from Astragalus root (*Radix astragalin seu* heydsari) and chinese

Angelica root (*Radix angelicae sinensis*) at a ratio 5:1 (w/w). *Labeo rohita* fed with 0.5% of *Achyranthes aspera* seed extracts for 4 weeks was shown to enhance lysozyme activity (Rao et al., 2006).

5.8 Micronuclei (MN) and nuclear abnormality (NA) studies: Giemsa staining

No any changes in blood morphology in all groups as shown in Fig.15. Normal erythrocyte approximately diameter 7 µm was contained mainly elliptical nuclei.

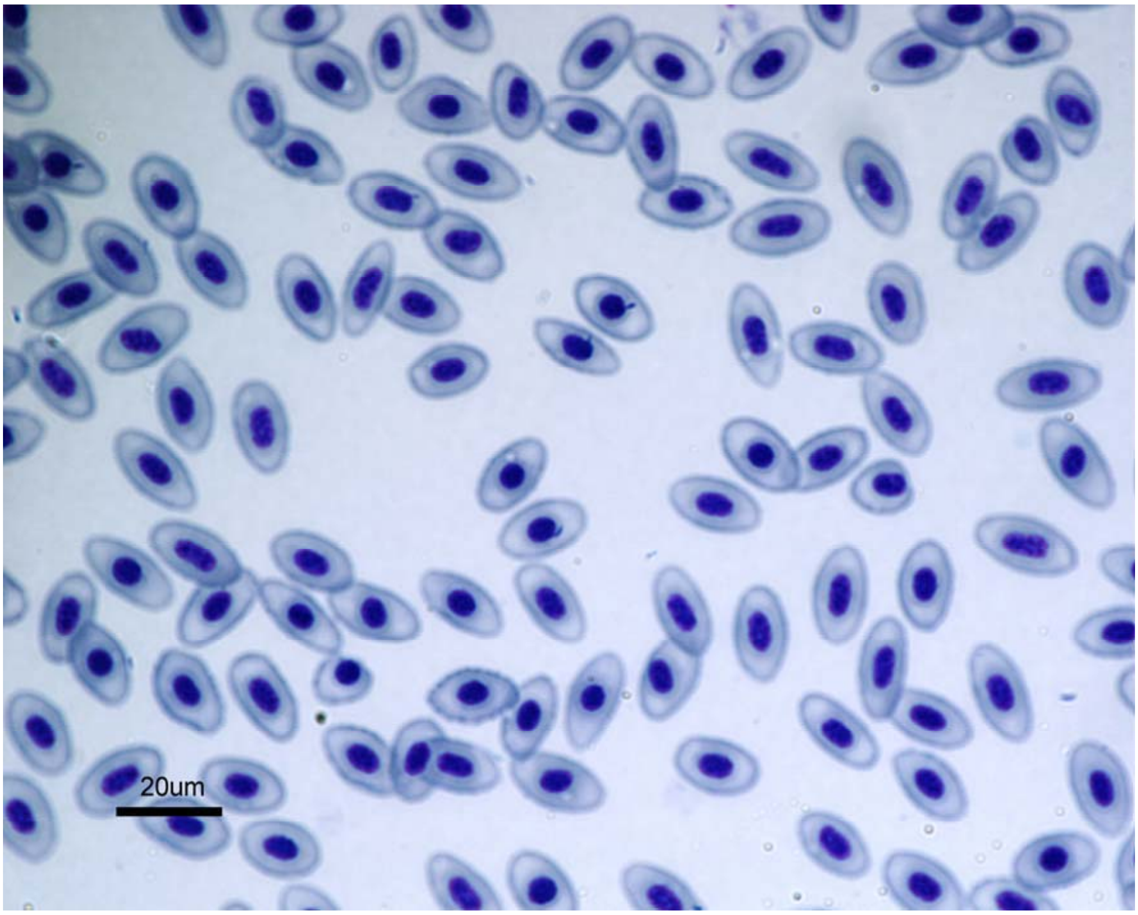


Figure 15. Fish blood smear showing normal appearance of erythrocytes.

5.9 Hematological studies

The study on fish hematology is important to known factors concerning its physiologic capacity, being also a helpful tool in the evaluation of fish immune system. Blood parameters i.e., hematocrit, hemoglobin and red cell indices indicate fish oxygen carrying capacity (Tavares-dias et al., 2008). Hematological analysis in the present study had shown in Table 31-72 and blood cell morphology as in Fig. 16.

5.9.1 Hematocrit

The result of hematocrit in experiment I and II are shown in Table 31-36.

Table 31. Hematocrit (%) after 48h and 7d in each treatment (n=5, mean ± S.D.).

Hematocrit (%)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	32.76 ±3.12	34.12 ±3.01	31.84 ±2.11	33.14 ±3.09	32.66 ±2.48	34.48 ±2.67	28.18 ±5.24	30.52 ±3.77	30.04 ±3.94	28.28 ±4.15	30.50 ±1.46	29.60 ±2.56
200 mg/kg BW	29.36 ±2.22	28.60 ±2.86	30.58 ±3.70	30.24 ±3.84	32.10 ±3.01	32.88 ±2.66	24.48 ±4.56	28.60 ±2.86	28.84 ±5.52	26.28 ±3.29	29.50 ±1.80	28.60 ±1.47
600 mg/kg BW	30.70 ±2.67	27.82 ±4.47*	26.60 ±3.08*	26.42 ±2.20*	30.12 ±3.09	30.74 ±3.57	22.62 ±2.43	25.80 ±1.89	26.60 ±3.08	22.38 ±5.19	28.10 ±2.19	28.80 ±3.15

Table 32. Hematocrit (%) after 1m and 2m in each treatment (n=5, mean ± S.D.).

Hematocrit (%)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	35.50 ±3.31	34.36 ±2.44	33.56 ±2.93	32.12 ±3.58	34.20 ±2.00	33.12 ±2.16	31.34 ±4.27	32.86 ±3.25	32.88 ±4.48	29.02 ±1.63	31.80 ±4.27	31.92 ±3.28
20 mg/g	32.98 ±1.07	28.10 ±1.34*	28.36 ±1.83*	28.38 ±2.39	33.90 ±1.54	32.98 ±3.25	29.88 ±4.22	28.24 ±2.39*	28.36 ±1.83	28.38 ±2.39	32.40 ±3.36	29.32 ±3.64
60 mg/g	31.04 ±4.15	27.24 ±2.28*	27.26 ±1.88*	26.86 ±1.84*	33.49 ±3.31	32.50 ±2.12	27.84 ±3.65	25.46 ±2.47*	27.26 ±1.88*	26.86 ±1.84	29.80 ±2.59	30.54 ±2.92

Table 33. Multiple comparisons in hematocrit between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: HCT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	2.1717*	.59847	.001	.7544	3.5890
	600 mg/kg	4.1183*	.59847	.000	2.7010	5.5356
200 mg/kg	0 mg/kg	-2.1717*	.59847	.001	-3.5890	-.7544
	600 mg/kg	1.9467*	.59847	.004	.5294	3.3640
600 mg/kg	0 mg/kg	-4.1183*	.59847	.000	-5.5356	-2.7010
	200 mg/kg	-1.9467*	.59847	.004	-3.3640	-.5294

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 34. Multiple comparisons in hematocrit between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: HCT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	.1933	.59847	.944	-1.2240	1.6106
	T. laurifolia	-2.0433*	.59847	.002	-3.4606	-.6260
P. granatum	P. guajava	-.1933	.59847	.944	-1.6106	1.2240
	T. laurifolia	-2.2367*	.59847	.001	-3.6540	-.8194
T. laurifolia	P. guajava	2.0433*	.59847	.002	.6260	3.4606
	P. granatum	2.2367*	.59847	.001	.8194	3.6540

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 35. Multiple comparisons in hematocrit between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: HCT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	2.6167*	.52331	.000	1.3774	3.8560
	60 mg/g	3.8778*	.52331	.000	2.6385	5.1171
20 mg/g	0 mg/g	-2.6167*	.52331	.000	-3.8560	-1.3774
	60 mg/g	1.2612*	.52331	.045	.0219	2.5005
60 mg/g	0 mg/g	-3.8778*	.52331	.000	-5.1171	-2.6385
	20 mg/g	-1.2612*	.52331	.045	-2.5005	-.0219

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 36. Multiple comparisons in hematocrit between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: HCT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	1.2950*	.52331	.038	.0557	2.5343
	T. laurifolia	-1.7605*	.52331	.003	-2.9998	-.5212
P. granatum	P. guajava	-1.2950*	.52331	.038	-2.5343	-.0557
	T. laurifolia	-3.0555*	.52331	.000	-4.2948	-1.8162
T. laurifolia	P. guajava	1.7605*	.52331	.003	.5212	2.9998
	P. granatum	3.0555*	.52331	.000	1.8162	4.2948

Based on observed means.

*. The mean difference is significant at the .05 level.

5.9.2 Hemoglobin

The result of hemoglobin in experiment I and II are shown in Table 37-42.

Table 37. Hemoglobin (mg/dL) after 48h and 7d in each treatment (n=5, mean ± S.D.).

Hemoglobin (mg/dL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	11.18 ±1.19	12.31 ±1.81	11.23 ±0.83	10.86 ±1.01	12.17 ±1.92	11.62 ±0.87	10.25 ±2.03	10.37 ±1.35	9.93 ±1.33	9.50 ±0.89	10.21 ±.57	9.77 ±1.58
200 mg/kg BW	9.59 ±0.91	10.53 ±1.07	10.76 ±1.59	10.52 ±1.19	10.61 ±1.93	11.39 ±1.38	8.85 ±1.46	9.52 ±1.19	9.72 ±1.24	9.24 ±1.09	9.82 ±0.81	9.96 ±1.03
600 mg/kg BW	10.99 ±1.17	9.64 ±1.46*	9.09 ±1.09*	8.98 ±0.7*	10.56 ±1.32	10.82 ±1.72	7.81 ±0.83	8.68 ±0.41	8.90 ++0.67	7.56 ±1.56	9.76 ±0.92	9.99 ±1.42

Table 38. Hemoglobin (mg/dL) after 1m and 2m in each treatment (n=5, mean ± S.D.).

Hemoglobin (mg/dL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	11.02 ±1.31	12.18 ±0.97	11.17 ±1.47	10.77 ±1.25	11.38 ±0.95	11.35 ±1.13	11.15 ±1.10	10.98 ±1.11	11.01 ±1.53	9.91 ±0.20	11.53 ±1.47	10.40 ±1.52
20 mg/g	11.32 ±0.91	9.50 ±0.62*	9.56 ±0.84	9.45 ±0.85	11.06 ±1.21	11.09 ±0.91	10.06 ±1.45	9.87 ±1.40	9.66 ±0.94	9.79 ±0.83	10.88 ±1.16	9.97 ±1.24
60 mg/g	11.26 ±2.00	9.42 ±1.12*	9.22 ±0.78*	9.07 ±0.50*	11.08 ±1.36	10.73 ±0.44	9.41 ±1.62	8.73 ±0.85*	9.18 ±0.55*	9.19 ±0.62	10.10 ±0.83	10.04 ±0.88

Table 39. Multiple comparisons in hemoglobin between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: HB
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	.7365*	.23221	.005	.1866	1.2864
	600 mg/kg	1.3817*	.23221	.000	.8317	1.9316
200 mg/kg	0 mg/kg	-.7365*	.23221	.005	-1.2864	-.1866
	600 mg/kg	.6452*	.23221	.017	.0952	1.1951
600 mg/kg	0 mg/kg	-1.3817*	.23221	.000	-1.9316	-.8317
	200 mg/kg	-.6452*	.23221	.017	-1.1951	-.0952

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 40. Multiple comparisons in hemoglobin between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: HB
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.2848	.23221	.439	-.2651	.8348
	T. laurifolia	-.5812*	.23221	.036	-1.1311	-.0312
P. granatum	P. guajava	-.2848	.23221	.439	-.8348	.2651
	T. laurifolia	-.8660*	.23221	.001	-1.4159	-.3161
T. laurifolia	P. guajava	.5812*	.23221	.036	.0312	1.1311
	P. granatum	.8660*	.23221	.001	.3161	1.4159

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 41. Multiple comparisons in hemoglobin between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: HB
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	.8868*	.20383	.000	.4041	1.3695
	60 mg/g	1.2850*	.20383	.000	.8023	1.7677
20 mg/g	0 mg/g	-.8868*	.20383	.000	-1.3695	-.4041
	60 mg/g	.3982	.20383	.128	-.0845	.8809
60 mg/g	0 mg/g	-1.2850*	.20383	.000	-1.7677	-.8023
	20 mg/g	-.3982	.20383	.128	-.8809	.0845

Based on observed means.
*. The mean difference is significant at the .05 level.

Table 42. Multiple comparisons in hemoglobin between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: HB
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.5768*	.20383	.015	.0941	1.0595
	T. laurifolia	-.3920	.20383	.136	-.8747	.0907
P. granatum	P. guajava	-.5768*	.20383	.015	-1.0595	-.0941
	T. laurifolia	-.9688*	.20383	.000	-1.4515	-.4861
T. laurifolia	P. guajava	.3920	.20383	.136	-.0907	.8747
	P. granatum	.9688*	.20383	.000	.4861	1.4515

Based on observed means.
*. The mean difference is significant at the .05 level.

5.9.3 Red and white blood cell count

The result of RBC in experiment I and II are shown in Table 43-48.

Table 43. RBC (x10^6/cu.mm) after 48h and 7d in each treatment (n=5, mean ± S.D.).

RBC (x10^6/cu.mm)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	1.10 ±0.11	1.12 ±0.12	1.11 ±0.14	1.10 ±0.09	1.13 ±0.09	1.21 ±0.09	0.97 ±0.15	1.06 ±0.12	1.16 ±0.34	0.95 ±0.14	1.03 ±0.06	0.99 ±0.11
200 mg/kg BW	0.97 ±0.07	0.96 ±0.09	1.03 ±0.12	1.04 ±0.15	1.07 ±0.10	1.14 ±0.12	0.82 ±0.15	0.96 ±0.09	0.96 ±0.18	0.88 ±0.12	0.98 ±0.05	0.96 ±0.09
600 mg/kg BW	1.02 ±0.11	0.93 ±0.15	0.88 ±0.11*	0.86 ±0.06*#	1.00 ±0.10	1.03 ±0.12	0.74 ±0.06	0.87 ±0.06	0.88 ±0.10	0.75 ±0.17	0.93 ±0.07	0.97 ±0.13

Table 44. RBC (x10^6/cu.mm) after 1m and 2m in each treatment (n=5, mean ± S.D.).

RBC (x10^6/cu.mm)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	1.19 ±0.11	1.17 ±0.08	1.12 ±0.10	1.07 ±0.12	1.15 ±0.08	1.13 ±0.09	1.05 ±0.14	1.12 ±0.13	1.10 ±0.18	0.97 ±0.05	1.07 ±0.14	1.05 ±0.12
20 mg/g	1.11 ±0.05	0.94 ±0.05*	0.94 ±0.06*	0.94 ±0.08	1.13 ±0.05	1.11 ±0.12	1.00 ±0.13	0.94 ±0.08*	0.94 ±0.07	0.94 ±0.08	1.09 ±0.12	0.97 ±0.13
60 mg/g	1.04 ±0.15	0.92 ±0.10*	0.91 ±0.08*	0.89 ±0.06*	1.13 ±0.14	1.11 ±0.07	0.96 ±0.15	0.86 ±0.08*	0.91 ±0.07	0.91 ±0.05	1.13 ±0.32	1.03 ±0.10

Table 45. Multiple comparisons in RBC between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: RBC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	.0963*	.02284	.000	.0423	.1504
	600 mg/kg	.1722*	.02284	.000	.1181	.2262
200 mg/kg	0 mg/kg	-.0963*	.02284	.000	-.1504	-.0423
	600 mg/kg	.0758*	.02284	.003	.0218	.1299
600 mg/kg	0 mg/kg	-.1722*	.02284	.000	-.2262	-.1181
	200 mg/kg	-.0758*	.02284	.003	-.1299	-.0218

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 46. Multiple comparisons in RBC between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: RBC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-.0070	.02284	.950	-.0611	.0471
	T. laurifolia	-.0775*	.02284	.003	-.1316	-.0234
P. granatum	P. guajava	.0070	.02284	.950	-.0471	.0611
	T. laurifolia	-.0705*	.02284	.007	-.1246	-.0164
T. laurifolia	P. guajava	.0775*	.02284	.003	.0234	.1316
	P. granatum	.0705*	.02284	.007	.0164	.1246

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 47. Multiple comparisons in RBC between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: RBC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/g	20 mg/g	.0915*	.02104	.000	.0417	.1413
	60 mg/g	.1137*	.02104	.000	.0638	.1635
20 mg/g	0 mg/g	-.0915*	.02104	.000	-.1413	-.0417
	60 mg/g	.0222	.02104	.544	-.0277	.0720
60 mg/g	0 mg/g	-.1137*	.02104	.000	-.1635	-.0638
	20 mg/g	-.0222	.02104	.544	-.0720	.0277

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 48. Multiple comparisons in RBC between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: RBC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	.0527*	.02104	.036	.0028	.1025
	T. laurifolia	-.0670*	.02104	.005	-.1168	-.0172
P. granatum	P. guajava	-.0527*	.02104	.036	-.1025	-.0028
	T. laurifolia	-.1197*	.02104	.000	-.1695	-.0698
T. laurifolia	P. guajava	.0670*	.02104	.005	.0172	.1168
	P. granatum	.1197*	.02104	.000	.0698	.1695

Based on observed means.

*. The mean difference is significant at the .05 level.

The result of WBC in experiment I and II are shown in Table 49-54.

Table 49. WBC ($x10^3$/cu.mm) after 48h and 7d in each treatment (n=5, mean $\pm$ S.D.).

WBC ($x10^3$/cu.mm)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	9.66 $\pm$1.15	9.62 $\pm$1.19	10.25 $\pm$0.98	9.73 $\pm$1.33	9.47 $\pm$0.86	10.07 $\pm$1.07	10.11 $\pm$1.33	9.86 $\pm$1.35	9.42 $\pm$1.08	10.11 $\pm$1.08	8.94 $\pm$1.36	9.08 $\pm$0.82
200 mg/kg BW	10.21 $\pm$0.95	10.92 $\pm$1.44	10.65 $\pm$0.84	11.05 $\pm$1.13	10.42 $\pm$1.50	10.28 $\pm$0.70	10.39 $\pm$0.64	10.75 $\pm$0.81	10.82 $\pm$1.36	10.47 $\pm$1.31	10.49 $\pm$2.02	10.00 $\pm$0.47
600 mg/kg BW	10.84 $\pm$1.78	11.66 $\pm$0.89*	10.96 $\pm$1.17	11.32 $\pm$1.62	10.91 $\pm$1.69	11.01 $\pm$1.57	10.55 $\pm$1.45	10.31 $\pm$0.56	10.74 $\pm$1.61	10.93 $\pm$1.05	11.08 $\pm$1.76	11.21 $\pm$1.21*

Table 50. WBC ($x10^3$/cu.mm) after 1m and 2m in each treatment (n=5, mean $\pm$ S.D.).

WBC ($x10^3$/cu.mm)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	8.50 $\pm$0.85	9.38 $\pm$0.61	8.75 $\pm$1.66	9.07 $\pm$1.02	9.01 $\pm$1.13	9.06 $\pm$1.00	9.79 $\pm$0.89	8.92 $\pm$1.11	9.49 $\pm$0.41	9.85 $\pm$1.12	9.43 $\pm$0.90	9.63 $\pm$0.79
20 mg/g	11.54 $\pm$1.78*	12.34 $\pm$0.54*	12.20 $\pm$0.61*	11.84 $\pm$1.08*	11.11 $\pm$1.26*	11.06 $\pm$0.38*	11.47 $\pm$0.82*	11.62 $\pm$1.15*	11.92 $\pm$0.84*	11.80 $\pm$0.84*	12.77 $\pm$0.52*	12.67 $\pm$1.21*
60 mg/g	12.40 $\pm$1.26*	12.79 $\pm$0.76*	12.03 $\pm$1.32*	11.64 $\pm$1.25*	11.86 $\pm$1.11*	11.52 $\pm$1.28*	10.59 $\pm$0.65*	10.83 $\pm$0.22*	11.53 $\pm$1.34*	11.78 $\pm$0.70*	12.60 $\pm$0.51*	12.59 $\pm$0.88*

Table 51. Multiple comparisons in WBC between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: WBC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	-.8812*	.22983	.001	-1.4254	-.3369
	600 mg/kg	-1.2647*	.22983	.000	-1.8089	-.7204
200 mg/kg	0 mg/kg	.8812*	.22983	.001	.3369	1.4254
	600 mg/kg	-.3835	.22983	.221	-.9278	.1608
600 mg/kg	0 mg/kg	1.2647*	.22983	.000	.7204	1.8089
	200 mg/kg	.3835	.22983	.221	-.1608	.9278

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 52. Multiple comparisons in WBC between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: WBC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-.1688	.22983	.743	-.7131	.3754
	T. laurifolia	.1590	.22983	.769	-.3853	.7033
P. granatum	P. guajava	.1688	.22983	.743	-.3754	.7131
	T. laurifolia	.3278	.22983	.330	-.2164	.8721
T. laurifolia	P. guajava	-.1590	.22983	.769	-.7033	.3853
	P. granatum	-.3278	.22983	.330	-.8721	.2164

Based on observed means.

Table 53. Multiple comparisons in WBC between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: WBC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	-2.6223*	.18267	.000	-3.0549	-2.1897
	60 mg/g	-2.6065*	.18267	.000	-3.0391	-2.1739
20 mg/g	0 mg/g	2.6223*	.18267	.000	2.1897	3.0549
	60 mg/g	.0158	.18267	.996	-.4168	.4484
60 mg/g	0 mg/g	2.6065*	.18267	.000	2.1739	3.0391
	20 mg/g	-.0158	.18267	.996	-.4484	.4168

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 54. Multiple comparisons in WBC between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: WBC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-.1432	.18267	.714	-.5758	.2894
	T. laurifolia	-.2627	.18267	.324	-.6953	.1699
P. granatum	P. guajava	.1432	.18267	.714	-.2894	.5758
	T. laurifolia	-.1195	.18267	.790	-.5521	.3131
T. laurifolia	P. guajava	.2627	.18267	.324	-.1699	.6953
	P. granatum	.1195	.18267	.790	-.3131	.5521

Based on observed means.

5.9.4 Red cell indices

5.9.4.1 Mean corpuscular volume (MCV)

The result of MCV in experiment I and II are shown in Table 55-60.

Table 55. MCV (fL) after 48h and 7d in each treatment (n=5, mean ± S.D.).

MCV (fL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	1.10 ±0.11	1.12 ±0.12	1.11 ±0.14	1.10 ±0.09	1.13 ±0.09	1.21 ±0.09	0.97 ±0.15	1.06 ±0.12	1.16 ±0.34	0.95 ±0.14	1.03 ±0.06	0.99 ±0.11
200 mg/kg BW	0.97 ±0.07	0.96 ±0.09	1.03 ±0.12	1.04 ±0.15	1.07 ±0.10	1.14 ±0.12	0.82 ±0.15	0.96 ±0.09	0.96 ±0.18	0.88 ±0.12	0.98 ±0.05	0.96 ±0.09
600 mg/kg BW	1.02 ±0.11	0.93 ±0.15	0.88 ±0.11*	0.86 ±0.06*#	1.00 ±0.10	1.03 ±0.12	0.74 ±0.06*	0.87 ±0.06*	0.88 ±0.10	0.75 ±0.17	0.93 ±0.07	0.96 ±0.14

Table 56. MCV (fL) after 1m and 2m in each treatment (n=5, mean ± S.D.).

MCV (fL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	1.19 ±0.11	1.17 ±0.08	1.12 ±0.10	1.07 ±0.12	1.15 ±0.08	1.13 ±0.09	1.05 ±0.14	1.12 ±0.13	1.10 ±0.18	0.97 ±0.05	1.07 ±0.14	1.05 ±0.12
20 mg/g	1.11 ±0.05	0.94 ±0.05*	0.94 ±0.06*	0.94 ±0.08	1.13 ±0.05	1.11 ±0.12	1.00 ±0.13	0.94 ±0.08*	0.94 ±0.07	0.94 ±0.08	1.09 ±0.12	0.97 ±0.13
60 mg/g	1.04 ±0.15	0.92 ±0.10*	0.91 ±0.08*	0.89 ±0.06*	1.13 ±0.14	1.11 ±0.07	0.96 ±0.15	0.86 ±0.08*	0.91 ±0.07	0.91 ±0.05	1.13 ±0.32	1.03 ±0.10

Table 57. Multiple comparisons in MCV between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCV
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	.0963*	.02284	.000	.0423	.1504
	600 mg/kg	.1722*	.02284	.000	.1181	.2262
200 mg/kg	0 mg/kg	-.0963*	.02284	.000	-.1504	-.0423
	600 mg/kg	.0758*	.02284	.003	.0218	.1299
600 mg/kg	0 mg/kg	-.1722*	.02284	.000	-.2262	-.1181
	200 mg/kg	-.0758*	.02284	.003	-.1299	-.0218

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 58. Multiple comparisons in MCV between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCV
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	-.0070	.02284	.950	-.0611	.0471
	T. laurifolia	-.0775*	.02284	.003	-.1316	-.0234
P. granatum	P. guajava	.0070	.02284	.950	-.0471	.0611
	T. laurifolia	-.0705*	.02284	.007	-.1246	-.0164
T. laurifolia	P. guajava	.0775*	.02284	.003	.0234	.1316
	P. granatum	.0705*	.02284	.007	.0164	.1246

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 59. Multiple comparisons in MCV between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCV
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	.0915*	.02104	.000	.0417	.1413
	60 mg/g	.1137*	.02104	.000	.0638	.1635
20 mg/g	0 mg/g	-.0915*	.02104	.000	-.1413	-.0417
	60 mg/g	.0222	.02104	.544	-.0277	.0720
60 mg/g	0 mg/g	-.1137*	.02104	.000	-.1635	-.0638
	20 mg/g	-.0222	.02104	.544	-.0720	.0277

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 60. Multiple comparisons in MCV between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCV
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.0527*	.02104	.036	.0028	.1025
	T. laurifolia	-.0670*	.02104	.005	-.1168	-.0172
P. granatum	P. guajava	-.0527*	.02104	.036	-.1025	-.0028
	T. laurifolia	-.1197*	.02104	.000	-.1695	-.0698
T. laurifolia	P. guajava	.0670*	.02104	.005	.0172	.1168
	P. granatum	.1197*	.02104	.000	.0698	.1695

Based on observed means.

*. The mean difference is significant at the .05 level.

5.9.4.2 Mean corpuscular hemoglobin (MCH)

The result of MCH in experiment I and II are shown in Table 61-66.

Table 61. MCH (pg/cell) after 48h and 7d in each treatment (n=5, mean ± S.D.).

MCH (pg/cell)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	10.19 ±0.84	11.11 ±1.89	10.16 ±0.69	9.94 ±1.00	10.78 ±1.79	9.65 ±1.22	10.53 ±1.05	9.78 ±0.91	8.85 ±1.36	10.10 ±0.66	9.92 ±0.61	9.88 ±0.80
200 mg/kg BW	9.87 ±0.72	11.02 ±0.30	10.42 ±0.74	10.17 ±1.22	9.86 ±0.87	10.03 ±0.61	10.86 ±0.34	9.93 ±0.49	10.28 ±0.95	10.56 ±0.48	9.97 ±0.35	10.38 ±0.47
600 mg/kg BW	10.77 ±0.90	10.44 ±0.50	10.34 ±0.91	10.49 ±0.49	10.58 ±0.53	10.50 ±0.83	10.56 ±0.77	10.07 ±0.88	10.11 ±0.48	10.19 ±0.92	10.47 ±0.45	10.27 ±0.37

Table 62. MCH (pg/cell) after 1m and 2m in each treatment (n=5, mean ± S.D.).

MCH (pg/cell)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	9.33 ±1.61	10.44 ±0.43	9.96 ±0.54	10.05 ±0.78	9.93 ±0.61	10.08 ±0.67	10.70 ±0.82	9.88 ±0.74	10.06 ±0.68	10.28 ±0.59	10.89 ±1.42	9.91 ±0.57
20 mg/g	10.20 ±0.73	10.12 ±0.35	10.13 ±0.59	10.03 ±0.14	9.76 ±0.64	10.00 ±0.86	10.04 ±0.55	10.43 ±0.88	10.24 ±0.41	10.41 ±0.70	10.01 ±0.49	10.28 ±0.47
60 mg/g	10.81 ±0.46	10.27 ±0.69	10.10 ±0.67	10.20 ±0.73	9.79 ±0.65	9.69 ±0.73	9.86 ±0.87	10.15 ±0.37	10.10 ±0.19	10.08 ±0.51	9.31 ±1.64	9.77 ±0.56

Table 63. Multiple comparisons in MCH between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCH
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	-.2038	.15875	.406	-.5798	.1721
	600 mg/kg	-.3242	.15875	.106	-.7001	.0518
200 mg/kg	0 mg/kg	.2038	.15875	.406	-.1721	.5798
	600 mg/kg	-.1203	.15875	.729	-.4963	.2556
600 mg/kg	0 mg/kg	.3242	.15875	.106	-.0518	.7001
	200 mg/kg	.1203	.15875	.729	-.2556	.4963

Based on observed means.

Table 64. Multiple comparisons in MCH between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCH
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.2920	.15875	.161	-.0839	.6679
	T. laurifolia	.2345	.15875	.305	-.1414	.6104
P. granatum	P. guajava	-.2920	.15875	.161	-.6679	.0839
	T. laurifolia	-.0575	.15875	.930	-.4334	.3184
T. laurifolia	P. guajava	-.2345	.15875	.305	-.6104	.1414
	P. granatum	.0575	.15875	.930	-.3184	.4334

Based on observed means.

Table 65. Multiple comparisons in MCH between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCH
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	-.0123	.13639	.996	-.3353	.3107
	60 mg/g	.1150	.13639	.677	-.2080	.4380
20 mg/g	0 mg/g	.0123	.13639	.996	-.3107	.3353
	60 mg/g	.1273	.13639	.620	-.1957	.4503
60 mg/g	0 mg/g	-.1150	.13639	.677	-.4380	.2080
	20 mg/g	-.1273	.13639	.620	-.4503	.1957

Based on observed means.

Table 66. Multiple comparisons in MCH between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCH
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
P. guajava	P. granatum	.0503	.13639	.928	-.2727	.3733
	T. laurifolia	.2323	.13639	.207	-.0907	.5553
P. granatum	P. guajava	-.0503	.13639	.928	-.3733	.2727
	T. laurifolia	.1820	.13639	.379	-.1410	.5050
T. laurifolia	P. guajava	-.2323	.13639	.207	-.5553	.0907
	P. granatum	-.1820	.13639	.379	-.5050	.1410

Based on observed means.

5.9.4.3 Mean corpuscular hemoglobin concentration (MCHC)

The result of MCHC in experiment I and II are shown in Table 67-72.

Table 67. MCHC (g/dL) after 48h and 7d in each treatment (n=5, mean ± S.D.).

MCHC (g/dL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	0.34 ±0.02	0.36 ±0.05	0.35 ±0.01	0.33 ±0.02	0.37 ±0.06	0.34 ±0.04	0.36 ±0.03	0.34 ±0.03	0.33 ±0.03	0.34 ±0.02	0.33 ±0.02	0.33 ±0.03
200 mg/kg BW	0.33 ±0.02	0.37 ±0.01	0.35 ±0.03	0.35 ±0.04	0.33 ±0.03	0.35 ±0.03	0.36 ±0.01	0.33 ±0.02	0.34 ±0.03	0.35 ±0.02	0.33 ±0.01	0.35 ±0.02
600 mg/kg BW	0.36 ±0.02	0.35 ±0.02	0.34 ±0.03	0.34 ±0.02	0.35 ±0.02	0.35 ±0.03	0.35 ±0.02	0.34 ±0.03	0.34 ±0.02	0.34 ±0.03	0.35 ±0.01	0.35 ±0.02

Table 68. MCHC (pg/cell) after 1m and 2m in each treatment (n=5, mean + S.D.).

MCHC (g/dL)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	0.31 +0.05	0.35 +0.01	0.33 +0.02	0.34 +0.02	0.33 +0.02	0.34 +0.03	0.36 +0.03	0.33 +0.02	0.34 +0.02	0.34 +0.02	0.37 +0.05	0.32 +0.02
20 mg/g	0.34 +0.02	0.34 +0.01	0.34 +0.02	0.33 +0.00	0.33 +0.02	0.34 +0.02	0.34 +0.02	0.35 +0.03	0.34 +0.02	0.35 +0.02	0.34 +0.01	0.34 +0.01
60 mg/g	0.36 +0.02	0.35 +0.02	0.34 +0.02	0.34 +0.02	0.33 +0.02	0.33 +0.02	0.34 +0.03	0.34 +0.01	0.34 +0.00	0.34 +0.01	0.34 +0.02	0.33 +0.02

Table 69. Multiple comparisons in MCHC between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCHC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	.0002	.00507	.999	-.0119	.0122
	600 mg/kg	-.0005	.00507	.995	-.0125	.0115
200 mg/kg	0 mg/kg	-.0002	.00507	.999	-.0122	.0119
	600 mg/kg	-.0007	.00507	.991	-.0127	.0114
600 mg/kg	0 mg/kg	.0005	.00507	.995	-.0115	.0125
	200 mg/kg	.0007	.00507	.991	-.0114	.0127

Based on observed means.

Table 70. Multiple comparisons in MCHC between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: MCHC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.0062	.00507	.446	-.0059	.0182
	T. laurifolia	.0050	.00507	.587	-.0070	.0170
P. granatum	P. guajava	-.0062	.00507	.446	-.0182	.0059
	T. laurifolia	-.0012	.00507	.971	-.0132	.0109
T. laurifolia	P. guajava	-.0050	.00507	.587	-.0170	.0070
	P. granatum	.0012	.00507	.971	-.0109	.0132

Based on observed means.

Table 71. Multiple comparisons in MCHC between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCHC
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	.0007	.00423	.986	-.0093	.0107
	60 mg/g	.0002	.00423	.999	-.0098	.0102
20 mg/g	0 mg/g	-.0007	.00423	.986	-.0107	.0093
	60 mg/g	-.0005	.00423	.992	-.0105	.0095
60 mg/g	0 mg/g	-.0002	.00423	.999	-.0102	.0098
	20 mg/g	.0005	.00423	.992	-.0095	.0105

Based on observed means.

Table 72. Multiple comparisons in MCHC between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: MCHC
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	.0048	.00423	.489	-.0052	.0148
	T. laurifolia	.0065	.00423	.276	-.0035	.0165
P. granatum	P. guajava	-.0048	.00423	.489	-.0148	.0052
	T. laurifolia	.0017	.00423	.918	-.0083	.0117
T. laurifolia	P. guajava	-.0065	.00423	.276	-.0165	.0035
	P. granatum	-.0017	.00423	.918	-.0117	.0083

Based on observed means.

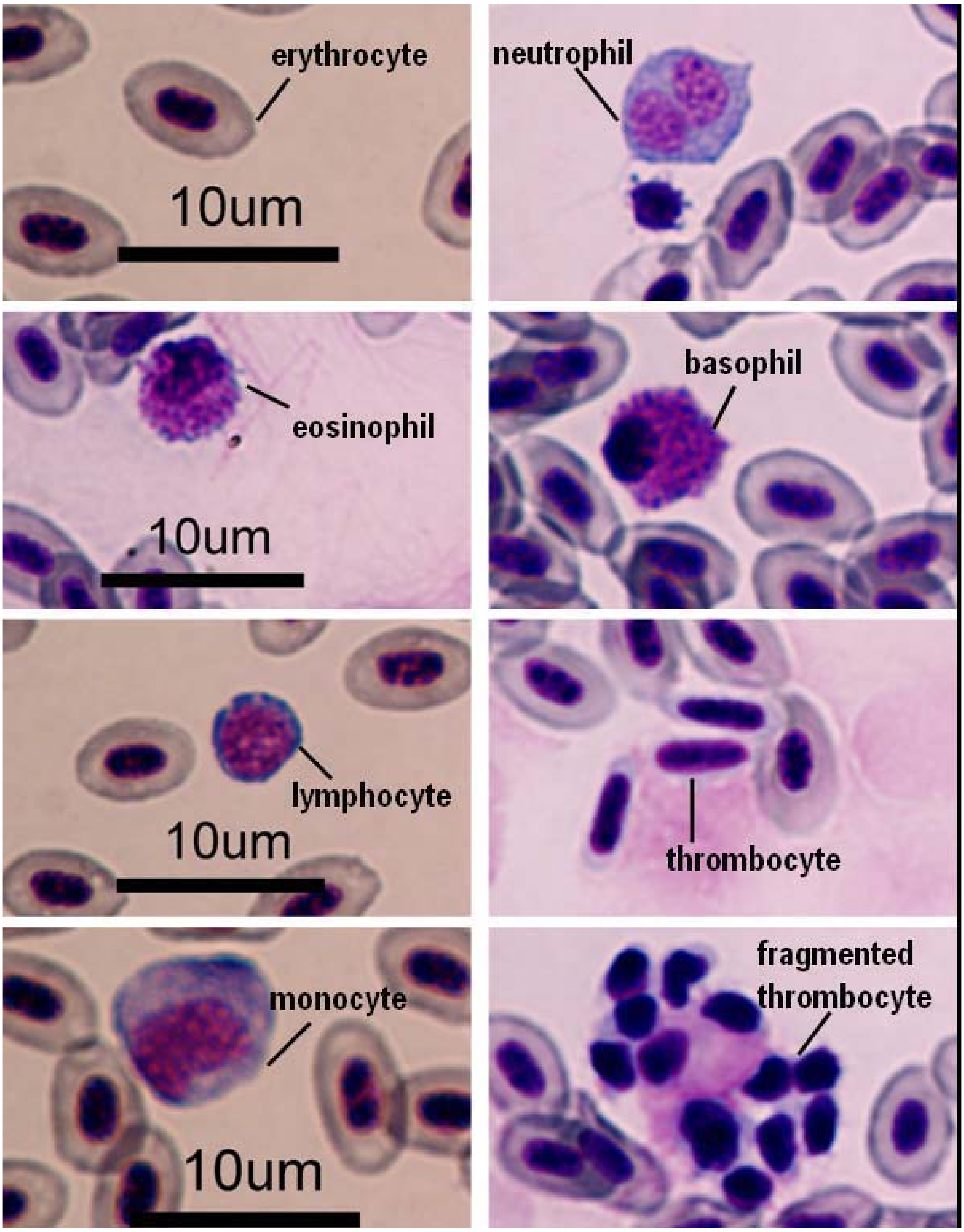


Figure 16. Fish blood cell morphology i.e., erythrocyte, neutrophil, eosinophil, basophil, lymphocyte and monocyte, including thrombocyte.

5.10 Biochemical studies

Demonstrations of increase or decrease in specific plasma activity with disease encourage researchers to evaluate a variety of enzyme systems looking for those which are organs or tissue specific. Nowadays, enzymogram plays an important role in diagnosis and prognosis of animal disease (Coles, 1989). Changes in plasma enzyme activity are used as indicators of tissue injury, environmental stress, or a diseased condition. The increase in enzyme activity depends on the enzyme concentration in cells, rate of leakage caused by injury and rate of clearance of the enzyme from plasma (Boyd, 1983).

5.10.1 Aspartate aminotransferase (AST)

The result of AST in experiment I and II are shown in Table 73-78.

Table 73. AST (U/L) after 48h and 7d in each treatment (n=5, mean ± S.D.).

AST (U/L)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	52.58 ±10.36	55.64 ±8.98	56.06 ±7.87	48.18 ±8.50	52.98 ±10.73	48.38 ±9.84	37.76 ±3.95	37.18 ±8.30	36.44 ±3.84	38.82 ±4.04	35.52 ±2.54	34.32 ±4.30
200 mg/kg BW	42.12 ±4.44	46.40 ±8.70	44.56 ±5.40	45.92 ±6.23	48.38 ±6.84	45.88 ±4.44	36.46 ±3.73	39.84 ±5.12	35.20 ±3.40	38.12 ±4.23	35.98 ±3.06	37.66 ±3.43
600 mg/kg BW	42.68 ±6.37	48.94 ±5.06	46.80 ±4.18	45.28 ±2.75	46.28 ±4.60	46.52 ±3.10	37.92 ±2.17	37.66 ±2.54	39.20 ±1.55	37.70 ±2.01	35.60 ±4.37	38.54 ±1.07

Table 74. AST (U/L) after 1m and 2m in each treatment (n=5, mean ± S.D.).

AST (U/L)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	46.30 ±4.10	48.74 ±3.13	46.18 ±4.22	46.80 ±6.61	49.20 ±5.05	47.68 ±9.22	41.44 ±5.51	38.54 ±7.39	40.92 ±7.59	39.90 ±9.55	37.90 ±3.47	41.68 ±5.18
20 mg/g	46.70 ±2.33	46.28 ±1.06	47.42 ±2.88	48.66 ±4.50	46.66 ±2.41	45.18 ±2.73	42.24 ±4.43	42.32 ±4.07	40.46 ±5.00	38.78 ±3.71	36.68 ±2.81	37.60 ±0.87
60 mg/g	49.08 ±2.72	47.24 ±2.33	48.96 ±1.54	49.36 ±4.41	48.82 ±3.36	48.98 ±2.71	43.06 ±3.55	45.06 ±1.17	43.02 ±2.79	45.04 ±2.55	40.68 ±3.75	40.48 ±2.80

Table 75. Multiple comparisons in AST between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: AST
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/kg	200 mg/kg	3.1117*	1.03414	.009	.6626	5.5607
	600 mg/kg	2.5617*	1.03414	.038	.1126	5.0107
200 mg/kg	0 mg/kg	-3.1117*	1.03414	.009	-5.5607	-.6626
	600 mg/kg	-.5500	1.03414	.856	-2.9991	1.8991
600 mg/kg	0 mg/kg	-2.5617*	1.03414	.038	-5.0107	-.1126
	200 mg/kg	.5500	1.03414	.856	-1.8991	2.9991

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 76. Multiple comparisons in AST between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: AST
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.2417	1.03414	.970	-2.2074	2.6907
	T. laurifolia	.7617	1.03414	.742	-1.6874	3.2107
P. granatum	P. guajava	-.2417	1.03414	.970	-2.6907	2.2074
	T. laurifolia	.5200	1.03414	.870	-1.9291	2.9691
T. laurifolia	P. guajava	-.7617	1.03414	.742	-3.2107	1.6874
	P. granatum	-.5200	1.03414	.870	-2.9691	1.9291

Based on observed means.

Table 77. Multiple comparisons in AST between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: AST
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 mg/g	20 mg/g	.5250	.80875	.793	-1.3903	2.4403
	60 mg/g	-2.0417*	.80875	.034	-3.9569	-.1264
20 mg/g	0 mg/g	-.5250	.80875	.793	-2.4403	1.3903
	60 mg/g	-2.5667*	.80875	.005	-4.4819	-.6514
60 mg/g	0 mg/g	2.0417*	.80875	.034	.1264	3.9569
	20 mg/g	2.5667*	.80875	.005	.6514	4.4819

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 78. Multiple comparisons in AST between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: AST
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P. guajava	P. granatum	.1250	.80875	.987	-1.7903	2.0403
	T. laurifolia	1.2883	.80875	.252	-.6269	3.2036
P. granatum	P. guajava	-.1250	.80875	.987	-2.0403	1.7903
	T. laurifolia	1.1633	.80875	.324	-.7519	3.0786
T. laurifolia	P. guajava	-1.2883	.80875	.252	-3.2036	.6269
	P. granatum	-1.1633	.80875	.324	-3.0786	.7519

Based on observed means.

5.10.2 Alanine aminotransferase (ALT)

The result of ALT in experiment I and II are shown in Table 79-84.

Table 79. ALT (U/L) after 48h and 7d in each treatment (n=5, mean ± S.D.).

Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	30.96 ±4.93	32.32 ±3.85	33.30 ±4.75	30.02 ±4.33	29.24 ±3.08	29.84 ±2.00	29.48 ±4.00	33.14 ±4.20	31.08 ±3.46	33.90 ±5.41	33.14 ±4.98	32.10 ±3.24
200 mg/kg BW	30.94 ±6.56	35.14 ±4.20	38.60 ±5.76	38.54 ±6.42	34.76 ±6.33	32.16 ±4.16	32.36 ±4.44	34.00 ±4.54	33.26 ±2.47	35.62 ±4.73	33.58 ±3.33	34.36 ±4.94
600 mg/kg BW	33.40 ±4.47	36.12 ±3.66	37.36 ±5.64	38.08 ±7.44	33.60 ±7.82	32.92 ±5.58	33.78 ±4.74	35.50 ±3.94	33.28 ±5.68	36.16 ±2.43	35.44 ±4.17	33.00 ±4.53

ALT (U/L)

Table 80. ALT (U/L) after 1m and 2m in each treatment (n=5, mean ± S.D.).

Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	33.76 ±3.03	35.14 ±3.38	34.86 ±3.60	38.70 ±6.09	33.38 ±3.48	34.34 ±4.59	35.26 ±4.66	35.82 ±3.33	34.30 ±4.16	32.64 ±4.85	33.60 ±4.12	34.34 ±4.34
20 mg/g	37.36 ±6.47	38.10 ±5.91	38.38 ±2.58	45.26 ±4.42	34.72 ±3.97	37.48 ±2.86	37.22 ±4.84	36.96 ±2.76	36.98 ±3.39	36.32 ±3.61	35.46 ±2.50	36.24 ±4.42
60 mg/g	40.92 ±7.08	42.64 ±4.15	42.10 ±5.30*	41.02 ±7.94	35.62 ±6.74	36.38 ±7.02	35.96 ±7.12	38.62 ±6.22	43.02 ±2.79*#	35.54 ±4.30	34.16 ±2.97	35.86 ±4.59

ALT (U/L)

Table 81. Multiple comparisons in ALT between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: ALT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	-2.9000*	.87537	.003	-4.9731	-.8269
	600 mg/kg	-3.3433*	.87537	.001	-5.4164	-1.2703
200 mg/kg	0 mg/kg	2.9000*	.87537	.003	.8269	4.9731
	600 mg/kg	-.4433	.87537	.868	-2.5164	1.6297
600 mg/kg	0 mg/kg	3.3433*	.87537	.001	1.2703	5.4164
	200 mg/kg	.4433	.87537	.868	-1.6297	2.5164

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 82. Multiple comparisons in ALT between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: ALT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-1.8383	.87537	.093	-3.9114	.2347
	T. laurifolia	.2500	.87537	.956	-1.8231	2.3231
P. granatum	P. guajava	1.8383	.87537	.093	-.2347	3.9114
	T. laurifolia	2.0883*	.87537	.048	.0153	4.1614
T. laurifolia	P. guajava	-.2500	.87537	.956	-2.3231	1.8231
	P. granatum	-2.0883*	.87537	.048	-4.1614	-.0153

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 83. Multiple comparisons in ALT between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: ALT
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/g	20 mg/g	-2.8617*	.87166	.004	-4.9259	-.7974
	60 mg/g	-3.8083*	.87166	.000	-5.8726	-1.7441
20 mg/g	0 mg/g	2.8617*	.87166	.004	.7974	4.9259
	60 mg/g	-.9467	.87166	.524	-3.0109	1.1176
60 mg/g	0 mg/g	3.8083*	.87166	.000	1.7441	5.8726
	20 mg/g	.9467	.87166	.524	-1.1176	3.0109

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 84. Multiple comparisons in ALT between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: ALT
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-.9467	.87166	.524	-3.0109	1.1176
	T. laurifolia	2.1817*	.87166	.036	.1174	4.2459
P. granatum	P. guajava	.9467	.87166	.524	-1.1176	3.0109
	T. laurifolia	3.1283*	.87166	.001	1.0641	5.1926
T. laurifolia	P. guajava	-2.1817*	.87166	.036	-4.2459	-.1174
	P. granatum	-3.1283*	.87166	.001	-5.1926	-1.0641

Based on observed means.

*. The mean difference is significant at the .05 level.

Aminotransferases are intracellular enzymes which are normally localized within the cells of the liver, heart, gills, kidney, muscle and other organs. Aspartate aminotransferase is present in high concentrations in the heart, liver, skeletal muscle, kidney and erythrocytes while alanine aminotransferase is present in high concentrations in liver and to a lesser extent in skeletal muscle, kidney and heart. The levels of these enzymes increase in the plasma when the cells are damaged or their membranes disrupted, allowing the enzymes to leak out of the cells. These enzymes are therefore of major importance in assessing and monitoring liver cytolysis (Ovie et al., 2010). Aminotransferases play vital roles in carbohydrate-protein metabolism in fish and other organisms' tissue. The aminotransferases occupy a central position in the amino acid metabolism as they help in retaining amino groups to form a new amino acid during the degradation of amino acid and also involved in the biochemical regulation of intracellular amino acid pool. They help in providing necessary intermediates for gluconeogenesis. The observed alterations in their activities in the exposed fish may therefore have adverse effect on the amino acid metabolism of the tissues and consequently the intermediates required for gluconeogenesis. Increased activities of both aminotransferases indicated amplified transamination processes. An increased in transamination occurs due to amino acid input into the TCA cycle in order to cope with the energy crisis during toxicant-based stress (Philip et al., 1995).

Pramyothin et al. (2005) reported that rat feeding with *T. laurifolia* at 25 mg/kg/day for 7 days after 14 days ethanol treatment, enhanced liver cell recovery by bringing serum transaminases back to normal. Kaur et al. (2006) reported that pretreatment with pomegranate flower extract at a dose regimen of 50-150 mg/kg BW in mice for a week significantly and dose dependently protected against ferric nitrilotriacetate induced oxidative stress as well as hepatic injury. A protection against ferric nitrilotriacetate induced liver injury was apparent as inhibition in the modulation of liver markers viz., aspartate and alanine aminotransferase, alkaline phosphatase, bilirubin and albumin in serum. Similar hepatoprotective effects have been reported with pomegranate extract, which inhibited carbon tetrachloride induced oxidative stress and hepatic injury (Chidambara Murthy et al., 2002), and also against trichloroacetic acid exposure in rat (Celik et al., 2009).

5.10.3 Alkaline phosphatase (ALP)

The result of ALP in experiment I and II are shown in Table 85-90.

Table 85. ALP (U/L) after 48h and 7d in each treatment (n=5, mean ± S.D.).

ALP (U/L)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d	48 h	7 d
0 mg/kg BW	35.60 ±4.37	36.16 ±2.43	33.02 ±2.46	33.32 ±2.97	32.10 ±3.24	32.24 ±2.28	34.86 ±3.60	34.00 ±4.54	31.08 ±3.46	33.90 ±5.41	33.14 ±4.98	32.10 ±3.24
200 mg/kg BW	33.60 ±4.12	35.14 ±2.86	34.00 ±3.18	35.74 ±4.66	34.76 ±6.33	32.16 ±4.16	32.36 ±4.44	34.40 ±4.37	33.26 ±2.47	35.62 ±4.73	33.58 ±3.33	34.36 ±4.94
600 mg/kg BW	33.40 ±4.47	36.12 ±3.66	37.36 ±5.64	36.08 ±6.65	33.60 ±7.82	32.92 ±5.58	33.78 ±4.74	35.50 ±3.94	33.28 ±5.68	36.16 ±2.43	33.84 ±3.48	33.00 ±4.53

Table 86. ALP (U/L) after 1m and 2m in each treatment (n=5, mean ± S.D.).

ALP (U/L)												
Concentration	*O. niloticus*						*P. altus*					
	P. guajava		*P. granatum*		*T. laurifolia*		*P. guajava*		*P. granatum*		*T. laurifolia*	
	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m	1 m	2 m
0 mg/g	33.76 ±3.03	35.14 ±3.38	34.86 ±3.60	32.70 ±4.81	33.38 ±3.48	34.34 ±4.59	33.26 ±5.20	35.82 ±3.33	34.30 ±4.16	32.64 ±4.85	33.60 ±4.12	34.34 ±4.34
20 mg/g	33.36 ±3.68	34.10 ±4.90	34.98 ±3.58	37.26 ±7.80	32.12 ±3.73	37.48 ±2.86	37.22 ±4.84	34.96 ±4.05	36.98 ±3.39	36.32 ±3.61	33.46 ±4.75	30.04 ±6.31
60 mg/g	34.92 ±8.18	36.64 ±7.13	38.10 ±6.79	37.02 ±8.38	35.62 ±6.74	32.38 ±4.91	35.96 ±7.12	35.62 ±6.17	37.02 ±6.41	35.54 ±4.30	34.16 ±2.97	33.86 ±3.15

Table 87. Multiple comparisons in ALP between concentration after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: ALP
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 mg/kg	200 mg/kg	-.6217	.80119	.718	-2.5190	1.2757
	600 mg/kg	-1.1267	.80119	.340	-3.0240	.7707
200 mg/kg	0 mg/kg	.6217	.80119	.718	-1.2757	2.5190
	600 mg/kg	-.5050	.80119	.804	-2.4024	1.3924
600 mg/kg	0 mg/kg	1.1267	.80119	.340	-.7707	3.0240
	200 mg/kg	.5050	.80119	.804	-1.3924	2.4024

Based on observed means.

Table 88. Multiple comparisons in ALT between plant after 48h and 7d in each treatment.

Multiple Comparisons

Dependent Variable: ALP
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	.1750	.80119	.974	-1.7224	2.0724
	T. laurifolia	1.4267	.80119	.180	-.4707	3.3240
P. granatum	P. guajava	-.1750	.80119	.974	-2.0724	1.7224
	T. laurifolia	1.2517	.80119	.265	-.6457	3.1490
T. laurifolia	P. guajava	-1.4267	.80119	.180	-3.3240	.4707
	P. granatum	-1.2517	.80119	.265	-3.1490	.6457

Based on observed means.

Table 89. Multiple comparisons in ALT between concentration after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: ALP
Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
0 mg/g	20 mg/g	-.8450	.93060	.636	-3.0488	1.3588
	60 mg/g	-1.5583	.93060	.218	-3.7622	.6455
20 mg/g	0 mg/g	.8450	.93060	.636	-1.3588	3.0488
	60 mg/g	-.7133	.93060	.724	-2.9172	1.4905
60 mg/g	0 mg/g	1.5583	.93060	.218	-.6455	3.7622
	20 mg/g	.7133	.93060	.724	-1.4905	2.9172

Based on observed means.

Table 90. Multiple comparisons in ALT between plant after 1m and 2m in each treatment.

Multiple Comparisons

Dependent Variable: ALP
Tukey HSD

(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
P. guajava	P. granatum	-.5800	.93060	.808	-2.7838	1.6238
	T. laurifolia	1.3317	.93060	.328	-.8722	3.5355
P. granatum	P. guajava	.5800	.93060	.808	-1.6238	2.7838
	T. laurifolia	1.9117	.93060	.103	-.2922	4.1155
T. laurifolia	P. guajava	-1.3317	.93060	.328	-3.5355	.8722
	P. granatum	-1.9117	.93060	.103	-4.1155	.2922

Based on observed means.

Alkaline phosphatase is a membrane bound enzyme found at bile pole of hepatoctyes and also found in pinocytic vesicle and golgi complex. It is present on all cell membranes where active transport occurs, and hydrolase and transphosphorylase in function. It is often employed to access the integrity of plasma membrane, since it is localized predominantly in the microvilli of the bile canaliculi, located in the plasma membrane (Kori-Siakpere et al., 2010). Decrease in ALP activity may be taken as an index of hepatic parenchymal damage and hepatocytic necrosis. Inhibition of ALP reflects alteration in protein synthesis and uncoupling of oxidative phosphorylation. The decreased of ALP activity in the plasma of the exposed fish was similar to the significant reduction in ALP in the liver and kidney of catfish, *Heterpneustes fossilis* after toxication with cadmium (Sastry and Subhadra, 1985). This decrease may be due to the damage and dysfunction of the liver. The decrease in ALP by stressors probably indicates an altered transport of phosphate and an inhibitory effect on the cell growth and proliferation (Rec, 1972). On the other hand, increase in the plasma alkaline phosphatase in *Clarias batrachus* following exposure to pesticide have reported as a consequence of osteoblastic activity increase or due to intra and extra hepatic obstructions of bilary passage (Jyothi and Narayan, 1999).

5.11 Light microscopic and scanning electron microscopic study

5.11.1 Gills

There were four gill arches on each side of the buccal cavity, which were termed from lateral to medial as first, second, third and fourth. Each gill arch was semilunar in shape consisting of numerous primary filaments, which had a cartilaginous central structure and highly vascularized. Each gill consisted of a primary filament and secondary lamellae. The primary filament had two rows of secondary lamellae that run perpendicular to each filament. No recognizable changes were observed in the gills of each group (Figs. 18-19). Briefly, the primary filament epithelium was one or two cell layers thick. The spaced of secondary lamellae were equally and its arrays of delicate secondary lamellae. Each secondary lamella was made up of two sheets of epithelium delimited by many pillar cells, which were contractile and separated the capillary channels. One to two erythrocytes were usually recognized within each capillary lumen. These secondary lamellae, together with the central vascular spaces, form the gaseous exchange barrier, or respiratory barrier. Chloride cells were identified as large epithelial cells with light cytoplasm, usually present at the base of secondary lamellae. Mucus cells and pavement cells were also present in the epithelium of the filament and at the base of lamellae, but they lacked the light cytoplasm and were smaller than chloride cells. For ultrastructure, gill filaments were covered with squamous pavement cells showing characteristic concentric patterns of microridges (Fig 20).

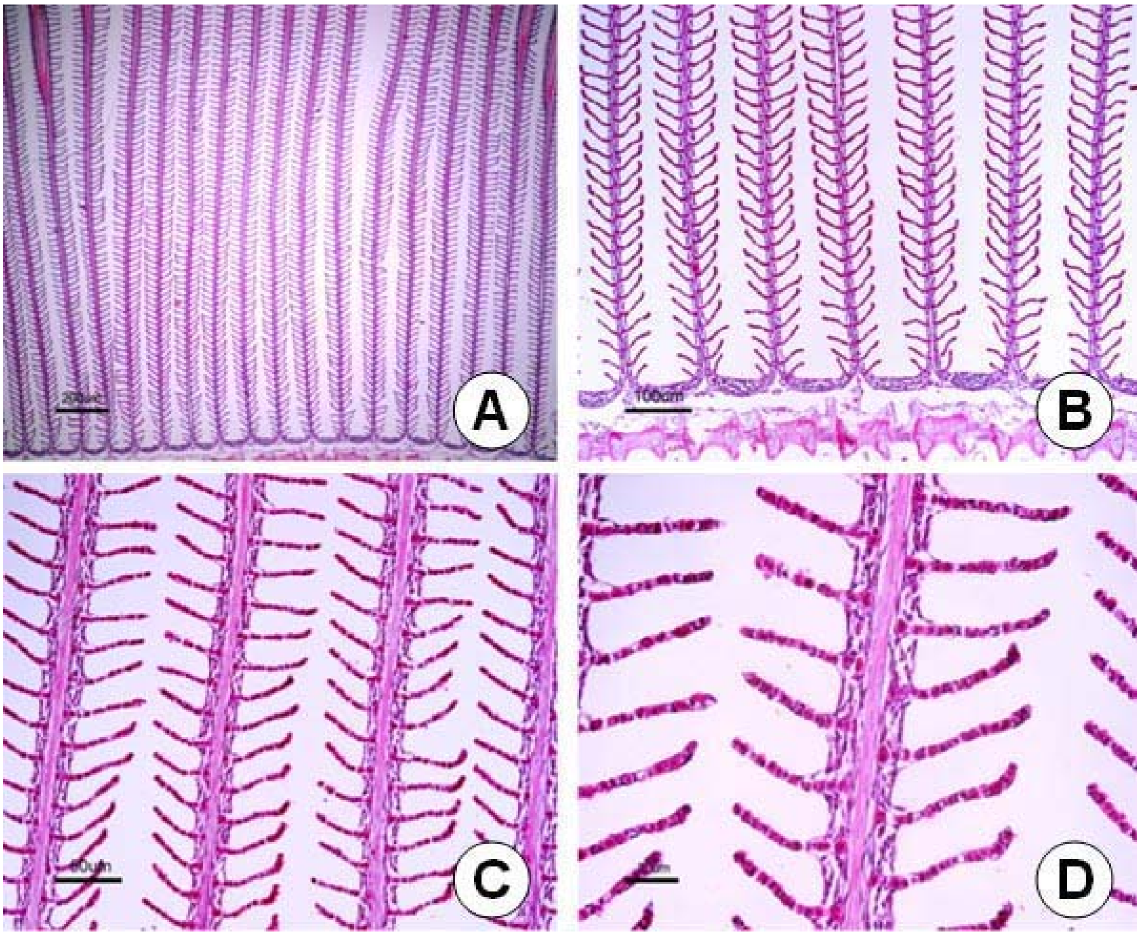


Figure 17. Light micrographs of transverse section of gills showing normal appearance of primary filament and secondary lamellae. Each primary filament has two parallel rows of secondary lamellae. Note: A: bar 200µm, B: bar 100µm, C: bar 50µm, and D: bar 20µm.

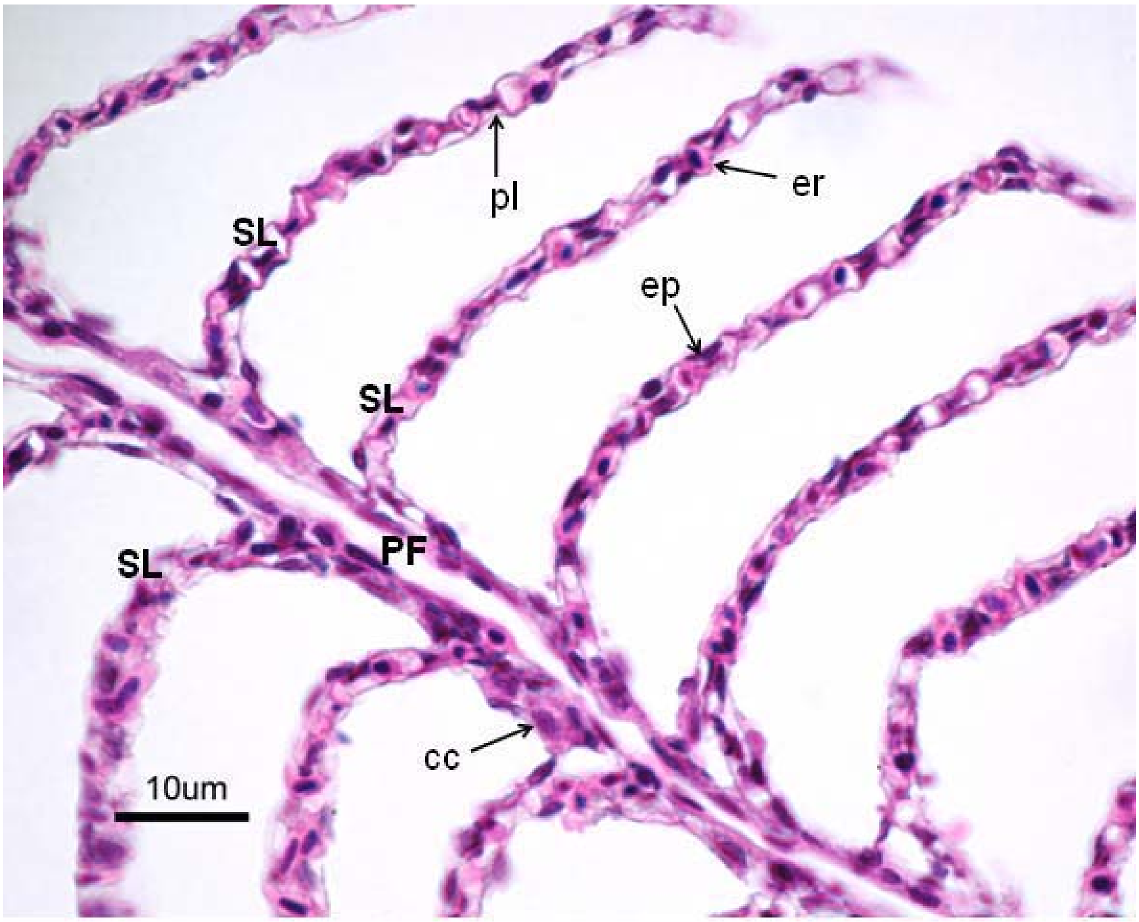


Figure 18. High magnification of transverse section of gills showing normal appearance of primary filament (PF), secondary lamellae (SL). Note: bar 10µm, ep=epithelial cell, er=erythrocyte, pl=pillar cell, cc=chloride cell.

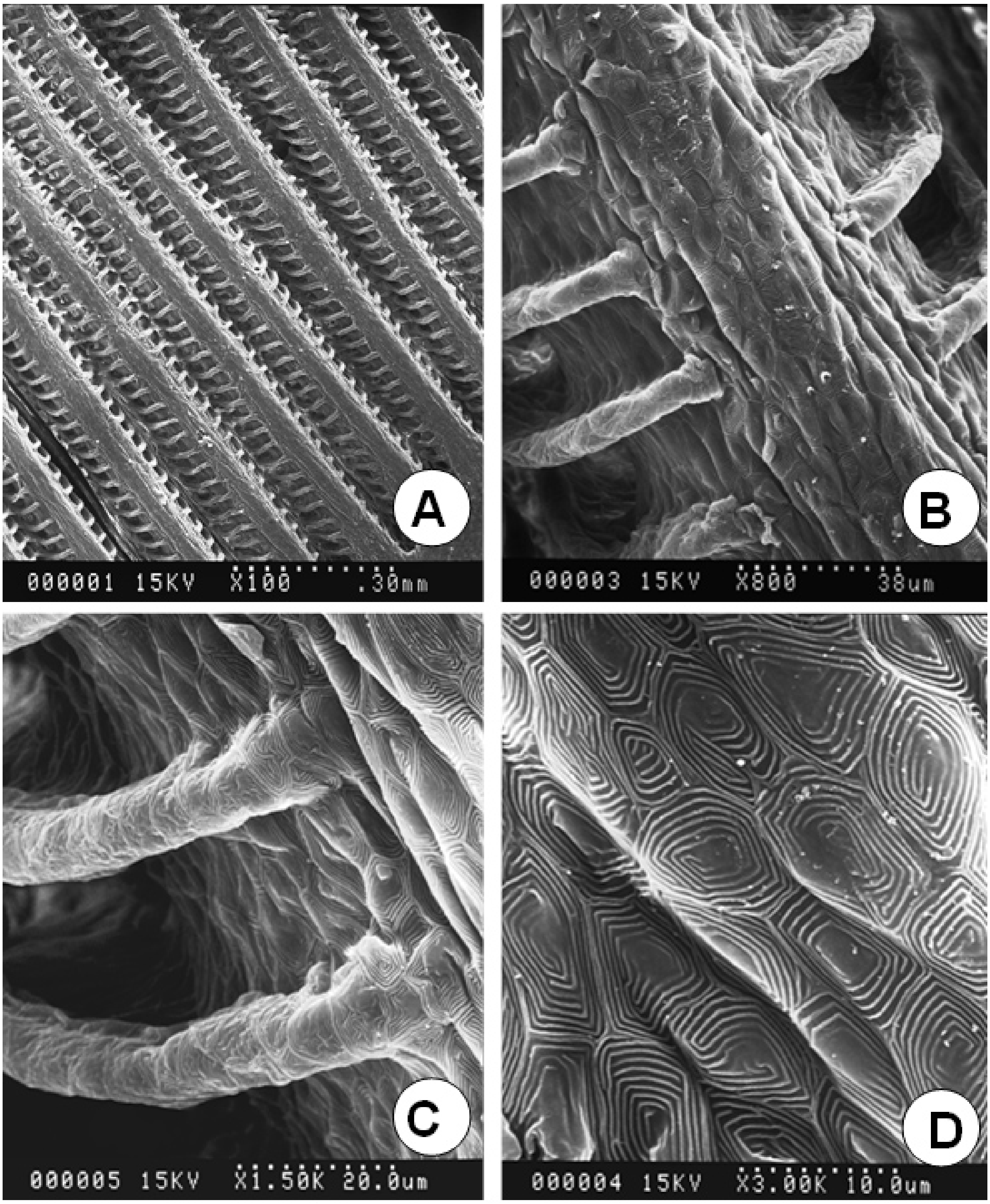


Figure 19. Scanning electron micrographs of gills showing normal appearance of primary filament, secondary lamellae and patterns of microridges.

5.11.2 Liver

Liver was a light brown organ, which extended the length of the abdomen and was closely applied to the other viscera. It was a compound organ in the form of a hepatopancreas. No recognizable changes were observed in the histology of the hepatocyte in each group (Fig. 20). The liver is the main organ which helps in the process of detoxification and deamination. Fish liver had a far less tendency for disposition of the hepatocytes in cords or lobules. Sinusoids, which are irregularly distributed between the polygonal hepatocytes, are fewer in number and are lined by endothelial cells with very prominent nuclei.

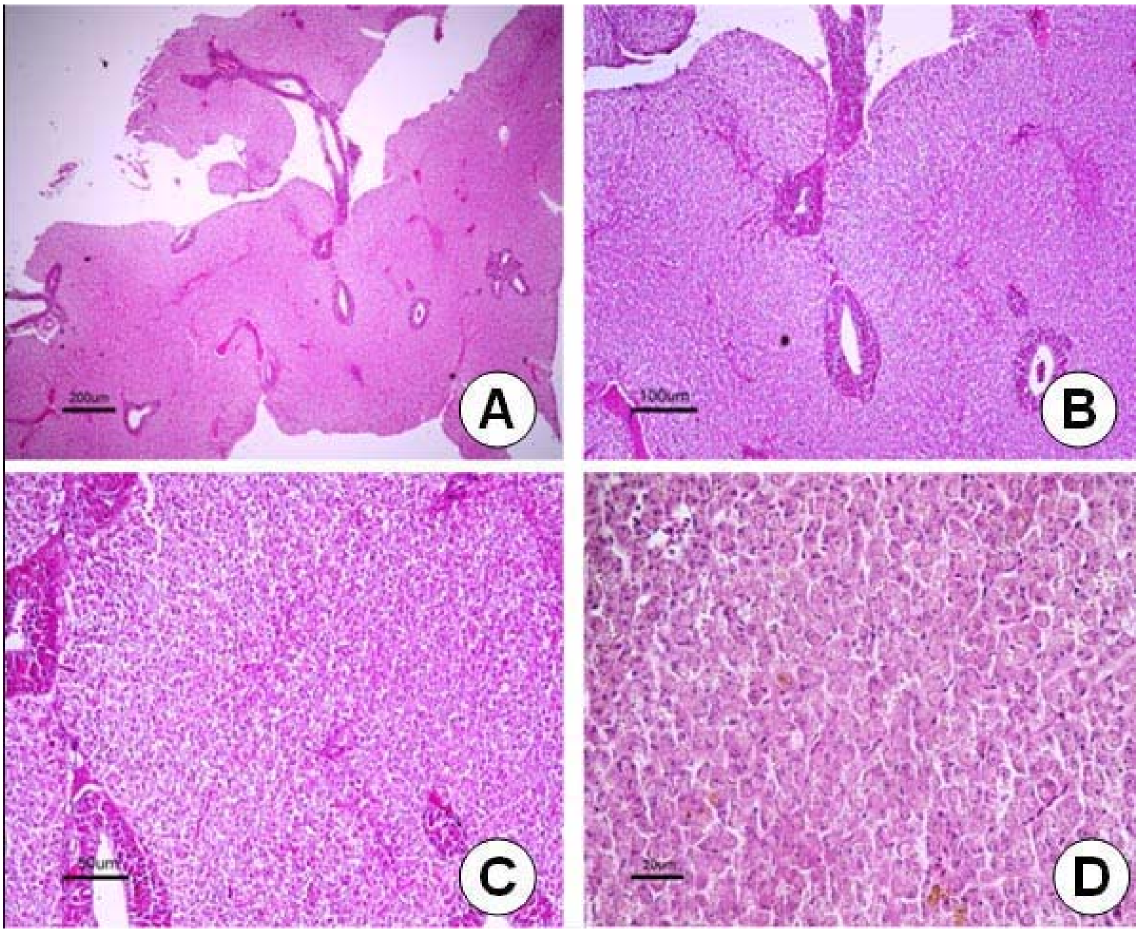


Figure 20. Light micrographs of transverse section of liver showing normal appearance of polygonal hepatocyte and sinusoids. Note: A: bar 200µm, B: bar 100µm, C: bar 50µm, and D: bar 20µm.

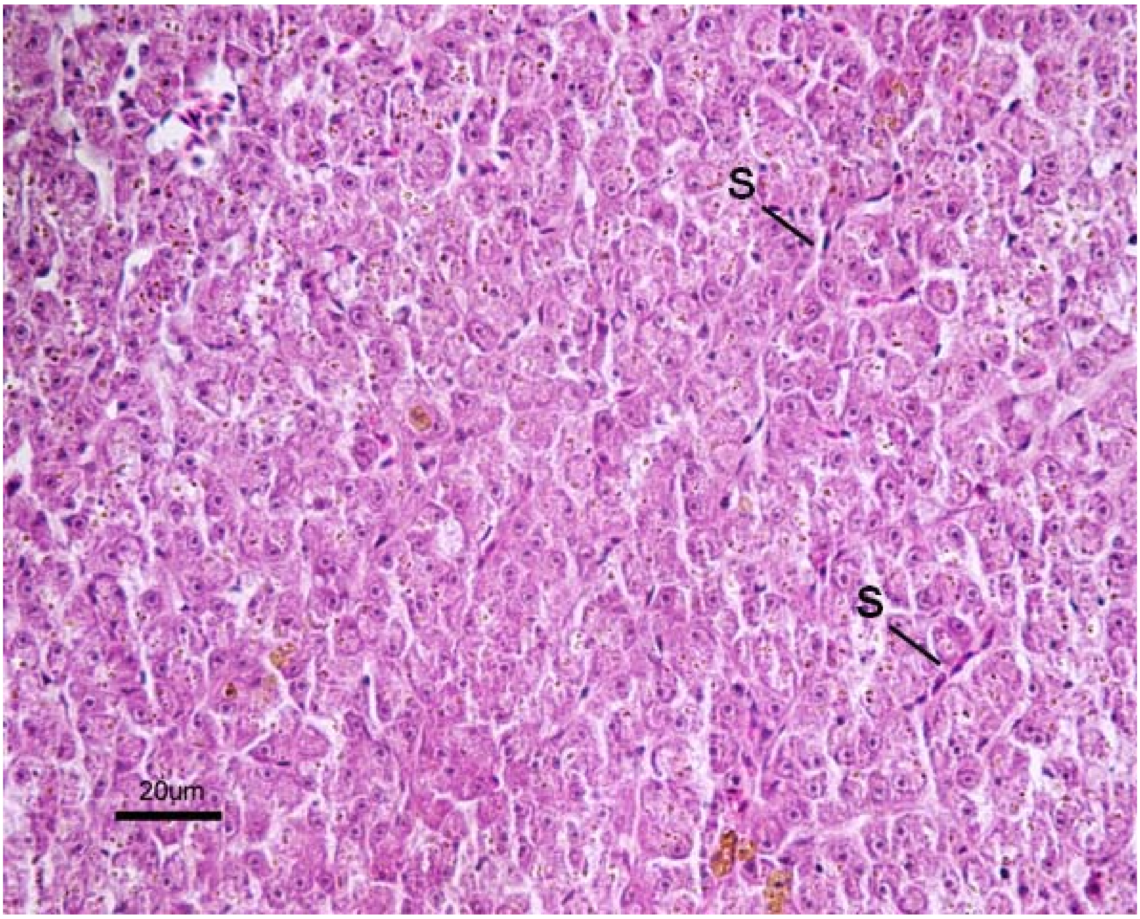


Figure 21. High magnification of transverse section of liver showing normal appearance of polygonal hepatocyte and sinusoids. Note: bar 20µm, S=Sinusoid.

The protective effect of pomegranate flower extract on ferric nitrilotriacetate induced hepatic injury in mice was apparent by histopathological examination (Kaur et al., 2006). In ferric nitrilotriacetate treated mice, there was an extensive loss of hepatic architecture and a large amount of fatty degeneration, necrosis and hemorrhage. In case of mice pretreated with pomegranate flower extract followed by ferric nitrilotriacetate, the liver retained almost normal hepatic architecture, with much less pathological changes (Kaur et al., 2006).

5.11.3 Kidney

In fish, the kidney is a mixed organ comprising hematopoietic, reticuloendothelial, endocrine and excretory elements. It performs an important function related to electrolyte and water balance and the maintenance of a stable internal environment. It was usually located in a retroperitoneal position up against the ventral aspect of the vertebral column. It was a dark brown or black organ normally extending the length of the body cavity. It was divided two parts; anterior or head kidney that composed of hemopoietic elements and posterior or tail or excretory kidney. There were no pathologic abnormalities in the renal corpuscles and tubules in each group (Figs. 22-23). The nephron of the typical freshwater fish was composed of a well-vascularized glomerulus, and renal tubule which were proximal, distal segments, and collecting duct system. The proximal tubule was covered by cuboidal or low columnar epithelial cells with round basal nuclei and brush border.

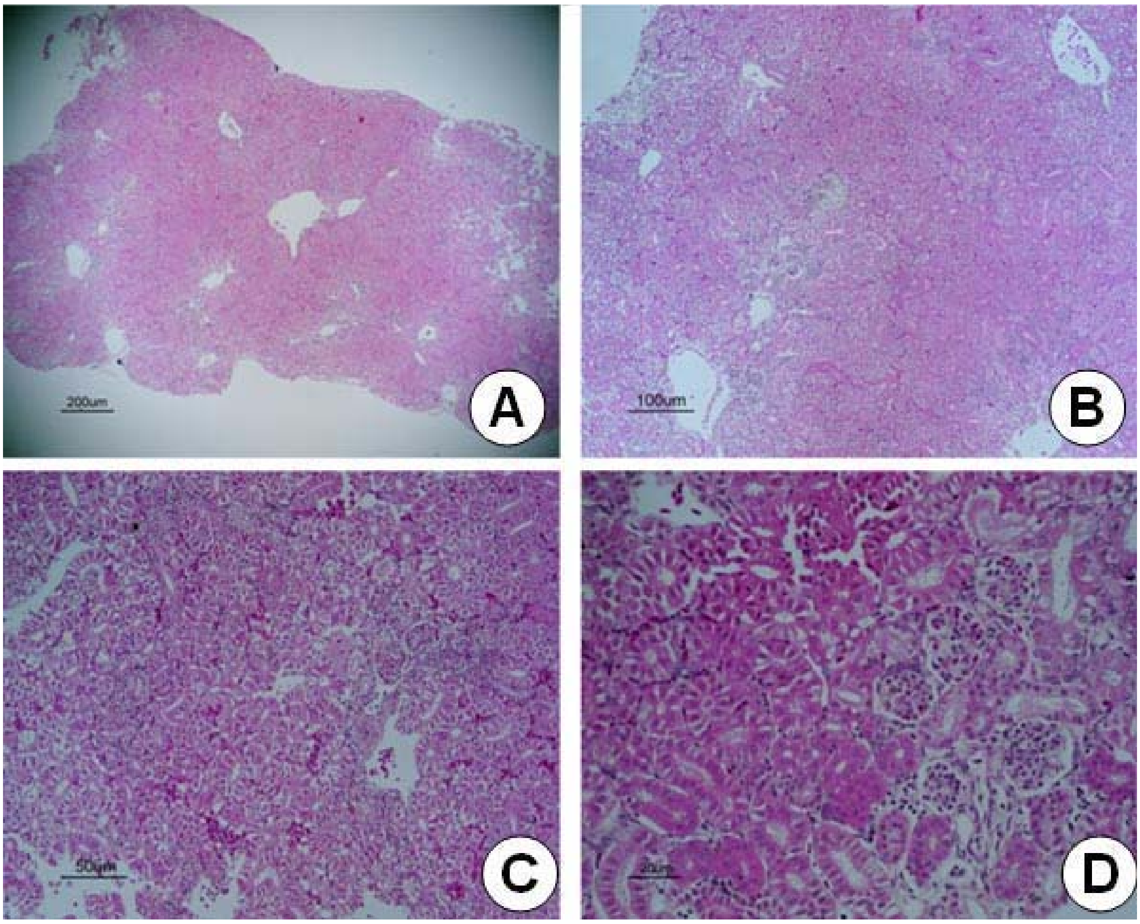


Figure 22. Light micrographs of transverse section of kidney showing normal appearance of renal corpuscles and tubules. Note: A: bar 200µm, B: bar 100µm, C: bar 50µm, and D: bar 20µm.

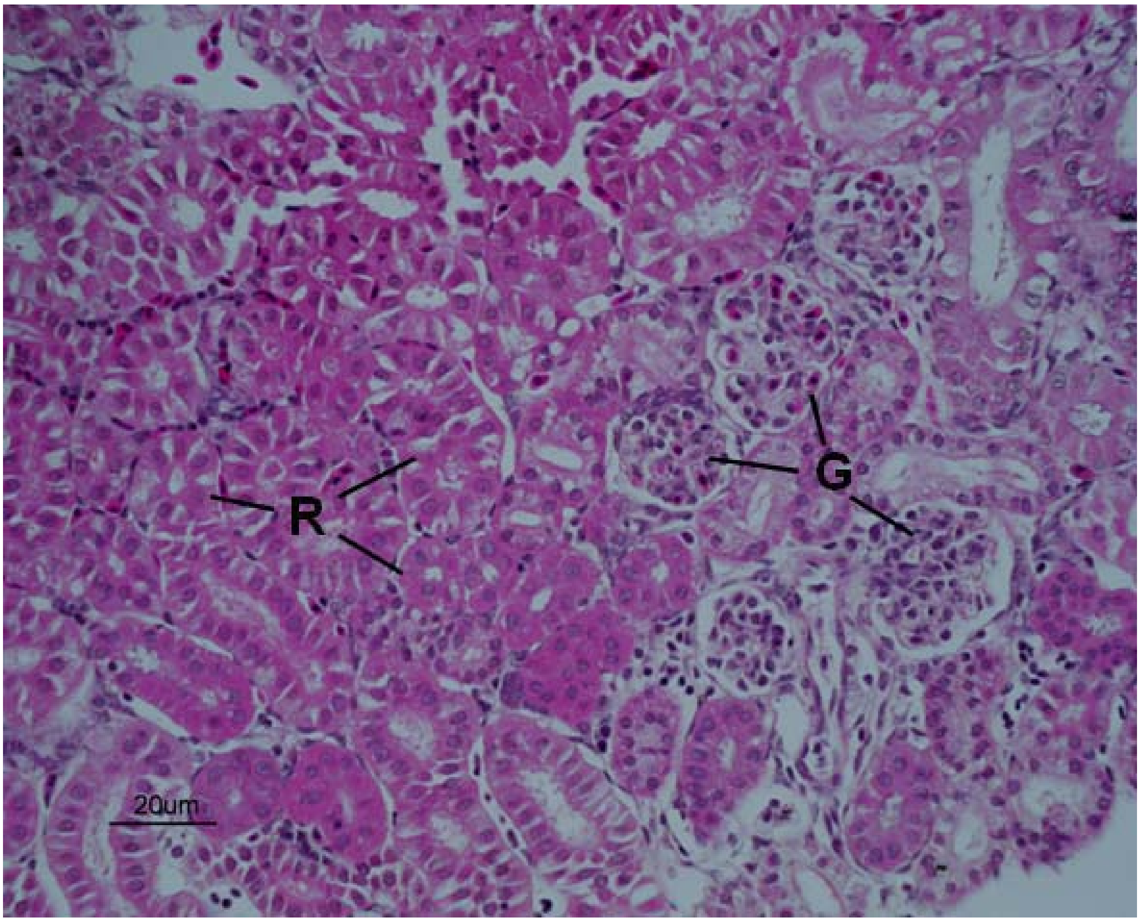


Figure 23. High magnification of transverse section of kidney showing normal appearance of renal corpuscles and tubules. Note: bar 20μm, G=Glomerulus, R=Renal tubule.

CHAPTER VII
CONCLUSION

Thailand is considered one of the countries, which bring medical plants into use, and sometimes they refer to it as "Thai Traditional Medicine (TTM)" which can be used to treat humans and animal diseases. The researchers have reported the efficacy of ascorbic acid in reducing genotoxicity in *O. niloticus* induced by lead (Jiraungkoorskul *et al.,* 2008); *Poronotus triacanthus* induced by copper (Jiraungkoorskul *et al.,* 2007a); and *P. altus* induced by cadmium (Jiraungkoorskul *et al.,* 2007b), using the micronucleus and nuclear abnormality tests. It has also been reported the efficacy of ascorbic acid in reducing the histopathological alterations in fish after cadmium exposure (Jiraungkoorskul *et al.,* 2006). Palipoch and college (2011) reported that *T. laurifolia* leaf extract-supplemented fish food was able to reduce lead concentration specifically in liver and muscle of *O. niloticus* upon $Pb(NO_3)_2$ exposure. It protects the lead-induced oxidative stress by reducing lipid peroxidation and increasing the reduced glutathione level and the activities of catalase, glutathione reductase and glutathione peroxidase in gill, kidney and liver of *O. niloticus*. Moreover, it was able to reduce gill pathology in lead-exposed *O. niloticus*. This data indicated that *T. laurifolia* leaf extract-supplemented fish food was able to protect *O. niloticus* against lead toxicity and able to apply as a fish food supplementation in *O. niloticus*. Jiraungkoorskul and coworker (2011) evaluated the efficiency of *P. guajava* on reducing ferric nitrilotriacetate (Fe-NTA) toxicity in *P. altus* via the histopathology analysis. The gills were observed epithelial lifting, lamellar cell hyperplasia. Blood congestion was seen in sinusoids and hepatocytes necroses were also observed. Renal tubular swelling and necrosis were seen. Some areas were found hemosiderin pigment accumulation. Fish with guava pre-treatment showed slightly alteration when compare those of non guava pre-treatment group. The results suggested that *P. guajava* leaf extract pre-obtained may play an important role in the reduction of Fe-NTA toxicity in fish.

The present research was first to investigate the effect of intraperitoneal injection of various doses of plant leaves extract at 48 hours and 7 days; second to investigate the effect of dietary supplement of various doses of dry plant leaves at 1 and 2 months in the term of: growth rate; macrophage studies; nitroblue tetra zolium test; lysozyme activity test; micronucleus and nuclear abnormality tests; hematology studies; liver function test; and histopathological and ultrastructural studies in gills, liver and kidney. The results had shown the time-dose dependent, the higher dose the more effect and the longer time the higher response. The administration route via the food supplement was the greater than the intraperitonial injection. The highest efficiency of leaf extract was *T. laurifolia* follow by *P. granatum* and *P. guajava.* In conclusion, the results presented in this study show no harmful of these leaf extracts.

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APPENDIX

Natt and Herrick's diluting fluid

NaCl	3.88 g
Na_2S0_4	2.50 g
Na_2HPO_4 $12H_2O$	2.91 g
KH_2PO_4	0.25 g
Formalin (37%)	7.50 ml
Methyl violet 2b	0.10 g

Preservative solution (10% Formalin)

1. Formalin (40% Formaldehyde)	1000 ml
2. Distilled water	9000 ml
3. Di-sodium-hydrogen-phosphate, anhydrous	65 g
4. Sodium-di-hydrogen-phosphate, monohydrate	40 g

Haris's haematoxylin Stain

1. Aluminium alum	40 g
2. Distilled water	400 ml
3. Haematoxylin	2 g
4. Absolute alcohol	20 ml
5. Mercuric Oxide	1 g
6. Glacial Acetic Acid	5 ml

Eosin Stain

1. 1% Eosin in water	100 ml
2. 1% Phloxin-B in water	20 ml
3. 95% alcohol	780 ml
4. Glacial Acetic Acid	4 ml

Schedule for histopathological process

No	Method	Duration	
1	Dissection		**First day**: Removal and fixing tissues in 10% buffered formaldehyde 24 hours.
2	10% buffered formaldehyde	24 hours	
3	70% alcohol	24 hours	**Second day**: Washing out the fixative
4	80% alcohol	40 minutes	**Third day**: Dehydration
5	95% alcohol	40 minutes	
6	95% alcohol	40 minutes	
7	Absolute alcohol	40 minutes	
8	Absolute alcohol	40 minutes	
9	Xylene	1 hour	
10	Xylene	1 hour	
11	Paraffin	40 minutes	
12	Paraffin	40 minutes	
13	Embedding		
14	Sectioning	24 hours	**Fourth day**: Sectioning, spreading on glass slides, drying overnight
15	Staining		**Fifth day**: Staining, mounting, and examination
16	Examination		

Schedule for staining sections

No	Method	Duration
1	Xylene (I)	5 minutes
2	Xylene (II)	5 minutes
3	Xylene (III)	5 minutes
4	Absolute alcohol (I)	3 minutes
5	Absolute alcohol (II)	3 minutes
6	Absolute alcohol (III)	3 minutes
7	95% alcohol	3 minutes
8	80% alcohol	3 minutes
9	70% alcohol	3 minutes
10	Running water	7 minutes
11	Hematoxylin	7 minutes
12	Running water	7 minutes
13	Eosin	3 minutes
14	95% alcohol (I)	3 minutes
15	95% alcohol (II)	3 minutes
16	95% alcohol (III)	3 minutes
17	Absolute alcohol (I)	3 minutes
18	Absolute alcohol (II)	3 minutes
19	Absolute alcohol (III)	3 minutes
20	Xylene (I)	5 minutes
21	Xylene (II)	5 minutes
22	Xylene (III)	5 minutes
23	Mounting	
24	Examination	

Schedule for scanning electron microscope procedures

No	Method	Duration
1	4% glutaraldehyde in 0.1M PBS pH 7.4	Prefix 2 h at 4°C
2	0.1M PBS pH 7.4	Wash 3 times at 4°C
3	1% osmium tetroxide in 0.1M PBS	Postfix 1 h at 4°C
4	Filtered water (cold)	Wash 3 times at 4°C
5	50% alcohol	Wash 2 times x 15 min at 4°C
6	70% alcohol	Wash 2 times x 15 min at 4°C
7	80% alcohol	Wash 2 times x 15 min at 4°C
8	90% alcohol	Wash 2 times x 15 min at 4°C
9	95% alcohol	Wash 2 times x 15 min at 4°C
10	100% alcohol	Wash 3 times x 15 min at 4°C
11	100% alcohol	Wash 3 times x 15 min at RT
12	Critical point drying	
13	Mounted and coated	
14	Examination	

APPENDIX
LIST OF MANUSCRIPTS

This research can be published in the international journals as following:

1. Sarawoot Palipoch, **Wannee Jiraungkoorskul*,** Tawewan Tansatit, Narin Preyavichyapugdee, Wipaphorn Jaikua, Piya Kosai. Effect of *Thunbergia laurifolia* (Linn.) Leaf extract dietary supplement against lead toxicity in Nile Tilapia (*Oreochromis niloticus*). World Journal of Fish and Marine Sciences 2011; 3(1): 1-9.
2. Sarawoot Palipoch, **Wannee Jiraungkoorskul*,** Tawewan Tansatit, Narin Preyavichyapugdee, Wipaphorn Jaikua, Piya Kosai. Protective efficiency of *Thunbergia laurifolia* leaf extract against lead (II) nitrate-induced toxicity in *Oreochromis niloticus*. Journal of Medicinal Plants Research 2011; 5(5): 719-728. Impact factor=0.590
3. **Wannee Jiraungkoorskul*,** Rachen Singhakumar, Kanitta Jiraungkoorskul, Piya Kosai. Dietary *Psidium guajava* supplementation reducing ferric nitrilotriacetate toxicity in *Puntius altus.* Research Journal of Medicinal Plant 2011, doi: 10.3923/rjmp.2011 (online first)

From this research result, the authors have applied to study the efficacy of another substance in reducing heavy metals toxicity. It can be published in the following journal:

1. Piyaporn Singhadach, **Wannee Jiraungkoorskul*,** Tawewan Tansatit, Piya Kosai, Chananya Ariyasrijit. Calcium pre-exposure reducing histopathological alteration in Nile Tilapia (*Oreochromis niloticus*) after lead exposure. Journal of Fisheries and Aquatic Science 2009; 4(5): 228-237.
2. Piya Kosai, **Wannee Jiraungkoorskul*,** Tawan Thammasunthorn, Kanitta Jiraungkoorskul. Reduction of copper-induced histopathological alterations by calcium exposure in Nile tilapia (*Oreochromis niloticus)*. Toxicology Mechanisms and Methods 2009, July-September vol. 19: No.6-7, 461-467. Impact factor=0.426
3. Piya Kosai, **Wannee Jiraungkoorskul*,** Apinya Synsatayakul, Kanitta Jiraungkoorskul. Efficacy of calcium reducing lead toxicity in hematology of *Oreochromis niloticus.* Journal of Fisheries and Aquatic Science 2011; 6(3): 346-355.

4. Piya Kosai, **Wannee Jiraungkoorskul***, Chaivira Sachamahithinant, Kanitta Jiraungkoorskul. Induction of testis-ova in Nile tilapia *(Oreochromis niloticus*) exposed to 17β-estradiol. Natural Science 2011; 3: 277-233.

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Effect of *Thunbergia laurifolia* (Linn) Leaf Extract Dietary Supplement Against Lead Toxicity in Nile Tilapia (*Oreochromis niloticus*)

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Abstract: This experiment was carried out to evaluate protective effects of *Thunbergia laurifolia* (Linn.) leaf extract against lead toxicity in *Oreochromis niloticus* (L.). Fish (n=120) were divided into 6 groups: groups 2, 4 and 6 treated with 45 mg L^{-1} of lead nitrate, whereas groups 1, 3 and 5 no exposed. Fish were fed basal fish food (groups 1 and 2), fish food supplemented with 0.2 mg of *T. laurifolia* leaf extract $g^{-1}$of fish food (groups 3 and 4) and 2 mg $g^{-1}$of fish food (groups 5 and 6). After 28 days of treatment, lead concentration, oxidative stress biomarkers and gill ultrastructure were investigated. Fish food supplemented with *T. laurifolia* leaf extract was able to reduce levels of lead in liver and muscle. Lead caused oxidative stress by reducing the content of reduced glutathione (GSH) and the activities of catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) and increasing lipid peroxidation (LPO). Fish food supplemented *T. laurifolia* leaf extracts were able to increase the activities of intrinsic antioxidant and able to deplete LPO in gill, kidney and liver upon lead exposure. Surprisingly, it was able to reduce gill pathology in lead-exposed *O. niloticus*.

Key words: *Thunbergia laurifolia* • Antioxidant • Lipid peroxidation • Fish • *Oreochromis niloticus*

INTRODUCTION

Heavy metals are metallic elements which present in both natural and contaminated environments. They show the ability to accumulate in long times resulting in the deleterious effects in various organs of fish and consequently, important risk for human safety [1]. Lead is one of the toxic heavy metals which distributed into aquatic environment by various sources such as mining and refining of ores. It has pointed to either elevated lipid peroxidation (LPO) or decreased intrinsic antioxidant defense in various tissues of animals [2-4]. It inhibits the intrinsic antioxidants such as reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT) [5,6].

Fish are a good model for assessing aquatic ecosystem health and in toxicology [7]. *Oreochromis niloticus* (L.) is an important economic cultured fish in worldwide [8]. It grows fast and is characterized by easily breeding. It also provides a high quality food source to humans. From a previous study performed in *O. niloticus* exposed for one month to sediments from Mae Klong River, Samutsongkram province, Thailand which contained elevated levels of heavy metals, lead and chromium, Peebua *et al*. [9] demonstrated abnormalities of gill, liver and kidney.

Thunbergia laurifolia (Linn.), a vine distributed in Southeast Asia, is a shrub with small oblong or ovate leaves. In Thai traditional medicine, *T. laurifolia* used as anti-inflammatory, anti-pyretic and anti-bacterial agents.

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It is also used as anti-dote against insecticides and toxic chemicals. Crude water extract of *T. laurifolia* leaf significantly increased K^+-stimulated dopamine release from rat striatal slices and synergized with amphetamine on K^+-stimulated dopamine release comparing with amphetamine alone [10]. *Thunbergia laurifolia* leaf extract increased up to 2-3 folds viability of primary cultures of rat hepatocyte upon ethanol treatment. It is promoted rat liver recovery decreasing the severity of rat liver injury and normalizing the levels of hepatic triglyceride, alanine aminotransferase and aspartate aminotransferase [11]. It is also exhibited strong anti-mutagenic activity [12]. In mice, co-treatment with aqueous *T. laurifolia* leaf extract at 100 mg kg^{-1} or 200 mg kg^{-1} body weight was found to restore the levels of caspase-3 activity and maintain total antioxidant capacity and antioxidant enzymes in the brain [13]. In the present study, we aimed to evaluate protect effect of *T. laurifolia* leaf extract-supplemented fish food against lead toxicity in *O. niloticus* for applied the extract as a fish food supplementation in the future.

MATERIALS AND METHODS

Plant Extraction: *Thunbergia laurifolia* leaves were collected in Ratchaburi province, Thailand and identified by at the Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand. Voucher specimen (Palipoch 001) was deposited at Suan Luang Rama IX herbarium, Bangkok. Fresh leaves of *T. laurifolia* were washed several times in running water, dried at 60°C for 48 h in the hot air oven (Thelco®, GCA/Precision scientific, USA) and made powder using a blender (Otto, Thailand). Ten grams of leaf powder extracted with 100 mL of 50% ethanol were incubated on shaker (Germmy Orbit Shaker model VRN-480, Taiwan) at 250 rpm and room temperature for 48 h and then centrifuged at 4,000 rpm for 10 min. Leaf extract was concentrated and dried under reduced pressure in a rotary evaporator (Rotavapor® R-200, BUCHI, USA). Samples were stored at -20°C until used.

Supplementation of Fish Food: The fish foods were grounded in blender, hydrated with distilled water 0.7 mL g^{-1} of fish food, mixed with leaf extract (0.2 and 2 mg g^{-1} of fish food) and extruded through a minced-meat processing machine. The mixture was break into small pellet and air-dried at 70°C for 48 h in hot air oven. Fish food was stored at room temperature.

Fish Treatments and Specimen Collections: *Oreochromis niloticus* (L.) from Chacheongsao province, Thailand with similar size was used. The average body weight was 54.18 g. The fish were kept in glass aquaria (50 x 50 x 120 cm) with continuous air and filled with 200 L of dechlorinated tap water. The temperature and pH of water were monitored. Light-dark cycles (16:8 h) were applied to the fish facilities. Fish were fed twice a day (2% of body weight per day) with commercial fish food containing 28% proteins, 4% fibers and 3% fats (Charoen Pokphand Group, Bangkok, Thailand). Fish were acclimatized and closely cared under laboratory condition for 28 days before performing the experiment.

The 96 h LC_{50} value of Nile tilapia (182.12 mg L^{-1}) exposed to lead nitrate [$Pb(NO_3)_2$] was determined in our laboratory [14]. In this study, fish were exposed to 45 mg L^{-1} of waterborne $Pb(NO_3)_2$ which corresponded to 25% of the 96 h LC_{50} Fish (n = 120) were divided into 6 groups and treated as following:

Group 1: Fed basal fish food
Group 2: Treated with 45 mg L^{-1} of $Pb(NO_3)_2$ and fed basal fish food
Group 3: Fed fish food supplemented with 0.2 mg of leaf extract g^{-1} of fish food
Group 4: Treated with 45 mg L^{-1} of $Pb(NO_3)_2$ and fed fish food supplemented with 0.2 mg of leaf extract g^{-1} of fish food
Group 5: Fed fish food supplemented with 2 mg of leaf extract g^{-1} of fish food Group 6: treated with 45 mg L^{-1} of $Pb(NO_3)_2$ and fed fish food supplemented with 2 mg of leaf extract g^{-1} of fish food

After 28 days of treatment, fish were euthanized with tricaine methane sulphonate (0.2 g L^{-1} of distilled water) by anesthetizing overdose. Operculum and peritoneal cavity were opened and collected the organs including gill, kidney, liver and muscle.

Determination of Lead Concentrations: For determining the concentration of lead, gill, kidney, liver and muscle were placed in a test tube and transferred to hot air oven at 65°C for 48 h. After adding 6 ml of solution containing nitric acid and perchloric acid (2:1) at 120°C for 3 h, small pieces of tissue samples were filtrated through a 0.45 ìm Millipore filter (Whatman, NJ, USA). The solution was then analyzed using the GBC 932 plus flame atomic absorption spectrophotometer [15].

Preparation of Post-mitochondrial Supernatant (PMS): Gill, kidney and liver were homogenized in chilled 0.1 M Phosphate buffer saline (PBS, pH 7.4) containing 1.17% potassium chloride using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 g, 4°C for 5 min to separate the nuclear debris. The supernatant was centrifuged at 10,500 g, 4°C for 30 min to obtain PMS.

GSH Level: GSH content was assayed by the method of Jollow *et al*. [16]. An aliquot of 1.0 mL of 10% PMS in distilled water was precipitated with 1.0 mL of 4% sulphosalicylic acid. The samples were kept at 4°C for 1 h and centrifuged at 1,200 g, 4°C for 15 min. The assay mixture contained 0.1 mL filtered aliquot, 2.7 mL of 0.1 M PBS (pH 7.4) and 0.2 mL of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, 40 mg in 10 mL of 0.1 M PBS, pH 7.4) in a total volume of 3 mL. The yellow color was measured at 412 nm.

CAT Activity: CAT activity was assayed by the method of Claiborne [17]. The mixture consisted of 1.95 mL of 0.05 M PBS (pH 7.0), 1.0 mL of 0.019 M H_2O_2 and 0.05 mL of 10% PMS. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed min^{-1}mg^{-1} protein.

GR Activity: GR activity was assayed according to Mohandas *et al*. [18]. The assay system consisted of 1.65 mL of 0.1 M PBS (pH 7.6), 0.1 mL of 0.5 mM EDTA, 0.05 mL of 1 mM oxidized glutathione, 0.1 mL of 0.1 mM NADPH and 0.1 mL of PMS in a total volume of 2.0 mL. The enzyme activity was measured by measuring disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

GPx Activity: GPx activity was assayed by the method of Mohandas *et al*. [18]. Reaction mixture consisted of 1.44 mL of 0.05 M PBS (pH 7.0), 0.1 mL of 1 mM EDTA, 0.1 mM sodium azide, 0.05 mL of GR (1 U ml^{-1}), 0.1 mL of 1 mM GSH, 0.1 mL of 2 mM NADPH, 0.01 mL of 0.25 mM H_2O_2 and 0.1 mL of 10% PMS in a total volume of 2 mL. Disappearance of NADPH was recorded at 340 nm. Enzyme activity was calculated as nmol NADP reduced min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1}cm^{-1}.

Protein Concentration: Protein content was estimated by Bradford assay (Sigma, USA) by using bovine serum albumin as protein standard.

LPO: LPO was measured by the procedure of Wright *et al*. [19]. The reaction mixture, in a total volume of 1.0 mL, contained 0.58 mL of 0.1 M PBS pH 7.4, 0.2 mL of 10% PMS, 0.2 mL of 100 mM ascorbic acid and 0.02 mL of 100 mM ferric chloride was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 mL of 10% trichloroacetic acid. Then, 1.0 mL of 0.67% TBA was added and all the tubes were placed in a boiling water bath for 20 min. Malondialdehyde (MDA) formed was measured at 535 nm. The rate of LPO was calculated as nmol of thiobarbituric reactive substances (TBARS) formed h^{-1} g^{-1} of tissue using a molar extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1}.

Determination of Gill Ultrastructure: Small pieces of gill were fixed with 4% glutaraldehyde in 0.1M PBS pH 7.4 for 24 h at 4°C, post-fixed in 1% osmium tetroxide for 1 h, dehydrated through series of alcohol and dried in a dryer machine (Hitachi HCP-2, Japan) using liquid carbon dioxide as a transitional medium. After drying, gill was mounted on aluminium stubs and coated with platinum and paladium in an ion-sputtering apparatus (Hitachi E-102, Japan) for 6 min at 10-15 mA. Then, gill was examined under the scanning electron microscope with a digital camera (Hitachi S-2500, Japan).

Pathological alterations of gill were evaluated semi-quantitatively by using modified method of Schwaiger *et al*. [20] were ranking from (-) no pathological alterations, (+) mild, (++) moderate and (+++) severe pathological alterations depend on the degree and extent of the alterations.

Statistical Analysis: All data were expressed as mean ± SD. Analysis of Variance (ANOVA) with Least Significant Difference (LSD) post-hoc test was performed for differences between each group. Significance of differences was considered at 5% level (P < 0.05).

RESULTS

Lead Concentrations: As shown in fig. 1, fish belonging to the group treated with $Pb(NO_3)_2$ and receiving not leaf extract (group 2) elicited the highest lead accumulation in kidney following gill, liver and muscle, respectively. Fish which were exposed to $Pb(NO_3)_2$ and fed fish food supplemented with 0.2 mg of *T. laurifolia* leaf extract g^{-1}of fish food (group 4) and 2 mg g^{-1}of fish food (group 6) were able to significantly reduce (P<0.05) lead concentration in liver and muscle, whereas in gill and kidney showed no significant difference of lead

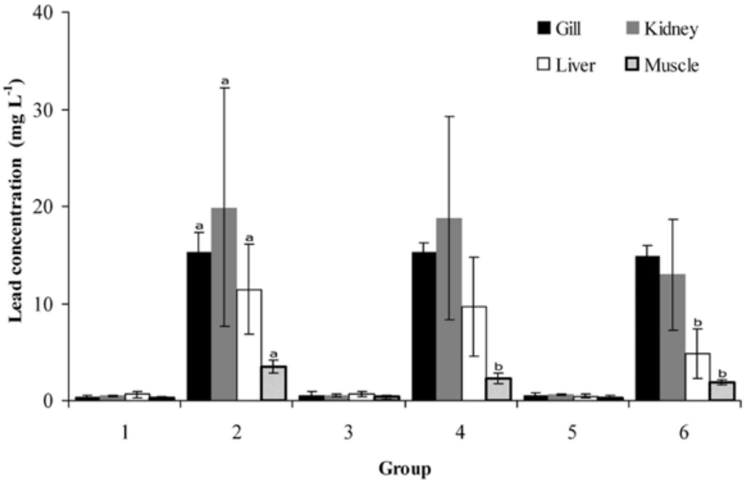

Fig. 1: Lead concentration in gill, kidney, liver and muscle of *O. niloticus*. The mean difference significant at (P>0.05) compared with fish belonging to group 1 (a) and group 2 (b).
Note: Group 1: Basal fish food
Group 2: $Pb(NO_3)_2$ + basal fish food
Group 3: 0.2 mg of leaf extract g•^{1} of fish food
Group 4: $Pb(NO_3)_2$ + 0.2 mg of leaf extract g•^{1} of fish food
Group 5: 2 mg of leaf extract g•^{1} of fish food
Group 6: $Pb(NO_3)_2$ + 2 mg of leaf extract g•^{1} of fish food

Table 1: LPO and the activities of antioxidants in gill, kidney and liver of *O. niloticus*. The mean difference significant at (P > 0.05) compared with fish belonging to group 1 (a) and group 2 (b).

		Group					
Parameter	Organ	1	2	3	4	5	6
LPO	Gill	2.48 ± 0.10	5.35 ± 0.69^{a}	2.12 ± 0.34	3.12 ± 0.19^{b}	1.40 ± 0.20^{a}	1.41 ± 0.05^{b}
(nmol TBARS	Kidney	2.13 ± 0.25	6.86 ± 0.21^{a}	1.40 ± 0.13^{a}	2.59 ± 0.26^{b}	2.26 ± 0.24	1.44 ± 0.34^{b}
formed h•^{1}g•^{1} tissue)	Liver	7.85 ± 0.12	12.64 ± 1.46^{a}	11.34 ± 1.65	8.41 ± 0.51^{b}	5.71 ± 0.39	7.21 ± 0.35^{b}
GSH	Gill	46.67 ± 3.21	28.33 ± 4.16^{a}	49.33 ± 0.58	32.00 ± 1.73	50.67 ± 1.53	43.00 ± 2.00^{b}
(mM)	Kidney	49.33 ± 5.86	32.00 ± 3.00^{a}	47.67 ± 2.89	33.00 ± 3.61	51.00 ± 5.29	40.33 ± 4.16^{b}
	Liver	68.33 ± 3.60	43.67 ± 3.06^{a}	70.67 ± 7.51	46.33 ± 8.08	73.67 ± 6.66	55.00 ± 3.46^{b}
CAT	Gill	189.23 ± 4.51	101.67 ± 5.86^{a}	192.33 ± 9.29	149.00 ± 10.58^{b}	197.67 ± 21.57	176.33 ± 13.20^{b}
(nmol H_2O_2 consumed	Kidney	101.33 ± 2.89	57.33 ± 3.06^{a}	113.00 ± 14.11	68.67 ± 8.96	150.67 ± 22.90^{a}	112.00 ± 6.00^{b}
min•^{1}mg•^{1} protein)	Liver	248.33 ± 7.51	112.67 ± 4.04^{a}	257.67 ± 6.03	143.00 ± 9.85^{b}	264.33 ± 10.12^{a}	221.33 ± 7.09^{b}
GR	Gill	111.33 ± 10.02	81.33 ± 2.08^{a}	118.67 ± 2.08	88.67 ± 9.07	120.67 ± 1.53	96.00 ± 2.65
(nmol NADPH oxidized	Kidney	81.33 ± 8.02	56.67 ± 4.73^{a}	81.67 ± 11.37	57.00 ± 7.94	86.33 ± 3.06	76.33 ± 7.51^{b}
min•^{1}mg•^{1} protein)	Liver	164.33 ± 6.11	131.00 ± 4.00^{a}	165.33 ± 6.51	133.33 ± 8.96	172.33 ± 3.06	145.33 ± 5.86^{b}
Gpx	Gill	122.67 ± 10.02	130.00 ± 6.24	126.00 ± 4.58	132.67 ± 3.21	133.67 ± 9.45	142.00 ± 4.58^{b}
(nmol NADP reduced	Kidney	99.67 ± 4.32	73.00 ± 6.00^{a}	100.33 ± 8.33	74.67 ± 11.24	107.67 ± 3.21	86.33 ± 10.50^{b}
min•^{1}mg•^{1} protein)	Liver	171.33 ± 2.52	189.00 ± 4.00^{a}	173.67 ± 13.32	193.00 ± 8.72	177.33 ± 7.02	191.00 ± 3.91

Note: Group 1: Basal fish food
Group 2: $Pb(NO_3)_2$ + basal fish food
Group 3: 0.2 mg of leaf extract g•^{1} of fish food
Group 4: $Pb(NO_3)_2$ + 0.2 mg of leaf extract g•^{1} of fish food
Group 5: 2 mg of leaf extract g•^{1} of fish food
Group 6: $Pb(NO_3)_2$ + 2 mg of leaf extract g•^{1} of fish food

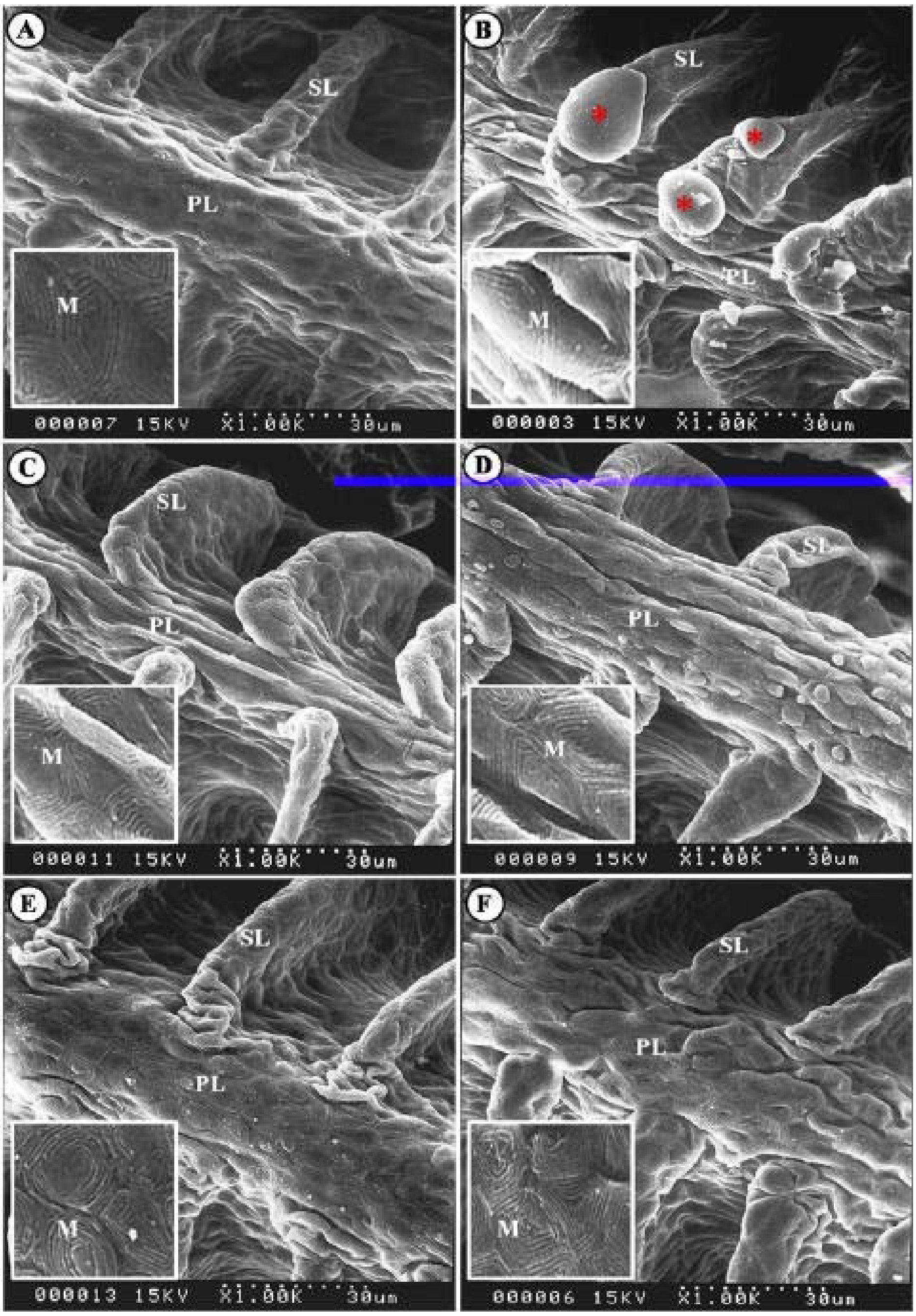

Fig. 2: Gill ultrastructure of *O. niloticus*. (A, C, D, E and F) indicated normal gill structure of primary lamellae (PL), secondary lamellae (SL) and microridges (M) of *O. niloticus* belonging to groups 1, 3, 4, 5 and 6, respectively; (B) demonstrated aneurisms in SL (red asterisk) of group 2.

Note: Group 1: Basal fish food

Group 2: $Pb(NO_3)_2$ + basal fish food

Group 3: 0.2 mg of leaf extract g^{-1} of fish food

Group 4: $Pb(NO_3)_2$ + 0.2 mg of leaf extract g^{-1} of fish food

Group 5: 2 mg of leaf extract g^{-1} of fish food

Group 6: $Pb(NO_3)_2$ + 2 mg of leaf extract g^{-1} of fish food

Table 2: Semi-quantitative scoring of gill lesions of *O. niloticus* where ranking from (-) no, (+) mild, (+ +) moderate and (+ + +) severe pathology.

	Group					
Lesion	1	2	3	4	5	6
Abnormal structure of secondary lamellae	-	+	-	+	-	-
Lamellar aneurism in secondary lamellae	-	+++	-	+	-	-
Thickness of the gill filament	-	+	-	-	-	-
Hypertrophy of secondary lamellae	-	++	-	+	-	-

Note: Group 1: Basal fish food

Group 2: $Pb(NO_3)_2$ + basal fish food

Group 3: 0.2 mg of leaf extract g• [1] of fish food

Group 4: $Pb(NO_3)_2$ + 0.2 mg of leaf extract g• [1] of fish food

Group 5: 2 mg of leaf extract g• [1] of fish food

Group 6: $Pb(NO_3)_2$ + 2 mg of leaf extract g• [1] of fish food

concentration compared with group 2. Fish belonging to group 6 significantly decreased (*P*<0.05) lead concentration in liver compared with group 2.

Effect of T. Laurifolia Leaf Extracts on Oxidative Stress Biomarkers: Oxidative stress biomarkers including GSH, CAT, GR, GPx and LPO in each group of *O. niloticus* were demonstrated in table 1, Fish belonging to group 2 significantly depleted (*P*<0.05) of the intrinsic antioxidant status and significantly elevated (*P*<0.05) LPO in gill, kidney and liver compared with group 1 which were no treated with $Pb(NO_3)_2$ and fed basal fish food.

In gill, comparing with group 2, fish belonging to groups 4 and 6 were able to significantly increase (*P*<0.05) the activity of CAT and to significantly reduce (*P*<0.05) LPO. They were able to increase the activity of GR, but the differences did not reach the significance level. Fish belonging to group 6 were able to significantly increase (*P*<0.05) the level of GSH and the activity of GPx.

In kidney, comparing with group 2, fish belonging to groups 6 were able to significantly increase (*P*<0.05) the level of GSH and the activities of CAT and GR and fish belonging to groups 4 and 6 significantly depleted (*P*<0.05) LPO. They increased the activity of GPx, but no differences were observed.

In liver, comparing with group 2, fish belonging to groups 4 and 6 were able to significantly increase (*P*<0.05) the activity of CAT and to significantly reduce (*P*<0.05) LPO. They were able to increase the activity of GPx, but no differences were detected. Fish belonging to group 6 were able to significantly increase (*P*<0.05) the level of GSH and the activity of GR.

Effect of *T. Laurifolia* Leaf Extracts on Gill Ultrastructure: Fish belonging to group 2 showed markedly pathological changes including abnormal structure of secondary lamellae, lamellar aneurism in secondary lamellae, thickness of the gill filament and hypertrophy of secondary lamellae. Surprisingly, fish belonging to groups 4 and 6 showed no or few pathological alterations observed on the gill epithelium of primary lamellae and secondary lamellae as well as groups 1, 3 and 5 (Fig. 2 and Table 2). Microridges of fish in all groups exhibited well-defined contours, concentric and long appearances.

DISCUSSION

Lead is a ubiquitous environmental metal which can induce a broad range of the physiological, biochemical and behavioral dysfunctions in fish [21,22]. Oxidative stress has been proposed as a possible pathogenesis of lead toxicity [23]. Previous studies reported that lead either decreased in antioxidant status such as CAT, GPx and GSH [24,25] or elevated LPO in lead-exposed animals [2,3,4,26]. Lead showed the inhibition of several enzymes having functional SH groups [27]. Reduced glutathione is a tripeptide containing cysteine that has a reactive SH group. Normally, GSH plays a key role in the cellular protection against oxidative stress by direct interaction of the SH group with reactive oxygen species (ROS) or involvement in the enzymatic detoxification reactions of ROS as a cofactor or a coenzyme [28]. Glutathione reductase possesses a disulfide at its active site [29] which was suggested as a lead target [2]. The inhibition of GR results in decreased GSH:GSSG ratio causing cells more susceptible to oxidative stress. On the other hand, GPx and CAT are metalloproteins which accomplish their antioxidant functions. Due to these enzymatic antioxidants depend on various essential trace elements for proper molecular structure and enzymatic activity, they are potential targets for lead toxicity [30].

Medicinal plant extracts are of gradually high interest for an alternative choice to treatment lead toxicity. In this experiment, *T. laurifolia* leaf extracts possess the chelating ability which demonstrated the depletion of lead levels in liver and muscle. Similarly to another plant, Zhang *et al*. [31] indicated that pre-germinated brown rice was able to decrease the lead accumulation in rats. Xia *et al*. [32] demonstrated *Smilax glabra* extract as chelating agent to reduce blood and tissue lead burden in rats. On the other hand, this experiment showed that *T. laurifolia* leaf extract- supplemented fish foods were not affected the levels of lead in gill and kidney. Agreement with previously reported of Tangpong and Satarug [13], they found that co-treatment with aqueous *T. laurifolia* leaf extract did not affect levels of lead in blood and brain of mice given lead in drinking water at 1 g L^{-1} for 8 weeks. This study demonstrated the protective regimen of *T. laurifolia* leaf extract-supplemented fish food by reducing LPO and increasing the intrinsic antioxidant status including GSH, CAT, GR and GPx in gill, kidney and liver of *O. niloticus* upon $Pb(NO_3)_2$ exposure. Similar results were illustrated in another extracts. *Smilax glabra* extract individually enhanced GSH content and co-treatment of *S. glabra* extract and meso-2,3-dimercaptosuccinic acid (DMSA) increased CAT activity and GSH level in brain, liver and kidney of lead-exposed rats [31]. The ethanol extract of *Aquailegia vulgaris* (L.) increased the level of GSH and improved the histological picture of liver and kidney of lead-treated rats [33]. Treatment with *Etlingera elatior* extract reduced lipid peroxides and protein carbonyl contents and increased antioxidant enzyme activities. Eventually, *E. elatior* extract was able to protect the lead acetate-induced bone marrow oxidative damage in rats [34].

From previous studies, 95% ethanol leaf extract of *T. laurifolia* was found to be good sources of chlorophyll derivatives, whereas water extract composed of apigenin and caffeic acid [14]. Endo *et al*. [35] suggested that chlorophyll derivatives may be acting as electron donors as evidenced by their ability to reduce free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Both porphyrin structure and nature of the central metal were considered important to antioxidant activity of chlorophyll derivatives. Apigenin was capable to inhibit LPO and to increase the antioxidant status [36]. Caffeic acid acts as natural antioxidant by both direct contribution and sparing á-tocopherol [37]. Moreover, it inhibited DNA fragmentation and caspase-3 activity during exposure to ROS [38]. Potential antioxidants of these substances in *T. lauriforia* leaf extract may play the key role to reduce LPO and increase the intrinsic antioxidant status against lead-induced oxidative stress and eventually, it was able to reduce gill pathology in lead-exposed *O. niloticus*.

In conclusion, Thunbergia laurifolia leaf extract-supplemented fish food was able to reduce lead concentration specifically in liver and muscle of *O. niloticus* upon $Pb(NO_3)_2$ exposure. It protects the lead-induced oxidative stress by reducing LPO and increasing the GSH level and the activities of CAT, GR and GPx in gill, kidney and liver of *O. niloticus*. Moreover, it was able to reduce gill pathology in lead-exposed *O. niloticus*. This data indicated that *T. laurifolia* leaf extract-supplemented fish food was able to protect *O. niloticus* against lead toxicity and able to apply as a fish food supplementation in *O. niloticus*.

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Full Length Research Paper

Protective efficiency of *Thunbergia laurifolia* leaf extract against lead (II) nitrate-induced toxicity in *Oreochromis niloticus*

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Thunbergia laurifolia **leaf was extracted by using 50% ethanol and supplemented with normal fish food. *Oreochromis niloticus* (n = 120) were divided into 6 groups by treating with or without 45 ppm of waterborne lead (II) nitrate and feeding normal fish food, fish food supplemented with *T. laurifolia* leaf extract in low or high dose. After 28 days of treatment, peripheral blood and organs were collected. Growth rate, blood chemistry, hematology and histology were investigated. Fish which were exposed to $Pb(NO_3)_2$ and were fed with fish food supplemented with *T. laurifolia* leaf extract especially in high dose, exhibited higher specific growth rate than fish which were exposed to $Pb(NO_3)_2$ and were fed with normal fish food. Moreover, *T. laurifolia* leaf extract can normalized blood chemistry, hematological and histological parameters in $Pb(NO_3)_2$-treated *O. niloticus*. We conclude that *T. laurifolia* leaf clearly reduced toxicity and is able to promote growth performance in *O. niloticus* after $Pb(NO_3)_2$ exposure.**

Key words: *Thunbergia laurifolia*, *Oreochromis niloticus*, lead, blood chemistry, hematology, histopathology.

INTRODUCTION

Heavy metal contamination in environment is one of the most important problems worldwide. It cannot only destroy through biological degradation, but heavy metals also have ability to accumulate for a long time leading to deleterious effect on the aquatic ecosystem and consequently possess serious health complications in human through food chain. In Thailand, heavy metal contamination in aquatic systems has extensively become the concern of this study. Heavy metals are reported as pollutants which caused the metabolic, physiological and structural alterations in fish (Jiraungkoorskul et al., 2006; 2007a; 2008). Lead (Pb) is an important pollutant in aquatic environment with increasing used in industry. Pb accumulation was found to increase in various organs including gills, liver, kidney, spleen and muscle of fish compared with control group leading to toxic effects in fish and reduced human food safety (Cicik et al., 2004; Jiraungkoorskul et al., 2008; Dai et al., 2009). Fish can uptake this metal through both gill and gastrointestinal tract. Gill is the primary site for Pb accumulation while digestive tract exhibited the greatest burden. Liver tissue always accumulated relatively high concentrations of Pb either through gill or gut (Rogers et al., 2003; Alves and Wood, 2006).

Thai traditional herb, *Thunbergia laurifolia*, which is a shrub with small oblong or ovate leaves dividing into 3 types including white, yellow and purple, of which the purple types are believed to possess health benefits. In Thai traditional medicine, *T. laurifolia* leaves are widely

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used as antipyretic and antidote against poisonous agents (Tejasen and Thongthapp, 1980). Previous studies had showed its therapeutic effect in drug addiction and hepatoprotective activity in ethanol-induced liver (Thongsaard and Marsden, 2002; Pramyothin et al., 2005). Aritajat et al. (2004) found that *T. laurifolia* extract decreased levels of blood glucose and also aids in the recovery of some β-cells in diabetic rats. It not only showed various pharmacological properties, but it also exhibited strong anti-mutagenic activity (Oonsivilai et al., 2007).

Fish are largely being used for assessment of aquatic environmental quality and can serve as bio-indicators of environmental pollution (Farombi et al., 2007). In the present experiment using freshwater fish, Nile tilapia (*Oreochromis niloticus*) as toxicological model due to its high efficiency to adapt in diverse diets, great resistance to diseases and handling practices as well as good tolerance to a wide variety of husbandry conditions. *O. niloticus* is a teleost fish, whose wide distribution around the world, is of economic importance for fishery and aquaculture. In this study, we aimed to evaluate the efficiency of *T. laurifolia* leaf extract to reduce pathotoxicological alterations in Nile tilapia after $Pb(NO_3)_2$ exposure.

MATERIALS AND METHODS

Plant extraction

Thunbergia laurifolia leaf was collected in Ratchaburi province, Thailand and was identified by Dr. Thaya Jenjittikul (Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand). Voucher specimen (Palipoch 001) was deposited at Suan Luang Rama IX herbarium, Bangkok. Fresh leaves of *T. laurifolia* were washed several times in running water, dried at 60°C for 48 h in the hot air oven (Thelco®, GCA/Precision scientific, USA) and it was made powder using blender (Otto, Thailand). Ten grams of leaf powder were extracted with 100 ml of 50% ethanol and incubated in room temperature on shaker (Germmy Orbit Shaker model VRN-480, Taiwan) at 250 rpm for 48 h, after that centrifuged at 4,000 rpm for 10 min. Supernatant was collected and dried under reduced pressure in rotary evaporator (Rotavapor® R-200, BUCHI, USA). Samples were stored at -20°C until use.

Fish food supplementation

The dietary supplementation was made by using the technique of Lamchumchang et al. (2007) with minor modification. The fish foods were grounded in blender, hydrated with distilled water 0.7 ml/g of fish food, mixed with *T. laurifolia* leaf extract and extruded through minced-meat processing machine. The mixture was break into small pellet and air-dried at 70°C for 48 h in hot air oven. Fish food was stored at room temperature.

Fish maintenance

Freshwater fish, Nile tilapia (*Oreochromis niloticus*) from Chacheongsao province, Thailand with similar size were used. The average body weight was 54.18 g, of which the length of the longest fish was not more than 1.5 times of the shortest fish. The fish were kept in the glass flow through aquaria (50 × 50 × 120 cm) with continuous air and filled with 200 L of dechlorinated tap water (APHA, 2005). The temperature and pH of water was monitored and light-dark cycles (16:8 h) were controlled in the fish facilities. The fish were fed twice a day (2% of body weight per day) with commercial fish food containing 28% proteins, 4% fibers and 3% fats (Charoen Pokphand Group, Bangkok, Thailand).The fish, *O. niloticus* were acclimatized and closely cared under laboratory condition for 4 weeks before performing the experiment.

Fish treatment and specimen collection

The 96 h LC_{50} value of Nile tilapia exposed to lead (II) nitrate ($Pb(NO_3)_2$) was determined in our laboratory as 182.12 ppm (Jiraungkoorskul et al., 2007b). In this study, fish were exposed to 45 ppm of waterborne $Pb(NO_3)_2$ which corresponded to 25% of the 96 h LC_{50}. Fish (n = 120) were weighted on day 0, randomly divided into 6 groups and treated as follows:

Group 1: Fed with normal fish food (control group).
Group 2: Treated with 45 ppm of $Pb(NO_3)_2$ and fed with normal fish food.
Group 3: Fed with Fish food mixed with 0.2 mg of leaf extract/g of fish food (low dose)
Group 4: Treated with 45 ppm of $Pb(NO_3)_2$ and fed with fish food mixed with 0.2 mg of leaf extract/g of fish food (low dose)
Group 5: Fed with fish food mixed with 2 mg of leaf extract/g of fish food (high dose)
Group 6: Treated with 45 ppm of $Pb(NO_3)_2$ and feeding fish food mixed with 2 mg of leaf extract/g of fish food (high dose).

On day 28 of treatment, fish were weighted, anesthetized with tricaine methan sulphonate (0.2 g/L of distilled water) and the peripheral blood on mid-ventral line was collected. After that fish were euthanized by anesthetizing overdose. Operculum and peritoneal cavity were opened and the organs including gills, kidney and liver were collected.

Growth measurement and biochemical studies

Specific growth rate was calculated using the following formula:
$(\ln W_f - \ln W_i)/ T \times 100$,

Where
ln = natural logarithm;
W_f = final weight (g);
W_i = initial weight (g);
T = time in days (Metwally, 2009).

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein and albumin in blood were measured using Random access chemistry analyzer (BT-2000 plus, Biotechnica instrument, Italy).

Hematological studies

Hematocrit (Hct) was determined by filling blood into the microhematocrit tube (Vitrex 0705427, Herlev, Denmark) by using capillary action. The tube was filled at least 5 cm, sealed with modeling clay and centrifuged in the microhematocrit centrifuge (BOECO, Germany) at 10,000 to 12,000 rpm for 5 min. Hct level was measured with hematocrit reader. Hemoglobin (Hb) was measured by using cyanmethemoglobin method. The 20 µl of blood was added into 5 ml of Drabkin's solution, measured with

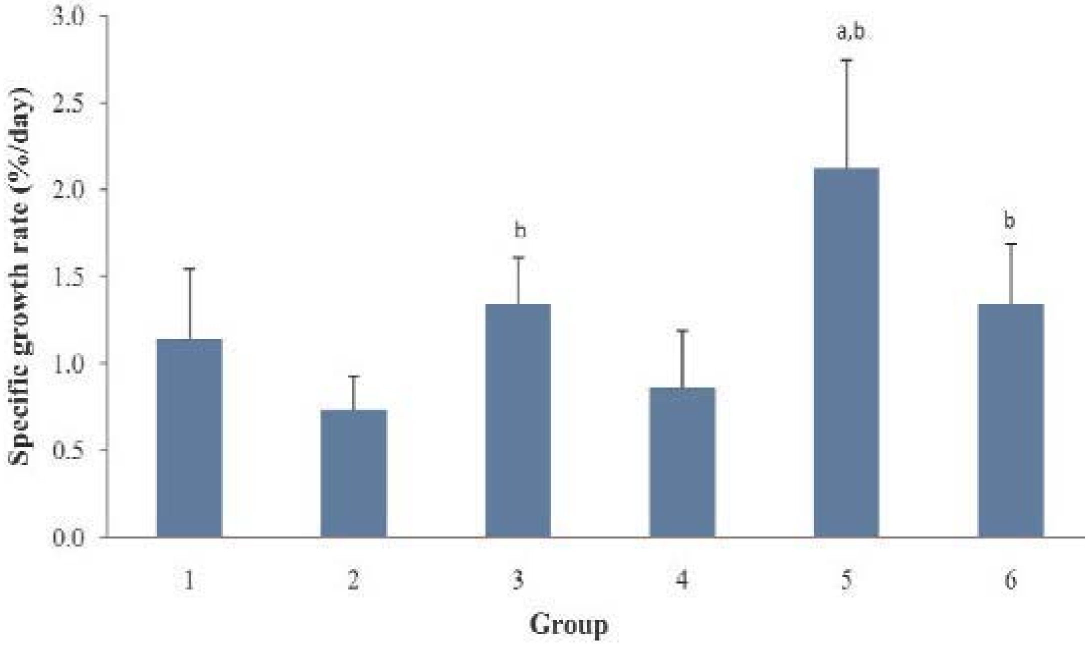

Figure 1. Specific growth rate of Nile tilapia (*Oreochromis niloticus*) in each group (n=20). Note: [a] and [b] were the mean difference significant at $P<0.05$ compared with group 1 and 2, respectively.

spectrophotometer at 530 nm and calculated from hemoglobin standard curve. For determining the number of red and white blood cells, 10 µl of blood was added into 2 ml of Natt and Herrick diluting solution (Natt and Herrick, 1952), mixed together, dropped into the hemocytometer and determined under light microscope.

Histological studies

Histopathology of the tissue organs was followed by the guidelines of Department of Pathobiology, Faculty of Science, Mahidol University, Thailand. Tissue organs including kidney, gills and liver were preserved in 10% formaldehyde for 24 h and washed with 70% ethanol.

Then the tissue organs were placed in small metal caskets, stirred by a magnetic stirrer (Lab-Line Pyro Magnestir, Melrose Park), they were dehydrated using alcohol series from 70 to 100% alcohol and embedded in paraffin (Paraplast X-TRA, Oxford Labware, 8889-503002, USA). The tissue organs were embedded in the paraffin using embedding machine (Axel Johnson Lab system, USA). Paraffin blocks were sectioned using a rotary ultra microtome (Histo STAT, Reichert, USA) and distributed onto glass slides with drying overnight. After staining with hematoxylin and eosin dyes and mounting, slides were observed under the light microscope (Humason, 1972).

Histological alterations were semi-quantitatively evaluated in terms of degree of tissue change (DTC) based on the severity of lesions. Organ changes were classified into 3 progressive stages:

Stage I = changes that do not damage the tissue to such an extent that the organ can repair itself.
Stage II = changes that are more severe and affect the associated tissue function.
Stage III = changes that preclude the restoration of the structure of the organ (Camargo and Martinez, 2007; Silva and Martinez, 2007).

The DTC was calculated by using the following mathematical equation (Poleksić and Mitrović-Tutundžić, 1994):

$$DTC = \sum I + 10\sum II + 100\sum III$$

where; I, II and III are the number of lesions of Stages I, II and III, respectively.

The average DTC was divided into five categories:

(i) 0 to 10: functionally normal.
(ii) 11 to 20: slightly to moderately damage.
(iii) 21 to 50: moderately to heavily damage.
(iv) 51 to 100: severely damage.
(v) >100: irreparable damage and the severity of tissue damage was compared among group.

Statistical analysis

All data were expressed as mean ± SD. Analysis of variance (ANOVA) with LSD test was performed for differences between each group. Significance of differences was considered at 5% level ($P<0.05$).

RESULTS

Specific growth rate

Growth rate of teleost fish, *O. niloticus* in each group was summarized in Figure 1. After 28 days of experimental period, fish in Group 6 which was exposed to $Pb(NO_3)_2$ and fed with fish food supplemented with high dose of *T.*

Table 1. Hematological parameters of Nile tilapia (*Oreochromis niloticus*) in each group expressed as mean ± SD. * The mean difference was significant at P<0.05 compared with Group 2.

Parameter	Group					
	1	2	3	4	5	6
Hct (%)	35.80 ± 0.84	34.00 ± 2.16	35.20 ± 2.28	33.00 ± 4.00	36.33 ± 7.64	33.80 ± 4.60
Hb (g/dl)	6.42 ± 0.74*	4.95 ± 0.51	6.35 ± 0.33*	5.68 ± 1.02	6.43 ± 1.69*	6.20 ± 0.29*
MCV (fl)	161.24 ± 11.54	177.88 ± 8.45	162.95 ± 12.03	162.48 ± 13.98	155.00 ± 1.42	160.38 ± 18.24
MCH (pg)	33.06 ± 4.20*	25.70 ± 2.07	32.43 ± 2.24*	32.63 ± 2.24*	31.13 ± 1.27*	31.28 ± 0.96*
MCHC (g/dl)	20.70 ± 1.39*	15.93 ± 0.61	20.18 ± 1.56*	20.88 ± 1.33*	20.93 ± 0.25*	20.80 ± 1.76*
RBC ($x10^6$ /cu.mm)	1.95 ± 0.24	1.74 ± 0.24	1.98 ± 0.27	1.87 ± 0.14	2.06 ± 0.54	1.85 ± 0.15
WBC ($x10^3$ /cu.mm)	7.00 ± 50.33	9.00 ± 34.64	7.50 ± 25.16	8.50 ± 50.00	7.33 ± 41.63	7.50 ± 44.33
Lymphocyte (%)	91.25 ± 1.26	92.75 ± 0.96	91.75 ± 1.26	91.75 ± 2.22	91.00 ± 1.29	91.75 ± 0.96
Neutrophil (%)	4.50 ± 0.58	3.75 ± 0.96	4.75 ± 0.50	4.75 ± 0.96	4.50 ± 1.29	4.50 ± 0.58
Monocyte (%)	4.25 ± 0.96	3.50 ± 1.29	3.50 ± 0.96	3.50 ± 1.29	4.50 ± 1.29	3.75 ± 0.50

Table 2. Plasma chemistry of Nile tilapia (*Oreochromis niloticus*) in each group expressed as mean ± SD. * The mean difference was significant at P<0.05 compared with Group 2.

Parameter	Group					
	1	2	3	4	5	6
Total protein (g/dl)	2.65 ± 0.30	2.45 ± 0.46	2.35 ±0.24	2.45 ± 0.13	2.67 ± 0.40	2.52 ± 0.22
Albumin (g/dl)	1.28 ±0.05	1.21 ± 0.10	1.23 ± 0.05	1.20 ± 0.08	1.30 ±0.10	1.26 ± 0.05
AST (U/L)	112.50 ± 46.87	139.00 ± 16.39	82.50 ± 20.79	135.50 ± 46.69	61.00 ±34.83*	62.20 ± 14.20*
ALT (U/L)	31.00 ± 9.83	39.00 ± 16.39	18.50 ± 3.87*	39.25 ± 14.43	19.67 ± 5.86*	25.60 ± 9.56

laurifolia leaf extract exhibited significantly (P<0.05) higher specific growth rate than fish in Group 2 which was exposed to $Pb(NO_3)_2$ and fed with normal fish food. Moreover, fish in Group 5 which were not exposed to $Pb(NO_3)_2$ and were fed with fish food supplemented with high dose of *T. laurifolia* leaf extract showed the highest growth rate and significant (P<0.05) difference compared to fish in Group1 (control group) and Group 2.

Blood chemistry and hematology

Results of blood chemistry were given in Table 1. Total protein and albumin revealed no significant difference when compared with each group. Fish in Groups 5 and 6 could significantly (P<0.05) reduce AST, while fish in Groups 3 and 5 significantly (P<0.05) reduce ALT compared with fish in Group 2.

Hematological parameters of *O. niloticus* in each group were illustrated in Table 2. Hct level, mean corpuscular volume (MCV), red blood cell (RBC) count, white blood cell (WBC) count and blood cell differential showed no significant difference when compared with fish in each group. Fish in Groups 3, 5 and 6 showed significantly (P<0.05) higher Hb level than fish in Group 2. Fish in Groups 3, 4, 5 and 6 revealed significantly (P<0.05) higher mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) level than fish in Group 2.

Histology

Efficiency of *T. laurifolia* leaf extract to reduce histological alterations was observed in kidney, gills and liver of *O. niloticus* (Table 3; Figures 2, 3 and 4). Fish in Group 2 were exposed to $Pb(NO_3)_2$ and were fed with normal fish food showed various histological alterations including: occlusion of proximal tubular lumen, cloudy swelling with cellular and nuclear hypertrophy, tubular necrosis and enlargement of glomerulus with reduction of Bowman's space in kidney (Figure 2B); hyperplasia of gill epithelium, epithelial lifting and lamellar fusion in gills (Figure 3B); cytoplasmic degeneration in hepatocyte, vacuolization of nucleus and irregular shape of hepatocyte (Figure 4B). Moreover, it showed narrowing of tubular lumens and dilatation of glomerulus capillaries in kidneys; hypertrophy of epithelium, blood congestion, lamellar disorganization, lamellar aneurysm and rupture of epithelial cells in gills; and nuclear and cellular hypertrophy, irregular shaped of nucleus and cellular rupture in liver (Table 3). Fish that were exposed to $Pb(NO_3)_2$ and were fed with fish food supplemented with *T. laurifolia* leaf extract (Groups 4 and 6) illustrated few pathological changes in kidney (Figure 2C and E), gills (Figure 3C and E) and liver (Figure 4C and E). Fish in

Table 3. Histopathological alterations of kidney, liver and gills in Nile tilapia (*Oreochromis niloticus*) in each group (n = 5) indicating the respective stage of tissue damage and frequency of occurrence. Stage I: do not alter normal physiology of tissue; stage II: more severe and causing abnormal physiology of tissue; stage III: marked severe and causing irreparable damage.

Histopathological alteration	Stage	Group					
		1	2	3	4	5	6
Kidney							
Nuclear hypertrophy of renal tubule	I	+	+++	+	++	0	+
Cellular hypertrophy of renal tubule	I	+	+++	+	++	+	+
Narrowing of tubular lumen	I	+	++	+	+	+	+
Enlargement of glomerulus	I	0	+++	0	0	0	0
Dilatation of glomerulus capillaries	I	+	++	0	+	0	0
Reduction of Bowman's space	II	0	+++	0	0	0	0
Occlusion of tubular lumen	II	0	++	0	+	0	0
Tubular necrosis	III	0	+++	0	0	0	0
Gills							
Hyperplasia of gill epithelium	I	+	+++	+	++	+	+
Hypertrophy of gill epithelium	I	0	++	0	+	0	+
Blood congestion	I	+	+	0	+	0	0
Lifting of lamellar epithelium	I	0	++	0	+	0	0
Lamellar fusion	I	0	+	0	+	0	0
Lamellar disorganization	I	0	++	0	+	0	0
Lamellar aneurysm	II	0	++	0	+	0	0
Rupture of epithelial cells	II	0	+	0	0	0	0
Liver							
Nuclear hypertrophy	I	0	++	0	+	0	0
Cellular hypertrophy	I	0	++	0	+	0	0
Irregular shaped nucleus	I	0	+++	0	0	0	0
Irregular shaped cell	I	+	+++	+	++	+	+
Cytoplasmic degeneration	II	0	+	0	+	0	0
Nuclear vacuolization	II	0	+	0	0	0	0
Cellular rupture	II	0	+	0	0	0	0

Note: absent (0); rare (+); frequency (++); very frequency (+++).

Groups 3 and 5 that were not exposed to Pb $(NO_3)_2$ but were fed with fish food supplemented with *T. laurifolia* leaf extract alone exhibited normal histology of kidney (Figure 2D and F), gills (Figure 3D and F) and liver (Figure 4D and F) as well as those of kidney (Figure 2A), gills (Figure 3A) and liver (Figure 4A) in control group.

In kidney, DTC of fish in Groups 3, 5 and 6 were 2.20 ± 0.84, 1.80 ± 0.45 and 2.80 ± 0.45, respectively, indicating normal function of kidney as well as fish in control group as shown in Table 4.

DTC of fish in Group 4 was 19.80 ± 5.76, indicating slightly to moderately damage of kidney but less pathological alteration than fish in Group 2 which shown irreparable damage. In gills, DTC of fish in Groups 3, 5 and 6 were 0.60 ± 0.55, 0.80 ± 0.45 and 1.40 ± 0.89, respectively, indicating normal function of gills as well as those in control group. DTC of Group 4 were 15.40 ± 0.89, indicating slightly to moderately damage of gills but less pathological alteration than fish in Group 2 which shown moderately to heavily damage. In liver, DTC of fish in Group 3, 5 and 6 were 0.60 ± 0.55, 0.80 ± 0.45 and 0.80 ± 0.45, respectively, and indicated normal function of liver as well as those in control group. DTC of fish in Group 4 were 12.40 ± 0.89, indicating slightly to moderately damage of liver but less pathological alteration than Group 2 which shown moderately to heavily damage.

DISCUSSION

From previous studies the leaf of *T. laurifolia* exhibited

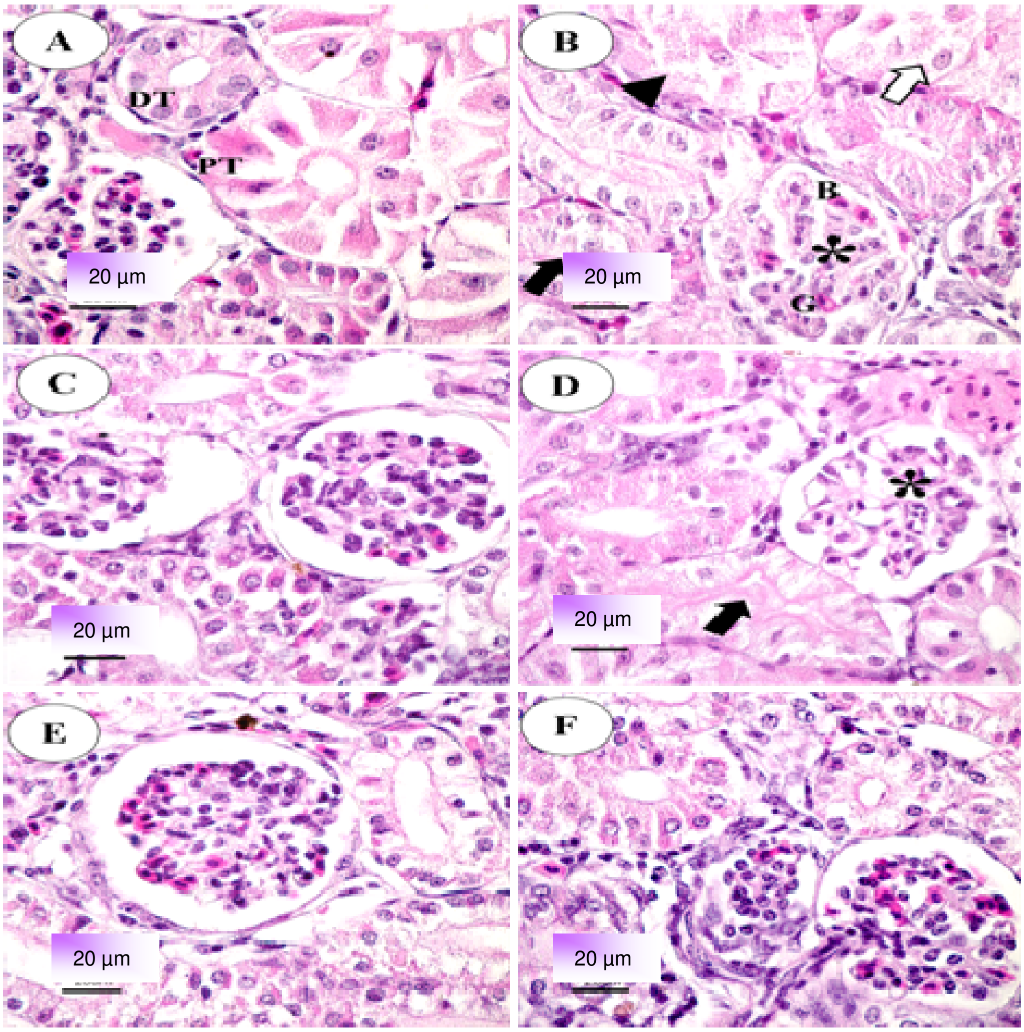

Figure 2. Histopathological alterations of kidney in Nile tilapia (*Oreochromis niloticus*) in each group. (A) Normal renal tubule both proximal (PT) and distal tubule (DT) and normal corpuscle of group 1 (control); (B) occlusion (black arrow) of proximal tubular lumen, cloudy swelling with cellular and nuclear hypertrophy (white arrow), tubular necrosis (arrowhead) and enlargement of glomerulus (G) with reduction of Bowman's space (B) (asterisk) of group 2; (D) narrowing of tubular lumen (arrow) and dilatation of glomerulus capillaries (asterisk) of group 4; (C, E and F) normal renal tubule and corpuscle of group 3, 5 and 6, respectively. Scale bar = 20 µm.

various pharmacological properties including antipyretic, antidote (Tejasen and Thongthapp, 1980), hepatoprotective activity (Pramyothin et al., 2005) and antioxidant (Oonsivilai et al., 2007). To our knowledge, the present study is the first report which clearly demonstrates the efficiency of *T. laurifolia* leaf extracted with 50% ethanol to reduce pathotoxicology of $Pb(NO_3)_2$ in blood chemistry, hematology and histology of freshwater fish, Nile tilapia (*O. niloticus*).

The evaluation of blood chemistry parameters is a routine and important tool which provides the vital information on the physiological status of animals (Chen et al., 2003). The present study showed no significant difference of total protein and albumin in each group indicating that $Pb(NO_3)_2$ at concentration of 45 ppm may not affect hepatic function of *O. niloticus*. AST and ALT are frequently used as biomarker of various tissue damages, such as liver, muscle and gills, but more specific in liver damage (De la Tore et al., 2000). Increased blood AST and ALT level of fish was caused mainly by the leakage of these enzymes from the hepatocyte into blood circulation as a result of liver damage. Fish that were exposed to $Pb(NO_3)_2$ and was fed with normal fish food significantly ($P<0.05$) demonstrated higher AST than fish in Groups 5 and 6 and higher ALT than fish in Groups 3 and 5. We thought

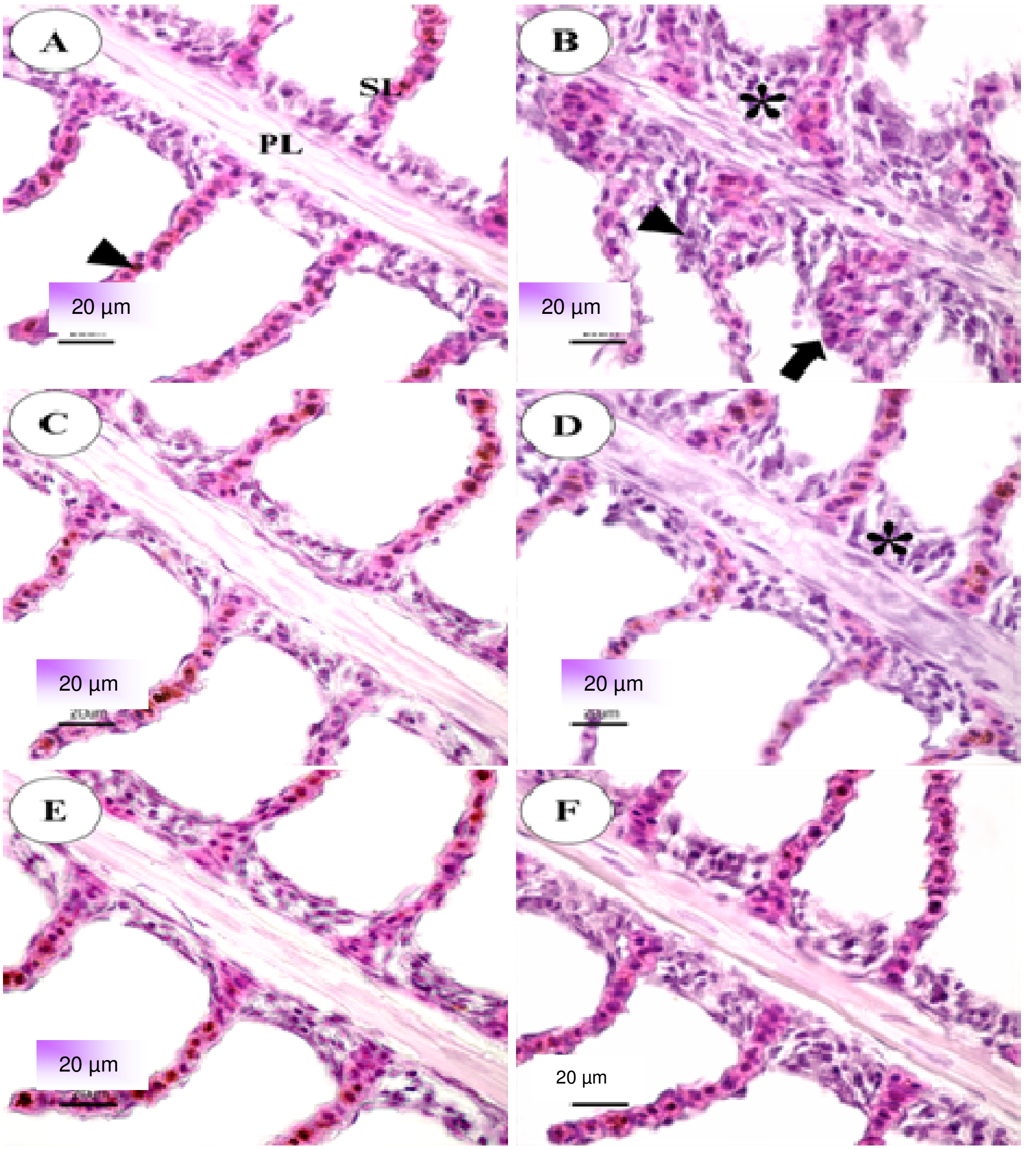

Figure 3. Histopathological alterations of gills in Nile tilapia (*Oreochromis niloticus*) in each group. (A) Normal primary lamellae (PL), lamellar epithelium (arrowhead) and secondary lamellae (SL) of Group 1 (control); (B) hyperplasia of gill epithelium (asterisk), epithelial lifting (arrowhead) and lamellar fusion (arrow) of Group 2; (D) hyperplasia of gill epithelium (asterisk) of group 4; (C, E and F) normal primary lamellae and secondary lamellae of Groups 3, 5 and 6, respectively. Scale bar = 20 µm.

that *T. laurifolia* leaf extract may act as hepatoprotective activity. Similar observations were reported in Wistar rats which demonstrated the ability of *T. laurifolia* leaf extract to reduce severity of rat liver injury and to normalize the level of ALT and AST after 14 days of ethanol exposure (Pramyothin et al., 2005).

Hematological indices are the vital parameters for evaluation of fish physiological status and they are more related to fish survival, reproduction and growth (Moiseenko, 1998). In the study, fish which exposed to $Pb(NO_3)_2$ and feeding normal fish food significantly ($P<0.05$) reduce Hb level, MCH and MCHC compared with fish in control group which were not exposed to $Pb(NO_3)_2$ but were feed with normal fish food alone (Table 1). Like other vertebrates, Pb-exposed fish provides abnormal heme synthesis. Normally, the erythrocyte enzyme delta-aminolevulinic acid dehydratase (ALA-D, E.C.4.2.1.24) catalyzes formation of porphobilinogen from aminolevulinic acid (ALA). Fish ALA-D is sensitive to the action of Pb, essential sulfhydryl groups in ALA-D are inactivated result in reduced hemoglobin formation (Sorensen, 1991). Fish that were

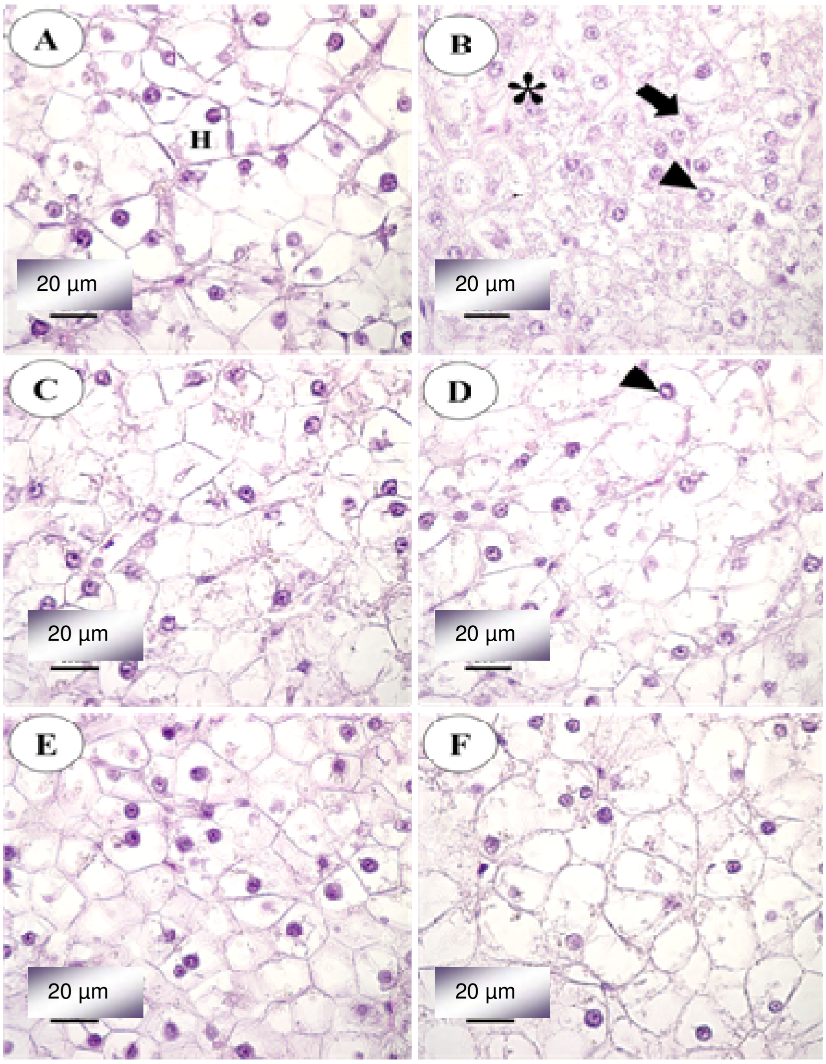

Figure 4. Histopathological alterations of liver in Nile tilapia (*Oreochromis niloticus*) in each group. (A) Normal hepatocyte (H) and showing sinusoid (S) of Group 1 (control); (B) cytoplasmic degeneration in hepatocyte (asterisk), vacuolization of nucleus (black arrowhead) and irregular shape of hepatocyte (arrow) of Group 2; (D) vacuolization of nucleus (arrowhead) of group 4; (C, E and F) normal hepatocyte of group 3, 5 and 6, respectively. Scale bar = 20 µm.

exposed to $Pb(NO_3)_2$ and fed with fish food supplemented with high dose of *T. laurifolia* leaf extract (Group 6) revealed significantly ($P<0.05$) higher Hb level, MCH and MCHC than fish in Group 2 (Table 1). The exact mechanism of action is still unknown. We hypothesize that *T. laurifolia* leaf extract may play a role in Pb chelation.

Histological analysis is a very sensitive parameter which is crucial for determining the cellular change generated by pollutant exposure in target organs (Hinton et al., 2001). In our study, it provides information of $Pb(NO_3)_2$-induced toxicity in target organs of fish including kidney, gills and liver. $Pb(NO_3)_2$ toxicity in kidney of *O. niloticus* demonstrated several pathological alterations such reduction of Bowman's capsule and occlusion of tubular lumen. Since kidney is the organ that filters the large volume of blood, $Pb(NO_3)_2$ present in blood can caused some pathological changes in Bowman's capsule, such as epithelial cell proliferation and thickening of basal lamina leading to the reduction of Bowman's capsule (Table 3; Figure 2D) similar to result which was reported by Silva and Martinez (2007). Occlusion of proximal and distal tubules can occur by the accumulation of some materials in lumen and also consequence of epithelial cells swelling (Takashima and Hibiya, 1995). Gills are participated in various vital functions including respiration, osmoregulation and excretion and considered as the main target of Pb uptake causing various pathologies (Table 3). Lifting of lamellar epithelium, hypertrophy and hyperplasia of gill epithelium indicated defense mechanism of gills. Hypertrophy of gill epithelium may be caused by an increase in the cellular metabolism resulting in an imbalance of osmotic regulation by impairing ionic active transport (Mazon et al., 2002). Liver is the essential organ for the metabolism and detoxification which is also one of the organs most affected by contaminants in water (Rodrigues and Fanta, 1998; Crestani et al., 2007). After $Pb(NO_3)_2$ exposure, liver exhibited several lesions as shown in Table 3 which has also been described in freshwater fish, *Prochilodus lineatus*, subjected to *in situ* tests along the upper reaches of Cambé stream (Camargo and Martinez, 2007). Increased nuclear vacuolization of hepatocytes indicated a signal of degenerative process that suggested metabolic damage possibly related to exposure of contaminated water (Pacheco and Santos, 2002). In the study, fish that were exposed to $Pb(NO_3)_2$ and were feed with fish food supplemented with *T. laurifolia* leaf extract could reduce histopathology in kidney, gills and liver. Normally, Pb has the ability to produce toxic by both direct and indirect pathways. In the indirect pathway, it can induce oxidative stress through either overproduction of free radical especially reactive oxygen species (ROS) or accompanying change of enzymatic antioxidants (Shi et al., 2005). Oxidative stress caused tissue damage leading to various diseases in humans (Young and Woodside, 2001; Valko et al., 2007). An accumulation of ROS caused deleterious effect on cell structure including DNA, lipids and proteins (Evans et al., 2004). Furthermore, ALA-D inactivation may also result in the accumulation of δ-aminolevulinic acid which can cause overproduction of ROS (Bhadauria and Flora, 2003). The present study revealed the health benefit of *T. laurifolia* leaf extracted to reduce histopathological alterations in fish, but actual mechanism of action is still unknown. HPLC analysis of *T. laurifolia* leaf extract identified caffeic acid and apigenin as primary constituents of water extracts, while acetone and ethanol extracts contained primarily chlorophyll a and b, pheophorbide a, pheophytin a, and lutein (Oonsivilai et al., 2007). These substances can act as antioxidant which indicated that substances in *T. laurifolia* leaf extract may play a key role to scavenge Pb-generated free radical resulting in reduce histopathological changes of fish after Pb exposure. This study selects 0.2 mg of leaf extract/g of fish food as low dose due to this concentration which is enough to reduce free radical including ROS *in vitro* (Data not shown). Further studies are necessary to investigate HPLC analysis for identified substance in 50% ethanol extract of *T. laurifolia* leaf. Moreover, Tejasen and Thongthapp (1980) reported that *T. laurifolia* leaf had the ability to antidote the poisonous agents which may involve in the reduction of Pb toxicity in fish.

Comparing specific growth rate in each group, fish which were not exposed to $Pb(NO_3)_2$ while been fed with fish food supplemented with high dose of *T. laurifolia* leaf extract showed the highest growth rate. Moreover, fish which were exposed to $Pb(NO_3)_2$ and were been fed with fish food supplemented with high dose of *T. laurifolia* leaf extract exhibited significantly ($P<0.05$) higher specific growth rate than fish which were exposed to $Pb(NO_3)_2$ and fed with normal fish food. We suggest that *T. laurifolia* leaf extract supplemented in fish food had the ability to promote growth performance and protect fish against $Pb(NO_3)_2$ toxicity. Thus, it may be use as food supplementation of economic fish, *O. niloticus* which is important for fishery and aquaculture in worldwide. We conclude that *T. laurifolia* leaf extract clearly improved growth performance, blood chemistry, hematology and histology against $Pb(NO_3)_2$ toxicity in Nile tilapia (*O. niloticus*).

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Dietary *Psidium guajava* Supplementation Reducing Ferric Nitrilotriacetate Toxicity in *Puntius altus*

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ABSTRACT

The efficiency of *Psidium guajava* was studied to illustrate the reduction of ferric nitrilotriacetate (Fe-NTA) toxicity in *Puntius altus* via the histopathology analysis. The fish (n = 40) were randomly divided into four groups. Each fish was transferred to each aquarium as follows: G1 and G2 were obtained normal fish food; G3 and G4 were obtained guava leaf extract 60 mg g^{-1} fish food. After 28 days dietary supplement, fish in G2 and G4 were injected intraperitoneal of 9 mg Fe kg^{-1} b.wt. Twenty-four hour after injection, lesions were especially most evident in the G2. The gills were observed epithelial lifting, lamellar cell hyperplasia. Blood congestion was seen in sinusoids and hepatocytes necroses were also observed. Renal tubular swelling and necrosis were seen. Some areas were found hemosiderin pigment accumulation. Fish with guava pre-treatment (G4) showed slightly alteration when compare those of G2 group. The results suggested that *P. guajava* leaf extract pre-obtained may play an important role in the reduction of Fe-NTA toxicity in fish.

Key words: *Psidium guajava*, herb, *Puntius altus*, toxicology, histopathology, food supplementation

INTRODUCTION

Thailand is considered one of the countries which bring medical plants into use and sometimes they refer to it as “Thai Traditional Medicine (TTM)” which can be used to treat humans and animal diseases. Ascorbic Acid (AA) is an essential vitamin for normal growth and physiological functions in animals including fishes. Most teleosts are unable to synthesize AA because of the lack of L-gulonolactone oxidase (Fracalossi *et al*., 2001). Therefore, an exogenous source of AA is required in fish diets. It functions as a general water-soluble reagent, on collagen formation, iron metabolism and the response to stress (Vijayavel *et al*., 2006). Many authors have reported the efficacy of AA in reducing genotoxicity in *Oreochromis niloticus* induced by lead (Jiraungkoorskul *et al*., 2008); *Poronotus triacanthus* induced by copper (Jiraungkoorskul and Sahaphong, 2007) and *Puntius altus* induced by cadmium (Jiraungkoorskul *et al*., 2007b), using the micronucleus and nuclear abnormality tests. It has also been reported the efficacy of AA in reducing the histopathological alterations in fish after cadmium exposure (Jiraungkoorskul *et al.*, 2006). Edema, lamellar cell hyperplasia, epithelial lifting and aneurysm were observed in the gills. There were blood congestion in sinusoids, vacuolation of hepatocytes, hemosiderin accumulation, apoptosis and nuclear pyknosis. Glomerulus atrophy, hydropic swelling, hyaline casts and necrosis were seen. Fortunately, in the combination of cadmium and AA treated group, they showed similar alterations as those observed in the cadmium treated alone group but they were less severe. The findings of this study can be used as guidelines for developing programs to help the fish which are cultured near heavy metal contaminated areas (Jiraungkoorskul *et al.*, 2006). However, the uses of natural origin are preferred over the others because they are safe and non-toxic and have no resistance problems.

Psidium guajava Linn. ("Farang" in Thai, or "Guava" in English) constituents include ascorbic acid, triterpenes (Begum *et al.*, 2004), carotenoids (Mercadante *et al.*, 1999) and flavonoids (Rattanachaikunsopon and Phumkhachorn, 2007). The leaves are elliptic to ovate about 5-15 cm in length. The flowers are white, with five petals and numerous stamens. This plant produces edible round or pear shaped fruit which contains high amounts of calcium. Guava leaves are used for medicinal purposes, as a remedy to diarrhea. Recent studies prove that guava has sugar lowering properties to help diabetics lower their sugar count (Gutierrez *et al.*, 2008). In addition, crushed leaves from guava plants can be applied on wounds, ulcers and rheumatic places and toothache can be relieved by chewing the leaves. Lastly, leaves extracts can also have antimicrobial properties and can also be for coughs (Jaiarj *et al.*, 1999). The study survey revealed that there are no scientific studies carried out regarding the efficiency of this plant in fish. Ferric nitrilotriacetate (Fe-NTA) has been chosen as a model organic compound to study the protective effect of this herb in its toxicity. Awai *et al.* (1979) first reported glycosuria and hepatic parenchymal iron deposits in rats following intraperitoneal injections with Fe-NTA. Its toxicity is assumed to be caused by the elevation of free serum iron concentration, following its reduction at the luminal side of the proximal tubule (Liu *et al.*, 1991), generating reactive oxygen species, leading to lipid peroxidation and induce oxidative stress in liver (Iqbal *et al.*, 1995). This present study was designed to assess the ability of the extract from *P. guajava* against ferric nitrilotriacetate induced toxicity by using histopathological analysis.

MATERIALS AND METHODS

Animal model: This study was performed at the Department of Pathobiology, Faculty of Sciences, Mahidol University, Bangkok, Thailand in 2010. Red tailed tinfoil barb, *P. altus*, 16.76±1.72 g in b.wt. and 9.85±0.50 cm in total length, were purchased from a commercial hatchery in Bangkok, Thailand. Tap water was filtered with activated charcoal to eliminate chemical contamination. The physicochemical characteristics of water were measured daily, according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Under laboratory condition, fish were acclimated for 30 days at 28.5±1.0°C, pH = 6.8-7.0, total hardness = 70-80 mg L^{-1} (as $CaCO_3$), alkalinity = 75-80 mg L^{-1} and conductivity = 190-210 μmhos cm^{-1}. A 16:8 h light-dark cycle was maintained throughout. Chlorine residual and ammonia were below detection limits. Fish were fed twice a day with 37%-protein commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day. The animal care and handling in this research was performed following the instruction of the Mahidol University-Institutional Animal Care and Use Committee (MU-IACUC). Therefore, this research was followed the mammal animal care and use i.e., (1) Use, care and transportation of fish for toxicopathological testing was complied with all applicable animal welfare laws. (2) Number of fish was kept to the minimum requirement for achieve scientifically valid results. (3) All protocols were taken to avoid the discomfort, distress or pain in the fish. (4) The appropriate dosage of the anesthesia was 200 mg L^{-1} ethyl-3-aminobenzoate methanesulfonate salt (MS222, Sigma) and the euthanasia was overdose of this chemical.

Preparation of dry leaf with fish food: Guava was collected from local area in Nakorn Prakom Province, Thailand. Fresh leaves were washed several times in water, dried at 45°C for 72 h and made semi powder by crushing using a mortar and pestle. The extraction was done by following the method of Winkaler *et al.* (2007) with slight modifications. Fish food was grounded in a blender and hydrated with 0.7 mL g^{-1} distilled water, mixed with the leaf semi powder extract and extruded through a minced-meat processing machine. Later, the mixture was broken into small pellets by hands and dried at 60°C for 48 h. The fishes were fed twice a day (2% b.wt.) with the prepared dry leaf food supplementation within 28 days.

Preparation of Ferric Nitrilotriacetate (Fe-NTA) solution: A solution of Fe-NTA was prepared by the method of Awai *et al.* (1979).

Experimental design: The fish (n = 40) were randomly divided into four groups. Each fish group was transferred to each aquarium as follows: G1 and G2 were obtained normal fish food; G3 and G4 were obtained guava leaf extract 60 mg g^{-1} fish food. After 28 days dietary supplement, fish in G2 and G4 were injected intraperitoneal of 9 mg Fe kg^{-1} b.wt. given in a volume of 10 mL kg^{-1} b.wt. Twenty-four h after injection, fish from each group was taken and anesthetized with 0.2 g L^{-1} MS-222, weighed and measured. The fish was dissected and the organs (gill, liver and kidney) were removed and prepared for histopathological analysis.

Specimen preparation for light microscopic study: The procedures for light microscopy were modified by Humason (1972). Briefly, the tissues were fixed in the 10% buffered formaldehyde for 24 h, dehydrate through a graded series of ethanol and clear with xylene solutions. They were embedded in a block using melted paraffin at the embedding station (Axel Johnson Lab System, USA). The paraffin blocks were sectioned at 4-5 µm thickness using a rotary microtome (HistoSTAT, Reichert, USA) and stained with Harris hematoxylin and eosin. The tissue glass slides were examined for abnormalities by a Nikon E600 light microscope and photographed by a Nikon DXM 1200 digital camera (Tokyo, Japan).

RESULTS

Gills

Control group: The gills consisted of a row of long thin filaments, the primary lamellae which projected from the arch like the teeth of a comb. The surface area of each primary lamella was increased further by the formation of regular semilunar folds across its dorsal and ventral surface, the secondary lamellae. The primary lamellar epithelium was one or two cell layers thick. Each secondary lamella was made up of two sheets of epithelium delimited by many pillar cells which were contractile and separated the capillary channels. One to two erythrocytes were usually observed within each capillary lumen. No recognizable changes were observed in the gills of the control (G1) and guava supplement group (G3) throughout this experiment (Fig. 1a, c).

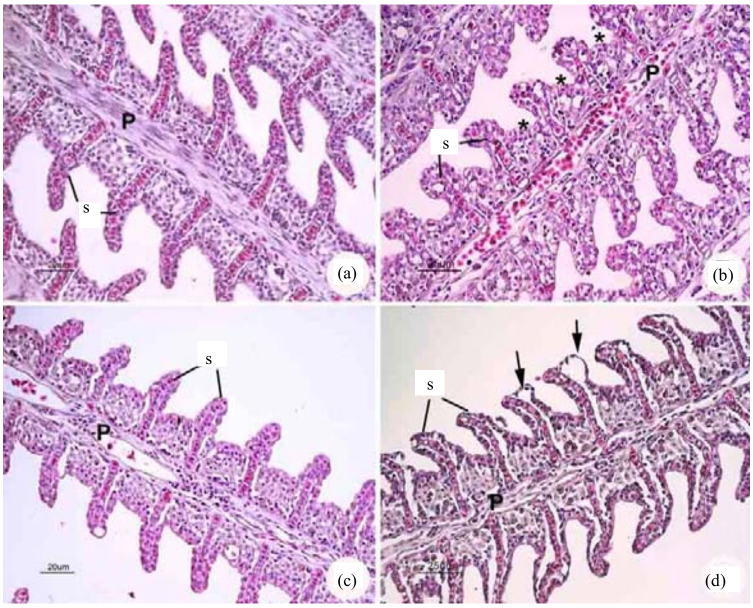

Fig. 1: Gills of *P. altus* in (a) G1, showing normal arrangement of primary (P) and secondary (s) lamellae (b) G2, showing epithelial hyperplasia (*) and blood congestion (c) G3, no recognition change was observed (d) G4, showing edema or epithelial lifting (arrows)

Treated groups: A variety of histological studies revealed that Fe-NTA affected gill tissue in G2. The lesions included hyperplasia, epithelial lifting or cell swelling and congestion. The lesion was started with the bending of the distal extremities of secondary lamellae, followed by a lifting of the outer layer of the lamellar epithelium, the formation of edematous spaces between the layers of epithelium which may become infiltrated with red blood cells and leukocytes. Finally, hyperplastic tissues were observed in the primary epithelial cells (Fig. 1b). Eventually, the whole epithelium sloughed off and the lamella lost its rigidity. Guava pre-obtained group (G4) showed similar but less severe alterations than those of G2 (Fig. 1d).

Liver

Control: Hepatocytes were polygonal and had a distinct central nucleus with densely staining chromatin margins and a prominent nucleolus. Sinusoids which were irregularly distributed between hepatocytes, were fewer in number and were lined by endothelial cells. No recognizable changes were observed in the hepatocytes of the control (G1) and guava supplement group (G3) throughout this experiment (Fig. 2a, c).

Treated groups: The hepatocytes in G2 were swelling and numerous vacuolization were also observed. Blood congestion and exhibited increasing size and pyknotic nuclei were seen (Fig. 2b). However, the histological alterations were less severe in G4 (Fig. 2d).

Kidney

Control: The kidney was divided into anterior and posterior portions. The nephron of the freshwater fish was composed of a well-vascularized glomerulus, proximal segments, distal