EFFICACY OF CRUDE EXTRACTS OF *ALLIUM SATIVUM*, *THUNBERGIA LAURIFOLIA*, AND *EURYCOMA LONGIFOLIA* AGAINST NEWLY EXCYSTED JUVENILE AND ADULT STAGES OF LIVER FLUKE, *OPISTHORCHIS VIVERRINI*

PHORNPHITCHA PECHDEE

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor Philosophy in Translational Medicine
Suranaree University of Technology
Academic Year 2023

ประสิทธิภาพของสารสกัดอย่างหยาบของ กระเทียม รางจืด และ ปลาไหลเผือก ต่อการต้านระยะตัวอ่อนและระยะตัวเต็มวัย ของพยาธิใบไม้ตับ ออร์พิสทอร์คิส วิเวอร์รินิ

นางสาวภรณ์พิชชา เพชรดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาวิชาเวชศาสตร์ปริวรรต
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2566

EFFICACY OF CRUDE EXTRACTS OF *ALLIUM SATIVUM*, *THUNBERGIA LAURIFOLIA*, AND *EURYCOMA LONGIFOLIA* AGAINST NEWLY EXCYSTED JUVENILE AND ADULT STAGES OF LIVER FLUKE, *OPISTHORCHIS VIVERRINI*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

.....

(Asst. Prof. Dr. Prasert Saichua)

Chairperson

.....

(Assoc. Prof. Schawanya Rattanapitoon, MD)

Member (Thesis Advisor)

.....

(Asst. Prof. Dr. Nathkapach Rattanapitoon)

Member (Thesis Co-advisor)

.....

(Dr. Wiwat Nuansing)

Member (Thesis Co-advisor)

.....

(Dr. Patpicha Arunsan)

Member

.....

(Dr. Sanong Suksaweang)

Member

.....

(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)

Vice Rector for Academic Affairs and Quality Assurance

.....

(Assoc. Prof. Sutham Pinjaroen, MD)

Dean of Institute of Medicine

ภรณ์พิชชา เพชรดี : ประสิทธิภาพของสารสกัดอย่างหยาบของ กระเทียม รางจืด และปลาไหลเผือก ต่อการต้านระยะตัวอ่อนและระยะตัวเต็มวัย ของพยาธิใบไม้ตับ ออร์พิสทอร์คิส วิเวอร์รินิ (EFFICACY OF CRUDE EXTRACTS OF *ALLIUM SATIVUM*, *THUNBERGIA LAURIFOLIA*, AND *EURYCOMA LONGIFOLIA* AGAINST METACERCARIA AND ADULT STAGES OF LIVER FLUKE, *OPISTHORCHIS VIVERRINI*) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ แพทย์หญิงชวัลญ์ญา รัตนพิทูลย์, 193 หน้า

คำสำคัญ: กระเทียม/รางจืด/ปลาไหลเผือก/พยาธิใบไม้ตับ/ต้านปรสิต

พยาธิใบไม้ตับ *Opisthorchis viverrini* นับว่าปัญหาทางสาธารณสุขและเป็นปัจจัยเสี่ยงที่สำคัญในการเกิดมะเร็งท่อน้ำดี [cholangiocarcinoma (CCA)] ในประเทศไทย ในขณะที่ยา Praziquantel (PZQ) ยังคงเป็นหลักที่ใช้ในการรักษาโรคติดเชื้อพยาธิใบไม้ตับในปัจจุบัน แต่การใช้ยายังคงมีปัญหาอยู่ โดยเฉพาะการใช้ยาในการติดเชื้อซ้ำการติดเชื้อเรื้อรัง การใช้ยาต่อเนื่องระยะยาว และการดื้อยา ซึ่งย้ำถึงความจำเป็นในการค้นหาสารสกัดจากสมุนไพร เพื่อเป็นทางเลือกที่ในการใช้เป็นยารักษาทดแทนยาแผนปัจจุบันที่ยังคงมีปัญหาอยู่

วัตถุประสงค์ของการศึกษานี้ เพื่อประเมินประสิทธิภาพของสารกัดหยาบ กระเทียม (*Allium sativum*) รางจืด (*Thunbergia laurifolia*) และ ปลาไหลเผือก (*Eurycoma longifolia*) ต่อตัวอ่อนและตัวเต็มวัยของพยาธิใบไม้ตับ *O. viverrini* โดยตัวอ่อนและตัวเต็มวัยของพยาธิถูกบ่มด้วยสารสกัดกระเทียม รางจืด และปลาไหลเผือก ที่ความเข้มข้นต่าง ๆ (ตัวอ่อน: 5 10 20 และ 40 mg/ml ตัวเต็มวัย: 20 30 และ 40 mg/ml) ซึ่งกลุ่มที่บ่มด้วย PZQ และ อาหารเลี้ยงเชื้อ Roswell Park Memorial Institute 1640 medium (RPMI-1640) ให้เป็นกลุ่มควบคุมเชิงบวกและกลุ่มควบคุมเชิงลบ จากนั้นประเมินการเคลื่อนไหวที่ช่วงเวลาต่าง ๆ ให้คะแนนและวิเคราะห์หาค่าเคลื่อนไหวที่สัมพันธ์ [Relative Motility value (RM)] และดัชนีการอยู่รอด [Survival index (SI)] ตรวจสอบความเสียหายของผิวหนังตัวอ่อนและตัวเต็มวัยของพยาธิ *O. viverrini* โดยใช้กล้องจุลทรรศน์อิเล็กตรอนสแกนนิ่ง [Scanning electron microscope (SEM)] นอกจากนี้ ยังทำการประเมินการแสดงออกของความเครียดด้วยการทดสอบหา Reactive oxygen species (ROS) และ Nitric oxide (NO) ในตัวเต็มวัยของพยาธิใบไม้ตับ *O. viverrini* ใช้เทคนิคการย้อมด้วยสีฟลูออเรสเซนต์จำเพาะ 2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA) และวิธีการตรวจสอบปฏิกิริยาด้วย Griess reagent

ผลการศึกษาพบว่า กลุ่มสารสกัดกระเทียม รางจืด และปลาไหลเผือก สามารถลดความเคลื่อนไหวในตัวอ่อนและตัวเต็มวัยของพยาธิใบไม้ตับ *O. viverrini* โดยขึ้นอยู่กับความเข้มข้นและเวลาที่เพิ่มขึ้น ส่งผลต่อการลดลงของค่า RM และ SI โดยพบว่ากลุ่มสารสกัดกระเทียมและบางกลุ่มสารสกัดรางจืด อัตราการเคลื่อนไหวลดลงมีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ในขณะที่กลุ่มสารสกัดปลาไหลเผือกพบว่าไม่มีความแตกต่างทางสถิติ เมื่อเทียบกับกลุ่มควบคุมเชิงบวก สกัดกระเทียม รางจืด และปลาไหลเผือก ส่งผลต่อการบวมและความเสียหายของผิวพยาธิใบไม้ตับ *O. viverrini* ตัวอ่อนและตัวเต็มวัย โดยเฉพาะในกลุ่มสารสกัดกระเทียม นอกจากนี้ยังทุกสารสกัดยังส่งผลต่อการแสดงออกของระดับ ROS และ NO ที่สูงขึ้นเมื่อเทียบกับกลุ่มควบคุมเชิงบวก การศึกษานี้จึงชี้ให้เห็นว่า สารสกัดหยาบกระเทียม รางจืด และปลาไหลเผือก มีศักยภาพในการต้านตัวอ่อนและตัวเต็มวัยของพยาธิใบไม้ตับ *O. viverrini* โดยเฉพาะสารสกัดกระเทียมที่ส่งผลค่อนข้างโดดเด่น อย่างไรก็ตาม การศึกษาเพิ่มเติมถึงกลไกของสารประกอบสำคัญที่มีในแต่ละสารสกัด จำเป็นต้องมีการศึกษาต่อไปในอนาคต เพื่อเข้าใจรายละเอียดและสามารถที่จะพัฒนาสารสกัดจากสมุนไพรไปสู่ยาทางเลือกเพื่อกำจัดหรือรักษาการติดเชื้อโรคทางปรสิตต่อไป

สาขาวิชาเวชศาสตร์ปริวรรต

ปีการศึกษา 2566

รายมือชื่อนักศึกษา .....

รายมือชื่ออาจารย์ที่ปรึกษา.....

รายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

รายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

PHORNPHITCHA PECHDEE : EFFICACY OF CRUDE EXTRACTS OF *ALLIUM SATIVUM*, *THUNBERGIA LAURIFOLIA*, AND *EURYCOMA LONGIFOLIA* AGAINST NEWLY EXCYSTED JUVENILE AND ADULT STAGES OF LIVER FLUKE, *OPISTHORCHIS VIVERRINI.* THESIS ADVISOR : ASSOC. PROF. SCHAWANYA RATTANAPITOON, MD, 193 PP.

Keyword: *Allium sativum/Thunbergia laurifolia/Eurycoma longifolia/Opisthorchis viverrini/*Against parasite

Opisthorchis viverrini presents a significant risk factor for cholangiocarcinoma (CCA) in Thailand. While praziquantel (PZQ) remains the mainstay treatment for opisthorchiasis, its linkage to adverse effects and the potential development of CCA during extended treatment, especially in instances of reinfection and chronic infection, highlights the critical need for alternative herbal interventions with anthelmintic properties.

The aim of this study was to assess the impact of crude extracts from *Allium sativum*, *Thunbergia laurifolia*, and *Eurycoma longifolia* on the *O. viverrini* newly excysted juveniles (NEJs) and adult worms. NEJs and adult worms were subjected to varying concentrations of crude extract (NEJs: 5, 10, 20, and 40 mg/ml; adult worms: 20, 30, and 40 mg/ml). As controls, another group with PZQ and Roswell Park Memorial Institute 1640 medium (RPMI-1640) (NEJs: 2 mg/ml; adult worms: 20 mg/ml), serving as positive and negative control groups, respectively. Motility assessment was conducted at different exposure times by determining relative motility values (RM) and survival index (SI). Morphological damage of NEJs and adult worms was evaluated using scanning electron microscopy (SEM). Additionally, the quantification of reactive oxygen species (ROS) and Nitric oxide (NO) as markers of oxidative stress was performed using 2',7'- Dichlorodihydrofluorescein diacetate (H2DCFDA) staining and the Griess reagent detection assay in adult worms.

Treatment with *A. sativum*, *T. laurifolia*, and *E. longifolia* effectively suppressed motility in *O. viverrini* NEJs and adult worms, leading to decreased RM values and SI.

Notably, significant differences were observed in the *A. sativum* and certain concentrations of *T. laurifolia* treated groups, while the groups treated with *E. longifolia* showed no significant variance compared to the negative control. Morphological damage, especially tegumental degradation and swelling, was evident across all treatment groups. Additionally, there were significantly elevated levels of ROS and NO generation, particularly severe in the *A. sativum* treated group. These findings suggest the potential effectiveness of crude extracts of *A. sativum*, *T. laurifolia*, and *E. longifolia* against both the *O. viverrini* NEJs and adult worm, particularly notable in the *A. sativum* treated groups, demonstrates their significant efficacy. However, further investigations are necessary to understand their mechanisms and key bioactive compounds for developing effective anti-parasitic agents against helminthic infections.

School of Translational Medicine
Academic Year 2023

Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....
Co-advisor's Signature .....

ACKNOWLEDGEMENT

I would like to express my gratitude to Assoc. Prof. Schawanya Rattanapitoon MD, my advisor, Asst. Prof. Dr. Nathkapach Rattanapitoon, Co-advisor, Dr. Wiwat Nuansing, Co-advisor, and Dr. Patpicha Arunsan for providing guidance, valuable advice, endless kindness, and supporting my research to succeed.

I would like to express my gratitude to my thesis committee members, Asst. Prof. Dr. Prasert Saichua, serving as the Chairperson, and Dr. Sanong Suksaweang, for their invaluable suggestions and constructive criticisms, which have greatly contributed to my growth and development for the future.

Additionally, I would like to thank Prof. Dr. Thewarach Laha for valuable suggestions and facilitating access to animal within the Animal Unit, Faculty of Medicine, Khon Kaen University.

I also appreciate to all members of the Parasite Research Disease Center (PDRC) for their assistance and support throughout the process of conducting my research experiments.

I am very grateful to Suranaree University of Technology for providing me with the laboratory facilities for conducting my research.

I would like to express gratitude to the Translational Medicine International Programs (TM), Institute of Medicine, Suranaree University of Technology, as well as external grants and scholarships for graduate students (OROG), Thailand Science Research and Innovation (TSRI), and National Science, Research, and Innovation Fund (NSRF) (NRIIS number 179304) for supporting my research.

Finally, I wish to thank my parents and all my friends for being with me and for their support, understanding, inspiration, and encouragement throughout my study.

PHORNPHITCHA PECHDEE

CONTENTS

		Page
ABSTRACT IN THAI		I
ABSTRACT IN ENGLIST		III
ACKNOWLEDGEMENT		V
CONTENTS		VI
LIST OF TABLES		IX
LIST OF FIGURES		XI
LIST OF ABBREVIATIONS		XXII
CHAPTER		
I	**INTRODUCTION**	1
	1.1 Thesis title	1
	1.2 Rational and Background	1
	1.3 Research Gap	4
	1.4 Research Objectives	4
	1.5 Research Hypotheses	5
	1.6 Scope and Limitations	5
	1.7 Expected Result	6
II	**LITERATURE REVIEWS**	7
	2.1 *Opisthorchis viverrine*	7
	2.2 Tegumental surface of *O.viverrini*	11
	2.3 Anthelminthic drugs	12
	2.4 The effects of herbal on parasite	14
	2.5 Oxidative stress	22
	2.6 Scanning electron microscopy	29
	2.7 Related research	34
	2.8 Conceptual Framework	47

CONTENTS (Continued)

		Page
III	**RESERCH METHODOLOGY**	48
	3.1 Experimental design	48
	3.2 Location of research	49
	3.3 Animal ethic and biosafety ethic (IBC) preparation	49
	3.4 Plant crude extraction	49
	3.5 Parasite preparation	53
	3.6 Anthelmintic activity on *O. viverrini* NEJs	59
	3.7 Anthelmintic activity on *O. viverrini* adult worms	60
	3.8 Motility activity	62
	3.9 Viability assay	63
	3.10 Measurement of Reactive oxygen species generation (ROS)	63
	3.11 Measurement of nitric oxide generation (NO)	64
	3.12 Morphological study by scanning electron microscope (SEM)	65
	3.13 Data analysis	66
IV	**RESULTS AND DISCUSSION**	67
	4.1 Results of Motility test and viability assay of *O. viverrini* NEJs	67
	4.2 Result of surface morphological changes of *O. viverrini* NEJs	74
	4.3 Result of motility test and viability assay of *O. viverrini* adult worm	79
	4.4 Result of ROS generation of *O. viverrini* adult worm	86
	4.5 Result of NO generation of *O. viverrini* adult worm	91
	4.6 Result of surface morphological changes of *O. viverrini* adult worm	94
	4.7 Discussion	98

CONTENTS (Continued)

		Page
IV	**CONCLUSION AND RECOMMENDATION**	105
	5.1 Conclusion on the Efficacy of Crude Extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* NEJs	105
	5.2 Conclusion on the Efficacy of Crude Extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* adult worms	105
	5.3 Recommendation and possible future studies	107
REFERENCES		109
APPENDIX		133
	APPENDIX A THE PREPARATIONS OF REAGENTS	134
	APPENDIX B SUPPLEMENTARY DATA	138
	APPENDIX C RESEARCH ETHICS DOCUMENTS	152
	APPENDIX D RESEARCH DISTIBUTIONS	155
	APPENDIX E RESEARCH ARTICLE ACHIEVEMENT	160
	CURRICULUM VITAE	171

LIST OF TABLES

Table		Page
2.1	The efficacy of herbal remedies against *O. viverrini*.....	15
2.2	Characteristics of NOS Isoforms [modified from (Khazan and Hedayati, 2015)].....	27
2.3	Difference between light microscope and electron microscope [modified from (Kannan, 2018)].....	30
2.4	Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.....	35
2.5	Prior studies of the efficacy of *T. laurifolia* extract in exerting anti-parasitic effects.....	42
2.6	Prior studies of the efficacy of *E. longifolia* in exerting anti-parasitic effects.....	44
1B	Motility score of *A. sativum* treated groups on *O. viverrini* NEJs.....	138
2B	Motility score of *T. laurifolia* treated groups on *O. viverrini* NEJs.....	140
3B	Motility score of *E. longifolia* treated groups on *O. viverrini* NEJs.....	141
4B	Motility score of negative control and positive control group on *O. viverrini* NEJs.....	143
5B	Motility score of *A. sativum* treated groups on *O. viverrini* adult worms.....	144
6B	Motility score of *T. laurifolia* treated groups on *O. viverrini* adult worms.....	145
7B	Motility score of *E. longifolia* treated groups on *O. viverrini* adult worms.....	146
8B	Motility score of negative control and positive control group on *O. viverrini* adult worms.....	147

LIST OF TABLES (Continued)

Table		Page
9B	The CTWF fluorescence intensity of *A. sativum* groups treated on *O. viverrini* adult worms	148
10B	The CTWF fluorescence intensity of *T. laurifolia* groups treated on *O. viverrini* adult worms	148
11B	The CTWF fluorescence intensity of *E. longifolia* groups treated on *O. viverrini* adult worms	149
12B	The concentration of NO generation in *A. sativum* groups treated on *O. viverrini* adult worms	150
13B	The concentration of NO generation in *T. laurifolia* treated groups on *O. viverrini* adult worms	150
14B	The concentration of NO generation in *E. longifolia* groups treated on *O. viverrini* adult worms	151
1E	The copyrights and patents to this research	161

LIST OF FIGURES

Figure		Page
2.1	*O. viverrini* adult worm (Pechdee, 2016).....	8
2.2	*O. viverrini* cercaria (Pechdee, 2016).....	9
2.3	*O. viverrini* metacercaria (Pechdee, 2016).....	10
2.4	Life cycle of *O. viverrini* (CDC, 2018).....	11
2.5	Thai *A. sativum*, cloves (A), bulbs (B), and peel (C). (Cr. Pechdee, 2024).....	17
2.6	*T. laurifolia* leaves (Cr. Pechdee, 2024).....	19
2.7	*E. longifolia*, Root (A) and Tree (B). (Pharmaceutical Sciences, Ubon Ratchathani University, 2010a).....	21
2.8	Main pathways for the formation of ROS and NOS. Mitochondria are the major source of cellular ROS during respiratory processes (Tell et al., 2013).....	25
2.9	The components of SEM (Kannan, 2018).....	32
2.10	Conceptual framework of this research.....	47
3.1	Experimental study flowchart of this study.....	48
3.2	*A. sativum* cloves (A), *T. laurifolia* leaves (B), *E. longifolia* dried roots (C). (Pharmaceutical Sciences, Ubon Ratchathani University, 2010b).....	50
3.3	*A. sativum* crude extract method.....	51
3.4	*T. laurifolia* and *E. longifolia* crude extraction method.....	52
3.5	*A. sativum* crude extract powder (A), *T. laurifolia* crude extract powder (B), *E. longifolia* crude extract powder (C).....	53
3.6	Naturally freshwater cyprinoid fish samples.....	54
3.7	The procedure of the Pepsin-HCl digestion method for the retrieval of *O. viverrini* metacercaria.....	54

LIST OF FIGURES (Continued)

Figure		Page
3.8	The *O. viverrini* metacercaria were observed under a stereomicroscope (A) scale bar = 400 µm, revealing the presence of oral and ventral suckers as well as an excretory bladder (B). Scale bar= 50 µm.....	55
3.9	The excysted *O. viverrini* metacercaria were observed under a stereomicroscope (A) scale bar= 400 µm, *O. viverrini* NEJs revealing the presence of oral and ventral suckers as well as an excretory bladder (B) scale bar= 50 µm.....	56
3.10	The golden syrian hamsters (*M. auratus*) used in this study.	57
3.11	The procedures for infecting hamsters with *O. viverrini* metacercaria.....	58
3.12	The procedures for euthanizing and dissecting hamsters to collect *O. viverrini* adult worms.....	59
3.13	Anthelmintic activity on *O. viverrini* NEJs.....	60
3.14	Anthelmintic activity on *O. viverrini* adult worms.....	61
3.15	Viability assay utilizing Trypan blue stain.....	63
3.16	Measurement of ROS generation in *O. viverrini* adult worms.....	64
3.17	Measurement of NO generation using the Griess Reagent reaction.....	65
3.18	Morphological study of *O. viverrini* NEJs and adult worms using SEM.....	66
4.1	The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *A. sativum* crude extract, compared to both negative and positive controls, across various time intervals.....	68
4.2	The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *A. sativum* crude extract, compared to both negative and positive controls, across various time intervals.....	69

LIST OF FIGURES (Continued)

Figure		Page
4.3	Motility scores showed significant variations between the negative control group and all *A. sativum* treated groups. However, there were no notable differences in mean motility scores between the positive control group and the *A. sativum* treated groups ($P < 0.05$).	69
4.4	The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *T. laurifolia* crude extract, compared to both negative and positive controls, across various time intervals.....	70
4.5	The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *T. laurifolia* crude extract, compared to both negative and positive controls, across various time intervals.....	71
4.6	Motility scores exhibited significant differences between the negative control group and the groups treated with 20 and 40 mg/ml of *T. laurifolia*. However, there were no significant differences in mean motility scores between the positive control group and the *T. laurifolia* treated groups ($P < 0.05$).....	71
4.7	The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *E. longifolia* crude extract, compared to both negative and positive controls, across various time intervals.....	72
4.8	The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *E. longifolia* crude extract, compared to both negative and positive controls, across various time intervals.....	73
4.9	Motility scores showed no significant differences between the negative control group and *E. longifolia* treated groups, as well as between the positive control group and *E. longifolia* treated groups ($P < 0.05$).....	73

LIST OF FIGURES (Continued)

Figure		Page
4.10	Viability of *O. viverrini* NEJs was assessed using Trypan blue staining. In the negative control group (A, G, and M), no staining was observed. Conversely, extensive Trypan blue staining was evident in the positive control group (B, H, and N), as well as in all groups treated with *A. sativum* (D, E, and F), *T. laurifolia* (J, K, and L), and *E. longifolia* (P, Q, and R).....	74
4.11	The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the negative control group, with a scale bar of 40 µm.....	76
4.12	The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the positive control group, with a scale bar of 40 µm. *= Alteration of tegumental surface by swelling expansion of tegumental surface.....	76
4.13	The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *A. sativum* treated groups with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 80, 80, 70, and 70 µm, respectively. * = Alteration of tegumental surface by degradation or damage of tegumental surface.....	77
4.14	The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *T. laurifolia* treated groups with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 40 µm for each. * = Alteration of tegumental surface by swelling (A II, B and C I) and degradation (D, I and II).....	78
4.15	The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *E. longifolia* treated groups with concentrations of 20 and 40 mg/ml (A and B), respectively, with scale bars of 40 µm and 80 µm, respectively. * = Alteration of tegumental surface by swelling (A I and II) and degradation (B I and II).....	78

LIST OF FIGURES (Continued)

Figure		Page
4.16	The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *A. sativum* treated groups, compared to both negative and positive controls, across various time intervals	80
4.17	The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *A. sativum* treated groups, compared to both negative and positive controls, across various time intervals	80
4.18	The mean motility scores were significantly different between the negative control and all groups treated with *A. sativum*. However, no significant difference was noted between the positive control and *A. sativum* treated groups ($p < 0.05$)	81
4.19	The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *T. laurifolia* treated groups, compared to both negative and positive controls, across various time intervals	82
4.20	The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *T. laurifolia* treated groups, compared to both negative and positive controls, across various time intervals	82
4.21	The mean motility scores were not significantly different between both the negative control and positive control groups compared to the *T. laurifolia* treated groups ($p < 0.05$)	83
4.22	The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *E. longifolia* treated groups, compared to both negative and positive controls, across various time intervals	84

LIST OF FIGURES (Continued)

Figure		Page
4.23	The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *E. longifolia* treated groups, compared to both negative and positive controls, across various time intervals.....	84
4.24	The mean motility scores were not significantly different between the negative control and *E. longifolia* treated groups. However, there was a significant difference observed between the positive control groups and the 20 mg/ml *E. longifolia* treated groups ($p < 0.05$).....	85
4.25	Viability of *O. viverrini* adult worms were assessed using Trypan blue staining. the negative control group (A, F, and K), positive control group (B, G, and L), *A. sativum* treated groups (C, D, and E), *T. laurifolia* treated groups (H, I, and J), and *E. longifolia* treated groups (M, N, and O). Scale bar = 2 mm.....	86
4.26	The ROS generation image of *A. sativum* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.....	87
4.27	The CTWF of fluorescence intensity in the *A. sativum* treated groups.....	88
4.28	The ROS generation image of *T. laurifolia* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.....	89
4.29	The CTWF of fluorescence intensity in the *T. laurifolia* treated groups.....	89
4.30	The ROS generation image of *E. longifolia* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.....	90

LIST OF FIGURES (Continued)

Figure		Page
4.31	The CTWF of fluorescence intensity in the *E. longifolia* treated groups	91
4.32	The alteration in NO levels due to *A. sativum* treatment in *O. viverrini* adult worms	92
4.33	The alteration in NO levels due to *T. laurifolia* treatment in *O. viverrini* adult worms	93
4.34	The alteration in NO levels due to *E. longifolia* treatment in *O. viverrini* adult worms	94
4.35	The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the negative control group, with a scale bar of 80 µm	96
4.36	The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the positive control group treated with 20 mg/ml PZQ. Images (A, B, C, and D) show various aspects of the tegumental surface morphology, with respective scale bars of 40 µm, 4 µm, 4 µm, and 40 µm.* = Swelling of tegumental surface (A and C), alteration of tegumental surface, swelling until bursting (B)	96
4.37	The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *A. sativum* treated groups at concentrations of 20, 30, and 40 mg/ml. Images (A, B, and C, D) correspond to these concentrations, with scale bars of 400 µm, 400 µm, 400 µm, and 60 µm, respectively. * = Alteration of tegumental surface by degradation (D, I and II), damage and blister until bursting (A, I, B, I and II) of tegumental surface	97

LIST OF FIGURES (Continued)

Figure		Page
4.38	The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *T. laurifolia* treated groups at concentrations of 30 and 40 mg/ml. Images A and B correspond to these concentrations, with scale bars of 400 µm each. Unfortunately, samples at 20 mg/ml were lost during sample preparation for SEM and are not shown. = Alteration of tegumental surface by degradation (B, II and III), swelling of tegumental surface (A, I, II and III, B, I).....	97
4.39	The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *E. longifolia* treated groups at concentrations of 20, 30, and 40 mg/ml. Images (A, B, and C, D) correspond to these concentrations, with scale bars of 400 µm each. *= Alteration of tegumental surface, swelling of tegumental surface.....	98
1A	Nitrite concentrations standard curve.....	135
1C	The animal ethics protocols were approved by the Animal Care and Use Committee of the Institute of Research and Development, Suranaree University of Technology.....	152
2C	The biosafety and ethics protocols were approved by the Biosafety and Ethics Committee of the Institute of Research and Development, Suranaree University of Technology.....	153
3C	Certificate of completion for permission to use animals for scientific purposes from the National Research Council of Thailand No.: U1-09993-2565.....	154
4C	Certificate of completion for biosafety and biosecurity course.....	154

LIST OF FIGURES (Continued)

Figure		Page
1D	Certificate of appreciation for poster presentation titled "Efficacy of *Allium sativum* crude extract against the human liver fluke, *Opisthorchis viverrini"* at The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023.....	156
2D	Certificate of appreciation for attending The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023.....	157
3D	Certificate of participation for oral presentation titled Effects of garlic crude extract (*Allium sativum* Linn.) against *Strongyloides stercoralis* larva at the 2nd international virtual conference on science and technology (SUT-IVCST 2021) hosted by Suranaree University of Technology, Thailand on 6th August 2021.....	158
4D	Certificate of best presentation award for oral presentation titled Effects of garlic crude extract (*Allium sativum* Linn.) against *Strongyloides stercoralis* larva at the 2nd international virtual conference on science and technology (SUT-IVCST 2021) hosted by Suranaree University of Technology, Thailand on 6th August 2021....	159
1E	Acceptance email from the Tropical Biomedicine Journal for the manuscript titled "Anthelmintic activity and pathophysiological effect of *Allium sativum* crude extract against the carcinogenic liver fluke, *Opisthorchis viverrini*," for publication.....	162
2E	Acceptance email from The Natural Products Journal for the manuscript titled "Papaya Seeds Extract Combined Lauryl Glucoside Against Human Parasites Contaminated in Fresh Vegetables," for publication.....	163

LIST OF FIGURES (Continued)

Figure		Page
3E	Acceptance email from the Tropical Biomedicine Journal for the manuscript titled “Diagnosis of the carcinogenic liver fluke, *Opisthorchis viverrini*: comparison of two coprological methods versus the automatic feces analyzer” for publication.....	164
4E	Acceptance email from the Tropical Biomedicine Journal for the manuscript titled “Anthelmintic activity andpathophysiological effect of anthelmintic drugs against carcinogenic liver fluke, *Opisthorchis viverrini.”* for publication.....	165
5E	Research article titled 'Oral parasitic protozoan *Entamoeba gingivalis* in periodontal disease patients, northeastern Thailand' has been published in the Tropical Biomedicine Journal.....	166
6E	Research article titled “Molecular detection of oral *Trichomonas tenax* in periodontal disease patients by polymerase chain reaction based 18S rRNA gene.” has been published in the Tropical Biomedicine Journal.....	167
7E	Research article titled “Molecular identification of *Opisthorchis viverrini* among the northeastern Cambodian population by internal transcribed spacer 2 based polymerase chain reaction.” has been published in the Tropical Biomedicine Journal.....	168
8E	Research article titled “Intestinal parasitic infections and risk factors among the population in Cambodia.” has been published in the Tropical Biomedicine Journal.....	169
9E	The copyright pertaining to this research, titled "คู่มือสมุนไพรไทยต้านปรสิต (Thai Herbs of Anti-Parasite)," has Copyright ID: ว .0445972021....	170

LIST OF ABBREVIATIONS

An	=	Anterior part
As	=	*Allium sativum* crude extract
CCA	=	Cholangiocarcinoma
CTWF	=	Corrected total worm fluorescence
DW	=	Distilled water
EB		Excretory blader
El	=	*Eurycoma longifolia* crude extract
H2DCFDA	=	2′,7′-dichlorofluorescein diacetate staining
kV	=	Kilovolt
MI	=	Motility index
Mv	=	Microvilli
NEJs	=	Newly excysted juvenile
NO	=	Nitric oxide synthese
OS	=	Oral sucker
Pa	=	Papillae
PBS	=	Phosphate buffer saline
Po	=	Posterior part
PZQ	=	Praziquantel
RM	=	Relative motility
ROS	=	Reactive oxygen species
RPMI	=	Roswell Park Memorial Institute
SEM	=	Scanning electron microscope
SI	=	Survival indix
Tl	=	*Thunbergia laurifolia* crude extract
VS	=	Ventral sucker

CHAPTER I
INTRODUCTION

1.1 Thesis title

Efficacy of crude extracts of *Allium sativum*, *Thunbergia laurifolia*, and *Eurycoma longifolia* against newly excysted juvenile and adult stages of liver fluke, *Opisthorchis viverrini*

1.2 Rational and Background

Opisthorchis viverrini, a parasitic liver fluke, it causes of opisthorchiasis, prevalent in northern and northeastern Thailand, with an overall infection rate of 2.2%, escalating to 14.3% in high-risk areas (Wattanawong et al., 2021). The persistent inflammatory response induced by *O. viverrini* in the bile ducts poses a substantial long-term risk for cholangiocarcinoma (CCA) (Hanpanich et al., 2017; Pinlaor et al., 2009) Currently, the drug of choice for treating opisthorchiasis is praziquantel (PZQ). PZQ was initially selected for its anti-helminthic properties in the mid-1970s and has since been utilized to treat various human trematode infections (Cioli and Pica-Mattoccia, 2003). However, repeated treatment with PZQ and subsequent reinfection with *O. viverrini* are associated with an increased risk of developing cholangiocarcinoma (CCA) (Lawson et al., 2015; Pinlaor et al., 2009). Inflammation and the resulting stress induced by *O. viverrini* antigens are statistically associated with papillary and intrahepatic CCA, along with the repeated use of PZQ treatment (Luvira et al., 2018). The emergence of drug resistance and the side effects associated with PZQ treatment raise significant concerns (Cioli and Pica-Mattoccia, 2003; Erko et al., 2012; Fallon and Doenhoff, 1994). The importance of investigating alternative herbal remedies is underscored. Bioactive compounds such as tannins, flavonoids, and alkaloids, recognized for their

effectiveness against diverse parasites (Mushtaq et al., 2018), could serve as guiding principles for identifying key bioactive compounds for parasite elimination in future research endeavors.

Several herbal remedies for *O. viverrini* liver fluke have been investigated. For instance, studies have shown that extracts of *Garcinia mangostana* and *Thunbergia laurifolia* possess anti-inflammatory and antioxidant properties against *O. viverrini* infection (Aukkanimart et al., 2015; Wonkchalee et al., 2.12, 2013). Additionally, Intuyod (2014) suggested that an anthocyanin complex (AC) derived from blue butterfly pea, turmeric, and purple waxy corn cobs exhibits anti-inflammatory and anti-periductal fibrosis effects against *O. viverrini* infection. Furthermore, Wannachat in 2020 demonstrated the effects of extracts from *Areca catechu* on the motility of *O. viverrini* NEJs and adult worms.

Allium sativum (Garlic or Ka-team in Thai), a traditional remedy with a rich historical usage against various ailments, possesses primary bioactive compounds including diallyl disulfide, s-allyl cysteine, methyl cysteine, and allicin, which exhibit robust antibacterial, anticancer, and antiparasitic properties. Particularly, allicin, extensively researched, showcases therapeutic potential against cardiovascular diseases, exerts antioxidant effects, and confers hepatoprotection against toxins and alcohol alcohol (Corzo-Martínez et al., 2007; Rabinkov et al., 1998). The effectiveness of *A. sativum* is extensively documented in both *in vivo* and *in vitro* studies, notably highlighting its inhibitory effects on helminths. *A. sativum* has demonstrated efficacy against *Haemonchus contortus* nematodes in ruminant animals (Palacios-Landín et al., 2015), *Anisakis* sp. in marine fish (Morsy et al., 2021) Previous research has indicated the influence of *A. sativum* on *Schistosoma mansoni*, including adult worms, cercaria, schistosomula, and miracidia stages (Aly et al., 2017; Cortés et al., 2017; Mantawy et al., 2012; Riad et al., 2009; Schelkle et al., 2013;). *A. sativum* has been shown to enhance the immune response in mice infected with *S. mansoni* (El Shenawy et al., 2008; Mantawy et al., 2011, 2012; Metwally et al., 2018). Furthermore, *A. sativum* extract has exhibited an impact on the motility of *Fasciola gigantica* (Singh et al., 2009).

In contrast, *Thunbergia laurifolia* (Rang Chuet, in Thai) is widely utilized for detoxification from lead poisoning or other toxins (Palipoch et al., 2011). Significant compounds such as rosmarinic acid (RA), apigenin, caffeic acid, allic acid, protocatechuic acid, and various vitamins have been identified in it. Several reports have indicated that apigenin, a bioactive compound in *T. laurifolia*, possesses antioxidant and anti-cancer properties (Chan et al., 2010; Oonsivilai et al., 2007). A previous study demonstrated that both fresh and dried *T. laurifolia* solutions significantly reduced inflammatory cells in hamsters infected with *O. viverrini.* Furthermore, the combination of *T. laurifolia* extract with praziquantel reduced inflammatory cell aggregation and inhibited the development of cholangiocarcinoma (CCA), which correlated with the serum Alanine transaminase (ALT) levels in hamsters infected with *O. viverrini* (Wonkchalee et al., 2012, 2013).

Additionally, *Eurycoma longifolia* (Pla Lai Puek, in Thai), one of the tropical herbal medicinal plants in Southeast Asian countries, shows potential in aphrodisiac, anti-malarial, anti-cancer, and anti-microbial properties (Ur Rehman et al., 2016). Quassinoids, cathin-6-one alkaloids, squalene type triterpenes, tirucallane-type triterpenes, and biphenylneolignans are among the major bioactive compounds reported in *E. longifolia* (Bhat and Karim, 2010). Previous studies have reported the effects of *E. longifolia* extract on the glutathione protein of *Toxoplasma gondii* and *Plasmodium falciparum* (Hout et al., 2006; Kavitha et al., 2012; Mohd Ridzuan et al., 2005; Sriwilaijaroen et al., 2010).

Therefore, the aim of this investigation is to assess the impact of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts on the human liver fluke, *O. viverrini* newly excysted juvenile (NEJs), and adult worms. The protective properties of the extracts against *O. viverrini* NEJs and adult worms were studied through relative motility values (RM) and % survival index (SI), alongside viability confirmation. Additionally, for *O. viverrini* adult worms, the assessment included evaluating oxidative stress generation and morphological tegumental damage. Thus, the examination of crude extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* constitutes a critical step in

identifying promising herbal candidates for the potential development of alternative therapeutic agents for parasitic infections.

1.3 Research Gap

Currently, long term and repeated treatment with PZQ is associated with intrahepatic and papillary CCA. The side effects and drug resistance of PZQ treatment are concerning, highlight the significance of exploring alternative herbal remedies. The *A. sativum, T. laurifolia*, and *E. longifolia* are widely used herbal remedies in Thailand, with a rich tradition. These herbals may serve as valuable guides for identifying key bioactive compounds for future parasite elimination strategies. This underscores the possibility of developing alternative and combined standard drugs, reducing reliance on chemical drugs, and enhancing the added value of Thai herbal treatments.

1.4 Research Objectives

1.4.1 General objective

This study aims to evaluate the effects of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts against the human liver fluke, *O. viverrini* NEJs, and adult worms.

1.4.2 Specific objectives

1.4.2.1 Crude extracts were obtained from *A. sativum* bulbs, of *T. laurifolia* leaves, and *E. longifolia* roots.

1.4.2.2 *O. viverrini* metacercaria were collected from naturally cyprinoid-infected fish. These metacercaria were then excysted to obtain *O. viverrini* NEJs.

1.4.2.3 *O. viverrini* adult worms were obtained by infecting hamsters with these metacercaria.

1.4.2.4 The effects of crude extracts of *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* NEJs and adult worms were evaluated.

1.4.2.5 The motility values (RM and SI) of *O. viverrini* NEJs and adult worms were evaluated at different time points following specific scoring criteria. Worm viability was confirmed through Trypan blue staining.

1.4.2.6 The morphological surface of *O. viverrini* NEJs and adult worms was evaluated after 12 hours of incubation with crude extracts using SEM.

1.4.2.7 Oxidative stress detection of ROS and NO in *O. viverrini* adult worms was performed using H2DCFDA staining and the Griess reagent reaction assay.

1.5 Research Hypotheses

Is it possible to investigate the efficacy of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts against *O. viverrini* NEJ and adult worms?

Recurrent treatment with Praziquantel (PZQ) is associated with intrahepatic and papillary CCA, while emerging drug resistance raises concerns. The persistent concerns regarding the side effects of PZQ highlight the significance of exploring alternative herbal remedies. *A. sativum*, *T. laurifolia*, and *E. longifolia* are widely used herbal remedies in Thailand, with a rich tradition. Therefore, these herbs may provide valuable insights into identifying key bioactive compounds for future parasite elimination strategies.

1.6 Scope and Limitations

The research was conducted at the Parasitic Disease Research Center laboratory (PDRC), Institute of Medicine, Suranaree University of Technology. *A. sativum* bulbs, *T. laurifolia* leaves, and *E. longifolia* dried roots were collected from the fresh market or around Nakhon-Ratchasima province, northeastern Thailand. Natural cyprinoid fish were gathered from water reservoirs in endemic areas of northeastern Thailand to obtain *O. viverrini* metacercariae. These metacercariae were then excysted to obtain *O. viverrini* NEJs. Male hamsters were procured from the Laboratory Animal Facility, Faculty of Medicine, Khon Kaen University, Thailand, and were infected with *O. viverrini*

metacercariae to obtain adult worms. The hamsters were housed for 2-3 months to allow for the development of *O. viverrini* adults. The experiment focused on assessing the antiparasitic activities of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts. The antiparasitic activities of the extracts were assessed through *in vitro* assays on *O. viverrini* NEJs and adult worms. The protective effects of the extracts against *O. viverrini* NEJs and adult worms were investigated using relative motility values (RM) and % survival index (SI), as well as viability confirmation. For *O. viverrini* adult worms, assessment included the evaluation of oxidative stress generation and morphological tegumental damage.

1.7 Expected Result

The effectiveness of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts against *O. viverrini* NEJs and adult worms suggests the potential for alternative treatments for opisthorchiasis. Assessing the feasibility, safety, and efficacy of these crude extracts as alternative therapeutics is crucial for preventing, treating, and controlling human liver fluke infections in the future. The anticipated outcomes include the introduction of alternative opisthorchiasis treatments, the elevation of the value of Thai herbs, and the recognition of the high impact of related publications. Although this study has shown promising results *in vitro*, further extensive research is needed, particularly regarding their practical application in the treatment and prevention of human parasite infections.

CHAPTER II
LITERATURE REVIEWS

2.1 *Opisthorchis viverrini*

Opisthorchis viverrini is a fish-borne trematode liver fluke. It belongs to the Opisthorchiidae family, which includes human liver flukes such as *O. viverrini*, *O. felineus*, and *Clonorchis sinensis*. In Thailand, at least 6 million people are infected with the liver fluke *O. viverrini*, with a particularly high prevalence in northern and northeastern regions, where the overall infection rate is 2.2%, escalating to 14.3% in high-risk areas (Jongsuksuntigul et al., 2003; Wattanawong et al., 2021). *O. viverrini* has been categorized as a group I biological carcinogen by the WHO International Agency for Research on Cancer (IARC, 1994). The persistent inflammatory response triggered by *O. viverrini* in the bile ducts poses a significant long-term risk for cholangiocarcinoma (CCA) (Hanpanich et al., 2017; Pinlaor et al., 2009; Sripa et al., 2007; 2011). *O. viverrini* infection and opisthorchiasis pose significant public health challenges in northeastern Thailand. The economic burden linked to *O. viverrini* infection in Thailand amounts to approximately USD $120 million annually, encompassing medical expenses and loss of wages (Kaewpitoon et al., 2015). Human acquisition of *O. viverrini* infection occurs through the consumption of raw or undercooked cyprinid fish containing the infective stage, metacercaria. The life cycle of *O. viverrini* involves a snail or first intermediate host, *Bithynia* spp., and a second intermediate host consisting of various species of cyprinid fish (Harinasuta and Harinasuta, 1984; Wykoff et al., 1965).

2.1.1 *O. viverrini* adult worm

Adult worms of *O. viverrini* are monoecious trematodes characterized by their dorso-ventrally flattened, lancet-shaped, thin, and transparent bodies. Freshly obtained worms from bile ducts exhibit a reddish-bile coloration, with an average size of approximately 7.0 (range: 5.4 – 10.2) × 1.5 (range: 0.8 – 1.9) mm. The oral sucker,

surrounding the mouth, is situated at the subterminal anterior position, while the ventral sucker is blind-ended at approximately the anterior one-fifth of the body length. The digestive system comprises a mouth, pharynx with a short esophagus, which then separates into two intestinal ceca extending close to the posterior end of the body. Two deeply multilobed testes are in the posterior body. The multilobed ovary is positioned in front of the anterior testis, close to the seminal receptacle and Laurer's canal. Vitelline glands are irregularly arranged in many groups on the lateral side of the body The excretory bladder is a sac-like tube that is long and S-shaped, running between the two testes (Harinasuta and Harinasuta, 1984; Kaewkes, 2003; Wykoff et al., 1965).

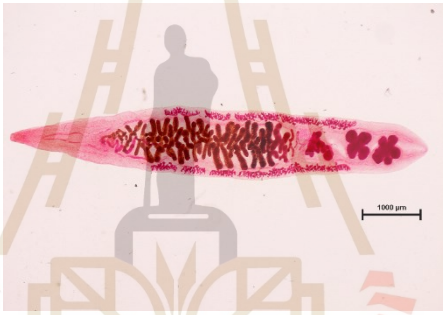


Figure 2.1 *O. viverrini* adult worm (Pechdee, 2016)

2.1.2 *O. viverrini* cercaria

The cercariae of *O. viverrini* emerge from infected *Bithynia* spp. snails in response to light stimulation and subsequently swim freely to find their second intermediate host, cyprinid fish. These cercariae have an average body size of 154 × 75 µm, covered with minute spines, and a tail size of 392 × 26 µm, featuring a dorso-ventral fin-fold. At rest or when hanging head down in water, *O. viverrini* cercariae exhibit an oculate, pleurolophocercus, and tobacco pipe-formed shape. Two eyespots are located laterally between the oral sucker and pharynx, with brownish pigments dispersed in the body portion. The oral sucker is subterminally anteriorly positioned and bears several rows of tooth-like structures, while the ceca are not well developed.

The tail of the cercariae exhibits transversely striated tegument at the proximal third and a fin fold that extends from the dorsal to ventral surface of the distal part. Cystogenous glands are dorsolateral on both sides of the body (Arunsan et al., 2014; Kaewkes, 2003; Wykoff et al., 1965)

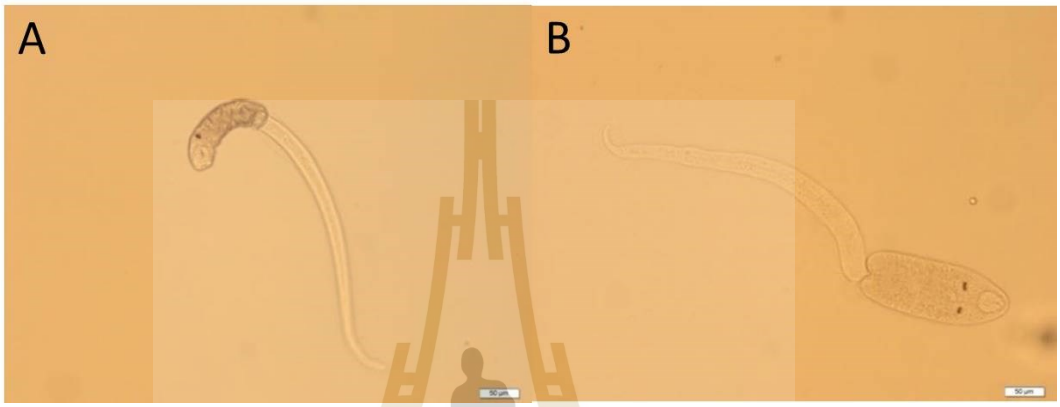


Figure 2.2 *O. viverrini* cercaria (Pechdee, 2016)

2.1.3 *O. viverrini* metacercaria

The *O. viverrini* metacercaria are formed after the penetration of cercariae underneath the skin of cyprinid fish. These mature encysted metacercariae have an average size of 201 × 167 µm. Typically, *O. viverrini* metacercariae are oval-shaped, although some may occasionally be round. The cyst walls consist of two layers, with the outer wall being thicker than the inner wall. Oral and ventral suckers are clearly visible in the encysted larva. The excretory bladder contains a dense mass of dark granules, and brownish-yellow pigments are scattered throughout the body. (Komiya, 1966; Scholz et al., 1991; Vajrasthira et al., 1961).

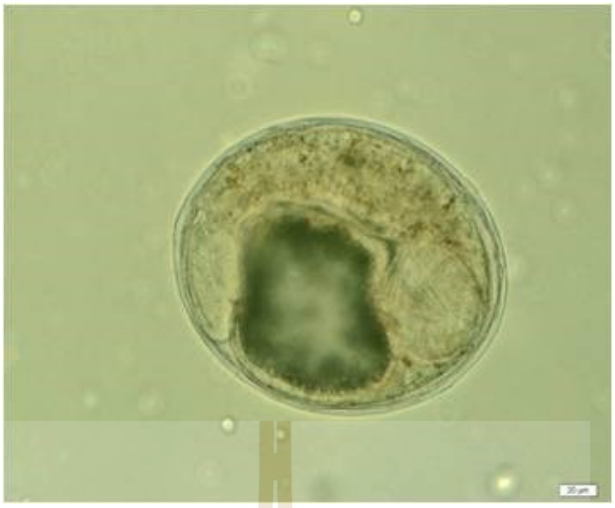


Figure 2.3 *O. viverrini* metacercaria (Pechdee, 2016)

2.1.4 Life cycle of *O. viverrini*

O. viverrini adult worms primarily inhabit the secondary bile ducts of human of and definitive host. They attach to the walls of these ducts using their oral and ventral suckers. The adult worms lay embryonated eggs containing miracidia, which are passed with bile into the duodenum and excreted with feces into the external environment. These embryonated eggs are then ingested by *Bithynia* spp. snails, where egg-hatching occurs in the digestive tract to release miracidia. These miracidia penetrate the snail tissue and transform into sporocysts. Rediae and cercariae are multiplied through asexual reproduction of germinal cells in sporocysts and rediae, respectively. Upon exiting the snail as free-swimming cercariae, they penetrate and transform into metacercaria by encystation in cyprinid fishes. Metacercariae become infective to definitive hosts, including humans, dogs, and cats, when these hosts ingest raw, fermented, or inadequately cooked cyprinid fish. After ingestion, the metacercaria is digested by gastric and intestinal juices. Juvenile excysted in duodenum then migrate up through the common bile duct into the intra-hepatic bile ducts. (Harinasuta, 1984; Waikakul et al., 1998).

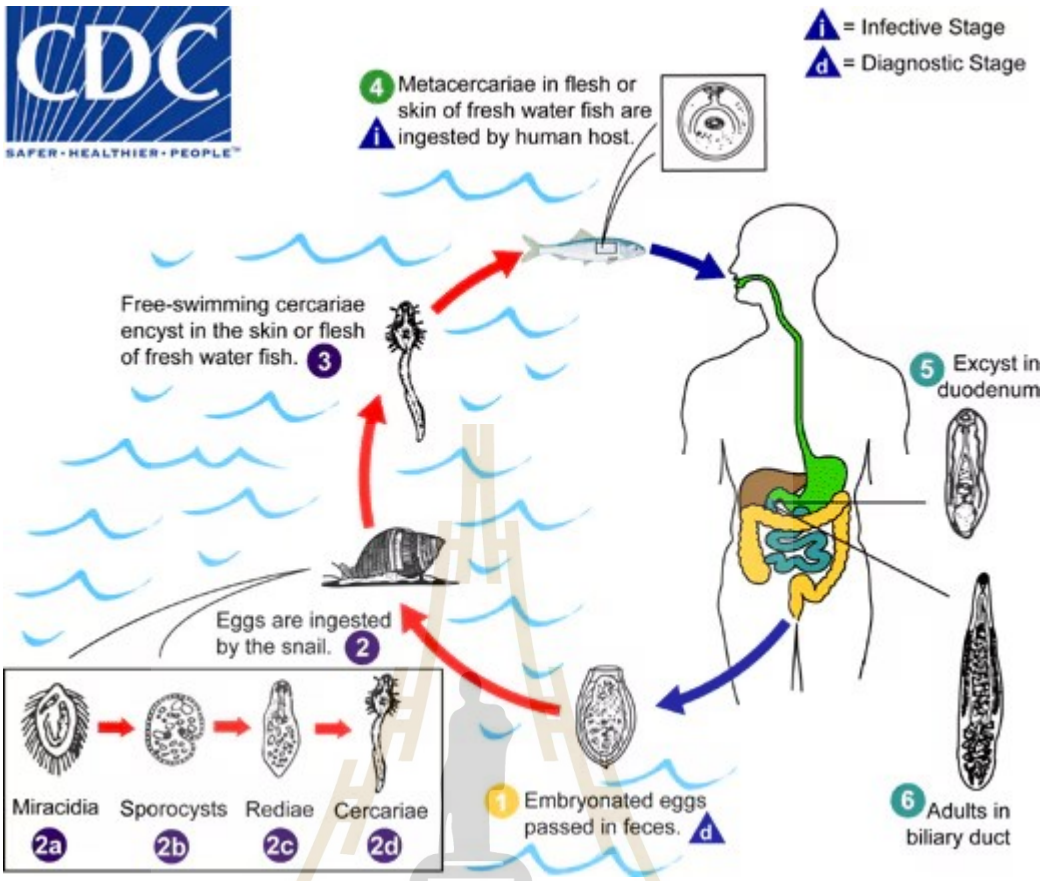


Figure 2.4 Life cycle of *O. viverrini* (CDC, 2018)

2.2 Tegumental surface of *O.viverrini*

The tegumental surface of *O. viverrini* newly excysted juvenile (NEJ) and adult stages was examined using scanning electron microscopy by Apinhasmit et al. (1993, 1994. The NEJ surface exhibits circumferentially arranged ridges alternating with troughs, bearing rows of spines encircling the body. These spines vary in edge shapes: serrated edges on the anterior part and single sharp edges on the middle part, with no spines on the posterior body. Three types of presumed sensory papillae are present: type A, a single small cone-shaped ciliated papilla; type B, a group of ciliated papillae on a common dome-shaped base; and type C, a large papilla with a non-ciliated bulb. Type A papillae are evenly scattered but more concentrated around oral and ventral suckers and the excretory pore. Pairs of both type A and B papillae line the body's

lateral surfaces, while type C papillae are solely on the ventral sucker's lip. In adult flukes, the surface appears highly corrugated with wave-like folds encircling the body, covered in closely packed stubby microvilli. Microvilli are more numerous and taller on the ventral surface than on the dorsal surface. Among microvilli on both stages, three types of sensory papillae like those in the NEJ were observed, but they are larger in size. The number of type A papillae increases, while that of type B papillae decreases during maturation (Apinhasmit et al.1993, 1994).

2.3 Anthelminthic drugs

2.3.1 Praziquantel

The general information of Praziquantel (PZQ), is 2- (cyclohexylcarbonyl) -1, 2, 3, 6, 7, 11b-hexahydro-4H-pyrazino [2,1-a] isoquinolin-4-one. Initially chosen for its anthelmintic properties in the mid-1970s, praziquantel was first utilized in the treatment of veterinary cestode and trematode infections. Subsequently, its application expanded to various human trematode infections, and it is currently regarded as the primary therapeutic agent for all forms of schistosomiasis (Cioli and Pica-Mattoccia, 2003). In its physical state, the drug appears as a white solid with a bitter taste, demonstrating stability under normal conditions. (Gonnert and Andrews, 1977). The pharmacokinetics of PZQ indicate that after oral administration, praziquantel appears in the bloodstream within approximately 15 minutes and reaches its peak concentration after 1-2 hours (Leopold et al., 1978; Valencia et al., 1994). Metabolism of praziquantel, when intravenously injected in animals, results in the rapid elimination of the sum of unchanged drug and metabolites from the intravascular space, with a half-life of 3 hours (phase I) and 8 hours (phase II). However, elimination of praziquantel itself is considerably shorter, approximately 1 hour. Following oral administration, praziquantel is almost entirely absorbed from the gastrointestinal tract, with maximum serum concentrations reached within 30 minutes to 1 hour (Steiner et al., 1976). The primary enzyme involved in praziquantel metabolism is microsomal

cytochrome P450, with main metabolites represented by mono-, di-, and tri-hydroxylated compounds (Cioli and Pica-Mattoccia, 2003).

2.3.2 Praziquantel opisthorchiasis treatment

Praziquantel (PZQ) has emerged as a highly effective chemotherapy option and is now the preferred treatment for opisthorchiasis. Opisthorchiasis treatment, the World Health Organization (WHO) recommends a praziquantel regimen of 25 mg/kg three times a day for 2 days (CDC, 2024). Further investigations into optimal dosing have been conducted. A systematic review and network meta-analysis found that administering praziquantel at doses of 50 mg/kg and 25 mg/kg of praziquantel given in a single day showed the highest predicted cure rate [93·8% (85·7–97·5)], while a single dose of 50 mg/kg praziquantel also resulted in a high predicted cure rate [92·1% (64·9–98·6)] (Qian et al., 2022).

The PZQ treatment, despite its effectiveness, can lead to adverse effects, primarily due to the induction of inflammation and subsequent oxidative and nitrative stress caused by the release of *O. viverrini* antigens. This presents a significant risk factor for the development of cholangiocarcinoma (CCA) in infected individuals (Pinlaor et al., 2009). Moreover, repeated treatment with PZQ, and consequently repeated infection with *O. viverrini*, is correlated with an elevated risk of CCA occurrence (Kamsa-Ard et al., 2015). Statistically, repeated PZQ treatment is associated with papillary and intrahepatic CCA (Luvira et al., 2018). Additionally, recurrent infections with *O. viverrini* followed by repeated PZQ treatment are linked to a high reinfection rate of nearly 90%, further heightening the risk of cholangiocarcinoma development (Hanpanich et al., 2017).

However, concerns are mounting due to the emergence of drug resistance and the side effects associated with PZQ treatment (Cioli and Pica-Mattoccia, 2003; Erko et al., 2012; Fallon and Doenhoff, 1994). Previous reports indicate PZQ resistance among certain strains of *S. mansoni* (Doenhoff et al., 2008; Gryseels et al., 2001; Stelma et al., 1995), as well as its ineffectiveness against schistosomula and the early developmental

stages of *Schistosoma* spp. (Sabah et al., 1986), potentially diminishing its efficacy in regions where schistosomiasis is endemic (Cioli and Pica-Mattoccia, 2003).

2.3.3 Praziquantel mechanism of action

The mechanism of action of praziquantel (PZQ) remains unclear. Evidence suggests that PZQ disrupts calcium-mediated processes in adult schistosomes, potentially by antagonizing (or partially agonizing, resulting in dysregulation) the parasite's voltage-gated calcium (Cav) channels and/or molecules that interact with and regulate these channels (Greenberg et al., 2005). Previous reports have linked exposure of schistosomes to the drug with calcium influx and muscular contraction, suggesting that calcium channels may be the target (Angelucci et al., 2007). Despite its high effectiveness, the molecular-level mechanism of action of PZQ remains incompletely understood. However, studies have observed that schistosomes exposed to the drug, both in laboratory settings and within living organisms, experience paralysis of their musculature. These findings suggest that PZQ induces a rapid influx of calcium ions (Ca^{2+}) within the schistosome, disrupting its inorganic ion transport mechanism and ultimately leading to the contraction of its musculature (Becker et al., 1980; Pax et al., 1978).

2.4. The effects of herbal on parasite

2.4.1 The herbal study on *O. viverrini*

Previous research has investigated herbal remedies against *O. viverrini* both *in vitro* and *in vivo*. Wannachat et al. (2020) reported similar findings using a crude extract from Areca catechu on *O. viverrini*, indicating a rapid decrease in motility with increasing concentrations of the *A. catechu* extract. Aukkanimart et al. (2015) examined the antioxidant, anti-inflammatory, and anthelmintic effects of the traditional folk medicine *Garcinia mangostana* pericarp extract in hamsters with opisthorchiasis. Intuyod et al. (2014) studied the activity of an anthocyanin complex containing cyanidin and delphinidin-rich extracts derived from turmeric against inflammation and periductal fibrosis in hamsters infected with *O. viverrini*. Additionally, Wonkchalee et al. (2012,

2013) demonstrated the efficacy of *T. laurifolia* in reducing inflammation resulting from pathological alterations in *O. viverrini*-infected hamsters.

Table 2.1 The efficacy of herbal remedies against *O. viverrini*

Herbs	Concentration/ dose	Result	Reference
Areca catechu crude extract	1, 5,10 mg/ml (*in vitro*) and 100 mg/kg 12 week (*in vitro*)	A rapid decrease in motility of *O. viverrini* worms is observed with increasing concentrations of the *A. catechu* extract. The reduction in egg production and worm burden	(Wannachat,et al., 2020)
Garcinia mangostana pericarp extract powder	100 mg/kg/day administered to *O. (in vivo)*	Anti-inflammatory interfere with parasite development	(Aukkanimart et al., 2015)
Anthocyanins extract from purple waxy corn, petals of blue butterfly pea and turmeric	175 and 700 mg/kg body weight every day for 1 month (*in vivo*)	Increases free-radical scavenging capacity. Decreases inflammation suppresses oxidative/nitrative stress. Reduces liver injury and periductal fibrosis.	(Intuyod et al., 2014)
Thunbergia laurifolia Fresh and dried leaves extract	100 mg/kg/day and 100 mg/kg/dose combination with PZQ (*in vivo*)	Antioxidant Antiinflammatory properties	(Wonkchalee et al., 2013; 2012)

2.4.2 *Allium sativum*

A. sativum (Garlic) belongs to the family *Liliaceae*, is grown globally primarily for the fleshy segments, known as cloves, within its bulbs (Figure 2.5). The garlic bulb is rounded and comprised of about 15 smaller bulblets called cloves, all enveloped

in a whitish or pinkish papery coat. It features four to twelve long, sword-shaped leaves attached to an underground stem. The flowers are densely packed in a spherical cluster on a spike that can extend up to 25 cm in length. Typically, the flowers fail to mature to a stage conducive for fertilization, resulting in abortion. While seeds are not commonly found in the wild, they have been successfully cultivated under laboratory conditions, exhibiting a black coat similar onion seed (Alam et al., 2016).

A. sativum contains an abundance of sulfur-based compounds, which impart its distinctive flavor and aroma, and are also associated with its beneficial effects (Butt et al., 2009). *A. sativum* is among the plants utilized for maintaining health and treating various ailments, known for its minimal or absence of side effects. *A. sativum* composition includes water (constituting 65% of its fresh weight), along with carbohydrates, proteins, lipids, fiber, vitamins, and minerals. The primary compounds found in garlic comprise organic sulfides, saponins, phenolic compounds, and polysaccharides (Zhong et al., 2019). One of its bioactive compounds, particularly allicin, is an organic sulfide predominantly present in freshly crushed garlic (comprising 1.1-3.5% of its fresh weight) (Hirsch et al., 2000). Most of the biological effects of garlic are attributed to its characteristic organosulfur compounds. Allicin (diallyl thiosulfate) has been reported to exhibit various biological activities, including antibacterial, antifungal, antiviral, antiparasitic, anticancer, and immunomodulatory effects on parasite-host interactions (Arreola et al., 2015; Cortes et al., 2017; Metwally et al., 2018; Liu et al., 2009). Furthermore, *A. sativum* has shown therapeutic potential against cardiovascular diseases, provides antioxidant effects, and offers protection to the liver from toxins and alcohol (Corzo-Martínez et al., 2007; Rabinkov et al., 1998).

Figure 2.5 Thai *A. sativum*, cloves (A), bulbs (B), and peel (C) (Cr. Pechdee, 2024).

2.4.3 The phytochemistry compound of *A. sativam*

The potential bioactive compounds found in *A. sativam* were categorized into several groups, depending on whether the garlic is intact or processed (Rana et al., 2011). Key bioactive compounds in garlic include thiosulfinates, which consist of δ-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin). These compounds serve as precursors to various compounds. Typically, when raw garlic is cut or crushed, these sulfoxides are converted into thiosulfinates (such as allicin) through enzyme-catalyzed reactions (Fujiwara et al., 1958). Other thiosulfinates found in garlic include allylmethyl-, methylallyl-, and trans-1-propenyl-thiosulfinate, which, like allicin, are all unstable (Lawson et al., 1991).

Organosulfur volatiles, *A. sativam* contains a wider variety of organosulfur volatiles than intact *A. sativam*. Major sulfides in *A. sativam* oil include diallyl disulfide (DADS) and diallyl sulfide (DAS), which result from the breakdown of allicin. The main volatiles identified in disrupted garlic and garlic essential oil include DAS, DADS, diallyl trisulfide, methylallyl disulfide, methylallyl trisulfide, vinyldithiins, and ajoenes. These are also significant components of steam-distilled *A. sativam* oil and oil-soluble *A. sativam* extracts, but with varying compositions (Amagase, 2006; Moutia et al., 2018).

Water-soluble organosulfur compounds, such as those found in aqueous and alcoholic *A. sativam* extracts, primarily consist of S-allyl-L-cysteines (SAC) derived from δ-glutamyl-S-allyl-L-cysteines. Additionally, these extracts contain trans-S-1-propenyl-L-cysteine and a small amount of S-methyl-L-cysteine (Amagase, 2006).

Furthermore, *A. sativam* contains other compounds such as lectins, prostaglandins, pectin, adenosine, fructan, vitamins B1, B2, B6, C, and E, biotin, nicotinic acid, glycolipids, phospholipids, fatty acids, and essential amino acids. Additionally, sapogenins and steroid saponins in garlic exhibit important pharmacological activities, including antifungal, antitumor, antithrombotic, and hypocholesterolemic effects (Moutia et al., 2018).

2.4.4 *Thunbergia laurifolia*

T. laurifolia (Rang Chuet in Thai), is a climbing plant characterized by smooth, opposing leaves along the stem. These leaves measure 8-10 cm in length and 4-5 cm in width, with a broad base that tapers to a pointed tip, often featuring scalloped lobes near the base. The flowers of this plant are trumpet-shaped, while the seed pod takes on a cone-shaped form, measuring approximately 1 cm in length with a rounded base. Notably, the purple flower variant of *T. laurifolia* has been noted for its diverse pharmacological properties, particularly in extracts derived from its stems, roots, and leaves (Junsi and Siripongvutikorn, 2016).

T. laurifolia is extensively utilized for its detoxifying properties against lead poisoning and other toxins, as well as for its antioxidant, anti-inflammatory, hepatoprotective, antitumor, and antihyperglycemic activities (Palipoch et al., 2011; Oonsivilai et al., 2007). In Thailand, *T. laurifolia* was included in the "National List of Essential Herbal Drugs A.D. 2011" by the Ministry of Public Health for the treatment of poisoning symptoms and fever (Thai-FDA, 2020). The leaf extract of *T. laurifolia* is primarily employed as an antidote for intoxications caused by insecticides, herbicides, lead, alcohol, and chemical toxins (Palipoch et al., 2011; Rocejanasaroj et al., 2014). Furthermore, it has demonstrated various pharmacological activities including antiproliferative, hepatoprotective, detoxifying, antimicrobial, antidiabetic, anti-inflammatory, and non-toxic effects (Chan et al., 2011).

Figure 2.6 *T. laurifolia* leaves (Cr. Pechdee, 2024)

2.4.5 The phytochemistry compound of *T. laurifolia*

The phytochemical analysis of *T. laurifolia* leaves reveals the presence of compounds grouped into five categories. Group 1 comprises sterols, including beta-sitosterol, stigmasterol, and alphaspinasterol. Group 2 consists of phenolics such as rosmarinic acid, apigenin, caffeic acid, gallic acid, and protocatechuic acid. Group 3 is carotenoids such as lutein and group 4 are unclassified steroids. Group 5 consists of glycosides, including grandifloric acid, benzyl β-glucopyranoside, benzyl β-(2'-O-β-glucopyranosyl)-glucopyranoside, 6-C-glucopyranosyl apigenin, 6,8-di-C-glucopyranosyl apigenin, (E)-2-hexenyl-β-glucopyranoside, and hexanol-β-glucopyranoside (Chan et al., 2011; Junsi and Siripongvutikorn, 2016). Other components, such as fiber, ash, protein, fat, and carbohydrate percentages in the leaf were analyzed proximately, with values of 16.8, 18.8, 16.7, 1.68, and 46.0%, respectively, based on dry weight (Jaiboon et al., 2010).

Notably, rosmarinic acid is identified as the primary component in *T. laurifolia* leaf water extract. This compound has garnered significant attention due to its diverse

pharmacological effects, including anti-inflammatory, antioxidant, antidiabetic, and hepatoprotective properties. Its broad spectrum of pharmacological activities renders it promising for drug development. The efficacy and safety of rosmarinic acid are contingent upon its pharmacokinetics, with bioavailability being a critical factor in the successful utilization of medicinal herb extracts (Guan et al., 2022; Woottisin et al., 2022).

2.4.6 *Eurycoma longifolia*

E. longifolia (known as Plarai Pleak in Thai and Tongkat Ali in Malaysia) is a slender evergreen tree that grows wild at an altitude of approximately 500 meters above sea level in the forests spanning Malaysia, Borneo, southern Myanmar, Cambodia, Vietnam, Laos, Thailand, Indonesia, and the Philippines. It is an evergreen, slow-growing herbal plant, reaching a maximum height of 15-18 m and bearing fruit after nearly 2–3 years of cultivation. The fruits start green, measuring 2-3 cm in length, and mature into a dark red color upon ripening. Its leaves are pinnate, arranged spirally, and elongated (10-15 inches) with 10–30 leaflets. Large panicles bear its flowers, and the plant is dioecious, with male and female flowers appearing on separate trees. Naturally thriving on wild jungle slopes, this plant is widely sought after either on its own or as an essential component in herbal remedies for various ailments and as health supplements (Bhat and Karim, 2010; Wizneh and Asmawi, 2014). The roots of *E. longifolia* have exhibited various biological activities, including anti-cancer, antimicrobial, anti-parasitic, hypertension regulation, anti-pyretic properties, and enhancement of male testosterone levels (Al-Salahi et al., 2013; Hout et al., 2006; Rehman et al., 2016).

Figure 2.7 *E. longifolia*, Root (A) and Tree (B). (Pharmaceutical Sciences, Ubon Ratchathani University, 2010a).

2.4.7 The phytochemistry compound of *E. longifolia*

The *E. longifolia* serves as a rich source of various classes of bioactive compounds, including quassinoids, β-carboline alkaloids, canthin-6-one alkaloids, triterpene-type tirucallane, squalene derivatives, and a range of lactones such as eurycolactone, eurycomalactone, laurycolactone, biphenyl neolignan, and bioactive steroids (Bhat and Karim, 2010).

The major constituents in *E. longifolia* along with their secondary metabolites include: Quassinoids consist of various types of eurycomanone (pasakbumin-A), eurycomanols, pasakbumin-B, hydroxyklaineanones, eurycomalactones, eurycomadilactones, eurylactones, laurycolactones, longilactones, and hydroxyglaucarubol (Chan et al., 1989).

Squalene derivatives include Teurilene, eurylene, 14-deacetyleurylene, and longilene peroxide (Itokawa et al., 1991).

Biphenyl neolignans comprise 2-hydroxy-3,2,6-trimethoxy-4-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)-biphenyl; two isomeric 2,2-dimethoxy-4-(3-hydroxy-1-propenyl)-4-(1,2,3-trihydroxypropyl) diphenyl ethers; and 2-hydroxy-3,2-dimethoxy-4-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)biphenyl (Morita et al., 1992).

Alkaloids consist of 5,9-dimethoxycanthin-6-one; 9,10-dimethoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one; 10-hydroxy-9-methoxycanthin-6-one; and 9-methoxy-3-methylcanthin-5,6-dione (Mitsunaga et al., 1994).

The root, particularly, contains a high concentration of eurycomanone, a bioactive compound derived from quassinoid metabolites that holds significant medicinal value (Hada Masayu et al., 2017; Jusoh et al., 2015). The presence of eurycomanone and its derivatives in *E. longifolia* has shown promising effects, including energy enhancement, muscle mass augmentation, bone mass increase, and amelioration of sexual dysfunction (Abd Aziz et al., 2021).

2.5 Oxidative stress

Oxidative stress arises from an imbalance in the production of reactive oxygen species (ROS) within the body, disrupting its capacity to detoxify these reactive intermediates or repair resulting damage to cellular and organ systems. The intracellular redox balance, reflecting the equilibrium between oxidizing and reducing agents within cells, is closely tied to the antioxidant peptide glutathione. Enzymes regulate glutathione levels intracellularly to maintain a reducing environment. Although some ROS are continually generated at low levels during normal metabolic processes, enzymes typically regulate these levels. However, disturbances in the redox balance can lead these moderately reactive species to interact with transition metals or other components of the redox cycle, resulting in the formation of highly reactive oxygen species. These highly reactive species can potentially cause significant damage to cell membranes, lipids, proteins, DNA, and cellular organelles (Burton and Jauniaux, 2011; Storz and Imlayt, 1999).

2.5.1 Free radical

When cells utilize oxygen for energy production through ATP (Adenosine triphosphate) synthesis in mitochondria, they concurrently generate free radicals, namely ROS and Reactive Nitrogen Species (RNS). These by-products play a dual role as both beneficial and harmful compounds, significantly impacting cellular functions.

ROS and RNS at low or moderate levels support cellular responses and immune function. However, elevated concentrations lead to oxidative stress, damaging cell structures and contributing to chronic conditions such as cancer, arthritis, and neurodegenerative diseases. To counter oxidative stress, the body employs antioxidants, either produced internally or obtained from diet or supplements. These antioxidants neutralize ROS and RNS, bolstering immune defenses and potentially reducing the risk of degenerative diseases. (Pham-Huy et al., 2008).

ROS and RNS collectively encompass free radicals and other non-radical reactive derivatives, also referred to as oxidants. Radicals are less stable compared to non-radical species but demonstrate stronger reactivity. A molecule that contains one or more unpaired electrons in its outer shell is characterized as a free radical (Dröge, 2002; Valko et al., 2004; Bahorun et al., 2006). Free radicals encompass hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$), and lipid peroxyl ($LOO^{\bullet}$). Additionally, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and lipid peroxide (LOOH) are not free radicals but are generally classified as oxidants. Nevertheless, they can readily initiate free radical reactions within living organisms. Biological free radicals are thus highly reactive molecules with unpaired electrons capable of reacting with various organic substrates such as lipids, proteins, and DNA (Genestra, 2007; Pham-Huy et al., 2008).

2.5.2 Generation of free radicals and oxidants

ROS and RNS are formed within cells through enzymatic and non-enzymatic reactions. Enzymatic reactions, such as those in the respiratory chain, phagocytosis, prostaglandin synthesis, and cytochrome P450 system, generate free radicals. For instance, the superoxide anion radical ($O_2^{\bullet -}$) is produced by oxidase systems like NADPH oxidase and xanthine oxidase, leading to the formation of hydrogen peroxide, hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl). Hydroxyl radical, the most reactive free radical in vivo, forms via the Fenton reaction catalyzed by Fe_2+ or Cu+ ions. Hypochlorous acid (HOCl) is generated by myeloperoxidase in

neutrophils, while nitric oxide radical ($NO^{\bullet}$) arises from the oxidation of L-arginine by nitric oxide synthase in biological tissues (Bahorun et al., 2006; Dröge, 2002; Genestra, 2007; Pham-Huy et al., 2008) (Figure 2.8). Free radicals can originate from non-enzymatic reactions of oxygen with organic compounds, as well as from ionizing radiations. Nonenzymatic processes occur during oxidative phosphorylation (aerobic respiration) in mitochondria. ROS and RNS are produced from both endogenous and exogenous sources. Endogenous free radicals stem from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, and aging. Exogenous ROS/RNS arise from environmental factors such as air and water pollution, cigarette smoke, alcohol, heavy metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking processes (smoked meat, used oil, fat), and radiation. Upon entry into the body via various routes, these exogenous compounds decompose or metabolize into free radicals (Genestra, 2007; Valko et al., 2004, 2007).

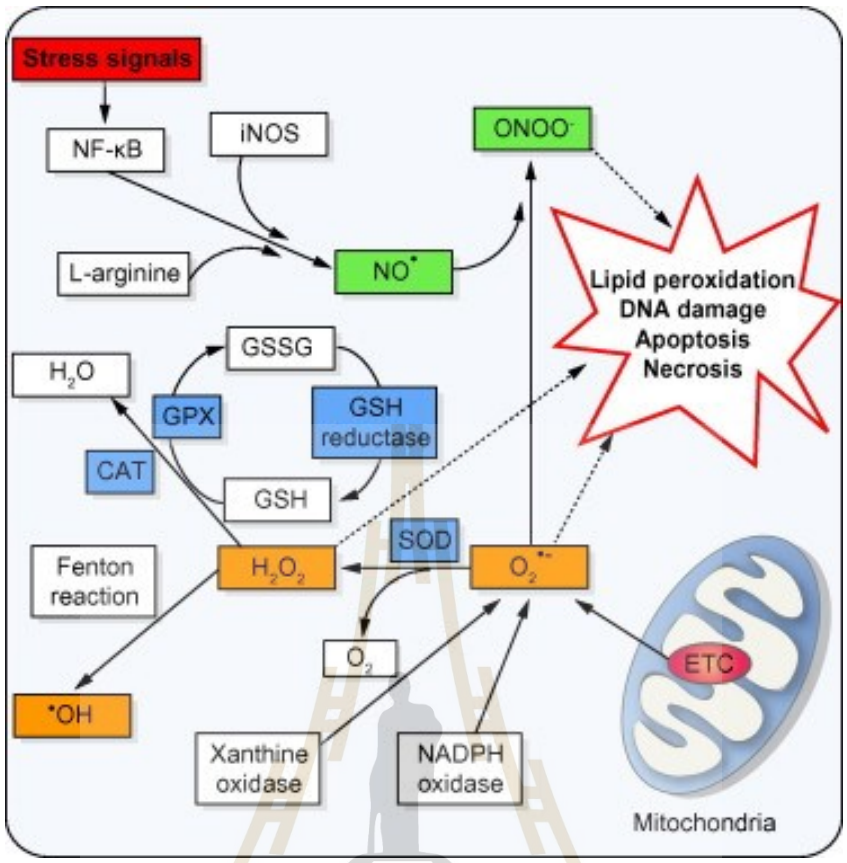


Figure 2.8 Main pathways for the formation of ROS and NOS. Mitochondria are the major source of cellular ROS during respiratory processes (Tell et al., 2013).

2.5.3 Nitric oxide (NO)

Nitric oxide (NO) serves as a vital biological mediator, playing crucial roles across various biological processes in fields such as biochemistry, physiology, immunology, and neuroscience. It is synthesized as a free radical from L-arginine by the enzyme Nitric Oxide synthase (NOS), which is encoded by separate genes and is highly regulated in biology. Several cofactors participate in this synthesis process, including oxygen, tetrahydrobiopterin (BH4), nicotinamide-adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Benz et al., 2002; Khazan and Hedayati, 2015).

There are three identified isoforms of Nitric Oxide synthase (NOS), as detailed in (Table 2.2). The neuronal isoform (nNOS or NOS1) and the endothelial isoform (eNOS

or NOS3 or cNOS) are constitutive enzymes that are calcium-dependent (cNOS). In contrast, the inducible isoform (iNOS or NOS2) is calcium-independent (Nahrevanian, 2009; Pacher et al., 2007). NO functions as a retrograde neurotransmitter and is produced by the neuronal isoform in both the central and peripheral nervous systems, suggesting its role in memory and learning processes. Additionally, nNOS regulates cardiac function, peristalsis, and sexual arousal in males and females. An alternatively spliced form of nNOS acts as a major muscle protein that responds to calcium release from the sarcoplasmic reticulum (SR). The inducible isoform iNOS produces large amounts of NO as part of a defensive mechanism. It is synthesized by various cell types in response to cytokines and plays a critical role in the body's defense against parasites, bacterial infections, and tumor growth. However, iNOS can also contribute to septic shock and may be involved in autoimmune diseases Endothelial NOS (eNOS) generates NO in blood vessels and is involved in regulating vascular function, cardiac function, angiogenesis, insulin secretion, and airway tone. eNOS is particularly crucial in controlling smooth muscle tone (Förstermann and Sessa, 2012; Khazan and Hedayati, 2015; Nahrevanian, 2009; Pacher et al., 2007).

Additionally, non-enzymatic production of NO occurs through the one-electron reduction of nitrite, a reservoir of NO in blood and tissues. This process appears to be widespread and is significantly accelerated under hypoxic conditions. This discovery challenges the traditional view that nitrate and nitrite are merely waste products of NO. NO can react with oxygen, metals, and free radicals. Biological transformations of NO into its forms (NOx) include NO_2, N_2O_3, NO_2 (nitrite), and NO_3 (nitrate) in blood, influenced by diffusion, convection, distribution coefficients, and chemical reactions across different compartments of human blood. The rates of NO formation and clearance determine its steady-state concentration. NO is beneficial at low concentrations (nanomolar) but can become toxic at higher levels (micromolar) or in the presence of ROS such as H2O2 and $O2^{\bullet-}$ (Förstermann and Sessa, 2012; Khazan and Hedayati, 2015).

Table 2.2 Characteristics of NOS Isoforms [modified from (Khazan and Hedayati, 2015)]

Name	Gene(s)	Location	Function	Ca2+ Dependency
Neuronal NOS (nNOS, NOS1)	NOS1, chromosome 12	nervous tissue, skeletal muscle type II	cell communication, signal transduction, neurotransmission	Ca2+ dependent
Inducible NOS (iNOS, NOS2)	NOS2, chromosome 17	Immune system, cardiovascular system	Immune defense against pathogens	Ca2+indepen dent
Endothelial NOS (eNOS, NOS3, cNOS)	NOS3, chromosome 7	endothelium	vasodilation, modulation of platelet aggregation, modulation of leukocyte endothelial interactions	Ca2+ dependent

2.5.4 The beneficial activities of free radicals

At low or moderate concentrations, ROS and RNS play essential roles in cellular structure maturation and act as defenders in the immune system. Phagocytes release these radicals to combat invading pathogens, crucial for protecting against disease . Patients with granulomatous disease demonstrate the necessity of ROS production through the NADPH oxidase system, as their impaired function leads to recurrent infections. Additionally, ROS and RNS contribute beneficially to cellular signaling systems, regulated by nonphagocytic NADPH oxidase isoforms in various cell types. For instance, nitric oxide (NO) serves as an intercellular messenger influencing blood flow, thrombosis, and neural function. Furthermore, these radicals contribute to nonspecific host defense, aiding in the elimination of pathogens and tumors, and can induce mitogenic responses. In summary, ROS/RNS at controlled levels are crucial for human health (Dröge, 2002; Pham-Huy et al., 2008; Valko et al., 2007).

2.5.5 The deleterious activities of free radicals

Excessive production of free radicals and oxidants results in oxidative stress, a harmful process that significantly impacts cell membranes and various structures such as proteins, lipids, lipoproteins, and DNA. Oxidative stress occurs when cells fail to adequately neutralize the excess free radicals generated, creating an imbalance between their formation and neutralization by antioxidants. For instance, excessive hydroxyl radicals and peroxynitrite can lead to lipid peroxidation, resulting in the production of cytotoxic and mutagenic compounds like malondialdehyde (MDA) and conjugated dienes. Lipid peroxidation spreads rapidly through a radical chain reaction, affecting numerous lipid molecules. Proteins are also susceptible to damage by ROS/RNS, causing structural alterations and loss of enzymatic function. DNA damage from oxidative stress results in various lesions that can induce mutations. To counteract these effects, the body employs DNA repair enzymes and antioxidants. Failure to regulate oxidative stress properly can contribute to chronic and degenerative diseases, accelerate the aging process, and exacerbate acute pathologies such as trauma and stroke (Halliwell, 2007; Pham-Huy et al., 2008; Willcox et al., 2004).

2.5.6 ROS detection methods

The diversity of free radicals and the various mechanisms of antioxidant action, multiple methods are employed to assess these capabilities. For instance, the ability to scavenge free radicals is evaluated using stable free radicals like DPPH$^{\bullet}$. The FRAP assay measures the ability to reduce ferric ions (FRAP). Additionally, the antioxidant properties of cell extracts are tested using the DCFH-DA assay. This assay involves the use of DCFH-DA (2', 7'-dichlorofluorescin-diacetate), a nonfluorescent compound that penetrates cells. Once inside, esterase enzymes cleave off the diacetate group from DCFH-DA, converting it into DCFH, which is more polar. Intracellular free radicals oxidize DCFH to produce the fluorescent compound DCF, emitting light. Therefore, the fluorescent signal from DCF reflects intracellular oxidative stress within the cell (Datta et al., 2019; Goel et al., 2020; Wolfe and Liu, 2007).

2.5.7 NO detection methods

NO exhibits extremely low stability in the body, degrading rapidly after synthesis with an average half-life of about 3-6 seconds in tissues and 1-2 seconds in blood. Upon synthesis, NO quickly oxidizes in the presence of oxygen to form nitrite and nitrate, metabolic by-products found in plasma and whole blood, respectively. Nitrite can further convert to nitrate under oxygen-sufficient conditions. The half-lives of nitrate and nitrite in the circulatory system are approximately 5-8 hours and 110 seconds, respectively. Because of NO short half-life, directly measuring its synthesis is challenging, prompting researchers to focus on measuring its metabolite products, nitrite and nitrate. Various methods are available for NO measurement, including UV absorbance, GC-MS, HPLC, ion-selective electrode, capillary electrophoresis, fluorescent assay, chemiluminescence assay, and electrochemical detection. Among these, the Griess assay is widely preferred due to its speed, simplicity, cost-effectiveness, and accuracy. It correlates well with measurements obtained using GC-MS for quantifying NO levels in serum (Ghasemi et al., 2007; Khazan and Hedayati, 2015; Romitelli et al., 2007).

2.6 Scanning electron microscopy

The concept of SEM dates back to the early 20th century, originating from the pioneering work of physicists Ruska and Knoll in 1933 (Abdullah and Mohammed, 2019). SEM has evolved into a crucial instrument for studying microstructural morphology and chemical compositions, replacing light sources with high-energy electron beams. This technique enables detailed examination of inorganic and biological materials at magnifications ranging from 1000 to over 500,000 times, with resolutions finer than 1 nm (Davies et al., 2022).

2.6.1 Principle and components in an SEM

SEM systems consist of essential components: an electron source, multiple electron lenses, a deflection system, a vacuum system, a control console, and various detectors for signal analysis. The electron gun generates and accelerates electrons

typically within the 0.1–30 kV energy range, selected based on considerations such as cost, longevity, and resolution requirements. To produce images, the beam crossover from the electron source must be demagnified to form a focused point or probe with a spot size smaller than 10 nm, achieved through electromagnetic lenses. The condenser lens reduces the beam size, while the objective lens focuses the electron probe onto the sample surface, a function controlled by the operator to achieve optimal image focus. Within the objective lens, additional components include the stigmator for correcting beam aberrations, scanning coils for moving the probe across the sample surface in a raster pattern with defined dwell times, and beam limiting apertures to control the beam size. The scanning coils create a raster pattern on both the sample and the viewing screen simultaneously, with the magnification ratio determined by the size of the sample raster projected onto the screen. For instance, a 10 μm wide sample raster projected at 100 mm on the screen results in a magnification of 10,000 times. A SEM requires a vacuum system capable of maintaining pressures ranging from 10-4 to 10-8 Pa, depending on the specific instrument's requirements and applications (Davies et al., 2022; Zhou et al., 2007). Differences between a light microscope and an electron microscope as detailed in (Table 2.3).

Table 2.3 Difference between light microscope and electron microscope [modified from (Kannan, 2018)].

No.	Feature	Light microscope	Electron microscope
1.	Electromagnetic spectrum	Visible light, 400-700 nm Colors visible	Electrons, app. 4nm Monochrome
2.	Maximum resolving power	app. 200nm	0.5nm with very fine detail
3.	Maximum magnification	x1000 to x1500	x500000

Table 2.3 (Continued) Difference between light microscope and electron microscope (Kannan, 2018)

No.	Feature	Light microscope	Electron microscope
4.	Radiation source	Tungsten or quartz halogen lamp	High voltage (50kV) tungsten lamp, Lanthanum hexaboride
5.	Lenses	Glass	Electro magnetics
6.	Interior	Air-filled	Vacuum
7.	Focusing screen	Human eye (retina), photographic film	Fluorescent (TV) screen, Photographic film
8.	Preparation of specimens	Temporary mounts living or dead	Tissues must be dehydrated = dead
9.	Fixation	Alcohol	OsO4 or KMnO4
10.	Embedding medium	Wax	Resin
11.	Sectioning of specimen	Hand or microtome sectioning < 20μm slice Whole cells visible	Ultra microtome sectioning < 50nm slices Parts of cells visible
12.	Staining	Water soluble dyes	Heavy metals
13.	Support for sample	Glass slide	Copper grid

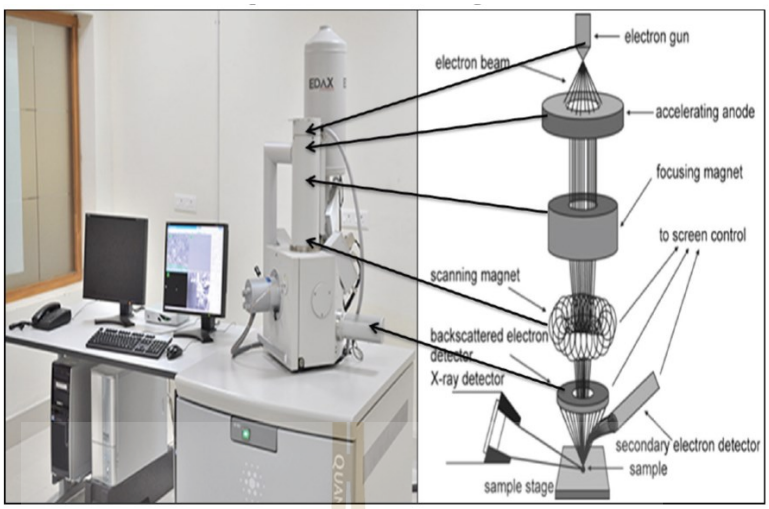


Figure 2.9 The components of SEM (Kannan, 2018)

2.6.2 Specimen Preparation for SEM

A scanning electron microscope (SEM) is utilized to capture three-dimensional images of specimen surfaces and to observe large objects. It functions by scanning the sample with a high-energy electron beam in a raster pattern. Electron interactions with atoms generate signals that convey details about the sample's surface topography, composition, and other characteristics. Ayub et al. (2017) have provided a detailed description of specimen preparation methods for SEM (Ayub et al., 2017).

2.6.2.1 Cleaning the specimen surface: Thorough cleaning is essential to remove contaminants like dust or soil.

2.6.2.2 Stabilizing the specimen: This step typically involves fixation using chemical solutions such as glutaraldehyde and osmium tetroxide to preserve the specimen's structure.

2.6.2.3 Rinsing: To remove excess fixative from the specimen.

2.6.2.4 Dehydrating: Gradually removing water from the specimen using acetone or ethanol.

2.6.2.5 Drying: Ensuring the specimen is completely dry to prevent damage in the high vacuum environment of the electron microscope chamber.

2.6.2.6 Mounting: Securing the specimen onto a holder using adhesive or conductive tape.

2.6.2.7 Coating: Applying a thin layer (20-30 nm) of conductive metal like gold or platinum to enhance specimen conductivity and prevent the buildup of electrical charges.

2.6.3 Advantages of SEM

SEM provides detailed 3D and topographical imaging, offering high-resolution views of surface details and morphology insights. It integrates various detectors such as secondary electron, backscattered electron, and energy dispersive X-ray spectroscopy (EDS) for comprehensive analysis of elemental composition and material properties. SEM operates swiftly, generating real-time or near real-time data to facilitate efficient analysis and experimentation. Modern SEMs produce digital data, facilitating convenient storage, analysis, and dissemination of research findings across academic communities. Moreover, SEM typically requires minimal sample preparation compared to other microscopy techniques, thereby reducing the time and effort involved in specimen preparation (Arenas-Alatorre et al., 2010; Kannan, 2018).

2.6.4 Disadvantages of SEM

SEM systems are costly to acquire, operate, and maintain, requiring substantial laboratory space due to their size and complexity. Proficiency in SEM operation necessitates specific training and expertise in intricate instrumentation and electron microscopy principles. The preparation of samples for SEM analysis can introduce artifacts or alter the natural state of the specimen, potentially impacting result accuracy. SEMs are predominantly tailored for examining solid samples and may require special preparation techniques to analyze liquids, gases, or delicate biological specimens effectively. Although minimal, there is a slight risk of radiation exposure associated with scattered electrons from beneath the sample surface during SEM operation (Arenas-Alatorre et al., 2010; Kannan, 2018).

2.7 Related research

2.7.1 Effects of *A. sativum* to parasite

A. sativum utility is documented in *in vivo* and *in vitro* studies, inhibiting protozoa, helminths and influencing immune responses. It has been observed that garlic demonstrates inhibitory effects on the development of various protozoa, including *Trypanosoma brucei brucei*, *T. congolense*, *T. vivax*, *Trypanosoma* spp., *Entamoeba histolytica*, *Leishmania major* and *L. infantum* (Behnia et al., 2008; Corral et al., 2016; Ghazanfari et al., 2006; Krstin et al., 2018; Nok et al., 1996; Vathsala et al., 2020). In infected mice, garlic has also shown enhance immune response against *Plasmodium yoelii* (Feng et al., 2012). Additionally, garlic has proven effective against *Haemonchus contortus*, a parasitic nematode in ruminant animals (Palacios-Landín et al., 2015), *Anisakis* sp., a nematode in marine fish (Morsy et al., 2021). Studies indicate that garlic influences *Schistosoma mansoni* at various stages, including adult worms, cercaria, schistosomula, and miracidia (Mantawy et al., 2012). It has enhanced the immune response in mice infected with *S. mansoni* (Aly et al., 2017; Cortes et al., 2017; El Shenawy et al., 2008; Mantawy et al., 2011; Metwally, 2006, 2018; Riad et al., 2013; Wan et al., 2017). Furthermore, garlic extract has been shown to affect the morphological damage caused by the liver fluke, *Fasciola gigantica* (Jeyathilakan et al, 2012; Singh et al., 2009).

Table 2.4 Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/*A. sativum* oil	*Anisakis* spp. infected rats	100 mg/kg BW daily for 21 days	Garlic oil exhibited a protective effect in rats inoculated with L3 larvae by ameliorating liver and kidney functions.	(Morsy et al., 2021)
Garlic pearl oil	*P. berghei* infected mice	100 ul/mouse on Day 3, 4 and 5	The garlic-arteether combination exhibited immunomodulatory and antiparasitic effects through the nitric oxide pathway in *P. berghei* infected mice.	(Vathsala et al., 2020)
Bulbs/dichloromethane extraction	*Trypanosoma brucei brucei*	Briefly, 2 x 10^6 *T. b. brucei* cells/ml were incubated with 3, 4 and 5 ug/ml	It inhibited *T. b. brucei* trypanothione reductase irreversibly and decreased the mitochondrial membrane potential	(Krstin et al., 2018)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/aqueous extract and Allicin standard extract	*S. mansoni* infected mice	50 mg/kg (Garlic extract) and 0.5 uM/mouse (Allicin) pretreatment and posttreatment on days 49	Garlic and allicin significantly reduced the worm burden and lowered serum concentrations of liver fibrosis markers and proinflammatory cytokines.	(Metwally et al., 2018)
Bulbs/ methanol extraction and and fractions	*S. mansoni* infected mice	2g/Kg BW daily for 45 days	*A. sativum* treatment had effective schistosomicidal activities, through reduction of worm burden and tissue eggs.	(Aly et al., 2017)
Bulbs/ *A. sativum* oil	*S. japonicum* cercariae and infected mice	for 30 min, 1h, and 4h (*in vitro)* and 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} (v/v) (*in-vivo*) on day 35	Garlic oil extract resulted in mortality of *S. japonicum* cercariae, exhibited preventive efficacy against of infection, and contributed to a reduction in worm burden.	(Wan et al., 2017)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Allicin standard extract	*Leishmania infantum*	15, 30, 60, 90 uM	Allicin Induces calcium and mitochondrial dysregulation causing necrotic death in *L. infantum*	(Corral et al., 2016)
Bulbs/n-hexane extraction	*Haemonchus contortus* larvae (L3) and *H. contortus* infected gerbils	40, 20, 10, 5, 2.5, 1.25, and 0.625 mg/ml (*in vitro*) and 40 mg/ml daily for 13 days (*in vivo*)	Garlic extract exhibited a 68% larvicidal activity (*in vitro)* and resulted in a 68.7% reduction of the parasitic burden (*in vivo).*	(Palacios-Landín et al., 2015)
Bulbs/crude juice extraction	*S. mansoni* infected mice	50 mg/kg BW/mouse daily for 7 weeks	Garlic extract was able to ameliorate genetic alterations in the DNA from schistosomal infection	(Riad et al., 2013)
Allicin standard extract	*P. yoelii* infected mice	3 and 9 mg/kg on day 0, 3, and 5 post-infections	Allicin could partially protect host against *P. yoelii* through enhancement of the host innate and adaptive immune responses.	(Feng et al., 2012)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/Aqueous extract	*F. gigantica* adult worms	1, 2.5 and 5% in RPMI-1640 culture media	The flukicidal effect was observed through a reduction in both motility and mortality at a concentration of 5%	(Jeyathilakan et al, 2012)
Bulbs/garlic powder	*S. mansoni* at different stages and infected mice	0.5-5 ppm (*in vitro*) 2 g/100 g BW daily for 45 days (*in vivo*)	*In vitro*, they demonstrated strong biocidal effects against all stages of *S. mansoni*. *In vivo*, there was significant ihibition observed in SOD, CAT, GR, TrxR, and SDH.	(Mantawy et al., 2012)
Bulbs/garlic powder	*S. mansoni* infected mice	2 g/100 g BW daily for 45 days	There was an increase in antioxidant enzyme activities, coupled with a significant reduction in worm burden, hepatic and intestinal eggs, as well as oogram count.	(Mantawy et al., 2011)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/crude juice extraction	*S. mansoni* infected mice	50 mg/kg BW daily for 7 weeks	Garlic treatment led to a significant reduction in egg and worm burden, accompanied by various ultrastructural alterations in the tegument	(Riad et al., 2009)
Bulbs/garlic essential oils ether extraction	*F. gigantica* adult worms	0.1, 0.3, 1 and 3 mg/ml for 15 min.	*A. sativum* essential oil induced an irreversible paralytic effect after exposure at a concentration of 3 mg/ml.	(Singh et al., 2009)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/ethanol: water (60:40) extraction and essential oil	*E. histolytica*	0-100 mg/ml	The antiamoebic activity of *A. sativum* extracts and essential oil was assessed at concentrations of 4 mg/ml and 0.4 mg/ml after 24 h, and at concentrations of 3 mg/ml and 0.3 mg/ml after 48 h, respectively.	(Behnia et al., 2008)
Bulbs/aqueous extraction	*S. mansoni* infected mice	125 mg/kg for 28 days (3 times/week)	Improved the antioxidant capacity of mice schistosomiasis. Reduced worm burden and alteration of tissue eggs	(El shenawy et al., 2008)
Bulbs/Aqueous extract and fractions	*L. major* infected mice	Garlic extract (20 mg/kg) or R10 fraction (0.04 mg/kg) Intraperitoneal injection on days 3, 5, 7 and 14	Augments parasite engulfment and destruction of intracellular amastigotes by macrophages.	(Ghazanfari et al., 2006)

Table 2.4 (Continued) Prior studies of the efficacy of *A. sativum* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Bulbs/*A. sativum* oil	*S. mansoni* infected mice	5ml/kg BW/day for 8 weeks	Garlic oils substantially normalized liver function enzymes and showed varying improvements in other parameters, notably reducing worm burden and ova count.	(Metwally, 2006)
Bulbs/ Cold extraction of *A. sativum* oil	*T. b. brucei, T. congolense,* and *T. vivax* infected mice	1.5, 3, and 5.0 mg/ml (*in vitro*) 40, 80, and 120 mg/kg/day 24 h until day 30 (*in vivo*)	The extract appears to consist of diallyl-disulfide (DAD), which could potentially interfere with the parasites' synthesis of membrane lipids.	(Nok et al., 1996)

2.7.2 *A. sativam* mechanism of action

The mechanism of action of *A. sativum* against parasites, as reported by Corral et al. in 2016, involves the bioactive compound allicin. Allicin induces calcium and mitochondrial dysregulation, leading to necrotic death in *L. infantum* parasites. Exposure of the parasites to allicin results in elevated Ca2+ levels and mitochondrial reactive oxygen species (ROS), along with the collapse of the mitochondrial membrane potential and decreased ATP levels. Importantly, these effects are not associated with

plasma membrane permeabilization and allicin exposure leads to increased cytosolic ROS levels and induces cell cycle arrest in the premitotic G2/M phase (Coral et al., 2016). Furthermore, the mechanism underlying the biological activity of garlic seems to be correlated with the quantity and composition of sulfur-containing compounds. It is probable that essential components within the parasitic cell, such as trypanothione reductase, are inhibited by the formation of disulfide bonds between the SH groups of crucial redox compounds and sulfur-containing secondary metabolites (Krstin et al., 2018).

2.7.3 Effects of *T. laurifolia* to parasite

The effects of *T. laurifolia* on parasites, it was demonstrated that solutions made from fresh and dried *T. laurifolia* effectively reduced inflammatory cells in *O. viverrini* infected hamsters. When combined with praziquantel, *T. laurifolia* extract further reduced inflammatory cell aggregation and inhibited the development of cholangiocarcinoma (CCA), which was correlated with the serum ALT level in *O. viverrini* infected hamsters, without notable toxic side effects. (Wonkchalee et al., 2012, 2013).

Table 2.5 Prior studies of the efficacy of *T. laurifolia* extract in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Leaves/Fresh and dried	*O. viverrini* infected hamster	100 mg/kg/day daily for 30 days	*T. laurifolia* fresh and dried solutions that clearly reduced the inflammatory cells in hamster infection with *O. viverrini*	(Wonkchalee et al., 2012)
Leaves/powder	*O. viverrini* infected hamster	100 mg/kg/dose daily for 60, 90, 120, and 180 days	*T. laurifolia* extract combined with PZQ reduced inflammatory cell aggregation and inhibiting CCA development	(Wonkchalee et al., 2013)

2.7.4 *T. laurifolia* mechanism of action

The mode of action through which *T. laurifolia* impacts parasites remains inadequately explored. However, insights into this mechanism can be deduced from the broader effects of phenolic compounds on bacterial cells, as outlined by Resende et al. (2015). Phenolic compounds are known to act effectively at the membrane interface of gram-positive bacteria, significantly compromising membrane plasticity and thereby undermining cell integrity. This disruption ultimately leads to destabilization of the cell membrane and its transport systems (Resende et al., 2015).

T. laurifolia leaf extract is considered an efficient antioxidant, as indicated by the study of Vale et al., 2020. They pointed out that using antioxidants, either alone or in combination with anthelmintic drugs, could mitigate tissue damage, infection-related complications, and even prevent the development of cancer associated with infections. Therefore, antioxidants represent a potential adjuvant approach during treatment to reduce morbidity and mortality. Despite some successful strategies, there is still a long way to go in implementing novel therapies for schistosomiasis or opisthorchiasis (Vale et al., 2020).

2.7.5 Effects of *E. longifolia* to parasite

Previous reports on the effects of *E. longifolia* on parasites have indicated its positive antimalarial properties through both *in vitro* and *in vivo* studies involving chloroquine-resistant *Plasmodium falciparum* and *P. yoelii*-infected mice (Chan et al., 2004; Hout et al., 2006; Kuo et al., 2004; Mohd Ridzuan et al., 2005, 2007; Sriwilaijaroen et al., 2010). *E. longifolia* extract has also shown potential as a therapeutic candidate against *Blastocystis* sp. and *Toxoplasma gondii* (Girish et al., 2015; Kavitha et al., 2012a, 2012b). Furthermore, it has been tested against schistosomes of *S. japonicum*, demonstrating an effect on adult schistosome movement (Jiwajinda et al., 2002).

Table 2.6 Prior studies of the efficacy of *E. longifolia* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Root/aqueous extraction	*Blastocystis sp.*	0.1 mg/ml and 1.0 mg/ml	Anti-protozoal activity at 1.0 mg/ml	(Girish et al., 2015)
Root/ methanol extraction and fractions	*Toxoplasma gondii*	1.125 ug/mL and 1.375 ug/mL	*T. gondii* cell wall alterations (SEM), decreased cytoplasmic volum (TEM) and rapid antiparasitic activity	(Kavitha et al., 2012)
Root/ methanol extraction and fractions	*T. gondii*	1.56–100 ug/ml	anti-*T. gondii* activity: TAF 355 fraction (EC50 = 0.369 ug/ml) and TAF 401 fraction (EC50 = 0.882 ug/ml).	(Kavitha et al., 2012)
Roots/ aqueous, ethyl alcohol, ethyl acetate, ethanol, and methanol extraction	*P. falciparum*	3.2 ng/ml-10.0 ug/ml	Ethanol and methanol extracts showed the higher antimalarial activities.	(Sriwilaijaroen et al., 2010)
E. longifolia standardized extract, TA164	*Plasmodium yoelii*-infected mice	10, 30, and 60 mg/kg	significant increase in the parasitemia suppression to 63, 67 and 80%.	(Mohd Ridzuan et al., 2007)
Root/aqueous extraction	*P. falciparum*	1, 2.5, 5, 10, 25 and 50 ug/ml	Antiplasmodial at IC50 values of ≤4 ug/ml	(Hout et al., 2006)
Root/ methanol extraction and fractions	*P. falciparum*	64 ug/ml - 0.03 ng/ml	TA164 Anti-parasite growth (IC50=0.17 ug/ml and IC75=6 ug/ml)	(Mohd Ridzuan et al., 2005)

Table 2.6 (Continued) Prior studies of the efficacy of *E. longifolia* in exerting anti-parasitic effects.

Part/Extraction	Parasites	Concentration/ dose	Result	Reference
Root /aqueous ethanol extract	*Plasmodium falciparum*	20 mg/ml were diluted with 10% human serum (22 concentrations of two-fold dilutions)	Quassinoids and alkaloid are cytotoxicity to antiplasmodial activity	(Chan et al., 2004)
Root/ methanol extraction and fractions	*P. falciparum*	1.0 ug with 10% nondialyzed human plasma	Eurycomanone and pasakbumi are antimalarial activity	(Kuo et al., 2004)
Leaves/ ethanol extract for isolate quassinoids	*S. japonicum* and *P. falciparum*	2, 20, and 200 uM	Longilactone: significant antischistosomal effect at 200 mg/ml. 11-Dehydroklaineanone) and 15b-O-acetyl-14-hydroxyklaineanone: plasmodicidal activity (IC50=2 ug/ml).	(Jiwajinda et al., 2002)

2.7.6 *E. longifolia* mechanism of action

The mechanism of action of *E. longifolia* against *T. gondii* was reported by Kavitha et al., 2012. They demonstrated that *E. longifolia* extract fractions act on *T. gondii* within the cytoplasmic region. They hypothesized that these extract fractions may induce intracellular oxidative stress through an indirect mechanism. Mitochondria are known as the primary source of reactive oxygen species (ROS) within cells. Additionally, uncontrolled superoxide flashes in mitochondria contribute to global

oxidative stress, which plays a crucial role in hypoxia/reoxygenation injury in cells. This model offers a rational explanation for the inhibition of *T. gondii* growth (Kavitha et al., 2012b).

2.8 Conceptual Framework

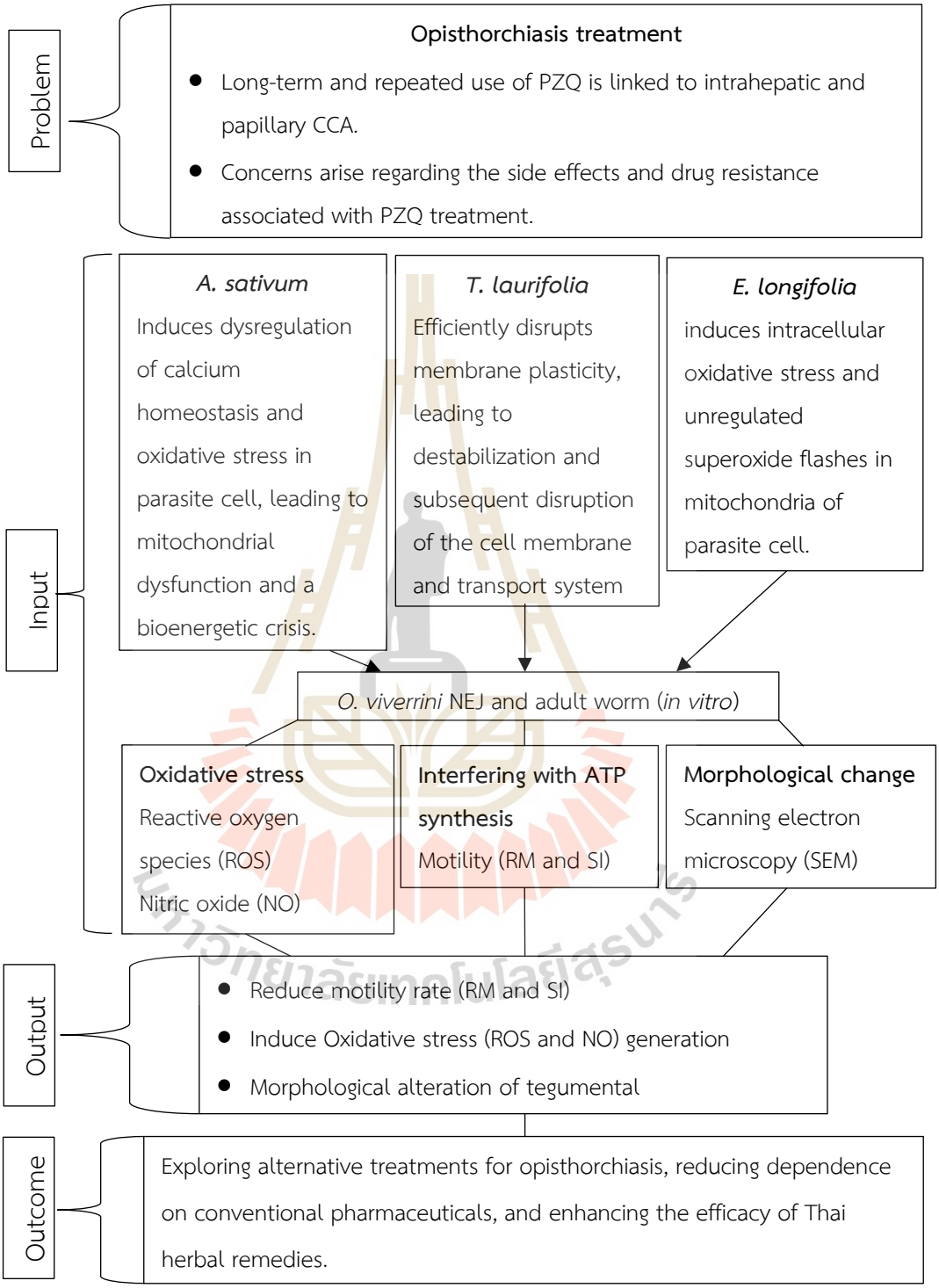


Figure 2.10 Conceptual framework of this research.

CHAPTER III

RESERCH METHODOLOGY

3.1 Experimental design

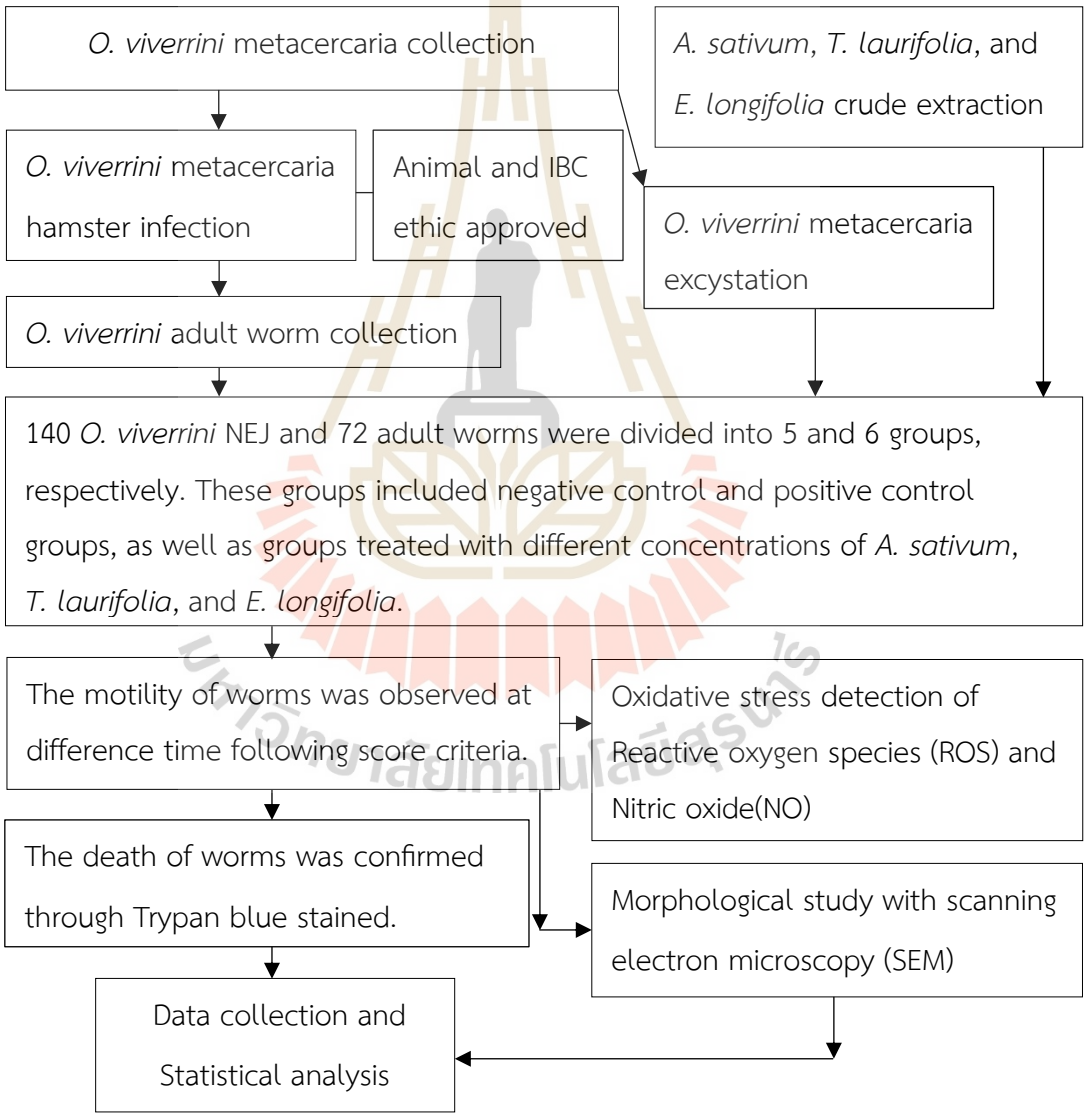


Figure 3.1 Experimental study flowchart of this study.

3.2 Location of research

The research was conducted at the Parasitic Disease Research Center (PDRC), Institute of Medicine, Suranaree University of Technology, located at 111 Maha Witthayalai Rd, Suranaree, Mueang Nakhon Ratchasima District, Nakhon Ratchasima 30000. The experiments were conducted between December 2022 and December 2023.

3.3 Animal ethic and biosafety ethic (IBC) preparation

The animal ethics protocols were approved by the Animal Care and Use Committee of the Institute of Research and Development, Suranaree University of Technology. Ethical Clearance No. SUT-IACUC-0013/2023. (Figure 1C, APPENDIX C)

The biosafety and ethics protocols were approved by the Biosafety and Ethics Committee of the Institute of Research and Development, Suranaree University of Technology. Ethical Clearance No. SUT-IBC-008-2023. (Figure 2C, APPENDIX C)

3.4 Plant crude extraction

3.4.1 *A. sativum*

A. sativum was collected from a local market in Nakhon Ratchasima Province. *T. laurifolia* leaves was harvested in the Nakhon Ratchasima Province. The roots of *E. longifolia* crude was purchased from local herbal shop that adheres to standards, with dried roots ground into 100 % powder.

Figure 3.2 *A. sativum* cloves (A), *T. laurifolia* leaves (B), *E. longifolia* dried roots (C). (Pharmaceutical Sciences, Ubon Ratchathani University, 2010b).

A. sativum cloves were meticulously peeled and washed with filtered water to remove any dust or particulate matter. One hundred fifty grams of cloves were ground, and *A. sativum* water was extracted by pressing it through three layers of gauze. *A. sativum* water underwent centrifugation at 5,000 RPM for 10 minutes to collect the supernatant, which was further filtered using Whatman filter No.1. The resulting liquid was then subjected to freeze-drying to yield a powdered extract (Labconco Frecz Dry®, Kansas, USA) under conditions of $133x10^{-3}$ mBar for 48 hours. The powdered extract was stored at -20 °C until used. (the percentage yield as shown in Appendix A3, APPENDIX A).

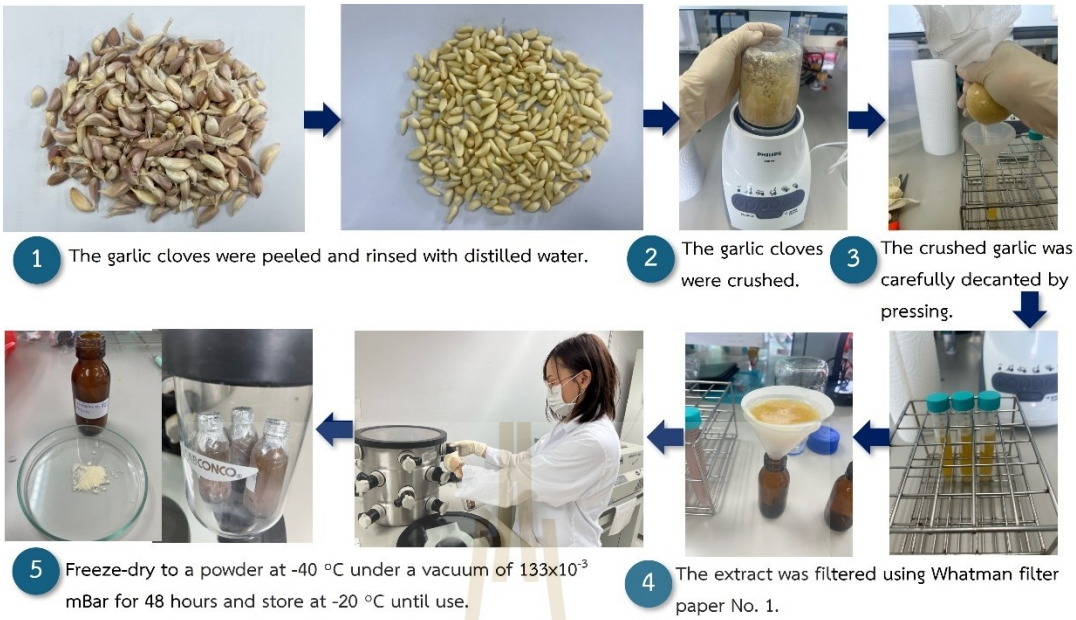


Figure 3.3 *A. sativum* crude extract method

3.4.2 *T. laurifolia* and *E. longifolia*

The fresh leaves of *T. laurifolia*, weighing 1 kg, were washed with distilled water, loosely labeled, and subsequently placed in a hot air oven at 60 °C for approximately 24 hours. Subsequently, the material was finely ground into a powder using a grinder. A mixture comprising 10 g each of *T. laurifolia* and *E. longifolia* root powder was prepared by combining them with 40 ml of distilled water. This blend underwent agitation in a shaking water bath at 140 rpm and 25 °C for 15 minutes (Memmert WTB50®, Schwabach, Germany). The resulting solution was then subjected to centrifugation at a speed of 3000 g for 3 minutes (Hermert Z446K®, Fujian, China). The supernatant was carefully collected, and an additional 40 ml of distilled water was introduced, repeating twice. The combined supernatant was subsequently processed through filtration using Whatman filter paper No. 1. The resultant extract underwent evaporation to eliminate water content utilizing (Rotavapor R-300® Flawil, Switzerland) until a concentrated extract was achieved, followed by freeze-drying to produce a powdered extract (Labconco FreeZone Dry®, Kansas, USA) under conditions

of 133x10^{-3} mBar for 48 hours. The resulting powdered extract was stored at -20 °C until used. (The percentage yield as shown in Appendix A4 and A5, APPENDIX A).

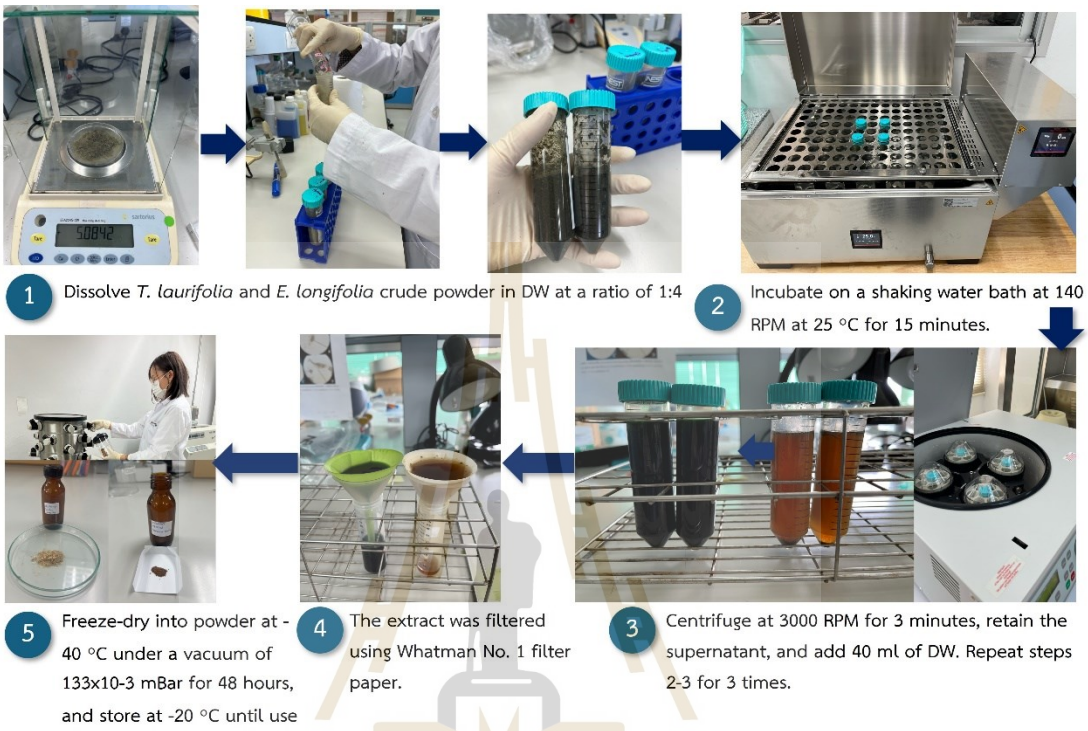


Figure 3.4 *T. laurifolia* and *E. longifolia* crude extraction method

The percentage yield of each crude extract was determined using the formula below (Abbas et al., 2021).

$$\text{Yield (\%)} = \frac{\text{Weight of the extract (g)} \times 100}{\text{Dried extract weight}} \quad (1)$$

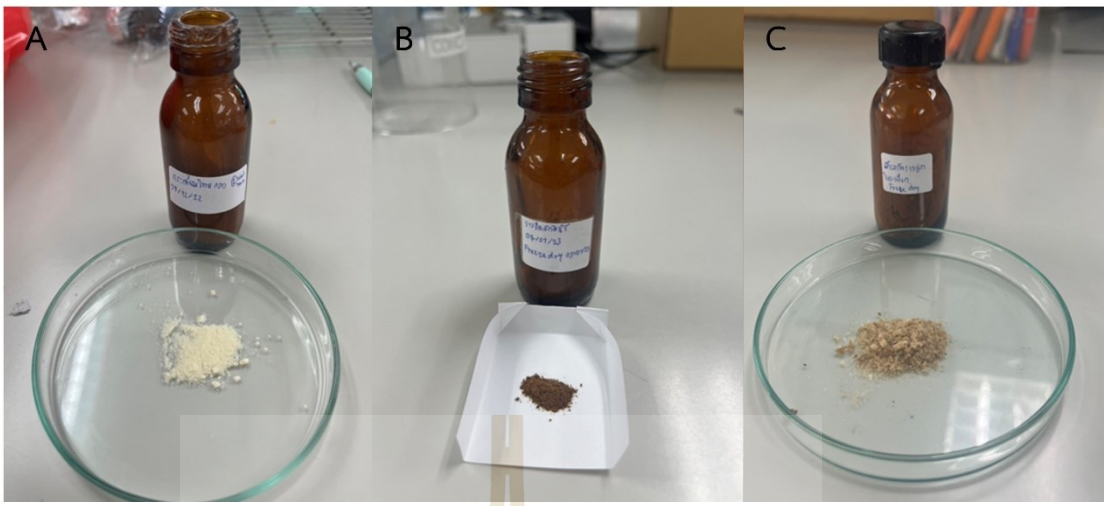


Figure 3.5 *A. sativum* crude extract powder (A), *T. laurifolia* crude extract powder (B), *E. longifolia* crude extract powder (C).

3.5 Parasite preparation

3.5.1 *O. viverrini* metacercaria collection

O. viverrini metacercaria were obtained from naturally infected cyprinid fish in an endemic region spanning Nakhon Ratchasima and Chaiyaphum Province, northeastern Thailand, between April 2023 and July 2023. Fresh cyprinid fish underwent digestion using the pepsin-HCl digestion method, as described below (Srisawangwong et al., 1997).

3.5.1.1 Crush and blend naturally infected cyprinid fish in 0.25% pepsin.

3.5.1.2 Incubate in shaking water bath at 37°C for 1-2 h.

3.5.1.3 Filter the digested samples with sieve size 1000, 300 µm (keep sediment) and 106 µm (keep debris on sieve), respectively.

3.5.1.4 The metacercaria and debris on 106 µm sieve will be washed for sedimentation several times with 0.85% NSS in a sedimentation jar until the supernatant clear and final step filter by sieve 250 µm and examine sediment under stereomicroscope.

3.5.1.5 Metacercariae identification was conducted based on their morphology under a stereomicroscope.

The morphology of the metacercariae was observed under a stereomicroscope, revealing distinct oral and ventral suckers, with the oral sucker positioned close to the posterior end of the worms. The excretory bladder appeared as an oval area containing a dense mass of dark granules, while brownish-yellow pigments were dispersed throughout the body (Scholz et al., 1991; Vajrasthira et al., 1961).

Figure 3.6 Naturally freshwater cyprinoid fish samples.

Figure 3.7 The procedure of the Pepsin-HCl digestion method for the retrieval of *O. viverrini* metacercaria.

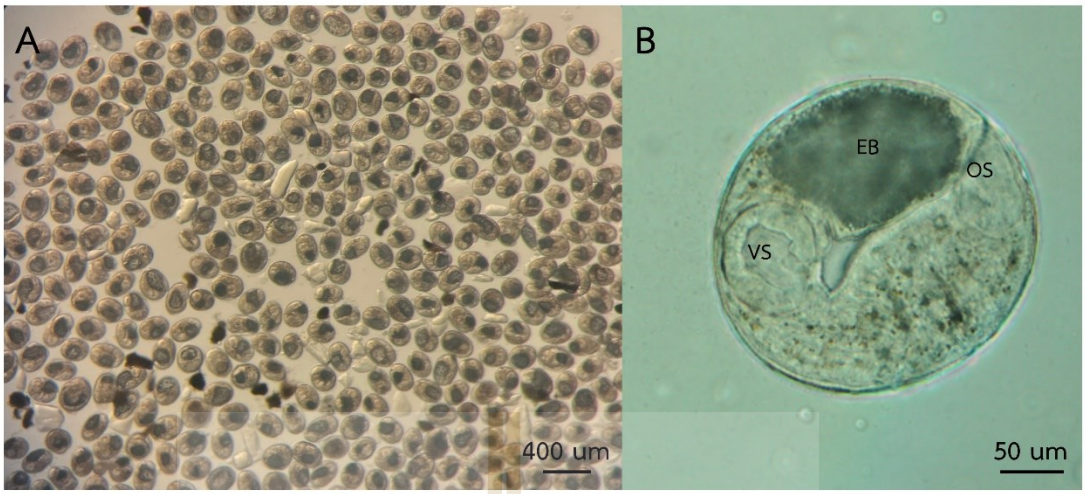


Figure 3.8 The *O. viverrini* metacercaria were observed under a stereomicroscope (A) scale bar = 400 µm, revealing the presence of oral and ventral suckers as well as an excretory bladder (B). Scale bar= 50 µm.

3.5.2 *O. viverrini* metacercaria excystation

O. viverrini metacercaria were excysted in 0.25% trypsin in 1x phosphate buffer saline (PBS) supplemented with 2x 200 U/ml penicillin, 200 µg/ml streptomycin for 5-15 mins at 37 ºC atmosphere to obtained *O. viverrini* NEJs for the experiment (Arunsan et al., 2019).

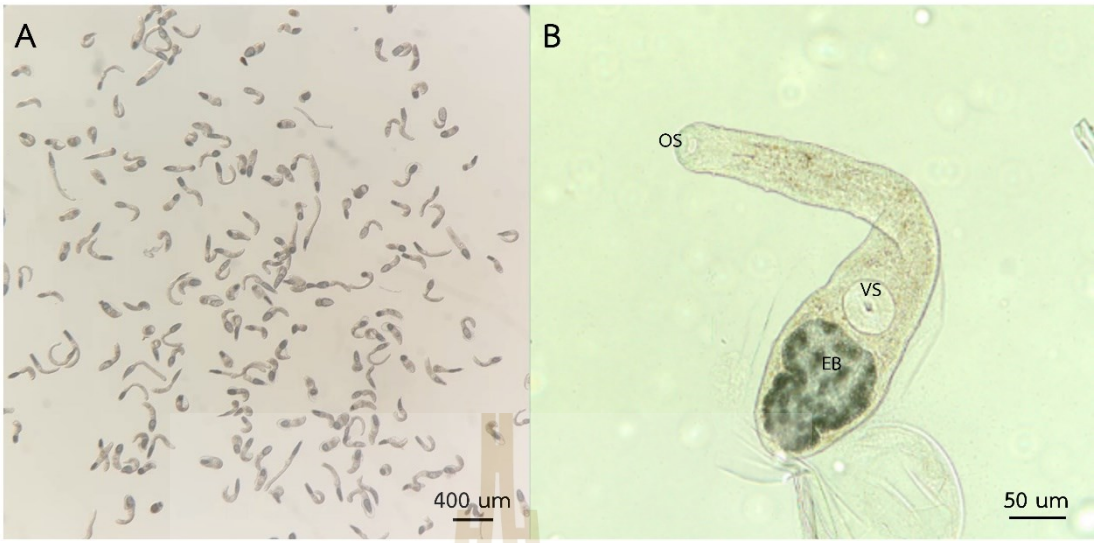


Figure 3.9 The excysted *O. viverrini* metacercaria were observed under a stereomicroscope (A) scale bar= 400 µm, *O. viverrini* NEJs revealing the presence of oral and ventral suckers as well as an excretory bladder (B) scale bar= 50 µm.

3.5.3 Animal

Male Golden Syrian hamsters (*Mesocricetus auratus*), aged 6 - 8 weeks and weighing 80 -100 g, were utilized in this experiment. These animals were procured from the Laboratory Animal Facility, Faculty of Medicine, Khon Kaen University, Thailand, and orally infected with 50 *O. viverrini* metacercaria through intragastric intubation. Post-infection, the hamsters were housed for 2 - 3 months at the Laboratory Animal Facility, Center for Scientific and Technological Equipment, Suranaree University of Technology. They were accommodated in stainless steel cages, with 2 - 3 hamsters per cage, and provided ad libitum access to a standard diet and water. The room temperature was maintained at 23 ± 2 ℃ atmosphere, with a relative humidity of 45 – 50 %, under a 12 - hour light-dark cycle.

Figure 3.10 The golden syrian hamsters (*M. auratus*) used in this study.

3.5.4 Hamsters *O. viverrini* infection

Hamsters were administered 50 *O. viverrini* metacercaria via oral intragastric intubation. The infection method is described below (Sripa and Kaewkes, 2002):

3.5.4.1 The hamsters were anesthetized using 1-3% Isoflurane to induce asphyxiation.

3.5.4.2 Fifty *O. viverrini* metacercaria were washed with 0.85% normal saline solution (NSS) and then drawn into a syringe with a gastric tube under a stereoscope.

3.5.4.3 The gastric tube containing the metacercaria was inserted orally, ensuring the passage of the metacercaria to the esophagus by oral administration, followed by gentle pressure applied with the syringe.

3.5.4.4 The hamsters infected were kept at the Laboratory Animal center for approximately 2-3 months to allow for the development of adult worms in the bile ducts.

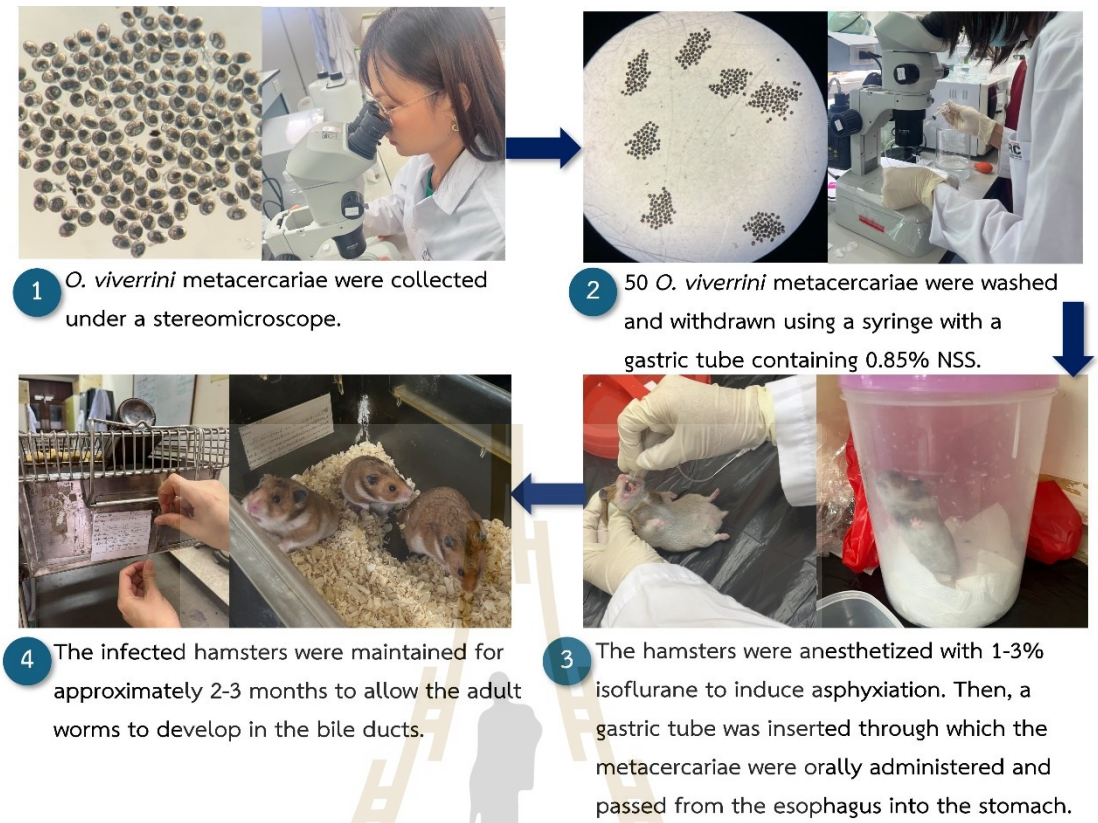


Figure 3.11 The procedures for infecting hamsters with *O. viverrini* metacercaria.

3.5.5 *O. viverrini* adult worm collection

Three months post-infection (p.i.) of *O. viverrini* infected hamsters, were euthanized, and their livers were dissected, along with cardiac puncture blood collection. Adult worms were then collected from the bile ducts of the livers. The process of adult worm collection is described below:

3.5.5.1 Two months post-infection, hamsters were euthanized by exposure to 1-3% Isoflurane (3-5 ml), ensuring indirect exposure by immersing gauze in a closed container and placing another layer of gauze on top to prevent direct contact.

3.5.5.2 Hamster were stretched with pin on platform and then hamsters were opened the abdomen until to pectoral and opened the midriff of hamster by surgical scissors.

3.5.5.3 The liver of the hamsters was dissected out with surgical scissors and immediately placed on a petri dish containing 0.85% normal saline solution (NSS).

3.5.5.4 Adult worms were pressed out from the liver bile ducts and incubated in culture media (Roswell Park Memorial Institute or RPMI-1640). Subsequently, actively motile adult worms were selected for further experimentation.

Figure 3.12 The procedures for euthanizing and dissecting hamsters to collect *O. viverrini* adult worms.

3.6 Anthelmintic activity on *O. viverrini* NEJs

O. viverrini NEJs were allocated into six distinct groups of each herb (5 NEJs/group, duplicate in each group): group 1 received the RPMI-1640 culture medium (negative control), group 2 was subjected to treatment with a standard drug of 2 mg/ml PZQ (Positive control), groups 3 to 6 involved treatment of *O. viverrini* NEJs with varied

concentrations of *A. sativum, T laurifolia, and E. longifolia* crude extract (5, 10, 20, and 40 mg/ml), with each group supplemented with 100 µg/ml of streptomycin antibiotic, respectively. All groups were exposed at different time intervals (0, 30 minutes, 1 hours, 3 hours, 6 hours, 12 hours, and 24 hours) under 37 °C atmosphere. The motility assessment included the evaluation of relative motility values (RM) and % survival index (SI) using predetermined scoring criteria at various exposure times. Ultimately, the mortality of *O. viverrini* NEJs was confirmed through the Trypan blue stain viability assay.

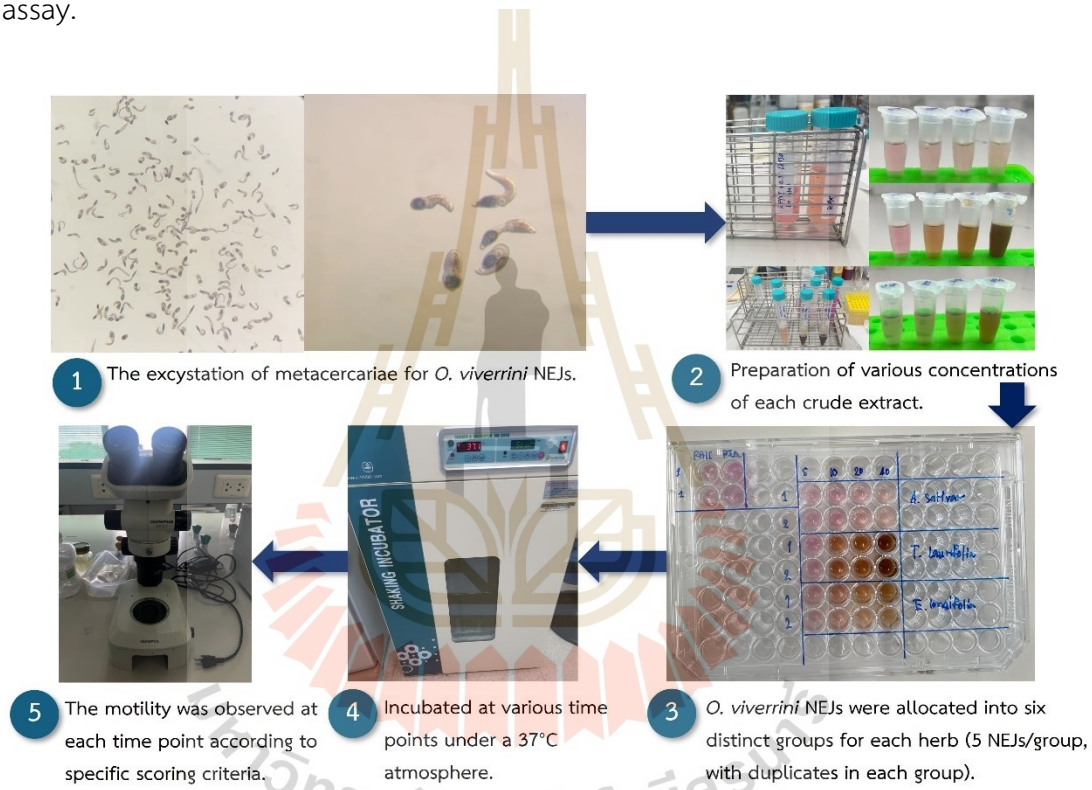


Figure 3.13 Anthelmintic activity on *O. viverrini* NEJs

3.7 Anthelmintic activity on *O. viverrini* adult worms

O. viverrini adult worms were pressed from the liver bile duct and incubated in culture media RPMI-1640. Subsequently, the actively adult worms were selected for experimentation. *O. viverrini* adult worms were allocated into five distinct groups of each herb (4 worms/group, duplicate in each group): group 1 received the RPMI-1640 culture medium, group 2 was subjected to treatment with a standard drug of 20 mg/ml

PZQ, groups 3 to 5 involved treatment of *O. viverrini* adult worms with varied concentrations of *A. sativum*, *T laurifolia*, and *E. longifolia* crude extract (20, 30, and 40 mg/ml), with each group supplemented with 100 µg/ml of streptomycin antibiotic. All groups exposed at different time intervals (0, 30 minutes, 1 hours, 3 hours, 6 hours, 12 hours, and 24 hours) under 37 °C atmosphere. The motility assessment included evaluating relative motility values (RM) and % survival index (SI) based on predefined scoring criteria at various exposure times. Quantification of reactive oxygen species (ROS) as indicators of oxidative stress was conducted using 2',7'-Dichlorodihydrofluorescein diacetate staining. Additionally, the morphological damage of *O. viverrini* adult worms was assessed using scanning electron microscopy (SEM). Ultimately, the mortality of *O. viverrini* adult worms was confirmed using the Trypan blue stain viability assay.

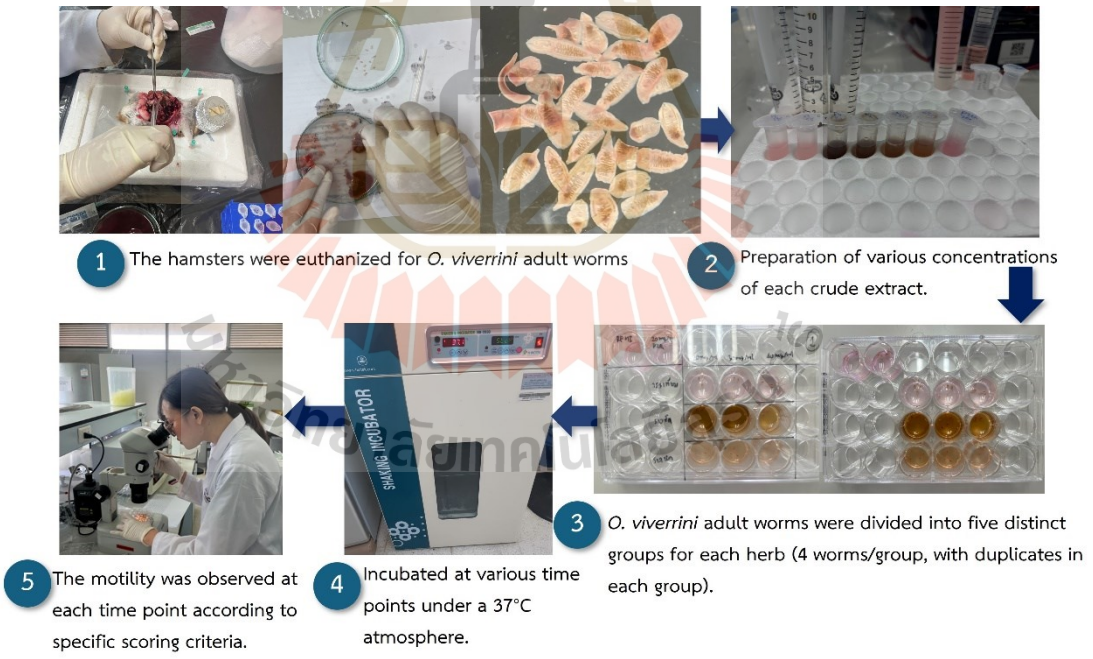


Figure 3.14 Anthelmintic activity on *O. viverrini* adult worms.

3.8 Motility activity

The motility was assessed by examining *O.viverrini* NEJs and adult worms under a stereomicroscope and scored based on the criteria established by Jiraungkoorskul and colleagues (2005).

3 = moving whole body

2 = moving only parts of the body

1 = immobile but alive

0 = died

In these experiments, there were three observers who participated in scoring. The relative motility value (RM) value was computed based on the motility scores across all experimental groups. Notably, the negative control group, where all parasites were scored 3, demonstrated an RM value of 100. Accordingly, a diminished RM value observed in the all-treated group suggested a more robust inhibition of motility due to the *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts. The RM values were determined employing the formula detailed below (Kiuchi et al., 1987; Lorsuwannarat et al., 2013).

$$\text{Motility index (MI)} = \frac{\sum nN}{N} \quad (2)$$

$$\text{Relative motility (RM) value} = \frac{\text{MI test} \times 100}{\text{MI control}} \quad (3)$$

n = motility score,

N = number of worms with the score of ‘‘n’’

The % Survival index (SI) was calculated to determine the percentage of live worms at a specific time after incubation. Worms that exhibited a motility score of 0 were classified as died, whereas those with other scores (3, 2, and 1) were regarded as still alive. The SI was calculated using the formula provided below (Kiuchi et al., 1987; Lorsuwannarat et al., 2013).

$$\% \text{ Survival index (SI)} = \frac{\text{Number of live worms (each group)} \times 100}{\text{Total worms (each group)}} \quad (4)$$

3.9 Viability assay

After 24 hours of incubation, the *O. viverrini* NEJs and adult worms were exposed to a 0.4 % Trypan blue stain at room temperature for 2-3 minutes. Following this, the worms underwent three washes with 1x PBS, and their viability was assessed under a light microscope.

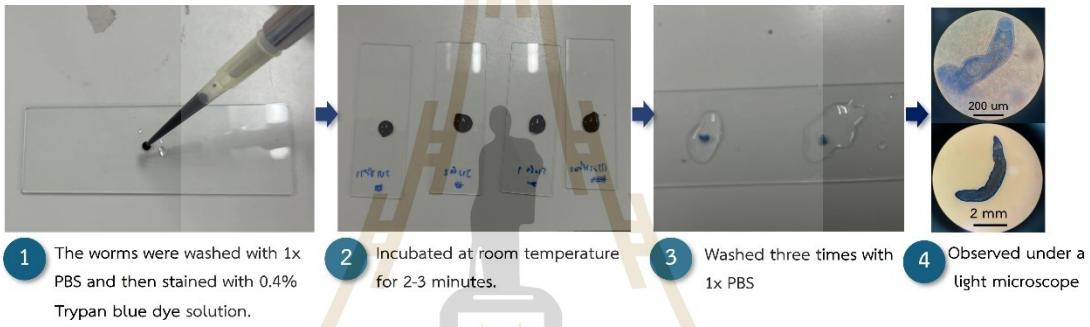


Figure 3.15 Viability assay utilizing Trypan blue stain.

3.10 Measurement of Reactive oxygen species generation (ROS)

The generation of stress induced by *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extract. initiated a cellular response after 6 hours of incubation, establishing an equilibrium between antioxidant defenses and ROS or free radicals. Subsequently, the subjected *O. viverrini* adult worms underwent thorough rinsing with DW. Following the rinsing procedure, the worms were exposed to a 30 µM fluorogenic dye, 2', 7'-Dichlorodihydrofluorescein diacetate (H2DCFDA) (Med Chem Express®, New Jersey, USA), and incubated in darkness at 37°C for 30 minutes. After incubation, the samples underwent additional washing with DW to eliminate any surplus fluorogenic dye. Slides were prepared for fluorescent imaging utilizing a fluorescence microscope (Ex/Em = 488/525 nm). The fluorescence levels generating from ROS were quantified by

analyzing fluorescence microscopy images through the ImageJ software. The corrected total worm fluorescence (CTWF) was determined by subtracting the integrated density from the product of the selected worm's area and the mean fluorescence of the background readings (El-Sharkawey, 2016). (The method as shown in Appendix A6. APPENDIX A).

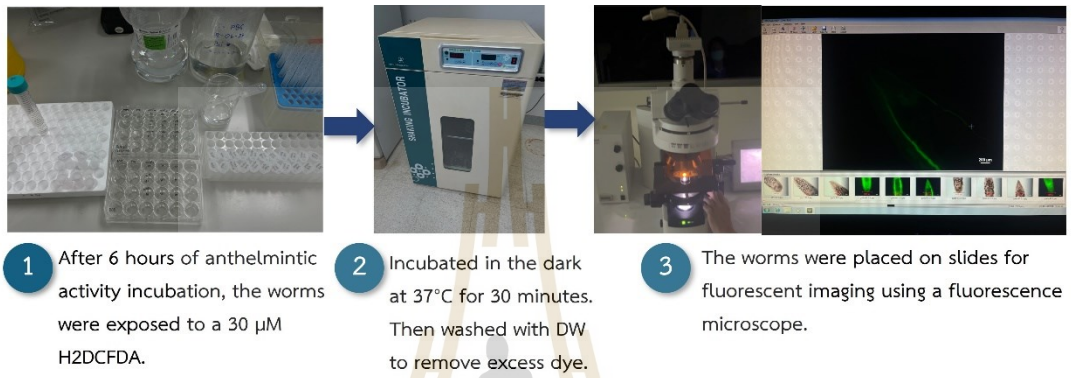


Figure 3.16 Measurement of ROS generation in *O. viverrini* adult worms.

3.11 Measurement of nitric oxide generation (NO)

3.11.1 *O. viverrini* adult worm protein crude extract

The protein crude extract of *O. viverrini* were extracted after 6 hours of incubation. The process of adult worm collection is described below:

3.11.1.1 Wash *O. viverrini* adult worms with NSS, then store in PBS at -20°C.

3.11.1.2 Place *O. viverrini* adult wo in PBS pH 7.4 with protease inhibitor and freeze at -80°C for 30 minutes. (Repeat and thaw 4-5 times).

3.10.1.3 Sonicate samples to disrupt cells and centrifuge at 15000xg for 30 minutes at 4°C.

3.11.1.4 Measure protein quantity of crude protein obtained.

3.11.2 Griess Reagent reaction by microplate assay

The crude protein was promptly utilized for NO detection using the Griess Reagent technique. In a microplate (with a sample capacity of at least 300 ul per well), combine the following in each well:

20 ul of Griess Reagent

150 ul of the nitrite-containing sample

130 ul of deionized water

Prepare a photometric reference sample by mixing 20 ul of Griess Reagent and 280 ul of deionized water. Incubate the mixture for 30 minutes at room temperature. Measure the absorbance of the nitrite-containing samples relative to the reference sample using a spectrophotometric microplate reader at time points 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours. The optimal measurement wavelength is 540 nm.

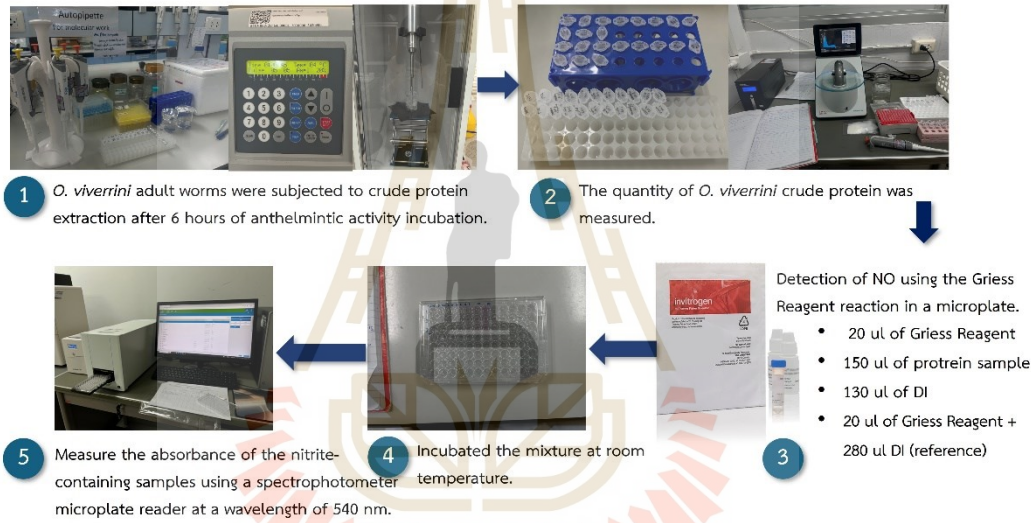


Figure 3.17 Measurement of NO generation using the Griess Reagent reaction.

3.12 Morphological study by scanning electron microscope (SEM)

The evaluation of morphological damage in *O. viverrini* NEJs and adult worms, following 12 hours of incubation, was performed using SEM. Subsequently, the NEJs and adult worms underwent multiple washes with DW. The NEJs and adult worms were fixed overnight in a glutaraldehyde fixative solution at 4 °C. Following fixation, the worms underwent three sequential 10-minute washes with DW. Post-fixation involved immersion in a 1% Osmium tetroxide fixative solution in 0.1 M phosphate buffer saline (PBS) with a pH of 7.2 for 1 hour, followed by three 10-minute DW washes. Subsequently, the samples were dehydrated through a series of graded acetone

solutions (30%, 50%, 70%, 90%, 95%, and 100% acetone) in two cycles. They were then desiccated using a critical point dryer (Leica CPD 300® Vienna, Austria), coated with a layer of gold using an Au ion sputtering device on conductive tape, and examined under scanning electron microscopy (FESEM/Carl Zeiss Auriga® Dresden, Germany) at an electric high tension 3.00 kV.

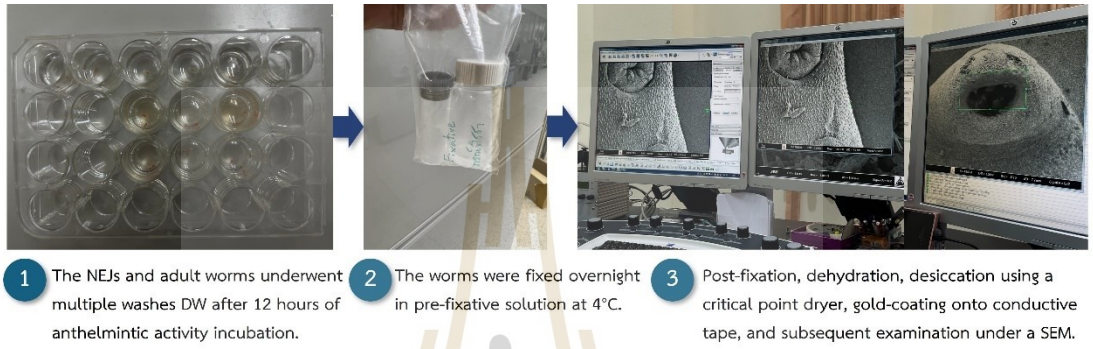


Figure 3.18 Morphological study of *O. viverrini* NEJs and adult worms using SEM.

3.13 Data analysis

The RM values and SI were analyzed through the respective formulas. Mean scores and standard deviations of motility were calculated for each group. Data analysis was performed using IBM SPSS Statistics 26 (SPSS Inc., Chicago, USA) with a One-Way ANOVA conducted among groups to compare the mean motility scores. Statistical significance was determined by a *P-value* < 0.05.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Results of Motility test and viability assay of *O. viverrini* NEJs

Upon reaching the specified time interval, *O. viverrini* NEJs were observed for motility scoring and calculation of RM and SI values. The negative control group displayed normal motility from 0 minutes to 12 hours (RM = 100 and SI = 100), with a slight decrease in motility observed at the conclusion of the experiment at 24 hours (RM = 100 and SI = 60).

The positive control group showed that *O. viverrini* NEJs displayed normal motility within the first 0 to 5 minutes (RM = 100 and SI = 100). However, after 1 hour, the parasites exhibited a rapid decrease in movement (RM = 53.57 and SI = 100), remaining relatively stable from 3 to 6 hours (RM = 51.85 and 53.85, SI = 100 and 100, respectively), and further declining at 12 hours (RM = 39.29 and SI = 100). Motility ceased entirely by the 24-hour mark (RM = 0 and SI = 0).

The *A. sativum* treated groups, at concentrations of 5, 10, 20, and 40 mg/ml, it was observed that the *O. viverrini* NEJs exhibited a decreasing motility rate at 1 hour in all groups. At a concentration of 5 mg/ml (RM = 42.86, 37.04, 38.46, and 35.71, SI = 100, respectively) and 20 mg/ml (RM = 35.71, 37.04, 38.46, and 35.71, SI = 100, respectively), the motility rate remained constant until 12 hours and ceased entirely at 24 hours (RM = 0 and SI = 0). In the group with a concentration of 10 mg/ml (RM = 35.71, 37.04, and 38.46, SI = 100, respectively), the motility rate decreased steadily from 1 to 6 hours and stopped entirely at 12 hours (RM = 0 and SI = 0). In the group with the highest concentration of 40 mg/ml (RM = 35.71 and 37.04, SI = 100, respectively), the motility rate decreased steadily from 1 to 3 hours and ceased entirely at 6 hours (RM = 0 and SI = 0) (Figure 4.1 and 4.2).

A comparative analysis revealed significant differences between the negative control group and all of *A. sativum* treated groups. However, there were no significant differences in mean motility scores between the positive control group and the *A. sativum* treated groups ($P < 0.05$) (Figure 4.3).

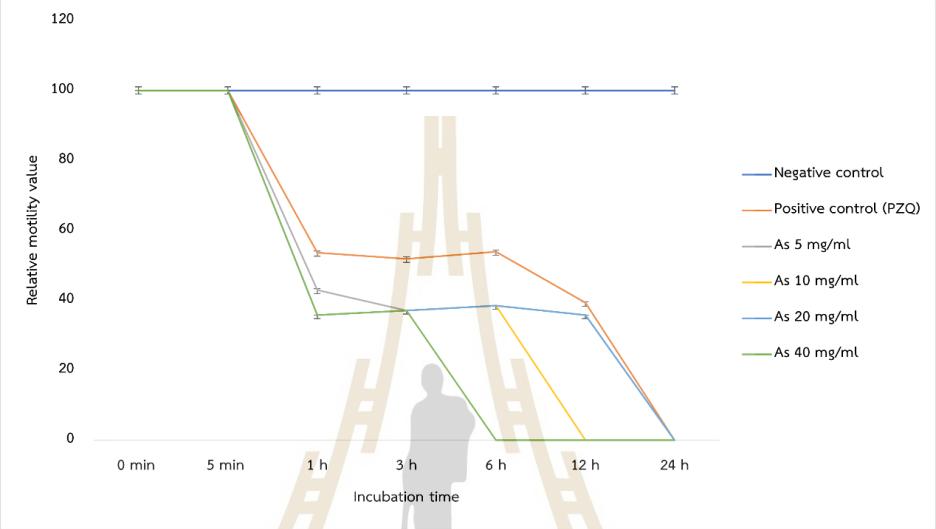


Figure 4.1 The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *A. sativum* crude extract, compared to both negative and positive controls, across various time intervals.

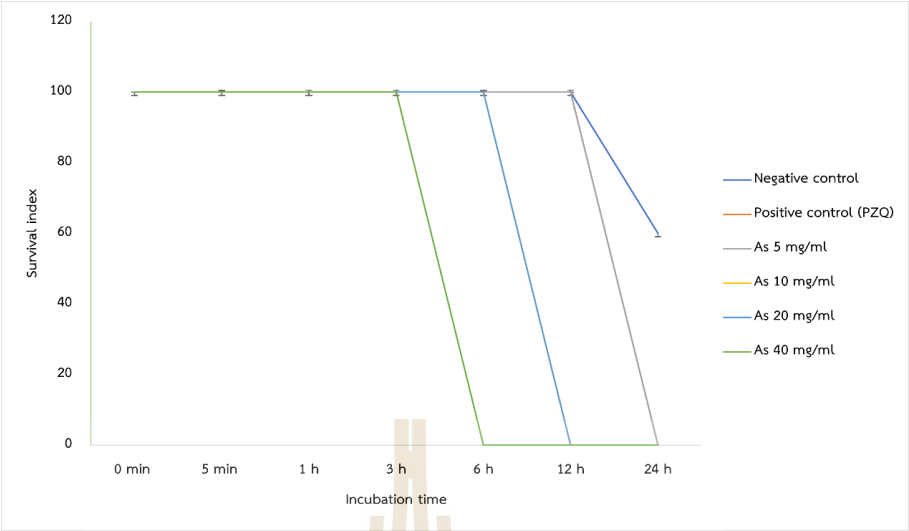


Figure 4.2 The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *A. sativum* crude extract, compared to both negative and positive controls, across various time intervals.

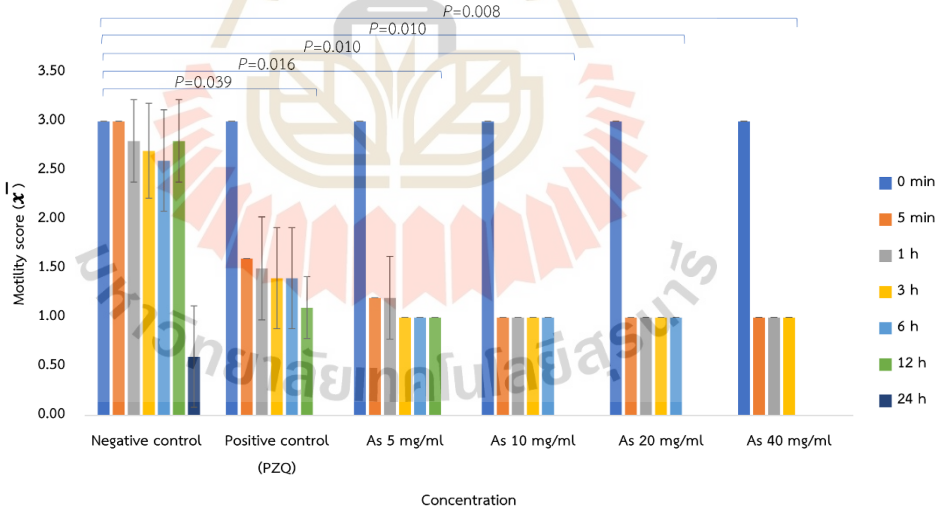


Figure 4.3 Motility scores showed significant variations between the negative control group and all *A. sativum* treated groups. However, there were no notable differences in mean motility scores between the positive control group and the *A. sativum* treated groups ($P < 0.05$).

The *T. laurifolia* treated groups, at concentrations of 5, 10, 20, and 40 mg/ml, it was observed that the parasites exhibited a decreasing motility rate at 1 hour in all groups, the motility rate remained constant until 12 hours (5 mg/ml: RM = 66.67, 74.07, 65.38, and 46.43, SI = 100, respectively. 10 mg/ml: RM = 66.67, 74.07, 50.00 and 46.43, SI = 100, respectively. 20 mg/ml: RM = 56.67, 59.26, 46.15, and 42.86, SI = 100, respectively. 40 mg/ml: RM = 36.67, 37.04, 38.46, and 35.71, SI = 100, respectively), and stopped entirely at 12 hours (RM = 0 and SI = 0) (Figure 4.4 and 4.5).

A comparative analysis was indicated significant differences between the negative control group and the groups treated with 20 and 40 mg/ml of *T. laurifolia*. However, there were no significant differences in mean motility scores between the positive control group and the *T. laurifolia* treated groups ($P < 0.05$) (Figure 4.6).

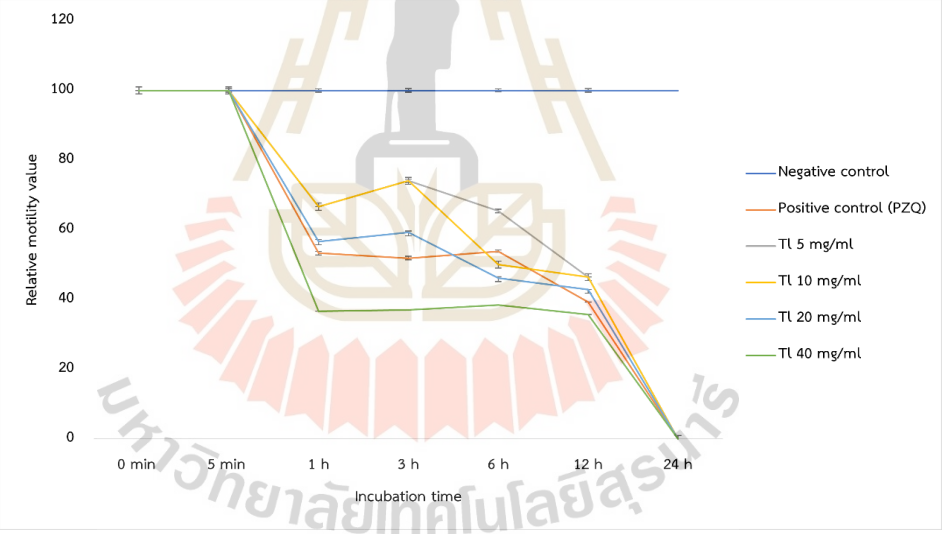


Figure 4.4 The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *T. laurifolia* crude extract, compared to both negative and positive controls, across various time intervals.

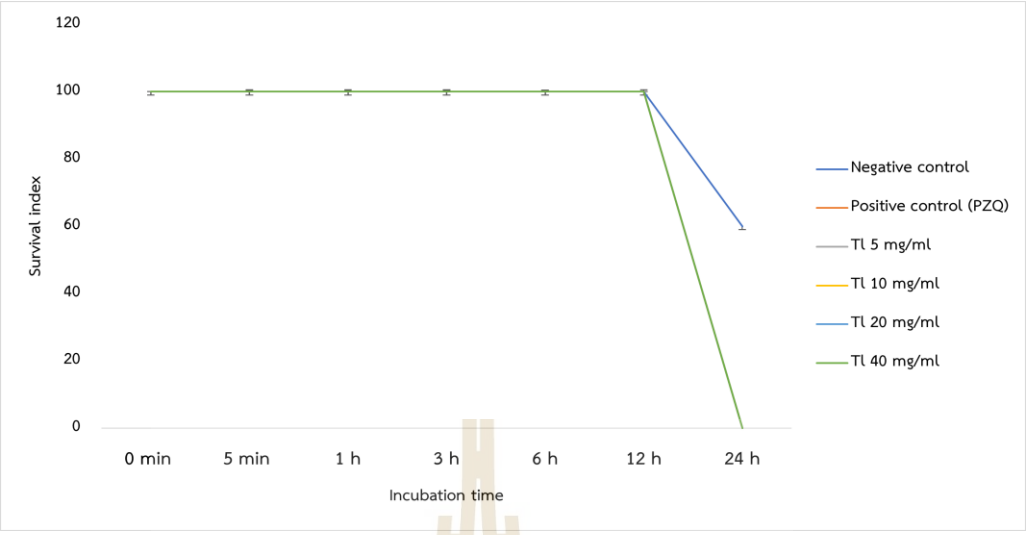


Figure 4.5 The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *T. laurifolia* crude extract, compared to both negative and positive controls, across various time intervals.

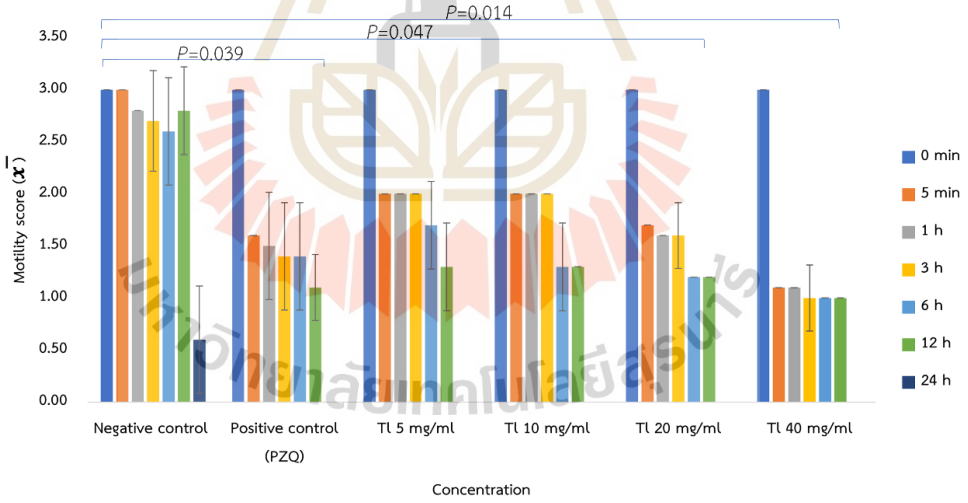


Figure 4.6 Motility scores exhibited significant differences between the negative control group and the groups treated with 20 and 40 mg/ml of *T. laurifolia*. However, there were no significant differences in mean motility scores between the positive control group and the *T. laurifolia* treated groups ($P < 0.05$).

The *E. longifolia* treated groups, at concentrations of 5, 10, 20, and 40 mg/ml, it was observed that the parasites exhibited a decreasing motility rate at 1 hour in all groups, the motility rate remained constant until 12 hours (5 mg/ml: RM = 66.67, 74.07, 46.15, and 42.86, SI = 100, respectively. 10 mg/ml: RM = 66.67, 74.07, 46.15, and 35.71, SI = 100, respectively. 20 mg/ml: RM = 66.67, 70.37, 38.46, and 35.71, SI = 100, respectively. 40 mg/ml: RM = 66.67, 70.37, 38.46, and 35.71, SI = 100, respectively), and stopped entirely at 12 hours (RM = 0 and SI = 0) (Figure 4.7 and 4.8).

A comparative analysis was indicated significant differences between the negative control group and *E. longifolia* treated groups, as well as between the positive control group and *E. longifolia* treated groups ($P < 0.05$) (Figure 4.9).

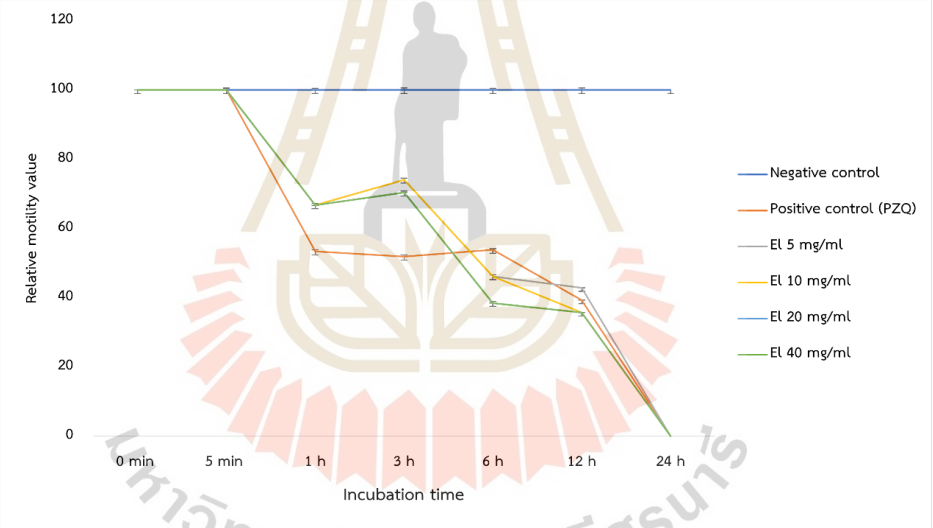


Figure 4.7 The RM values of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *E. longifolia* crude extract, compared to both negative and positive controls, across various time intervals.

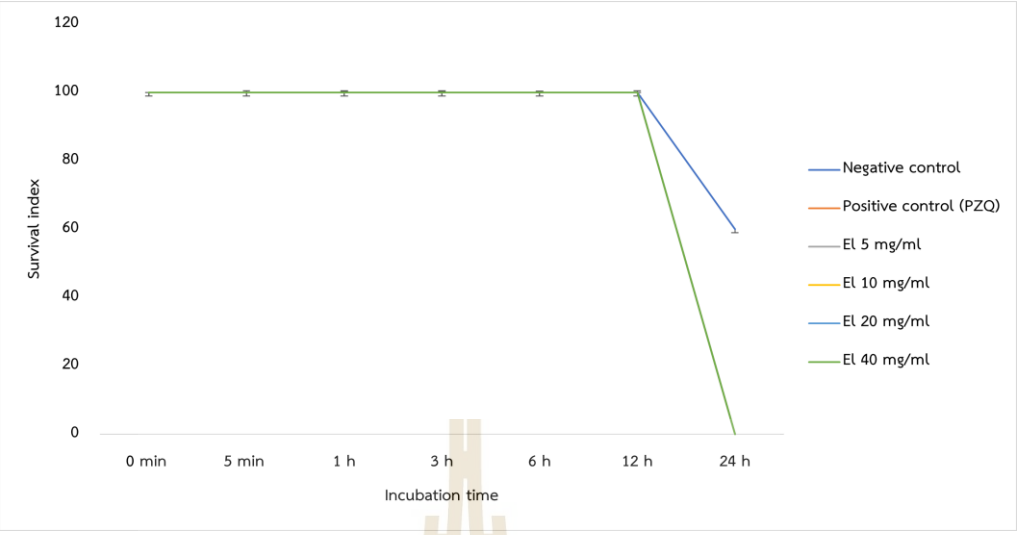


Figure 4.8 The SI of *O. viverrini* NEJs, were assessed after treatment with 5, 10, 20, and 40 mg/ml *E. longifolia* crude extract, compared to both negative and positive controls, across various time intervals.

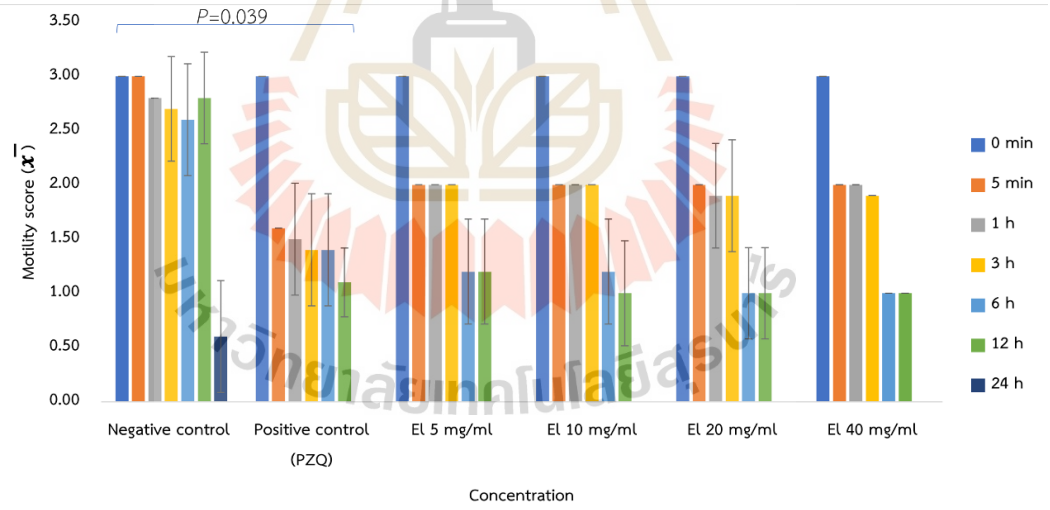


Figure 4.9 Motility scores showed no significant differences between the negative control group and *E. longifolia* treated groups, as well as between the positive control group and *E. longifolia* treated groups ($P < 0.05$).

Viability confirmation via Trypan blue staining showed no staining in the negative control group. In contrast, extensive Trypan blue staining was observed in the positive control group, as well as in all groups treated with *A. sativum*, *T. laurifolia*, and *E. longifolia* (Figure 4.10).

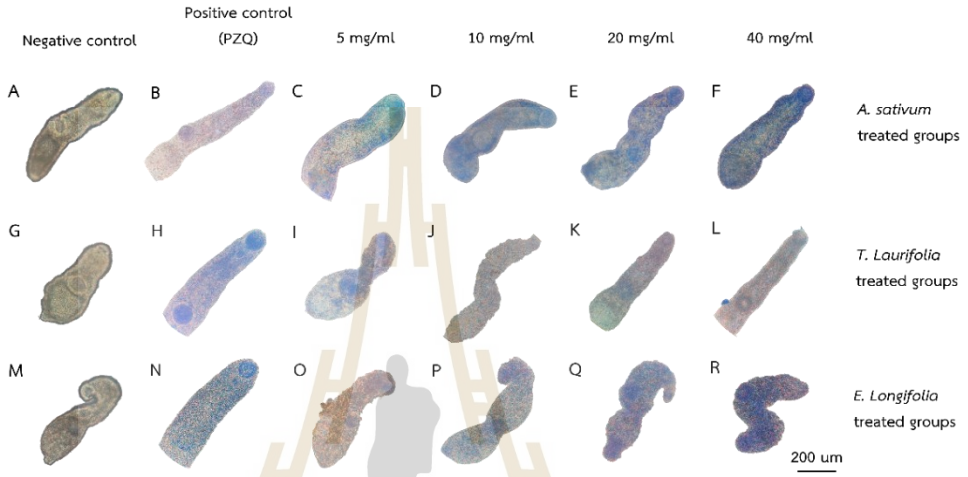


Figure 4.10 Viability of *O. viverrini* NEJs was assessed using Trypan blue staining. In the negative control group (A, G, and M), no staining was observed. Conversely, extensive Trypan blue staining was evident in the positive control group (B, H, and N), as well as in all groups treated with *A. sativum* (D, E, and F), *T. laurifolia* (J, K, and L), and *E. longifolia* (P, Q, and R).

4.2 Result of surface morphological changes of *O. viverrini* NEJs

The surface morphological changes of *O. viverrini* NEJs were assessed using SEM post of treatment for12-hour. The tegumental surface of *O. viverrini* NEJs in the negative control group were showed normal of tegumental sureface, presence the normal of sensory papillae (Pa), under of the ventral sucker (Vs) region displays the small spines (Sp). The tegumental morphology of *O. viverrini* NEJs in the negative control group (RPMI medium) (Figure 4.11), with a scale bar of 40 µm. Specific features are illustrated, including the anterior end (I, scale bar = 8 µm), spines around the

ventral sucker (III and IV, scale bars = 8 μm and 4 μm, respectively), and the normal tegumental surface of *O. viverrini* NEJs (II, scale bar = 4 μm).

The positive control group treated in the positive control group (2 mg/ml PZQ) (Figure 4.12), the images reveal swelling and stretching of the tegumental surface (I and III, with scale bars of 4 μm each), as well as tegumental alterations characterized by swelling and degradation around the ventral sucker (II, scale bar = 4 μm).

The tegumental morphology of *O. viverrini* NEJs in the *A. sativum* treated groups (Figure 4.13) with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 80, 80, 70, and 70 μm, respectively. They illustrate the severely damaged tegumental surfaces in these *A. sativum* treated groups, such as intense swelling leading to bursting and degradation, which cannot fully depict the true details of the skin surface of *O. viverrini* NEJs, particularly at the concentrations of 20 and 40 mg/ml.

The tegumental morphology of *O. viverrini* NEJs in the *T. laurifolia* treated groups (Figure 4.14) with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 40 μm for each. At 5 mg/ml (A), the tegumental surface appears relatively normal with slight swelling observed in the anterior part (A, II). At concentrations of 10 and 20 mg/ml (B and C), the tegumental surface shows widespread swelling throughout the body of *O. viverrini* NEJs. At 40 mg/ml (D), severely damaged tegumental surfaces are clearly visible, characterized by extensive degradation along the entire body of *O. viverrini* NEJs.

The tegumental morphology of *O. viverrini* NEJs in the *E. longifolia* treated groups (Figure 4.15) with concentrations of 20 and 40 mg/ml (A and B), respectively, with scale bars of 40 μm and 80 μm, respectively. (Due to the samples at 5 and 10 mg/ml being lost during sample preparation for SEM). At 20 mg/ml (A), the tegumental surface shows widespread swelling throughout the body of *O. viverrini* NEJs. At 40 mg/ml (B), extensive swelling and degradation are evident along the entire body, particularly around the anterior part near the oral sucker (B, I), where degradation extends to the spines due to dehydration of *O. viverrini* NEJs cells.

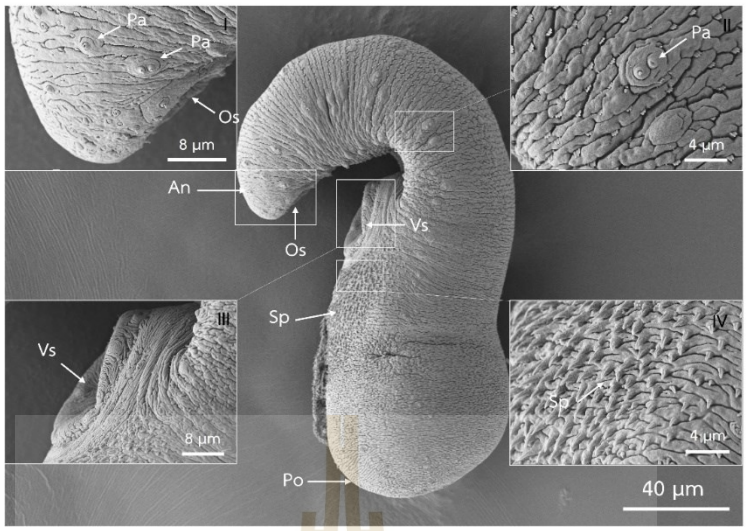


Figure 4.11 The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the negative control group, with a scale bar of 40 µm.

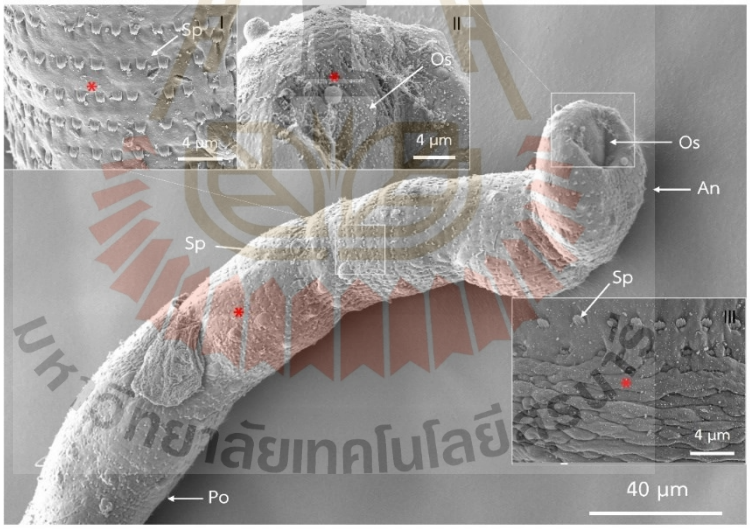


Figure 4.12 The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the positive control group, with a scale bar of 40 µm. *= Alteration of tegumental surface by swelling expansion of tegumental surface.

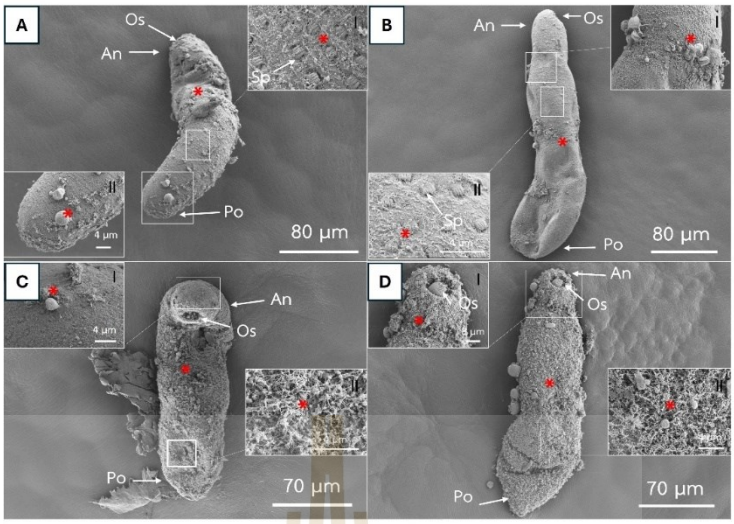


Figure 4.13 The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *A. sativum* treated groups with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 80, 80, 70, and 70 µm, respectively. * = Alteration of tegumental surface by degradation or damage of tegumental surface.

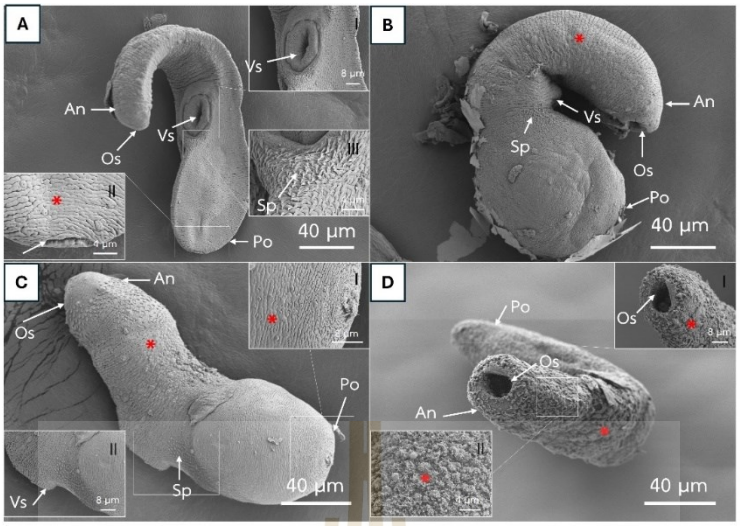


Figure 4.14 The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *T. laurifolia* treated groups with concentrations of 5, 10, 20, and 40 mg/ml (A, B, C, and D), respectively, with scale bars of 40 µm for each. * = Alteration of tegumental surface by swelling (A, II, B and C, I) and degradation (D, I and II).

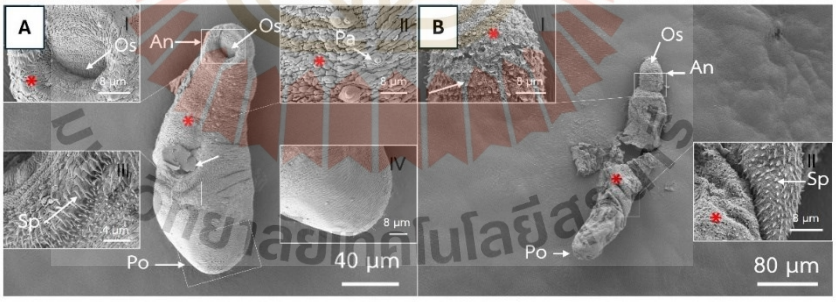


Figure 4.15 The SEM images depict the tegumental morphology of *O. viverrini* NEJs in the *E. longifolia* treated groups with concentrations of 20 and 40 mg/ml (A and B), respectively, with scale bars of 40 µm and 80 µm, respectively. * = Alteration of tegumental surface by swelling (A, I and II) and degradation (B, I and II).

4.3 Result of motility test and viability assay of *O. viverrini* adult worm

Throughout the entire experimental duration, adult worms in the negative control group exhibited continuous active movement and sustained vitality (RM = 100 and SI = 100).

The positive control, at 30 minutes, the *O. viverrini* adult worms began to exhibit a continuous decrease in motility, reaching 1 and 3 hours (RM = 51.77, 50.98, and 43.13, SI = 100, respectively). At 6 and 12 hours, there was another decrease in motility (RM = 39.02 and 35.00, SI = 75.00 and 25.00, respectively), culminating in complete cessation of movement at the 24-hour mark (RM = 0 and SI = 0).

The *A. sativum* treated groups with concentrations of 20, 30, and 40 mg/ml at the 0-minute mark, all worms set displayed active motility and a healthy appearance, resembling the negative control group, with RM = 100 and SI = 100, respectively. However, at the 30-minute mark, there was a rapid decrease in motility rate (RM) (RM = 50, SI = 100 in all concentrations, respectively), after 1 hour of exposure, the *A. sativum* treated groups at concentrations of 20, 30, and 40 mg/ml exhibited reduced motility, with RM values of 56.86, 47.06, and 47.06, respectively, while maintaining SI = 100 for all groups. By 3 hours, the *A. sativum* treated groups displayed a substantial reduction in both RM and SI (RM = 23.52, 17.64, and 15.68, SI = 50.00, 37.50, and 25, respectively). Consequently, all worms in these groups ceased movement or perished by the 6-hour assessment (RM = 0 and SI = 0) (Figure 4.16 and 4.17).

A comparative analysis utilizing One-Way ANOVA revealed significant differences between the negative control group, and the *A. sativum* treated groups at concentrations of 20, 30, and 40 mg/ml. Conversely, no significant differences were observed between the positive control group and the 20, 30, and 40 mg/ml *A. sativum* treated groups ($p < 0.05$) (Figure 4.18).

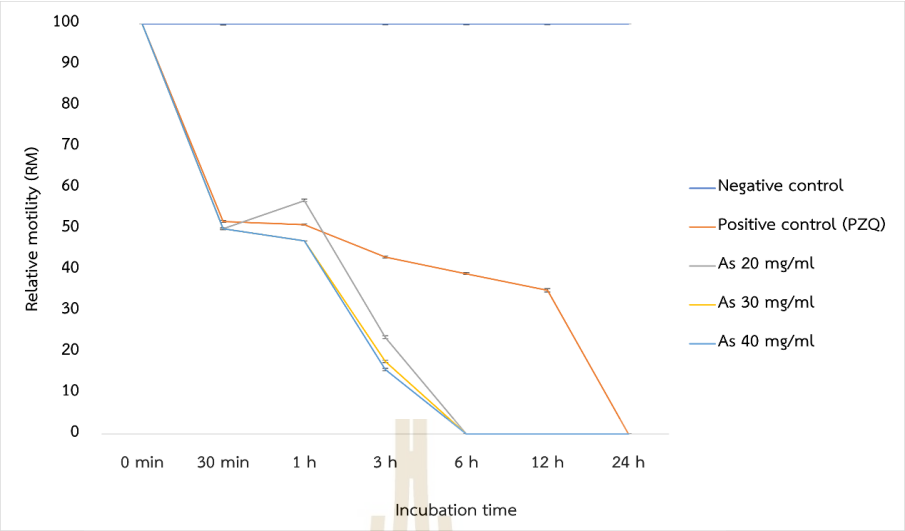


Figure 4.16 The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *A. sativum* treated groups, compared to both negative and positive controls, across various time intervals.

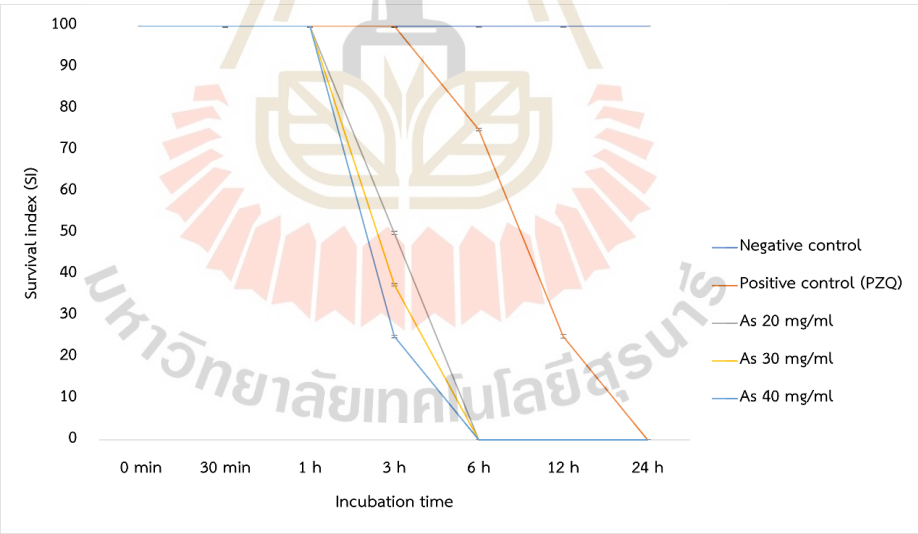


Figure 4.17 The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *A. sativum* treated groups, compared to both negative and positive controls, across various time intervals.

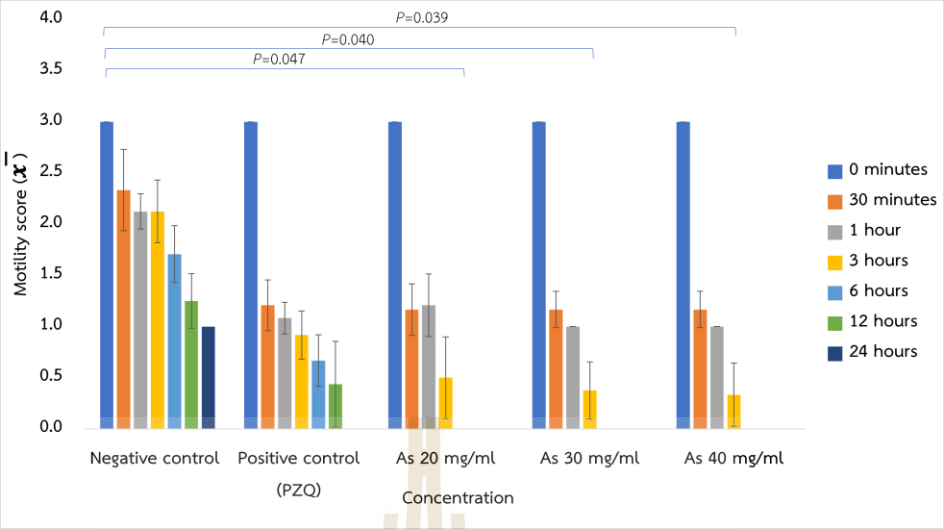


Figure 4.18 The mean motility scores were significantly different between the negative control and all groups treated with *A. sativum*. However, no significant difference was noted between the positive control and *A. sativum* treated groups ($p < 0.05$).

The *T. laurifolia* treated groups with concentrations of 20, 30, and 40 mg/ml at the 0-minute mark, all worm sets exhibited active motility and a healthy appearance, resembling the negative control group, with RM = 100 and SI = 100. However, it was observed that the parasites exhibited a decreasing motility rate at 30 minutes in all groups. This motility rate remained constant until 6 hours (20 mg/ml: RM = 48.21, 52.94, 50.97, and 60.98, SI = 100, respectively; 30 mg/ml: RM = 51.79, 64.71, 64.69, and 60.98, SI = 100, respectively; 40 mg/ml: RM = 50.00, 50.98, 49.01, and 63.41, SI = 100, respectively). Additionally, the motility rate of each group decreased once again at the 12-hour mark (RM = 40.00, SI = 50, respectively) and ceased entirely at 24 hours (RM = 0 and SI = 0) (Figure 4.19 and 4.20).

A comparative analysis was indicated not significantly different between both the negative control and positive control groups compared to the *T. laurifolia* treated groups ($p < 0.05$) (Figure 4.21).

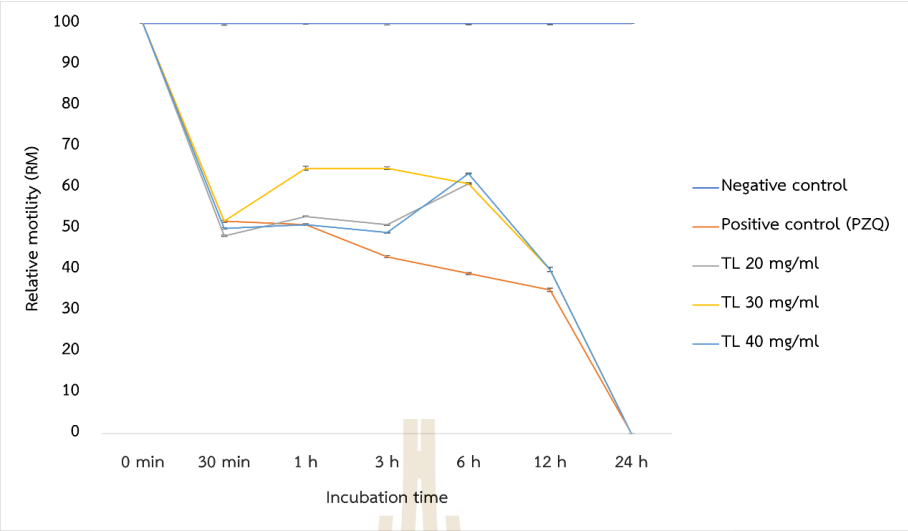


Figure 4.19 The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *T. laurifolia* treated groups, compared to both negative and positive controls, across various time intervals.

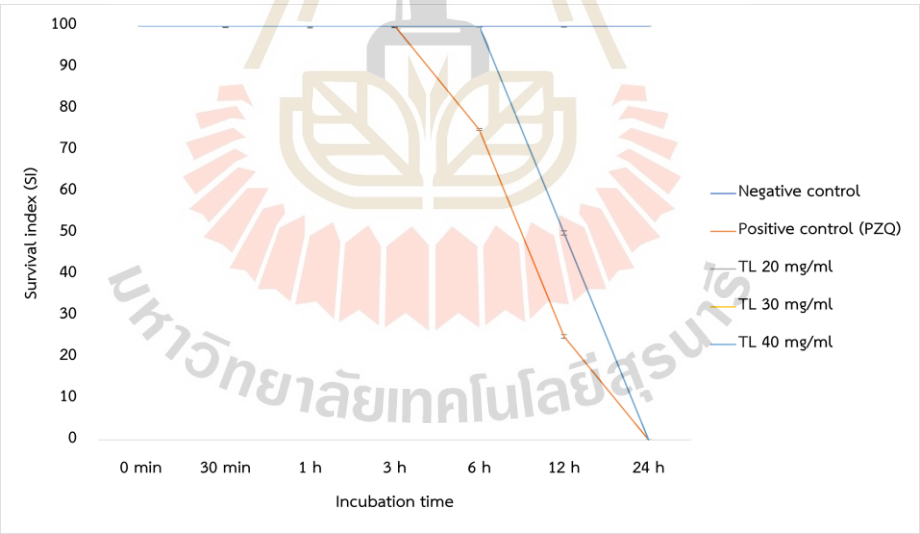


Figure 4.20 The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *T. laurifolia* treated groups, compared to both negative and positive controls, across various time intervals.

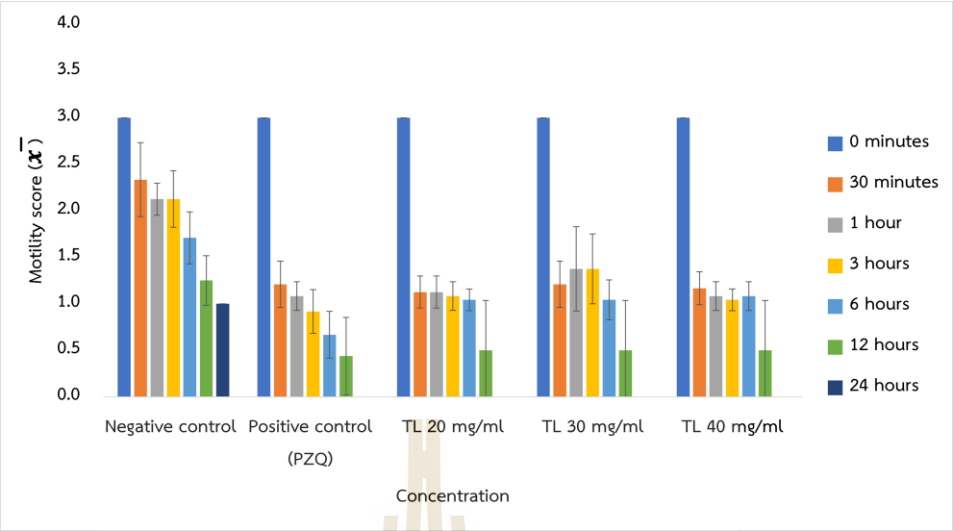


Figure 4.21 The mean motility scores were not significantly different between both the negative control and positive control groups compared to the *T. laurifolia* treated groups ($p < 0.05$).

The *E. longifolia* treated groups with concentrations of 20, 30, and 40 mg/ml at the 0-minute mark, all worm sets exhibited active motility and a healthy appearance, resembling the negative control group, with RM = 100 and SI = 100. However, it was observed that the *O. viverrini* adult worms exhibited a decreasing motility rate at 30 minutes in all groups (20 mg/ml: RM = 87.500, SI = 100; 30 mg/ml: RM = 75.00, SI = 100; 40 mg/ml: RM = 62.50, SI = 100, respectively). Interestingly, it was found that from hours 1 to 12, the parasites displayed unusually high levels of active motility (20 mg/ml: RM = 133.33, 125.47, 146.34, and 145.00, SI = 100; 30 mg/ml: RM = 115.69, 111.74, 143.90, and 120.00 SI = 100; and 40 mg/ml: RM = 100.00, 107.82, 148.78, 110.00, SI = 100, respectively). When the experiment concluded at the 24-hour mark, some individuals worm from each group remained alive, although their motility rates had significantly decreased (RM = 25 and SI = 25) (Figure 4.22 and 4.23).

A comparative analysis was indicated not significantly different between the negative control and *E. longifolia* treated groups. However, there was a significant difference observed between the positive control groups and the 20 mg/ml *E. longifolia* treated groups ($p < 0.05$) (Figure 4.24).

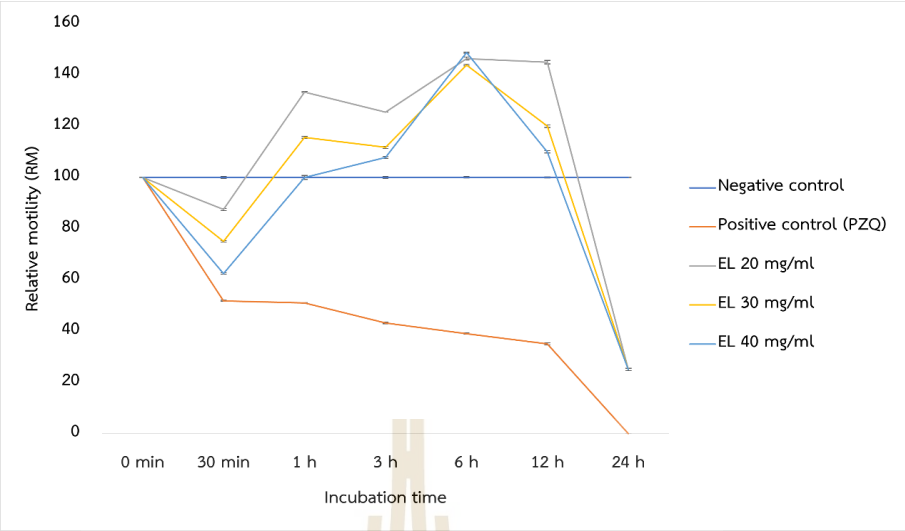


Figure 4.22 The RM values of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *E. longifolia* treated groups, compared to both negative and positive controls, across various time intervals.

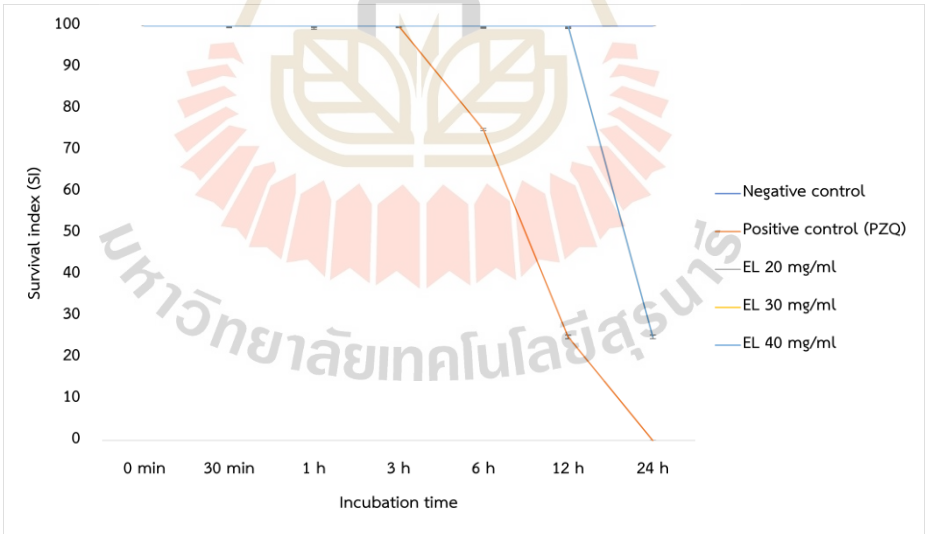


Figure 4.23 The SI of *O. viverrini* adult worms, were assessed after incubated with 20, 30, and 40 mg/ml *E. longifolia* treated groups, compared to both negative and positive controls, across various time intervals.

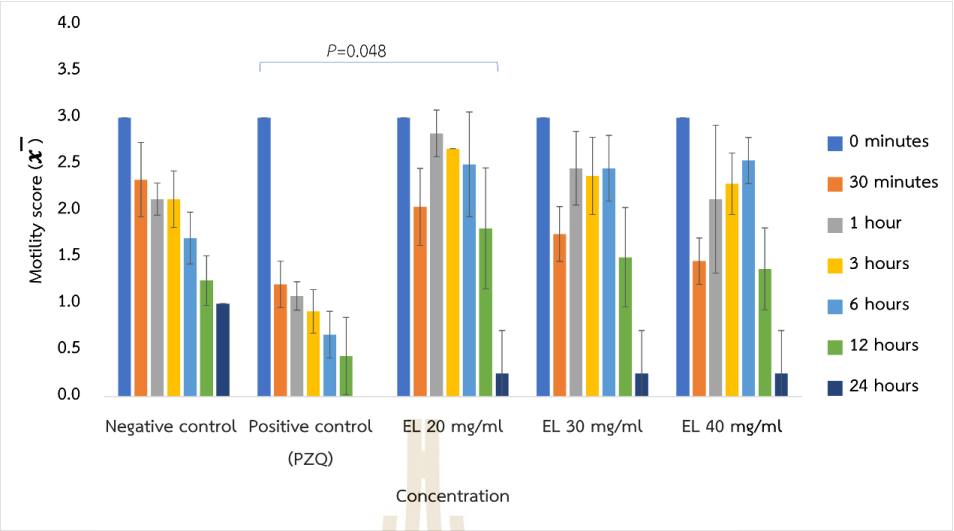


Figure 4.24 The mean motility scores were not significantly different between the negative control and *E. longifolia* treated groups. However, there was a significant difference observed between the positive control groups and the 20 mg/ml *E. longifolia* treated groups ($p < 0.05$).

The viability of *O. viverrini* adult worms was assessed using Trypan blue staining. In the negative control group, no staining was observed. Conversely, extensive Trypan blue staining was evident in the positive control group, as well as in all treated groups with *A. sativum*, *T. laurifolia*, and *E. longifolia* (Figure 4.25).

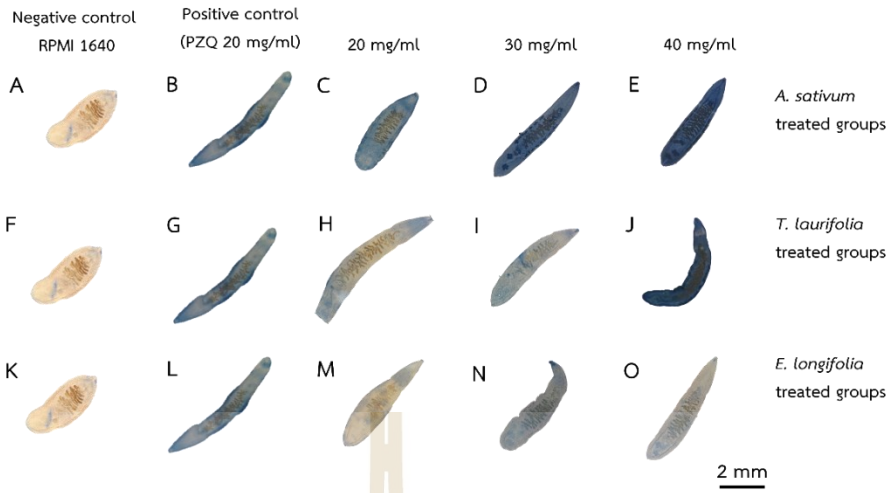


Figure 4.25 Viability of *O. viverrini* adult worms were assessed using Trypan blue staining. the negative control group (A, F, and K), positive control group (B, G, and L), *A. sativum* treated groups (C, D, and E), *T. laurifolia* treated groups (H, I, and J), and *E. longifolia* treated groups (M, N, and O). Scale bar = 2 mm.

4.4 Result of ROS generation of *O. viverrini* adult worm

A significant cellular stress response, resulting in ROS generation, was evident in all groups following a 6-hour incubation period. ROS presence was determined using H2DCFDA fluorescence dye, allowing for comprehensive ROS analysis. Minimal ROS generation was observed in the negative control group in the anterior, middle, and posterior regions of the *O. viverrini* adult worm. In contrast, the positive control group displayed partial ROS generation, primarily around the oral and ventral suckers in these regions.

The *A. sativum* treated groups exhibited significantly higher ROS levels throughout the entire body of the worms, including the anterior, middle, and posterior regions of the *O. viverrini* adult worm, at concentrations of 20 mg/ml, 30 mg/ml, and 40 mg/ml, respectively (Figure 4.26). The CTWF of the *A. sativum* treated groups (20, 30, and 40 mg/ml) displayed higher fluorescence intensity compared to the control groups (Figure 4.27).

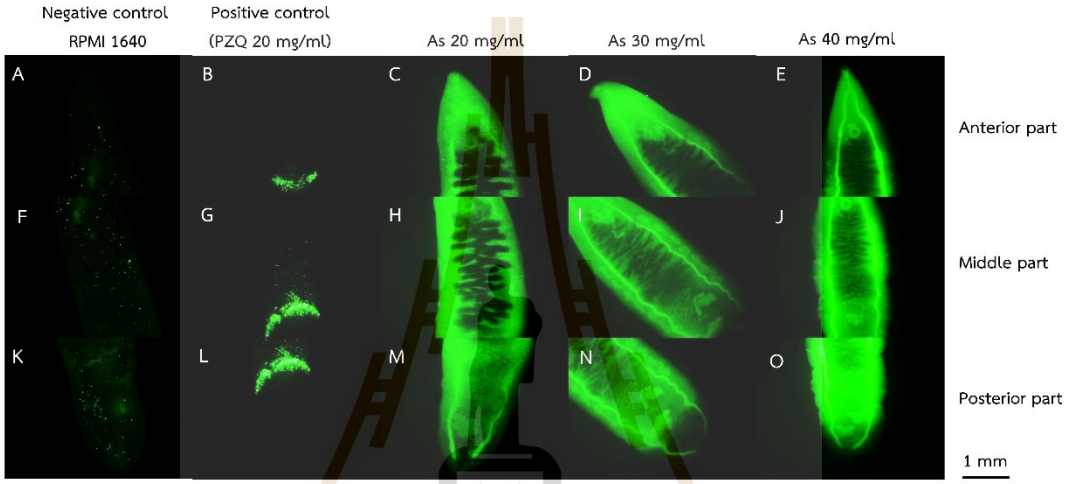


Figure 4.26 The ROS generation image of *A. sativum* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.

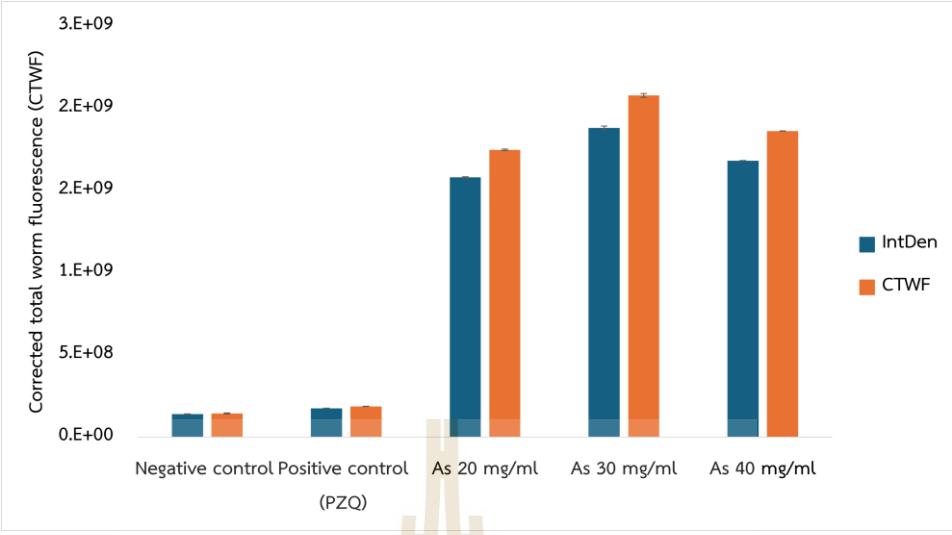


Figure 4.27 The CTWF of fluorescence intensity in the *A. sativum* treated groups.

The *T. laurifolia* treated groups was showed elevated levels of ROS throughout the entire body of the *O. viverrini* adult worms, encompassing the anterior, middle, and posterior regions, in comparison to the control group. This effect was particularly pronounced in certain areas such as the testes and genital pore, at concentrations of 20 mg/ml, 30 mg/ml, and 40 mg/ml respectively (Figure 4.28).

The CTWF was showed fluorescence intensity of *T. laurifolia* treated groups (20, 30, and 40 mg/ml) exhibited higher levels compared to the control groups (Figure 4.29).

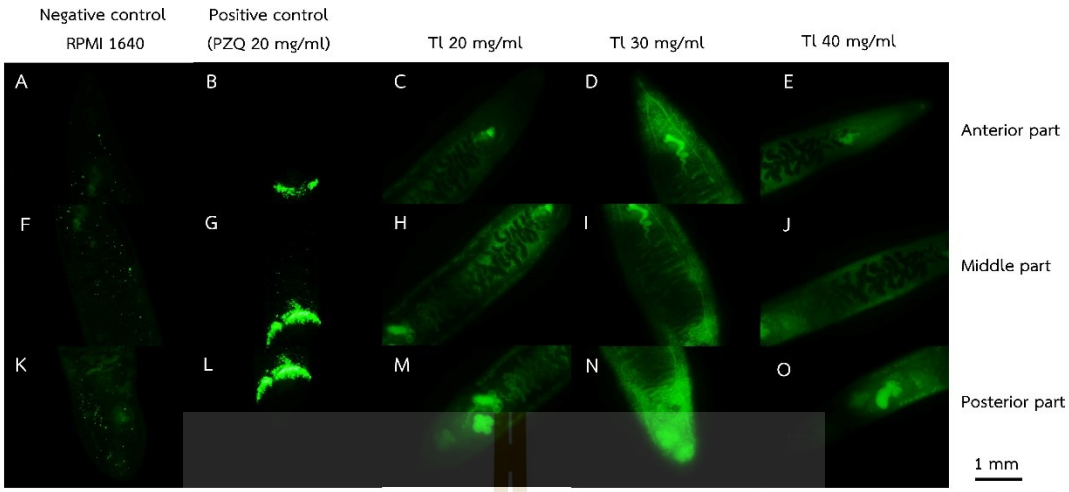


Figure 4.28 The ROS generation image of *T. laurifolia* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.

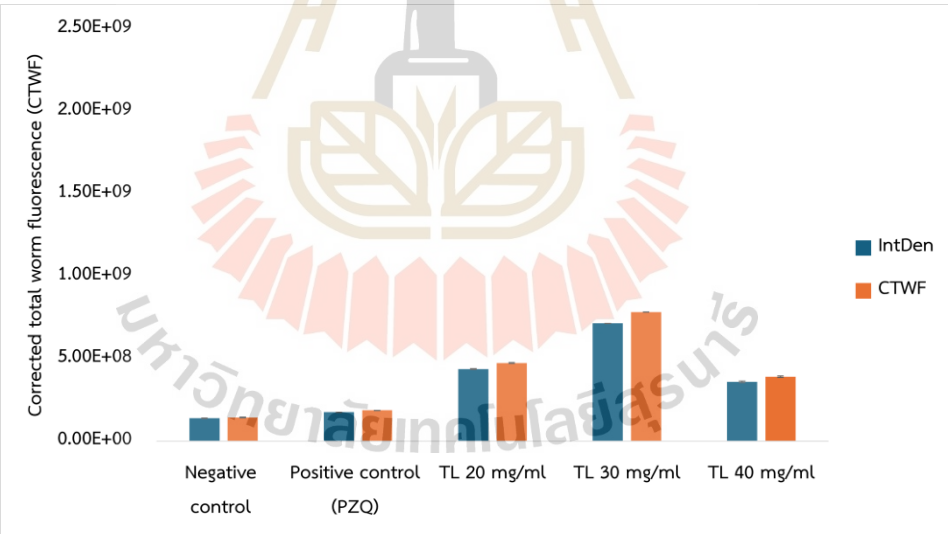


Figure 4.29 The CTWF of fluorescence intensity in the *T. laurifolia* treated groups.

The *E. longifolia* treated groups displayed comparatively reduced levels of ROS across the entire body of the worms, including the anterior, middle, and posterior regions, in contrast to the control group. However, in specific areas such as the testes and genital pore, the intensity of ROS appeared to be heightened. These effects were

observed at concentrations of 20 mg/ml, 30 mg/ml, and 40 mg/ml respectively (Figure 4.30).

The fluorescence intensity of *E. longifolia* treated groups (at concentrations of 20, 30, and 40 mg/ml) demonstrated relatively lower levels compared to the control groups, with an increase observed with higher concentrations (Figure 4.31).

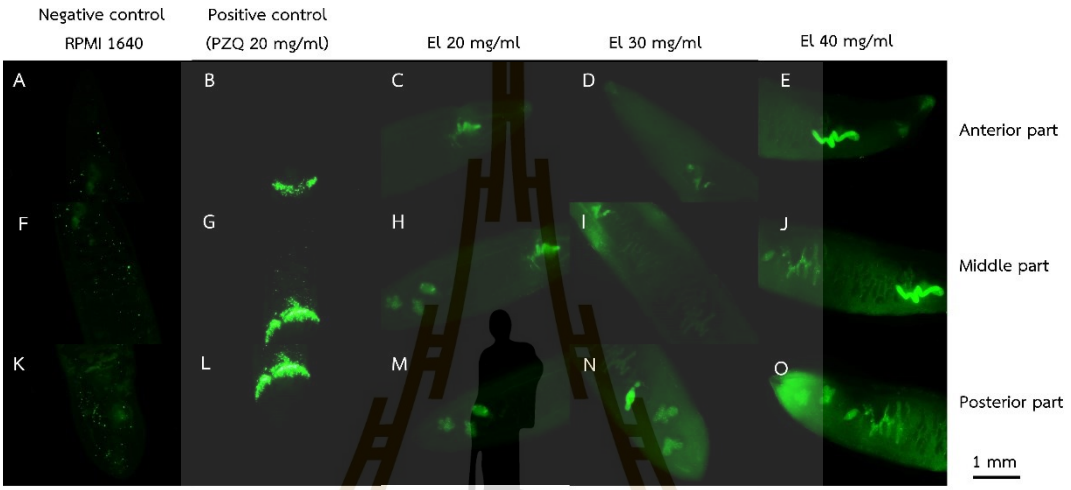


Figure 4.30 The ROS generation image of *E. longifolia* treated groups, 20 mg/ml (C, D, and E), 30 mg/ml (H, I, and J), and 40 mg/ml (M, N, and O) groups, respectively. Scale bar = 1 mm.

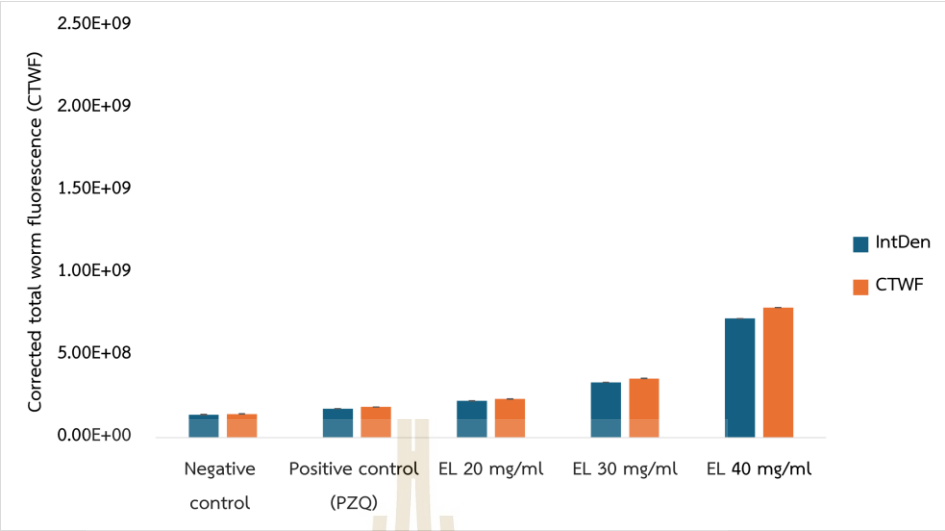


Figure 4.31 The CTWF of fluorescence intensity in the *E. longifolia* treated groups.

4.5 Result of NO generation of *O. viverrini* adult worm

The negative control groups, the NO levels increased from 1.454 uM to 1.526 uM within 24 hours, representing an increase of 0.372 uM during the observation period. Conversely, in the positive control group, NO levels increased from 1.514 uM to 1.599 uM within 24 hours, indicating an increase of 0.085 uM.

The *A. sativum* treated groups, at a concentration of 20 mg/ml, NO levels increased from 1.490 uM to 1.599 uM within 24 hours, signifying an increase of 0.109 uM. Similarly, at 30 mg/ml concentration, NO levels increased from 1.490 uM to 1.647 uM within 24 hours, with an increase of 0.157 uM observed. Furthermore, at a concentration of 40 mg/ml, NO levels increased from 1.478 uM to 1.719 uM within 24 hours, representing an increase of 0.372 uM. However, these changes in NO levels were not statistically significant when compared with the negative and positive control groups (Figure 4. 32).

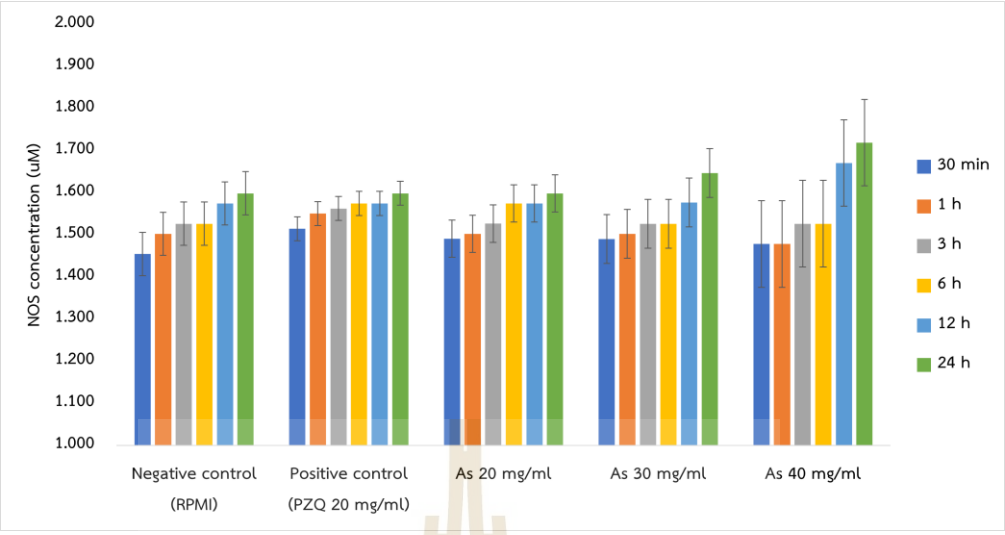


Figure 4.32 The alteration in NO levels due to *A. sativum* treatment in *O. viverrini* adult worms.

The *T. laurifolia* treated groups, at a concentration of 20 mg/ml, NO levels increased from 1.490 uM to 1.635 uM within 24 hours, resulting in a total increase of 0.145 uM. Similarly, at a concentration of 30 mg/ml, NO levels rose from 1.611 uM to 1.695 uM within the same timeframe, showing an increase of 0.084 uM. At the highest concentration tested, 40 mg/ml, NO levels increased from 1.538 uM to 1.647 uM within 24 hours, indicating a rise of 0.109 uM. These changes in NO levels were found to be statistically significant when comparing both the negative control and positive control groups with the 30 mg/ml and 40 mg/ml concentrations of *T. laurifolia* treated groups ($p < 0.05$) (Figure 4. 33).

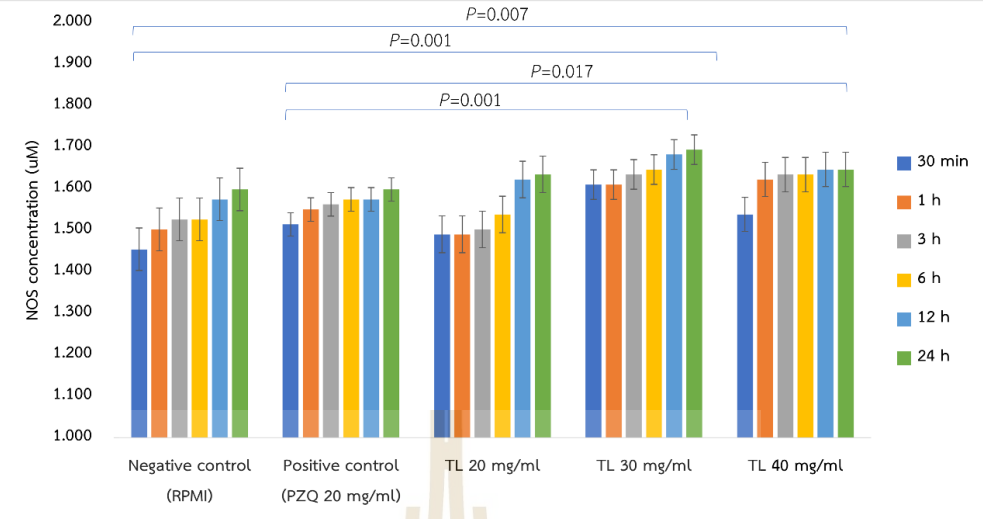


Figure 4.33 The alteration in NO levels due to *T. laurifolia* treatment in *O. viverrini* adult worms.

The *E. longifolia* treated groups, at a concentration of 20 mg/ml, NO levels increased from 1.454 uM to 1.647 uM within 24 hours, resulting in a total increase of 0.193 uM. Similarly, at a concentration of 30 mg/ml, NO levels rose from 1.430 uM to 1.647 uM within the same timeframe, showing an increase of 0.217 uM. At the highest concentration tested, 40 mg/ml, NO levels increased from 1.611 uM to 1.767 uM within 24 hours, indicating a rise of 0.156 uM.

These changes in NO levels were found to be significantly different between both the negative control and positive control groups when compared to the 40 mg/ml concentration of *E. longifolia* treated groups ($p < 0.05$) (Figure 4.34).

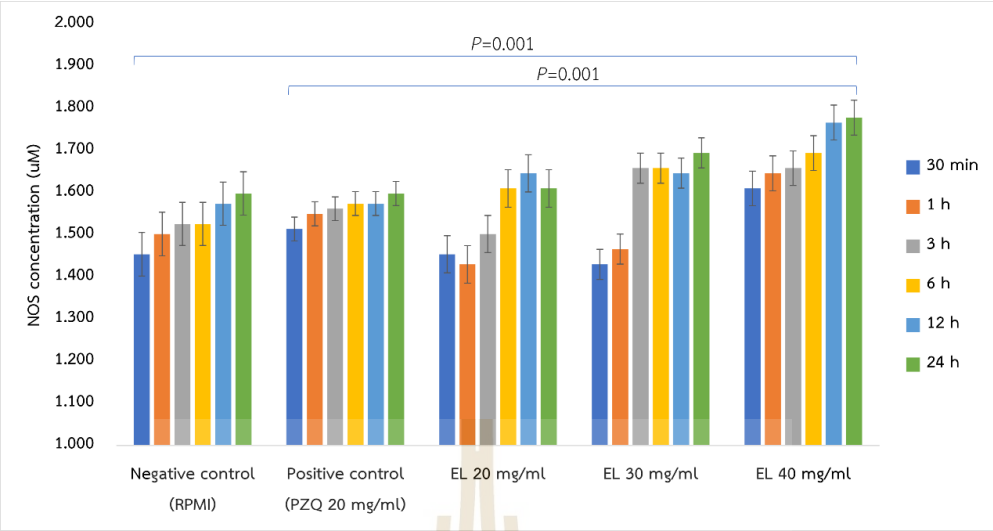


Figure 4.34 The alteration in NO levels due to *E. longifolia* treatment in *O. viverrini* adult worms.

4.6 Result of surface morphological changes of *O. viverrini* adult worm

The negative control group with RPMI medium, the worms exhibited a regular, smooth surface, numerous short microvilli (Mv) and the typical distribution of papillae (Pa) around the oral suckers (Os) and ventral suckers (Vs). The arrangement of papillae (Pa) among the microvilli was also normal (Figure 4.35).

The SEM images illustrate the tegumental morphology of *O. viverrini* adult worms in different treatment groups (Figure 4.36). In the positive control group treated with 20 mg/ml PZQ, the tegumental surface around the oral sucker appeared swollen (A, scale bar = 40 µm), with alterations showing swelling until bursting (B, scale bar = 4 µm) and stretched microvilli (Mv) (C, scale bar = 4 µm), along with swelling of papillae and microvilli around the ventral sucker (C, scale bar = 40 µm).

The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *A. sativum* treated groups (Figure 4.37). At 20 mg/ml (A, scale bar = 400 µm) showed irregular and non-smooth tegumental surfaces, significant morphological disruptions of blistered microvilli surfaces (A, I and III) and around the ventral sucker (Os) (A, II). At 30 mg/ml (B, scale bar = 400 µm), the tegumental surface exhibited

swelling throughout the entire body, significant morphological disruptions, blistering until bursting of microvilli surfaces (B, I and II), and around the ventral sucker (B, III). At 40 mg/ml (C and D, scale bar = 400 and 60 µm, respectively), considerable damage to the microvilli surfaces and papillae around the ventral sucker (D, scale bar = 60 µm) and oral sucker (C, I) was evident. Extensive degradation of the microvilli surfaces was observed throughout the worm's body (C, II and D, I, II).

The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *T. laurifolia* treated groups (Figure 4.38). Due to the loss of samples at 20 mg/ml during SEM preparation, images are only available for concentrations at 30 mg/ml (A, scale bar = 400 µm) and 40 mg/ml (B, scale bar = 400 µm). At 30 mg/ml (A), the tegumental surface of the adult worms showed slight swelling, particularly noticeable around the Oral sucker (A, I), ventral sucker (A, II), and microvilli surface across the entire body of the worm (A, III). At 40 mg/ml (B), the worm displayed a non-smooth surface across its body, with distinct swelling observed around the Oral sucker, degradation of the microvilli surface on the body surface, and around the ventral sucker (B, II and III).

The SEM images depict the tegumental morphology of O. viverrini adult worms in the *E. longifolia* treated groups (Figure 4.39). At 20 mg/ml (A, scale bar = 400 µm), the worm displayed a generally smooth body surface with minimal swelling of the microvilli surface (A, I). At 30 mg/ml (B, scale bar = 400 µm), the surface remained generally smooth, like the appearance at 20 mg/ml, showing tegumental surfaces around the Oral sucker (B, I) and ventral sucker (B, II and III) with minor and relatively normal swelling. Distinct papillae (Pa) were also clearly visible. At 40 mg/ml (C and D, scale bar = 400 µm each), the worm exhibited a non-smooth surface across its body, with noticeable swelling around the Oral sucker (C, II and D, I) and ventral suckers (C, III). Upon closer examination of the microvilli surface (C, I, and D, II), pronounced swelling in the form of nodules of microvilli was observed, indicating a relatively severe condition.

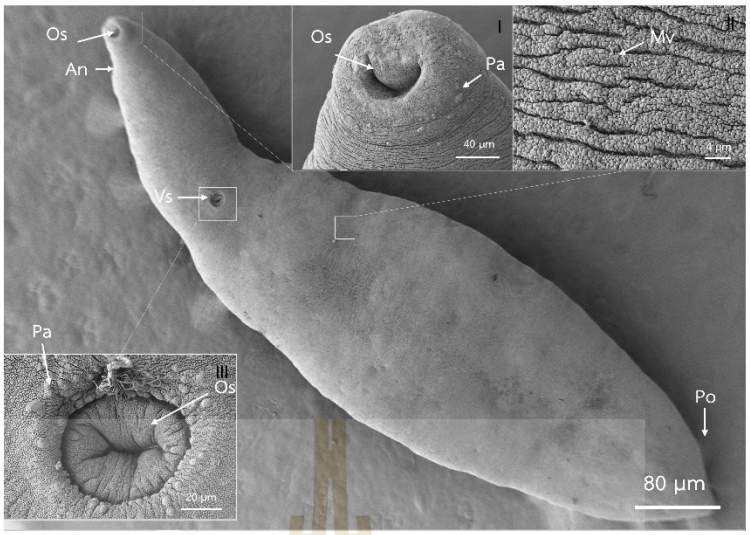


Figure 4.35 The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the negative control group, with a scale bar of 80 µm.

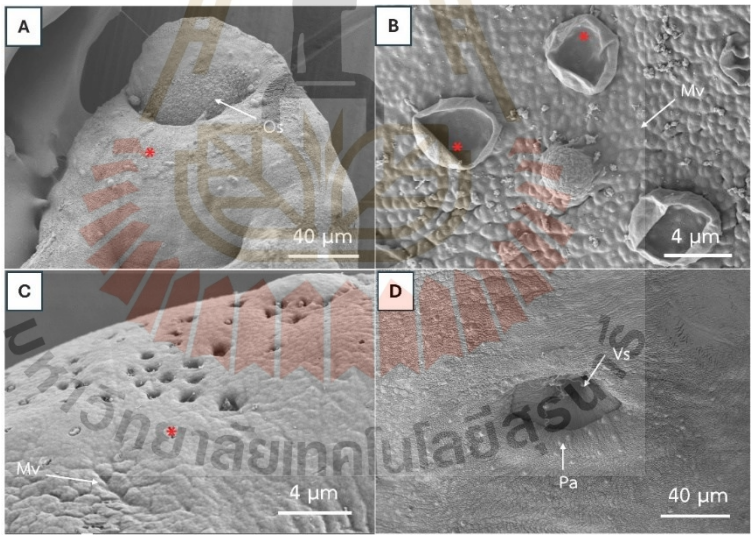


Figure 4.36 The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the positive control group treated with 20 mg/ml PZQ. Images (A, B, C, and D) show various aspects of the tegumental surface morphology, with respective scale bars of 40 µm, 4 µm, 4 µm, and 40 µm. * = Swelling of tegumental surface (A and C), alteration of tegumental surface, swelling until bursting (B).

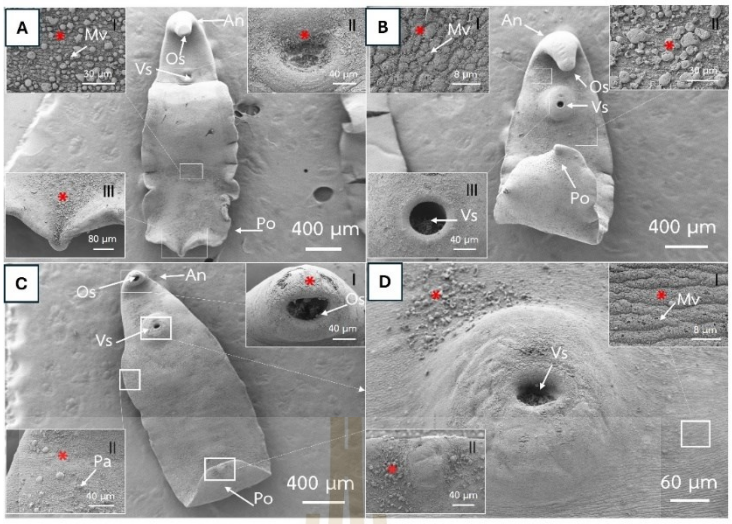


Figure 4.37 The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *A. sativum* treated groups at concentrations of 20, 30, and 40 mg/ml. Images (A, B, and C, D) correspond to these concentrations, with scale bars of 400 µm, 400 µm, 400 µm, and 60 µm, respectively. * = Alteration of tegumental surface by degradation (D, I and II), damage and blister until bursting (A, I, B, I and II) of tegumental surface.

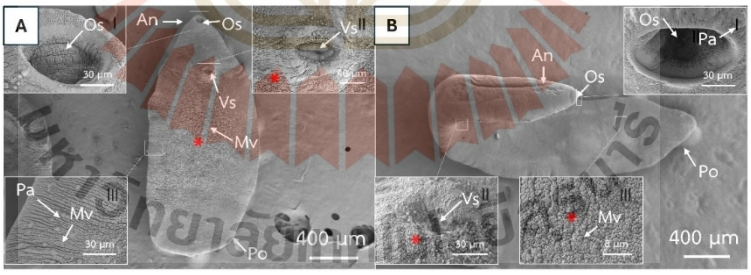


Figure 4.38 The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *T. laurifolia* treated groups at concentrations of 30 and 40 mg/ml. Images A and B correspond to these concentrations, with scale bars of 400 µm each. Unfortunately, samples at 20 mg/ml were lost during sample preparation for SEM and are not shown. = Alteration of tegumental surface by degradation (B, II and III), swelling of tegumental surface (A, I, II, and III, B, I).

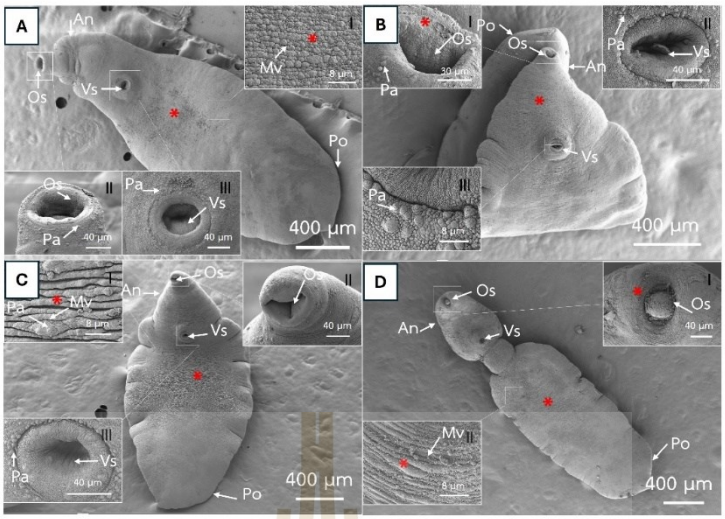


Figure 4.39 The SEM images depict the tegumental morphology of *O. viverrini* adult worms in the *E. longifolia* treated groups at concentrations of 20, 30, and 40 mg/ml. Images (A, B, and C, D) correspond to these concentrations, with scale bars of 400 µm each. *= Alteration of tegumental surface, swelling of tegumental surface.

4.7 Discussion

The efficacy against *O. viverrini* NEJs and adult worms were demonstrated the potential of crude extracts of *A. sativum*, *T. laurifolia*, and *E. longifolia* in reducing the RM and SI of *O. viverrini* NEJs and adult worms. depending on concentration and exposure time. In the *O. viverrini* NEJs, *A. sativum* treated groups was exhibited significant differences compared to the negative control group. In some group of *T. laurifolia* treated groups, significant differences were observed between the negative control group. Finally, in the *E. longifolia* treated groups, no significant differences between negative control group. In the *O. viverrini* adult worms, *A. sativum* treated groups was showed a significant difference compared to the negative control group. In contrast, in the *T. laurifolia* and *E. longifolia* treated groups, no significant difference compared to both the negative and positive control groups. When compared to the positive control group, it was found that the groups treated with extracts of *A. sativum*,

T. laurifolia, and *E. longifolia* did not exhibit statistically significant differences, indicating comparable efficacy to the PZQ standard drug.

The present findings are consistent with the research conducted by Lorsuwannarat and colleagues (2013), who investigated the *in vitro* anthelmintic effects of plumbagin on *S. mansoni*. In their study, the plumbagin-treated group exhibited a more rapid reduction in RM values compared to the group treated with PZQ (Lorsuwannarat et al., 2013). Similarly, Wannachat et al. (2020) reported comparable outcomes using a crude extract from *Areca catechu* on *O. viverrini* NEJs and adult worms, indicating a swift decline in motility with escalating concentrations of the *A. catechu* extract (Wannachat et al., 2020). Furthermore, *A. sativum* extract exhibited effective schistosomicidal activities by diminishing worm burden and tissue eggs (Aly et al., 2017; Wan et al., 2017). Its flukicidal effect was observed through a reduction in both motility and mortality of schistosome (Jeyathilakan et al., 2012), while *A. sativum* essential oil induced an irreversible *F. gigantica* paralytic effect after exposure at a concentration of 3 mg/ml (Singh et al., 2009). Furthermore, it has been found that the extract of *E. longifolia* reduces the movement of adult *S. japonicum* and their egg-laying activity (Jiwajinda et al., 2002).

The SEM analysis revealed morphological damage characterized by tegumental swelling evident across all treatment groups of *O. viverrini* NEJs and adult worm. In the groups treated with *A. sativum*, noticeable morphological abnormalities were observed on the tegumental surface of *O. viverrini* NEJs and adult worm, including degradation of the tegumental structure and extensive exfoliation. Conversely, in the *T. laurifolia* treated groups, exhibited slight swelling of the tegumental surface. Meanwhile, in the *E. longifolia* treated groups, displayed significant swelling of the tegumental surface. In contrast, in the negative control group showed a normal tegumental surface. These effects were found to be dependent on both exposure time and concentration. Investigations suggested that administering *A. sativum* extracts to mice infected with the *S. mansoni* blood fluke resulted in a significant reduction in egg and worm burden, accompanied by various ultrastructural alterations in the tegument (Riad et al., 2009)

and tissue *S. mansoni* eggs (El shenawy et al., 2008). Additionally, *A. sativum* treatment exhibited effective schistosomicidal activities by reducing worm burden and tissue eggs (Aly et al., 2017). This observation may correspond to the mechanism described by Corral et al., (2016), which explains that allicin in *A. sativum* induces calcium and mitochondrial dysregulation, disrupting calcium homeostasis and triggering oxidative stress. These processes overwhelm the antioxidant defense mechanisms of the cell, leading to mitochondrial dysfunction and a bioenergetic crisis in *Leishmania infantum*. Ultimately, this cascade results in necrotic death of the *Leishmania* parasite. Furthermore, studies on *E. longifolia* in *T. gondii* found that it caused cell wall alterations (SEM), decreased cytoplasmic volume (TEM), and rapid antiparasitic activity (Kavitha et al., 2012 a, 2012b). In the discussion by Ahmed et al., 2020, it is noted and referenced that the observed anthelmintic effect of plant extracts may be attributed to the presence of secondary metabolites. Specifically, tannins, alkaloids, and phenolics are mentioned as potentially exerting anthelmintic activity by inhibiting hatching, interfering with development into the infective larval stage, and reducing the motility of adult worms (Wang et al., 2010). Additionally, these metabolites have been shown to interfere with coupled oxidative phosphorylation and block ATP synthesis in adult worms, leading to the release of enzymes that degrade the worm membrane (Joshi et al., 2010; Martin et al., 1997; Wang et al., 2010). This biological cascade involves cellular death, particularly when respiration halts, inducing hypoxia and metabolic shifts in tissues. Decreased ATP production prompts anaerobic metabolism, generating intracellular lactate and lowering pH. Diminished ATP triggers lysosome swelling and enzyme release. Tissue hypoxia escalates calcium influx, activating enzymes and damaging membranes and organelles. These events lead to cellular self-digestion (autolysis) and tissue decay (Madea et al., 2014). This study indicated these processes through observed alterations in *O. viverrini* adult worm morphological surfaces.

This study revealed that *A. sativum* treated groups displayed significantly higher levels of reactive ROS across the entire body of the worms, exhibiting higher fluorescence intensity. In contrast, *T. laurifolia* and *E. longifolia* treated groups showed elevated ROS levels throughout the entire body, particularly prominent in specific regions such as the testes and genital pore. Notably, minimal ROS generation was observed in the regions of the negative control group. Furthermore, within 24 hours of treatment, *A. sativum* treated groups displayed increased levels of NO; however, these changes did not reach statistical significance when compared with both the negative and positive control groups. Conversely, in the *T. laurifolia* and *E. longifolia* treated groups, NO levels exhibited statistically significant differences when compared to both the negative control and positive control groups. This observation is consistent with the study conducted by Goel et al., (2020) on oxidative stress in *H. contortus* parasites treated with *Lansium parasiticum* aqueous extract-protected silver nanoparticles, indicating a metabolic shift in response to ROS-induced oxidative stress. Previous studies have demonstrated that *A. sativum* directly impacts stress generation. *A. sativum* has shown strong biocidal effects against all stages of *S. mansoni*, with significant inhibition observed in SOD, CAT, GR, TrxR, and SDH (Mantawy et al., 2012). Additionally, there was an increase in antioxidant enzyme activities, coupled with a significant reduction in worm burden, hepatic and intestinal eggs, as well as oogram count (Mantawy et al., 2011). Generally, the generation of stress-induced ROS entails the production of highly reactive molecules, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, within cells or organisms in response to various stressors. These stressors encompass physical, chemical, environmental, or biological factors capable of disrupting the normal balance in cells. Under stressful conditions, the overproduction of ROS or NO can lead to oxidative stress, which is associated with impairment of proteins, lipids, and DNA, thus affecting cellular functionality (Finkel, 2011). The generation of stress-induced ROS and NO involves a complex process with numerous cellular pathways and mechanisms, playing critical roles in cellular processes such as signaling and immune responses (Schieber and Chandel, 2014).

Therefore, the expression of ROS and NO in *O. viverrini* adult worms in this study depends on the cellular characteristics of each parasite.

Currently, the drug used for the treatment of Opisthorchiasis is PZQ. Its mechanism of action involves increasing calcium influx, which causes the muscles of parasites to contract, affecting their movement and inducing paralysis (Becker et al., 1980; Pax et al., 1978). Allicin, the major bioactive compound of *A. sativum*, has been studied for its mechanism of action on parasites. Research conducted by Corral and colleagues (2016) investigated the effects of allicin on protozoa *Leishmania* sp. Their findings revealed that allicin induces dysregulation of calcium homeostasis and oxidative stress, which cannot be countered by the antioxidant defense of the cell. Consequently, this leads to mitochondrial dysfunction and a bioenergetic collapse, resulting in cell necrosis and cell cycle arrest in the pre-mitotic phase (Corral et al., 2016). In addition. the *A. sativum* extract appears to contain diallyl-disulfide (DAD), which could potentially interfere with the parasites' synthesis of membrane lipids (Nok et al., 1996).

Rosmarinic acid, a major compound in *T. laurifolia* extract, has garnered significant interest due to its potential broad pharmacological effects (Guan et al., 2022; Woottisin et al., 2022). The mechanism through which *T. laurifolia* affects parasites has not been extensively studied. However, insights into this mechanism can be inferred from the general action of phenolic compounds on bacterial cells, as outlined by Resende et al. (2015). Phenolic compounds are known to effectively disrupt the membranes of gram-positive bacteria at the membrane-interface. This disruption can significantly compromise membrane plasticity, leading to weakened membrane integrity, destabilization, and subsequent disruption of the cell membrane and transport systems *T. laurifolia* leaf extract is recognized as an efficient antioxidant, indicating that antioxidants represent a promising adjuvant approach during treatment to reduce morbidity and mortality. Despite some successful strategies, there is still considerable progress needed in implementing novel therapies for schistosomiasis or opisthorchiasis (Vale et al., 2020).

The bioactive compounds known as quassinoids are significant constituents of *E. longifolia*, as highlighted by Chan et al. (1989). The mechanism of action of *E. longifolia* was elucidated by Kavitha et al. (2012), who demonstrated that extract fractions from *E. longifolia* act on *T. gondii* within the cytoplasmic region. They proposed that these fractions might induce intracellular oxidative stress through an indirect mechanism. Mitochondria, being the primary source of ROS within cells, play a pivotal role in this process. Uncontrolled superoxide flashes in mitochondria contribute to global oxidative stress, which is critical in hypoxia/reoxygenation injury in cells. This model provides a plausible explanation for the inhibition of *T. gondii* growth (Kavitha et al., 2012b).

Studies on the elimination of parasites by *A. sativum* have demonstrated various effects. *A. sativum* oil displayed a protective effect in rats inoculated with L3 larvae by improving liver and kidney functions (Morsy et al., 2021). Allicin was shown to partially protect hosts against *P. yoelii* by enhancing innate and adaptive immune responses (Feng et al., 2012). The combination of *A. sativum* and arteether exhibited immunomodulatory and antiparasitic effects through the nitric oxide pathway in *P. berghei* infected mice (Vathsala et al., 2020). Additionally, it irreversibly inhibited *T. b. brucei* trypanothione reductase and decreased mitochondrial membrane potential (Krstin et al., 2018). *A. sativum* and allicin significantly reduced worm burden and decreased serum concentrations of liver fibrosis markers and proinflammatory cytokines (Metwally et al., 2018). They also enhanced parasite engulfment and destruction of intracellular amastigotes by macrophages (Ghazanfari et al., 2006). *A. sativu*m oils substantially normalized liver function enzymes and showed varying improvements in other parameters, notably reducing worm burden and ova count (Metwally, 2006). *A. sativum* oil extract resulted in mortality of *S. japonicum* cercariae, exhibited preventive efficacy against infection, and contributed to a reduction in worm burden (Wan et al., 2017). Furthermore, *A. sativum* extract exhibited larvicidal activity and reduction in parasitic burden (Palacios-Landín et al., 2015), along with demonstrating antiamoebic activity (Behnia et al., 2008). Studies on parasite eradication

in *T. laurifolia* have shown promising results. Wonkchalee et al. (2012, 2013) demonstrated the effectiveness of *T. laurifolia* in reducing inflammation caused by pathological alterations in *O. viverrini*-infected hamsters. In the case of *E. longifolia*, various studies have highlighted its potential in combating parasites. Girish et al. (2015) reported anti-protozoal activity at a concentration of 1.0 mg/ml, while Kavitha et al. (2012a) demonstrated anti-*T. gondii* activity. Additionally, ethanol and methanol extracts exhibited higher antimalarial activities (Sriwilaijaroen et al., 2010), and there was a significant increase in parasitemia suppression (Mohd Ridzuan et al., 2007). Other studies have shown anti-parasite growth effects (Mohd Ridzuan et al., 2005), with quassinoids and alkaloids exhibiting cytotoxicity and antiplasmodial activity (Chan et al., 2004; Hout et al., 2006; Jiwajinda et al., 2002). Furthermore, compounds like eurycomanone and pasakbumi have displayed antimalarial activity (Kuo et al., 2004).

Furthermore, prior investigations have explored herbal remedies against *O. viverrini* both *in vitro* and *in vivo*. Aukkanimart et al. (2015) investigated the antioxidant, anti-inflammatory, and anthelmintic effects of the traditional folk medicine Garcinia mangostana pericarp extract in hamsters with opisthorchiasis. Additionally, Intuyod et al. (2014) examined the activity of an anthocyanin complex containing cyanidin and delphinidin-rich extracts derived from turmeric against inflammation and periductal fibrosis in hamsters infected with *O. viverrini*. However, the specific use of garlic for eliminating *O. viverrini* liver flukes has not been previously reported. This indicates that *A. sativum*, *T. laurifolia*, and *E. longifolia* have efficacy against various types of parasites. Therefore, detailed studies in the future are of interest for the development of these as alternative drugs for parasite eradication, replacing chemical substances in the future.

CHAPTER V
CONCLUSION AND RECOMMENDATION

5.1 Conclusion on the Efficacy of Crude Extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* NEJs

The efficacy against *O. viverrini* NEJs demonstrated the potential of crude extracts of *A. sativum*, *T. laurifolia*, and *E. longifolia* in reducing the RM and SI of *O. viverrini* NEJs, depending on concentration and exposure time, while the negative control group displayed normal motility. The *A. sativum* treated groups, which exhibited significant differences compared to the negative control group. In some group of *T. laurifolia* treated groups, significant differences were observed between the negative control group. Finally, in the *E. longifolia* treated groups, no significant differences between negative control group.

In the groups treated with *A. sativum*, noticeable morphological abnormalities were observed on the tegumental surface of *O. viverrini* NEJs, including degradation of the tegumental structure and extensive exfoliation. Conversely, in the *T. laurifolia* treated groups, NEJs exhibited slight swelling of the tegumental surface. Meanwhile, in the *E. longifolia* treated groups, NEJs displayed significant swelling of the tegumental surface. In contrast, NEJs in the negative control group showed a normal tegumental surface.

5.2 Conclusion on the Efficacy of Crude Extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* adult worms

In the groups treated with *A. sativum*, a rapid decrease in motility rate was observed at the 30-minute mark, and after 1 hour of exposure, all worms in these groups ceased movement or perished by the 6-hour assessment, indicating a significant

difference compared to the negative control group. In contrast, in the *T. laurifolia* treated groups, the motility rate remained constant from 30 minutes until 6 hours, with another decrease observed at the 12-hour mark and complete cessation of movement at 24 hours, showing no significant difference compared to both the negative and positive control groups. Similarly, in the *E. longifolia* treated groups, a decrease in motility rate was noted at 30 minutes, followed by unusually high levels of active motility from hours 1 to 12. At the conclusion of the 24-hour experiment, some worms remained alive in each group, with no significant difference compared to the negative control group. Throughout the entire experimental duration, adult worms in the negative control group exhibited continuous active movement and sustained vitality.

The *A. sativum* treated groups demonstrated significantly elevated levels of ROS across the entire body of the worms, with higher fluorescence intensity compared to the control groups. In the *T. laurifolia* treated groups, elevated ROS levels were observed throughout the entire body of the *O. viverrini* adult worms, particularly pronounced in certain regions such as the testes and genital pore, surpassing those of the control groups. Conversely, the *E. longifolia* treated groups exhibited comparatively reduced ROS levels across the entire body of the worms, although certain regions such as the testes and genital pore showed heightened ROS intensity. In contrast, minimal ROS generation was observed in the regions of the negative control group.

In the *A. sativum* treated groups, NO levels increased within 24 hours; however, these changes were not statistically significant when compared with the negative and positive control groups. In contrast, in the *T. laurifolia* and *E. longifolia* treated groups, NO levels were found to be statistically significant when comparing both the negative control and positive control groups.

The morphological surface alterations and damages observed in *A. sativum* treated groups exhibited a consistent pattern. *O. viverrini* adult worms treated with *A. sativum* displayed irregular and non-smooth surfaces, significant morphological disruption, and considerable damage throughout their bodies. Those treated with

T. laurifolia showed noticeable but slight swelling of the tegumental surface, while those treated with *E. longifolia* exhibited a generally smooth surface with pronounced swelling in the form of microvilli nodules. When compared to the negative control group, where worms exhibited regular, smooth surfaces, and normal tegumental structures.

This study unequivocally confirms the anthelmintic effectiveness of extracts from *A. sativum*, *T. laurifolia*, and *E. longifolia* against *O. viverrini* NEJs and adult worms, aligning with prior research. Notably, *A. sativum* extract exhibited remarkable efficacy in this study, as evidenced by the rapid reduction in RM and SI of *O. viverrini* NEJs and adult worms. Moreover, stress-induced ROS generation was significantly higher in *O. viverrini* adult worms treated with *A. sativum* extract, leading to morphological damage characterized by edema and disturbance. Furthermore, the groups treated with *T. laurifolia* and *E. longifolia* exhibited similar effects, although to a lesser extent. However, it was observed that the motility rate in the *E. longifolia* treated groups showed abnormal changes, indicating higher activity during the testing, which is not typical. Furthermore, the expression of stress-related NO in *T. laurifolia* treated groups showed significantly higher generation. Overall, the extract of *A. sativum* demonstrates relatively high efficacy against *O. viverrini* NEJs and adult worms. These findings collectively suggest that *A. sativum* crude extract demonstrates efficacy as an anthelmintic treatment for the liver fluke, *O. viverrini*, *in vitro*.

5.3 Recommendation and possible future studies

The knowledge acquired from herbal crude extracts, specifically those derived from *A. sativum*, *T. laurifolia*, and *E. longifolia*, which demonstrate anti-parasitic properties against the *o. viverrini* NEJ and adult worms (*in vitro*), holds considerable potential for the development of herbal extracts designed to control and treat infections caused by liver flukes. This approach presents a practical alternative to current pharmaceutical interventions. To further deepen our understanding and utilization of these herbal extracts, forthcoming research endeavors should encompass

comprehensive investigations involving both *in vivo* and *in vitro* studies. These studies indicated only the basic impacts, such as motility, morphological surface changes, and induction of stress, of *A. sativum*, *T. laurifolia*, and *E. longifolia* crude extracts on *O. viverrini* NEJs and adult worms. Despite the promising effects observed with these herbal extracts in combating *O. viverrini* liver fluke infections, it is imperative to underscore the necessity for future investigations into the specific mechanisms and identification of key bioactive compounds. This critical research step is essential for the development of effective anti-parasitic agents, positioning herbal extracts as viable alternatives for the treatment of liver fluke infections. As we progress, a more comprehensive understanding of the underlying biological processes and active components will pave the way for the formulation of targeted and efficacious treatments in the ongoing pursuit of addressing parasitic infections.

REFERENCES

REFERENCES

Abbas, A., Naqvi, S. A. R., Rasool, M. H., Noureen, A., Mubarik, M. S., & Tareen, R. B. (2021). Phytochemical Analysis, Antioxidant and Antimicrobial Screening of *Seriphidium oliverianum* Plant Extracts. *Dose-Response*, *19*, 1-9. doi:10.1177/15593258211004739

Abd Aziz, N. A., Hasham, R., Sarmidi, M. R., Suhaimi, S. H., & Idris, M. K. H. (2021). A review on extraction techniques and therapeutic value of polar bioactives from Asian medicinal herbs: Case study on *Orthosiphon aristatus*, *Eurycoma longifolia* and *Andrographis paniculata*. *Saudi Pharmaceutical Journal*, *29*(2), 143-165. doi:10.1016/j.jsps.2020.12.016

Abdullah, A., & Mohammed, A. In: Scanning electron microscopy (SEM): A review. Proceedings of 2018 International Conference on Hydraulics and Pneumatics-HERVEX, Băile Govora, Romania, November 7-9, 2018; Băile Govora, Romania, November 7-9, 2018; pp. 77-85. Retrieved from https://www.researchgate.net/publication/330169176

Ahmed, A. H., Ejo, M., Feyera, T., Regassa, D., Mummed, B., & Huluka, S. A. (2020). *In vitro* anthelmintic activity of crude extracts of *Artemisia herba-alba* and *Punica granatum* against *Haemonchus contortus*. *Journal of parasitology research*, *2020*(1), 4950196. doi:10.1155/2020/4950196

Alam, K., Hoq, O., & Uddin, S. (2016). Medicinal plant *Allium sativum*. A review. *Journal of Medicinal Plant Studies*, *4*(6), 72-79. Retrieved from https://www.researchgate.net/publication/342379203

Al-Salahi, O. S. A., Kit-Lam, C., Majid, A. M. S. A., Al-Suede, F. S. R., Saghir, S. A. M., Abdullah, W. Z., & Yusoff, N. M. (2013). Anti-angiogenic quassinoid-rich fraction from *Eurycoma longifolia* modulates endothelial cell function. *Microvascular research*, *90*, 30-39. doi:10.1016/j.mvr.2013.07.007

Aly, I., Taher, E. E., El-Sayed, H., Mohammed, F. A., Gehan, E. L., Hamad, R. S., & Bayoumy, E. M. (2017). Efficacy of soluble glycoprotein fraction from *Allium sativum* purified by size exclusion chromatography on murine *Schistosomiasis mansoni*. *Microbial Pathogenesis*, *107*, 243-248. doi:10.1016/j.micpath.2017.03.039

Amagase, H. (2006). Clarifying the Real Bioactive Constituents of Garlic. *American Society for Nutrition*, *136*(3), 716S-725S. doi:10.1093/jn/136.3.716s

Angelucci, F., Basso, A., Bellelli, A., Brunori, M., Mattoccia, L. P., & Valle, C. (2007). The anti-schistosomal drug praziquantel is an adenosine antagonist. *Parasitology, 134*(9), 1215-1221. doi:10.1017/s0031182007002600

Apinhasmit, W., Sobhon, P., Saitongdee, P., & Upatham, E. (1993). *Opisthorchis viverrini*: changes of the tegumental surface in newly excysted juvenile, first-week and adult flukes. *International journal for Parasitology*, *23*(7), 829-839. doi:10.1016/0020-7519(93)90046-2

Apinhasmit, W., Sobhon, P., Saitongdee, P., Menayotin, S., & Upatham, E. (1994). *Opisthorchis viverrini*: ultrastructure of the tegument of the first-week juveniles and adult flukes. *International journal for Parasitology*, *24*(5), 613-621. doi:10.1016/0020-7519(94)90113-9

Arenas-Alatorre, J., Silva-Velazquez, Y., Alva Medina, A., & Rivera, M. (2010). Advantages and limitations of OM, SEM, TEM and AFM in the study of ancient decorated pottery. *Applied Physics A*, *98*, 617-624. doi:10.1007/s00339-009-5451-4

Arreola, R., Quintero-Fabian, S., Lopez-Roa, R. I., Flores-Gutierrez, E. O., Reyes-Grajeda, J. P., Carrera-Quintanar, L., & Ortuno-Sahagun, D. (2015). Immunomodulation and anti-inflammatory effects of garlic compounds. *Journal of immunology research*, *2015*, 401630. doi:10.1155/2015/401630

Arunsan, P., Donthaisong, C., Suwannatrai, K., Prasopdee, S., Kulsantiwong, J., Suwannatrai, A., & Tesana, S. (2014). Localization of the cystogenous glands of *Opisthorchis viverrini* cercariae. *Southeast Asian Journal of Tropical Medicine and Public Health*, *45*(2), 276-289. Retrieved from https://pubmed.ncbi.nlm.nih.gov/24968667/

Arunsan, P., Ittiprasert, W., Smout, M. J., Cochran, C. J., Mann, V. H., Chaiyadet, S., ... Sotillo, J. (2019). Programmed knockout mutation of liver fluke granulin attenuates virulence of infection-induced hepatobiliary morbidity. *Elife*, *8*, e41463. doi:10.7554/eLife.41463

Aukkanimart, R., Boonmars, T., Sriraj, P., Songsri, J., Laummaunwai, P., Waraasawapati, S., & Boonjaraspinyo, S. (2015). Anthelmintic, anti-inflammatory and antioxidant effects of *Garcinia mangostana* extract in hamster opisthorchiasis. *Experimental parasitology*, *154*, 5-13. 10.1016/j.exppara.2015.03.007

Ayub, B., Wani, H., doi:Shoukat, S., Para, P. A., Ganguly, S., & Ali, M. (2017). Specimen preparation for electron microscopy: an overview. *Journal of environment and life sciences*, *2*(3), 85-88. Retrieved from https://www.researchgate.net/publication/319464131

Bahorun, T., Soobrattee, M., Luximon-Ramma, V., & Aruoma, O. (2006). Free radicals and antioxidants in cardiovascular health and disease. *Internet Journal of Medical Update*, *1*(2), 25-41. doi:10.1046%2Fj.1365-2125.1997.t01-1-00594.x

Becker, B., Mehlhorn, H., Andrews, P., Thomas, H., & Eckert, J. (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. *Zeitschrift fur Parasitenkunde, 63*(2), 113-128. doi:10.1007/BF00927527

Behnia, M., Haghighi, A., Komeilizadeh, H., Tabaei, S. J. S., & Abadi, A. (2008). *In vitro* antiamoebic activity of Iranian *Allium sativum* in comparison with Metronidazole against *Entamoeba histolytica*. *Iranian Journal of Parasitology*, *3*(4), 32-38. Retrieved from https://www.researchgate.net/publication/285206124

Benz, D., Cadet, P., Mantione, K., Zhu, W., & Stefano, G. (2002). Tonal nitric oxide and health–a free radical and a scavenger of free radicals. *Medical science monitor basic research*, *8*(1), 1-4. Retrieved from https://www.researchgate.net/publication/11574995

Bhat, R., & Karim, A. A. (2010). Tongkat Ali (*Eurycoma longifolia* Jack): A review on its ethnobotany and pharmacological importance. *Fitoterapia*, *81*(7), 669-679. doi:10.1016/j.fitote.2010.04.006

Burton, G. J., & Jauniaux, E. (2011). Oxidative stress. *Best practice & research Clinical obstetrics & gynaecology*, *25*(3), 287-299. doi:10.1016/j.bpobgyn.2010.10.016

Butt, M. S., Sultan, M. T., Butt, M. S., & Iqbal, J. (2009). Garlic: nature's protection against physiological threats. *Critical reviews in food science and nutrition*, *49*(6), 538-551. doi:10.1080/10408390802145344

Centers for Disease Control and Prevention. (2018). Opisthorchiasis. Retrieved from https://www.cdc.gov/dpdx/opisthorchiasis/index.html

Centers for Disease Control and Prevention. (2024). Clinical Overview of Opisthorchis. Retrieved from https://www.cdc.gov/liver-flukes/hcp/clinical-overview-opisthorchis/index.html

Chan, E. W. C., Lim, Y. Y., Chong, K. L., Tan, J. B. L., & Wong, S. K. (2010). Antioxidant properties of tropical and temperate herbal teas. *Journal of Food Composition and Analysis*, *23*(2), 185-189. doi:10.1016/j.jfca.2009.10.002

Chan, E. W. C., Suit Ying, E., Yuen Ping, T., & Zhiew Cheng, W. (2011). Phytochemistry and Pharmacological Properties of *Thunbergia laurifolia*: A Review. *Pharmacognosy*, *3*(24), 1-6. doi:10.5530/pj.2011.24.1

Chan, K. L., O'Neill, M. J., Phillipson, J. D., & Warhurst, D. C. (1986). Plants as sources of antimalarial drugs. Part 31 *Eurycoma longifolia*. *Planta Medica*, *52*(02), 105-107. doi:10.1055/s-2007-969091

Chan, K.-L., Choo, C.-Y., Abdullah, N. R., & Ismail, Z. (2004). Antiplasmodial studies of *Eurycoma longifolia* Jack using the lactate dehydrogenase assay of *Plasmodium falciparum*. *Journal of Ethnopharmacology*, *92*(2-3), 223-227. doi:10.1016/j.jep.2004.02.025

Cioli, D., & Pica-Mattoccia, L. (2003). Praziquantel. *Parasitology research*, *90*(1), S3-S9. doi:10.1007/s00436-002-0751-z

Corral, M. J., Jiménez-Antón, M. D., Alunda, J. M., Benito-Peña, E., Moreno-Bondi, M. C., & Cuevas, L. (2016). Allicin Induces Calcium and Mitochondrial Dysregulation Causing Necrotic Death in *Leishmania*. *PLoS Neglected Tropical Diseases*, *10*(3), e0004525. doi:10.1371/journal.pntd.0004525

Cortes, A., Garcia-Ferrus, M., Sotillo, J., Esteban, J. G., Toledo, R., & Munoz-Antoli, C. (2017). Effects of dietary intake of garlic on intestinal trematodes. *Parasitology Research*, *116*(8), 2119-2129. doi:10.1007/s00436-017-5511-1

Corzo-Martínez, M., Corzo, N., & Villamiel, M. (2007). Biological properties of onions and garlic. *Trends in food science & technology*, *18*(12), 609-625. doi:10.1016/j.tifs.2007.07.011

Datta, S., Choudhury, D., Das, A., Mukherjee, D. D., Dasgupta, M., Bandopadhyay, S., & Chakrabarti, G. (2019). Autophagy inhibition with chloroquine reverts paclitaxel resistance and attenuates metastatic potential in human nonsmall lung adenocarcinoma A549 cells via ROS mediated modulation of β-catenin pathway. *Apoptosis*, *24*, 414-433. doi:10.1007/s10495-019-01526-y

Davies, T. E., Li, H., Bessette, S., Gauvin, R., Patience, G. S., & Dummer, N. F. (2022). Experimental methods in chemical engineering: Scanning electron microscopy and X-ray ultra-microscopy SEM and XuM. *The Canadian Journal of Chemical Engineering*, *100*(11), 3145-3159. doi:10.1002/cjce.24405

Doenhoff, M. J., Cioli, D., & Utzinger, J. (2008). Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Current opinion in infectious diseases*, *21*(6), 659-667. doi:10.1097/qco.0b013e328318978f

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiological reviews*, *82*, 47-95. doi:10.1152/physrev.00018.2001

El Shenawy, N. S., Soliman, M. F., & Reyad, S. I. (2008). The effect of antioxidant properties of aqueous garlic extract and *Nigella sativa* as anti-schistosomiasis agents in mice. *Revista do Instituto de Medicina Tropical de São Paulo*, *50*(1), 29-36. doi:10.1590/s0036-46652008000100007

El-Sharkawey, A. (2016). Calculate the Corrected Total Cell Fluorescence (CTCF). Calculate the Corrected Total Cell Fluorescence (CTCF). doi:10.13140/RG.2.1.1307.8008

Erko, B., Degarege, A., Tadesse, K., Mathiwos, A. & Legesse, M. (2012). Efficacy and side effects of praziquantel in the treatment of *Schistosomiasis mansoni* in schoolchildren in Shesha Kekele Elementary School, Wondo Genet, Southern Ethiopia. *Asian Pacific Journal of Tropical Biomedicine*, *2*, 235-239. doi:10.1016%2FS2221-1691(12)60049-5

Fallon, P. G. & Doenhoff, M. J. (1994). Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. *The American Journal of Tropical Medicine and Hygiene*, *51*(1), 83-88. doi:10.4269/ajtmh.1994.51.83

Feng, Y., Zhu, X., Wang, Q., Cao, Y., Jiang, Y., Shang, H., & Cui, L. (2012). Allicin enhances host pro-inflammatory immune responses and protects against acute murine malaria infection. *Malaria Journal*, *11*(268), 1-9. doi:10.1186/1475-2875-11-268

Finkel, T. (2011). Signal transduction by reactive oxygen species. *Journal of Cell Biology, 194*(1), 7-15. doi:10.1083%2Fjcb.201102095

Förstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *European heart journal*, *33*(7), 829-837. doi:10.1093%2Feurheartj%2Fehr304

Fujiwara, M., Yoshimura, M., Tsuno, S., & Murakami, F. (1958). "allithiamine", a newly found derivative of vitamin B1: IV. on the alliin homologues in the vegetables. *Journal of Biochemistry*, *45*(3), 141-149. doi:10.1093/oxfordjournals.jbchem.a126850

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular signalling*, *19*(9), 1807-1819. doi:10.1016/j.cellsig.2007.04.009

Ghasemi, A., Hedayati, M., & Biabani, H. (2007). Protein precipitation methods evaluated for determination of serum nitric oxide end products by the Griess assay. *Journal of Medical and Scientific Research*, *2*(15), 29-32. Retrieved from https://www.researchgate.net/publication/259772709

Ghazanfari, T., Hassan, Z. M., & Khamesipour, A. (2006). Enhancement of peritoneal macrophage phagocytic activity against *Leishmania major* by garlic (*Allium sativum*) treatment. *Journal of Ethnopharmacology*, *103*(3), 333-337. doi:10.1016/j.jep.2005.08.026

Girish, S., Kumar, S., & Aminudin, N. (2015). Tongkat Ali (*Eurycoma longifolia*): a possible therapeutic candidate against B*lastocystis* sp. *Parasites & vectors*, *8*(1), 1-7. doi:10.1186/s13071-015-0942-y

Goel, V., Kaur, P., Singla, L. D., & Choudhury, D. (2020). Biomedical evaluation of *Lansium parasiticum* extract-protected silver nanoparticles against *Haemonchus contortus*, a parasitic worm. *Frontiers in Molecular Biosciences*, *7*, 595646. doi:10.3389/fmolb.2020.595646

Gonnert, R., & Andrews, P. (1977). Praziquantel, a new board-spectrum antischistosomal agent. *Zeitschrift fur Parasitenkunde*, *52*, 129-150. doi:10.1007/bf00389899

Greenberg, R. M. (2005). Are Ca2+ channels targets of praziquantel action?. *International journal for Parasitology*, *35*(1), 1-9. doi:10.1016/j.ijpara.2004.09.004

Gryseels, B., Mbaye, A., De Vlas, S., Stelma, F., Guisse, F., Van Lieshout, L., Tchuem-Tchuenté, L. (2001). Are poor responses to praziquantel for the treatment of *Schistosoma mansoni* infections in Senegal due to resistance? An overview of the evidence. *Tropical medicine & international health*, *6*(11), 864-873. doi:10.1046/j.1365-3156.2001.00811.x

Guan, H., Luo, W., Bao, B., Cao, Y., Cheng, F., Yu, S., & Shan, M. (2022). A comprehensive review of Rosmarinic acid: from phytochemistry to pharmacology and its new insight. *Molecules*, *27*(10), 3292. doi:10.3390%2Fmolecules27103292

Hada Masayu, I., Pin, K., Rabitah, Z., & Mohd Radzi, A. (2017). Effects of drying temperature on drying kinetics and eurycomanone content of *Eurycoma longifolia* roots. *Food Research*, *1*(6), 270-275. doi:10.26656/fr.2017.6.112

Halliwell, B. (2007). Biochemistry of oxidative stress. *Biochemical society transactions*, *35*(5), 1147-1150. doi:10.1007/5584_2015_161

Hanpanich, P., Laha, T., Sripa, B., Mairiang, E., Sereerak, P., Upontain, S., & Tangkawattana, S. (2017). Decreased risk of cholangiocarcinogenesis following repeated cycles of *Opisthorchis viverrini* infection-praziquantel treatment: Magnetic Resonance Imaging (MRI) and histopathological study in a hamster model. *Parasitology international*, *66*(4), 464-470. doi:10.1016/j.parint.2016.04.012

Harinasuta, C. (1984). Epidemiology and control of schistosomiasis in Southeast Asia. *Southeast Asian journal of tropical medicine and public health*, *15*(4), 431-438. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6535256

Harinasuta, C., & Harinasuta, T. (1984). *Opisthorchis viverrini*: life cycle, intermediate hosts, transmission to man and geographical distribution in Thailand. *Arzneimittelforschung*, *34*(9B), 1164-1167. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6542383

Hirsch, K., Danilenko, M., Giat, J., Miron, T., Rabinkov, A., Wilchek, M., Sharoni, Y. (2000). Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. *Nutrition and cancer*, *38*(2), 245-254. doi:10.1207/S15327914NC382_14

Hout, S., Chea, A., Bun, S. S., Elias, R., Gasquet, M., Timon-David, P., & Azas, N. (2006). Screening of selected indigenous plants of Cambodia for antiplasmodial activity. *Journal of Ethnopharmacology*, *107*(1), 12-18. doi:10.1016/j.jep.2006.01.028

IARC. (1994). Infection with liver flukes (*Opisthorchis viverrini*, *Opisthorchis felineus* and Clonorchis sinensis). *IARC monographs on the evaluation of carcinogenic risks to humans*, *61*, 121-175. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7715069

IARC. (2012). Biological agents. Volume 100 B. A review of human carcinogens. *IARC monographs on the evaluation of carcinogenic risks to humans*, *100*(Pt B), 1-441. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23189750

Intuyod, K., Priprem, A., Limphirat, W., Charoensuk, L., Pinlaor, P., Pairojkul, C., & Pinlaor, S. (2014). Anti-inflammatory and anti-periductal fibrosis effects of an anthocyanin complex in *Opisthorchis viverrini*-infected hamsters. *Food and Chemical Toxicology*, *74*, 206-215. doi:10.1016/j.fct.2014.09.021

Itokawa, H., Kishi, E., Morita, H., Takeya, K., & Iitaka, Y. (1991). Eurylene, a new squalene-type triterpene from *Eurycoma longifolia*. *Tetrahedron Letters*, *32*(15), 1803-1804. doi:10.1016/S0040-4039(00)74334-6

Jaiboon, V., Boonyanuphap, J., Suwansri, S., Ratanatraiwong, P., & Hansawasdi, C. (2010). Alpha amylase inhibition and roasting time of local vegetables and herbs prepared for diabetes risk reduction chili paste. *Asian Journal of Food and Agro-Industry*, *3*(1), 1-12. Retrieved from https://www.researchgate.net/publication/237764511

Jeyathilakan, N., Murali, K., Anandaraj, A. & Abdul Basith, S. (2012). *In vitro* evaluation of anthelmintic property of ethno-veterinary plant extracts against the liver fluke *Fasciola gigantica*. *Journal of Parasitic Diseases*, *36*(1), 26-30. doi:10.1007/s12639-011-0064-1

Jiraungkoorskul, W., Sahaphong, S., Tansatit, T., Kangwanrangsan, N., & Pipatshukiat, S. (2005). Eurytrema pancreaticum: the *in vitro* effect of praziquantel and triclabendazole on the adult fluke. *Experimental parasitology*, *111*(3), 172-177. doi:10.1016/j.exppara.2005.07.004

Jiwajinda, S., Santisopasri, V., Murakami, A., Kawanaka, M., Kawanaka, H., Gasquet, M., & Ohigashi, H. (2002). *In vitro* anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medicinal plant in Southeast Asia. *Journal of Ethnopharmacology*, *82*(1), 55-58. doi:10.1016/s0378-8741(02)00160-5

Jongsuksuntigul, P., & Imsomboon, T. (2003). Opisthorchiasis control in Thailand. *Acta tropica*, *88*(3), 229-232. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14611877

Joshi, B., Kommuru, D., Terrill, T., Mosjidis, J., Burke, J., Shakya, K., & Miller, J. (2011). Effect of feeding *Sericea lespedeza* leaf meal in goats experimentally infected with *Haemonchus contortus*. *Veterinary parasitology, 178*(1-2), 192-197. doi:10.1016/j.vetpar.2010.12.017

Junsi, M., & Siripongvutikorn, S. (2016). *Thunbergia laurifolia*, a traditional herbal tea of Thailand: botanical, chemical composition, biological properties and processing influence. *International Food Research Journal*, *23*(3), 923-927. Retrieved from https://www.researchgate.net/publication/316425599

Jusoh, S., Ghani, R. A., Ab Kadir, W. R. W., & Ishak, M. F. (2015). Phytochemical assessment of multi-locational Tongkat Ali (*Eurycoma longifolia*) in Peninsular Malaysia. *Jurnal Teknologi*, *77*(3), 87-91. Retrieved from https://www.researchgate.net/publication/283622940

Kaewkes, S. (2003). Taxonomy and biology of liver flukes. *Acta tropica*, *88*(3), 177-186. doi:10.1016/j.actatropica.2003.05.001

Kaewpitoon, N., Kootanavanichpong, N., Kompor, P., Chavenkun, W., Kujapun, J., Norkaew, J., ... Panpimanmas, S. (2015). Review and current status of *Opisthorchis viverrini* infection at the community level in Thailand. *Asian Pacific Journal of Cancer Prevention*, *16*(16), 6825-6830. doi:10.7314/apjcp.2015.16.16.6825

Kamsa-Ard, S., Luvira, V., Pugkhem, A., Luvira, V., Thinkhamrop, B., Suwanrungruang, K., & Bhudhisawasdi, V. (2015). Association between praziquantel treatment and cholangiocarcinoma: a hospital-based matched case-control study. *BMC cancer*, *15*, 1-13. doi:10.1186/s12885-015-1788-6

Kannan, M. (2018). Scanning electron microscopy: Principle, components and applications. A textbook on fundamentals and applications of nanotechnology, 81-92. Retrieved from https://www.researchgate.net/publication/341553212

Kavitha, N., Noordin, R., Chan, K. L., & Sasidharan, S. (2012a). *In vitro* anti-*Toxoplasma gondii* activity of root extract/fractions of *Eurycoma longifolia* Jack. *BMC complementary and alternative medicine*, *12*, 1-8. doi:10.1186/1472-6882-12-91

Kavitha, N., Noordin, R., Kit-Lam, C., & Sasidharan, S. (2012b). Real time anti-*Toxoplasma gondii* activity of an active fraction of *Eurycoma longifolia* root studied by in situ scanning and transmission electron microscopy. *Molecules*, *17*(8), 9207-9219. doi:10.3390/molecules17089207

Khazan, M., & Hedayati, M. (2015). The role of nitric oxide in health and diseases. *Scimetr*, *3*(1), e2098. doi:10.5812/scimetr.20987

Kiuchi, F., Miyashita, N., Tsuda, Y., Kondo, K. & Yoshimura, H. (1987). Studies on crude drugs effective on visceral larva migrant. I. Identification of larvicidal principles in betel nuts. *Chemical and Pharmaceutical Bulletin*, *35*(7), 2880-2886. doi:10.1248/cpb.35.2880

Komiya, Y. (1966). Clonorchis and clonorchiasis. *Advances in Parasitology*, *4*, 53-106. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4899990

Krstin, S., Sobeh, M., Braun, M. S., & Wink, M. (2018). Anti-Parasitic Activities of *Allium sativum* and *Allium cepa* against *Trypanosoma b. brucei* and *Leishmania tarentolae*. *Medicines*, *5*(2), 1-13. doi:10.3390/medicines5020037

Kuo, P. C., Damu, A. G., Lee, K. H., & Wu, T. S. (2004). Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. *Bioorganic & medicinal chemistry*, *12*(3), 537-544. doi:10.1016/j.bmc.2003.11.017

Lawson, L. D., Wang, Z. Y. J., & Hughes, B. G. (1991). Identification and HPLC quantitation of the sulfides and dialk (en) yl thiosulfinates in commercial garlic products. *Planta medica*, *57*(04), 363-370. doi:10.1055/s-2006-960119

Leopold, G., Ungethum, W., Groll, E., Diekmann, H. W., Nowak, H., & Wegner, D. H. (1978). Clinical pharmacology in normal volunteers of praziquantel, a new drug against schistosomes and cestodes. An example of a complex study covering both tolerance and pharmacokinetics. *European Journal of Clinical Pharmacology*, *14*, 281-291. doi:10.1007/bf00560463

Liu, C. T., Su, H. M., Lii, C. K., & Sheen, L. Y. (2009). Effect of supplementation with garlic oil on activity of Th1 and Th2 lymphocytes from rats. *Planta medica*, *75*(3), 205-210. doi:10.1055/s-0028-1088396

Lorsuwannarat, N., Saowakon, N., Ramasoota, P., Wanichanon, C., & Sobhon, P. (2013). The anthelmintic effect of plumbagin on *Schistosoma mansoni*. *Experimental Parasitology*, 133, 18-27. doi:10.1016/j.exppara.2012.10.003

Luvira, V., Kamsa-Ard, S., Kamsa-Ard, S., Luvira, V., Srisuk, T., Pugkhem, A., ... Bhudhisawasdi, V. (2018). Association between repeated praziquantel treatment and papillary, and intrahepatic cholangiocarcinoma. *Annals of Hepatology*, *17*(5), 802-809. doi:10.5604/01.3001.0012.3140

Madea, B., Henssge, C., Reibe, S., Tsokos, M. & Kernbach Wighton, G. (2014). Postmortem changes and time since death. In: Handbook of forensic medicine, Madea, B. (editor). New Jersey: John Wiley & Sons, pp.75-133. doi:10.1002/9781119648628.ch7

Mantawy, M. M., Ali, H. F., & Rizk, M. Z. (2011). Therapeutic effects of *Allium sativum* and *Allium cepa* in *Schistosoma mansoni* experimental infection. *Revista do Instituto de Medicina Tropical de São Paulo*, *53*(3), 155-163. doi:10.1590/s0036-46652011000300007

Mantawy, M. M., Aly, H. F., Zayed, N., & Fahmy, Z. H. (2012). Antioxidant and schistosomicidal effect of *Allium sativum* and *Allium cepa* against Schistosoma mansoni different stages. *European Review for Medical and Pharmacological Sciences*, *16*(3), 69-80. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/22957420

Mohd Ridzuan, M., Noor Rain, A., Zhari, I., & Zakiah, I. (2005). Effect of *Eurycoma longifolia* extract on the Glutathione level in *Plasmodium falciparum* infected erythrocytes *in vitro*. *Tropical biomedicine*, *22*(2), 155-163. Retrieved from https://www.researchgate.net/publication/6904038

Mohd Ridzuan, M., Sow, A., Noor Rain, A., Mohd Ilham, A., & Zakiah, I. (2007). *Eurycoma longifolia* extract-artemisinin combination: parasitemia suppression of *Plasmodium yoelii*-infected mice. *Tropical Biomedicine, 24*(1), 111-118. Retrieved from https://www.researchgate.net/publication/6269471

Martin, R. (1997). Modes of action of anthelmintic drugs. *The Veterinary Journal, 154*(1), 11-34. doi:10.1017/S0031182097001029

Metwally, N. S. (2006). Potency of *Allium sativum* and *Allium cepa* oils against *Schistosoma mansoni* infection in mice. *The Egyptian Journal of Hospital Medicine*, *23*(1), 319-332. doi:10.21608/ejhm.2006.17943

Metwally, D. M., Al Olayan, E. M., Alanazi, M., Alzahrany, S. B., & Semlali, A. (2018). Antischistosomal and anti-inflammatory activity of garlic and allicin compared with that of praziquantel *in vivo*. *BMC complementary and alternative medicine*, *18*(1), 1-11. doi:10.1186/s12906-018-2191-z

Mitsunaga, K., Koike, K., Tanaka, T., Ohkawa, Y., Kobayashi, Y., Sawaguchi, T., & Ohmoto, T. (1994). Canthin-6-one alkaloids from *Eurycoma longifolia*. *Phytochemistry, 35*(3), 799-802. doi:10.1016/S0031-9422(00)90609-5

Morita, H., Kishi, E., Takeya, K., & Itokawa, H. (1992). Biphenylneolignans from wood of *Eurycoma longifolia*. *Phytochemistry*, *31*(11), 3993-3995. doi:10.1016/S0031-9422(00)97570-8

Morsy, K., Ghamdi, A. A., Dajem, S. B., Bin-Meferij, M., Alshehri, A., El-Kott, A., ... Al-Doaiss, A. (2021). The oil of garlic, *Allium sativum* L. (Amaryllidaceae), as a potential protectant against *Anisakis* spp. Type II (L3) (Nematoda) infection in Wistar rats. *Revista Brasileira de Parasitologia Veterinária, 30*(1), e015920. doi:10.1590/s1984-296120201086

Moutia, M., Habti, N., & Badou, A. (2018). *In vitro* and *In vivo* Immunomodulator Activities of *Allium sativum* L. *Evidence-Based Complementary and Alternative Medicine*, *2018*, 1-10. doi:10.1155/2018/4984659

Mushtaq, S., Abbasi, B. H., Uzair, B. & Abbasi, R. (2018). Natural products as reservoirs of novel therapeutic agents. *Experimental and Clinical Sciences*, *4*(17), 420-451. doi:10.17179%2Fexcli2018-1174

Nahrevanian, H. (2009). Involvement of nitric oxide and its up/down stream molecules in the immunity against parasitic infections. *Brazilian Journal of Infectious Diseases*, *13*, 440-448. doi:10.1590/s1413-86702009000600010

Nok, A. J., Williams, S., & Onyenekwe, P. C. (1996). *Allium sativum*-induced death of African trypanosomes. *Parasitology Research*, *82*(7), 634-637. doi:10.1007/s004360050177

Oonsivilai, R., Cheng, C., Bomser, J., Ferruzzi, M. G., & Ningsanond, S. (2007). Phytochemical profiling and phase II enzyme-inducing properties of *Thunbergia laurifolia* Lindl. (RC) extracts. *Journal of ethnopharmacology*, *114*(3), 300-306. doi:10.1016/j.jep.2007.08.015

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiological reviews*, *87*(1), 315-424. doi:10.1152/physrev.00029.2006

Palacio Landin, J., Mendoza de Gives, P., Salinas Sanchez, D. O., Lopez-Arellano, M. E., Liebano-Hernandez, E., Hernandez-Velazquez, V. M., & Valladares-Cisneros, M. G. (2015). *In vitro* and *in vivo* Nematocidal Activity of *Allium sativum* and *Tagetes erecta* Extracts Against *Haemonchus contortus*. *Turkiye parazitolojii dergisi*, *39*(4), 260-264. doi:10.5152/tpd.2015.4523

Palipoch, S., Jiraungkoorskul, W., Jaikua, W., Kosai, P., Tansatit, T., & Preyavichyapugdee, N. (2011). Protective efficiency of *Thunbergia laurifolia* leaf extract against lead (II) nitrate-induced toxicity in *Oreochromis niloticus*. *Journal of Medicinal Plants Research*, *5*(5), 719-728. Retrieved from https://www.researchgate.net/publication/258358682

Pax, R., Bennett, J. L., & Fetterer, R. (1978). A benzodiazepine derivative and praziquantel: Effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *304*(3), 309-315. doi:10.1007/BF00507974

Pechdee, P. (2016) *Effects of Albendazole, Artesunate, Praziquantel and Miltefosine on Opisthorchis viverrini cercaria and metacercaria* (Master's thesis). Khon Kean University, Khon Kean, Thailand.

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International journal of biomedical science*, *4*(2), 89-96. doi:10.59566/IJBS.2008.4089

Pharmaceutical Sciences, Ubon Ratchathani University. (2010a). ฐานข้อมูลสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี; ปลาไหลเผือก. Retrieved from https://apps.phar.ubu.ac.th/phargarden/main.php?action=viewpage&pid=149

Pharmaceutical Sciences, Ubon Ratchathani University. (2010b). ฐานข้อมูลเครื่องยาสมุนไพรไทย คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี; ปลาไหลเผือก. Retrieved from https://apps.phar.ubu.ac.th/thaicrudedrug/main.php?action=viewpage&pid=82

Pinlaor, S., Prakobwong, S., Boonmars, T., Wongkham, C., Pinlaor, P., & Hiraku, Y. (2009). Effect of praziquantel treatment on the expression of matrix metalloproteinases in relation to tissue resorption during fibrosis in hamsters with acute and chronic *Opisthorchis viverrini* infection. *Acta tropica*, *111*(2), 181-191. doi:10.1016/j.actatropica.2009.04.011

Qian, M. B., Patel, C., Palmeirim, M. S., Wang, X., Schindler, C., Utzinger, J., Zhou, X. N., & Keiser, J. (2022). Efficacy of drugs against clonorchiasis and opisthorchiasis: a systematic review and network meta-analysis. *The Lancet Microbe*, *3*(8), e616-e624. doi:10.1016/s2666-5247(22)00026-x

Rabinkov, A., Miron, T., Konstantinovski, L., Wilchek, M., Mirelman, D. & Weiner, L. (1998). The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. *Biochimica et Biophysica Acta, 1379*(2), 233-244. doi:10.1016/s0304-4165(97)00104-9

Rana, S. V., Pal, R., Vaiphei, K., Sharma, S. K., & Ola, R. P. (2011). Garlic in health and disease. *Nutrition Research Reviews*, *24*(1), 60-71. doi:10.1017/s0954422410000338

Rehman, S. U., Choe, K., & Yoo, H. H. (2016). Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules*, *21*(3), 331. doi:10.3390%2Fmolecules21030331

Resende, F. A., Nogueira, L. G., Bauab, T. M., Vilegas, W., & Varanda, E. A. (2015). Antibacterial potential of flavonoids with different hydroxylation patterns. *Ecletica Quimica*, *40*, 173-179. doi:10.26850/1678-4618EQJ.V40.1.2015.P173-179

Riad, N. H., Taha, H. A., & Mahmoud, Y. I. (2009). Effects of garlic on albino mice experimentally infected with *Schistosoma mansoni*: a parasitological and ultrastructural study. *Tropical Biomedicine*, *26*(1): 40-50. Retrieved from https://www.researchgate.net/publication/26757175

Riad, N. H., Taha, H. A., & Mahmoud, Y. I. (2013). Effects of garlic on **Schistosoma mansoni** harbored in albino mice: molecular characterization of the host and parasite. *Gene*, *518*(2), 287-291. doi:10.1016/j.gene.2013.01.023

Rocejanasaroj, A., Tencomnao, T., & Sangkitikomol, W. (2014). *Thunbergia laurifolia* extract minimizes the adverse effects of toxicants by regulating P-glycoprotein activity, CYP450, and lipid metabolism gene expression in HepG2 cells. *Genetics and molecular research GMR*, *13*(1), 205-219. doi:10.4238/2014.January.10.12

Romitelli, F., Santini, S. A., Chierici, E., Pitocco, D., Tavazzi, B., Amorini, A. M., Lazzarino, G., & Di Stasio, E. (2007). Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC–MS: the importance of a correct removal of proteins in the Griess assay. *Journal of Chromatography B*, *851*(1-2), 257-267. doi:10.1016/j.jchromb.2007.02.003

Sabah, A., Fletcher, C., Webbe, G., & Doenhoff, M. (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. *Experimental parasitology*, *61*(3), 294-303. doi:10.1016/0014-4894(86)90184-0

Schelkle, B., Snellgrove, D., & Cable, J. (2013). *In vitro* and *in vivo* efficacy of garlic compounds against *Gyrodactylus turnbulli* infecting the guppy (*Poecilia reticulata). Veterinary Parasitology*, *198*(1-2), 96-101. doi:10.1016/j.vetpar.2013.08.027

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Current biology*, *24*(10), R453-R462. doi:10.1016%2Fj.cub.2014.03.034

Scholz, T., Ditrich, O., & Giboda, M. (1991). Differential diagnosis of opisthorchiid and heterophyid metacercariae (Trematoda) infecting flesh of cyprinid fish from Nam Ngum Dam Lake in Laos. *Southeast Asian journal of tropical medicine and public health*, *22*, 171-173. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1822880

Singh, T. U., Kumar, D., Tandan, S. K., & Mishra, S. K. (2009). Inhibitory effect of essential oils of *Allium sativum* and *Piper longum* on spontaneous muscular activity of liver fluke, *Fasciola gigantica*. *Experimental parasitology*, *123*(4), 302-308. doi:10.1016/j.exppara.2009.08.002

Sripa, B., & Kaewkes, S. (2002). Gall bladder and extrahepatic bile duct changes in *Opisthorchis viverrini*-infected hamsters. *Acta tropica*, *83*(1), 29-36. doi:10.1016/s0001-706x(02)00052-9

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Brindley, P. J. (2007). Liver fluke induces cholangiocarcinoma. *PLoS Medicine*, *4*(7), 1148-1155. doi:10.1371/journal.pmed.0040201

Sripa, B., Bethony, J. M., Sithithaworn, P., Kaewkes, S., Mairiang, E., Loukas, A., ... Brindley, P. J. (2011). Opisthorchiasis and *Opisthorchis*-associated cholangiocarcinoma in Thailand and Laos. *Acta tropica, 120*, S158-S168. doi:10.1016/j.actatropica.2010.07.006

Srisawangwong, T., Sithithaworn, P., & Tesana, S. (1997). Metacercariae isolated from cyprinoid fishes in Khon Kaen District by digestion technic. *Southeast Asian journal of tropical medicine and public health*, *28*, 224-226. Retrieved from https://pubmed.ncbi.nlm.nih.gov/9656383/

Sriwilaijaroen, N., Kondo, S., Nanthasri, P., Auparakkitanon, S., Suzuki, Y., & Wilairat, P. (2010). Antiplasmodial effects of Brucea javanica (L.) Merr. and *Eurycoma longifolia* Jack extracts and their combination with chloroquine and quinine on *Plasmodium falciparum* in culture. *Tropical medicine and health*, *38*(2), 61-68. doi:10.2149/tmh.2009-11

Steiner, K., Garbe, A., Diekmann, H.W., & Nowak, H. (1976). The fate of praziquantel in the organism I. Pharmacokinetics in animals. *European Journal of Drug Metabolism and Pharmacokinetics, 1*, 85-95. doi:10.1007/BF03189262

Stelma, F., Talla, I., Sow, S., Kongs, A., Niang, M., Polman, K., ... Gryseels, B. (1995). Efficacy and side effects of praziquantel in an epidemic focus of *Schistosoma mansoni*. *The American journal of tropical medicine and hygiene*, *53*(2), 167-170. doi:10.4269/ajtmh.1995.53.167

Storz, G., & Imlayt, J. A. (1999). Oxidative stress. *Current opinion in microbiology*, *2*(2), 188-194. doi:10.1016/S1369-5274(99)80033-2

Tell, G., Vascotto, C., & Tiribelli, C. (2013). Alterations in the redox state and liver damage: hints from the EASL Basic School of Hepatology. *Journal of hepatology*, *58*(2), 365-374. doi:10.1016/j.jhep.2012.09.018

Ur Rehman, S., Kevin, C., & Hye Hyun, Y. (2016). Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules*, *21*(3), 331-361. doi:10.3390/molecules21030331

Vajrasthira, S., Harinasuta, C., & Komiya, Y. (1961). The morphology of the metacercaria of *Opisthorchis viverrini*, with special reference to the excretory system. *Annals of tropical medicine and parasitology*, *55*, 413-418. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13924247

Vale, N., Gouveia, M. J., & Gärtner, F. (2020). Current and novel therapies against helminthic infections: The potential of antioxidants combined with drugs. *Biomolecules*, *10*(3), 1-27. doi:10.3390%2Fbiom10030350

Valencia, C.I., Catto, B.A., Webster, L.T., Jr., Barcelon, E., & Ofendo-Reyes, R. (1994). Concentration time course of praziquantel in Filipinos with mild *Schistosoma japonicum* infection. *The Southeast Asian Journal of Tropical Medicine and Public Health, 25*, 409-414. Retrieved from https://pubmed.ncbi.nlm.nih.gov/7855670/

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., & Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and cellular biochemistry*, *266*, 37-56. doi:10.1023/b:mcbi.0000049134.69131.89

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1), 44-84. doi:10.1016/j.biocel.2006.07.001

Vathsala, P., & Krishna Murthy, P. (2020). Immunomodulatory and antiparasitic effects of garlic-arteether combination via nitric oxide pathway in *Plasmodium berghei*-infected mice. *Journal of parasitic diseases*, *44*(1), 49-61. doi:10.1007/s12639-019-01160-0

Waikakul, S., Vanadurongwan, V., & Sakarnkosol, S. (1998). Relationship between foot length and the inter anterior superior iliac distance. *Injury*, *29*(10), 763-767. doi:10.1016/s0020-1383(98)00181-8

Wan, K., Wang, P., & Zhang, L. (2017). *In vivo* and *in vitro* activity of oil extract of garlic (*Allium sativum* Linnaeus) against *Schistosoma japonicum* cercariae. *Revista da Sociedade Brasileira de Medicina Tropical*, *50*, 126-129. doi:10.1590/0037-8682-0301-2016

Wang, R., Ding, Y., Liu, R., Xiang, L., & Du, L. (2010). Pomegranate: constituents, bioactivities and pharmacokinetics. *Fruit, vegetable and cereal science and biotechnology, 4*(2), 77-87. Retrieved from https://www.researchgate.net/publication/228474896

Wannachat, C.; Kumpangnil, K.; Krasuaythong, N.; Kanharat, N.; Leksomboon, R. In: Effects of *Areca catechu* Linn. extract on motility of *Opisthorchis viverrini*. Proceedings of RSU International Research Conference 2020, Pathum Thani, Thailand, May 1, 2020; Rangsit University: Thailand, 2020; pp. 298-304. Retrieved from https://rsucon.rsu.ac.th/files/proceedings/inter2020/IN20-190.pdf

Wattanawong, O., Iamsirithaworn, S., Kophachon, T., Nak-Ai, W., Wisetmora, A., Wongsaroj, T., & Sripa, B. (2021). Current status of helminthiases in Thailand: A cross-sectional, nationwide survey, 2019. *Acta Tropica*, *223*, 1-20. doi:10.1016/j.actatropica.2021.106082

Willcox, J. K., Ash, S. L., & Catignani, G. L. (2004). Antioxidants and prevention of chronic disease. *Critical reviews in food science and nutrition*, *44*(4), 275-295. doi:10.1080/10408690490468489

Wizneh, F. M., & Asmawi, M. Z. (2014). *Eurycoma longifolia* Jack (Simarubaceae); advances in its medicinal potentials. *Pharmacognosy Journal*, *6*(4), 1-9. doi:10.5530/pj.2014.4.1

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of agricultural and food chemistry*, *55*(22), 8896-8907. doi:10.1021/jf0715166

Wonkchalee, N., Boonmars, T., Laummaunwai, P., Aromdee, C., Hahnvajanawong, C., Wu, Z., & Sudsarn, P. (2013). A combination of praziquantel and the traditional medicinal plant *Thunbergia laurifolia* on *Opisthorchis viverrini* infection and cholangiocarcinoma in a hamster model. *Parasitology research*, *112*(12), 4211-4219. doi:10.1007/s00436-013-3613-y

Wonkchalee, O., Boonmars, T., Aromdee, C., Laummaunwai, P., Khunkitti, W., Vaeteewoottacharn, K., Sudsarn, P. (2012). Anti-inflammatory, antioxidant and hepatoprotective effects of *Thunbergia laurifolia* Linn. on experimental opisthorchiasis. *Parasitology research*, *111*(1), 353-359. doi:10.1007/s00436-012-2846-5

Woottisin, N., Sukprasert, S., Kulsirirat, T., Tharavanij, T., & Sathirakul, K. (2022). Evaluation of the Intestinal Permeability of Rosmarinic Acid from *Thunbergia laurifolia* Leaf Water Extract in a Caco-2 Cell Model. *Molecules*, *27*(12), 1-16. doi:10.3390/molecules27123884

Wykoff, D. E., Harinasuta, C., Juttijudata, P., & Winn, M. M. (1965). *Opisthorchis viverrini* in Thailand the life cycle and comparison with *O. felineus*. *Journal of parasitology research*, *51*, 207-214. doi:10.2307/3276083

Zhong, R., Xiang, H., Cheng, L., Zhao, C., Wang, F., Zhao, X., & Fang, Y. (2019). Effects of feeding garlic powder on growth performance, rumen fermentation, and the health status of lambs Infected by gastrointestinal nematodes. *Animals*, *9*(3), 1-10. doi:10.3390/ani9030102

Zhou, W., Apkarian, R., Wang, Z. L., & Joy, D. (2007). Fundamentals of scanning electron microscopy (SEM). Scanning microscopy for nanotechnology: techniques and applications, 1-40. doi:10.1007/978-0-387-39620-0_1

APPENDIX

APPENDIX A

THE PREPARATIONS OF REAGENTS

Appendix A1 Reagents of 0.25% pepsin

Pepsin powder	2.5 g
HCl	15 ml
NaCl	8.5 g
Distilled water	1000 ml

Appendix A2 Reagents of 0.85% NaCl

Filtered water	1000 ml
NaCl	85 g

Appendix A3 % Yield of crude extracts

% Yield of *A. sativum* crude extract

$$\text{Yield (\%)} = \frac{2.82 \text{ g} \times 100}{150 \text{ g}}$$

Yield (%) = 1.88

% Yield of *T. laurifolia* crude extract

$$\text{Yield (\%)} = \frac{0.37 \text{ g} \times 100}{10 \text{ g}}$$

Yield (%) = 3.70

% Yield of *E. longifolia* crude extract

$$\text{Yield (\%)} = \frac{0.97 \text{ g} \times 100}{10 \text{ g}}$$

Yield (%) = 9.70

Appendix A4 The calibration for sodium nitrite standard curve

Chemical: Griess reagent Kit for nitrite determination (G-7921)®, Thermo Fisher Scientific.

1. Prepare sodium nitrite solutions with concentrations ranging from 1 to 100 uM by diluting the nitrite standard solution (Component C) with deionized water.
2. Prepare samples and conduct absorbance measurements as detailed earlier, substituting the standard nitrite solutions (300 ul for the cuvette assay or 150 ul for the microplate assay) for the experimental samples.
3. Construct a standard curve of nitrite concentration (x-axis) against absorbance (y-axis).
4. Determine nitrite concentrations corresponding to the absorbance of experimental samples from the standard plot.

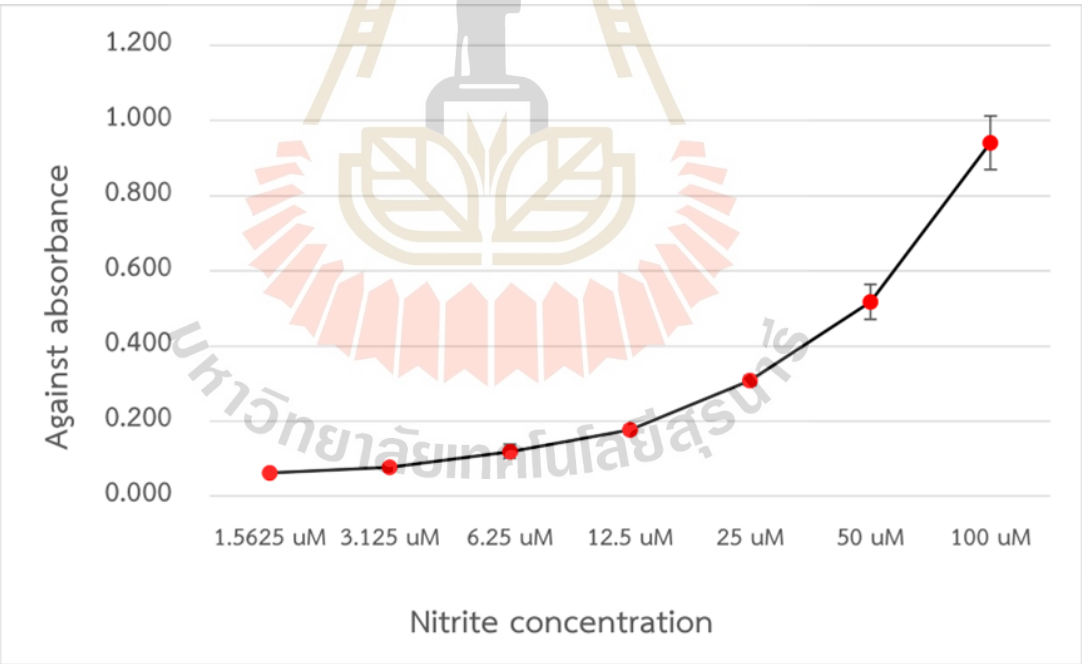


Figure 1A. Nitrite concentrations standard curve.

Appendix A5 Stock solution of H2DCFDA

Chemical: H2DCFDA® MCE MedChemExpress

H2DCFDA stock solution (10 mM)

DMSO	1.0261 ml
H2DCFDA mass	5 mg

30 µM of H2DCFDA

C1 V1 = C2 V2

10 mM x V1 = 30 µM x 10 ml

V1 = 30 µM x 10000 µl

10000 µM

V1 = 30 µl

30 µl of H2DCFDA stock solution + 9970 µl of PBS pH 7.4

Appendix A6 Quantification of worm fluorescence levels using the ImageJ program

This protocol follows the methodology outlined by El-Sharkawey (2016).

1. Open the image of interest and select the "Straight" tool to draw a line on the photo's scale. Then, select to "Analyze" and choose "Set Scale."
2. Input the "Known distance" based on the scale present in the original photo and specify the "Unit of length" as µm, then click "Global" to apply the settings and then click "Ok" to confirm.
3. Next, select "Analyze" and set measurements by choosing "Area," "Integrated intensity," and "Mean grey value."
4. select to "Image," then "Color," and select "Split channels" then analyze the green channel photo by using the "Freehand selection" tool to trace around the region of interest in the gray color. Then, choose "Measure."
5. Select a region without fluorescence (black) for background readings.
6. The results can be saved in an Excel program.
7. The formula for calculating the corrected total worm fluorescence (CTWF) is:

CTWF = Integrated Density – (Area of selected cell x Mean fluorescence of background readings)

APPENDIX B

SUPPLEMENTARY DATA

Table 1B Motility score of *A. sativum* treated groups on *O. viverrini* NEJs

Concentration			5 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.20	0.12	40.00	100.00	0.42
1 h	1.20	0.12	42.86	100.00	0.42
3 h	1.00	0.10	37.04	100.00	0.00
6 h	1.00	0.10	38.46	100.00	0.00
12 h	1.00	0.10	35.71	100.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			10 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.00	0.10	33.33	100.00	0.00
1 h	1.00	0.10	35.71	100.00	0.00
3 h	1.00	0.10	37.04	100.00	0.00
6 h	1.00	0.10	38.46	100.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00

Table 1B (Continued) Motility score of *A. sativum* treated groups on *O. viverrini* NEJs

Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.00	0.10	33.33	100.00	0.00
1 h	1.00	0.10	35.71	100.00	0.00
3 h	1.00	0.10	37.04	100.00	0.00
6 h	1.00	0.10	38.46	100.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.00	0.10	33.33	100.00	0.00
1 h	1.00	0.10	35.71	100.00	0.00
3 h	1.00	0.10	37.04	100.00	0.00
6 h	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00

Table 2B Motility score of *T. laurifolia* treated groups on *O. viverrini* NEJs

Concentration			5 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	2.00	0.20	71.43	100.00	0.00
3 h	2.00	0.20	74.07	100.00	0.00
6 h	1.70	0.17	65.38	100.00	0.48
12 h	1.30	0.13	46.43	100.00	0.48
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			10 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	2.00	0.20	71.43	100.00	0.00
3 h	2.00	0.20	74.07	100.00	0.00
6 h	1.30	0.13	50.00	100.00	0.48
12 h	1.30	0.13	46.43	100.00	0.48
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.70	0.17	56.67	100.00	0.48
1 h	1.60	0.16	57.14	100.00	0.52
3 h	1.60	0.16	59.26	100.00	0.52
6 h	1.20	0.12	46.15	100.00	0.42
12 h	1.20	0.12	42.86	100.00	0.42
24 h	0.00	0.00	0.00	0.00	0.00

Table 2B (Continued) Motility score of *T. laurifolia* treated groups on *O. viverrini* NEJs

Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.10	0.11	36.67	100.00	0.32
1 h	1.10	0.11	39.29	100.00	0.32
3 h	1.00	0.10	37.04	100.00	0.00
6 h	1.00	0.10	38.46	100.00	0.00
12 h	1.00	0.10	35.71	100.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00

Table 3B Motility score of *E. longifolia* treated groups on *O. viverrini* NEJs

Concentration			5 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	2.00	0.20	71.43	100.00	0.00
3 h	2.00	0.20	74.07	100.00	0.00
6 h	1.20	0.12	46.15	100.00	0.42
12 h	1.20	0.12	42.86	100.00	0.42
24 h	0.00	0.00	0.00	0.00	0.00

Table 3B (Continued) Motility score of *E. longifolia* treated groups on *O. viverrini* NEJs

Concentration			10 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	2.00	0.20	71.43	100.00	0.00
3 h	2.00	0.20	74.07	100.00	0.00
6 h	1.20	0.12	46.15	100.00	0.42
12 h	1.00	0.10	35.71	100.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	1.90	0.19	67.86	100.00	0.32
3 h	1.90	0.19	70.37	100.00	0.32
6 h	1.00	0.10	38.46	100.00	0.00
12 h	1.00	0.10	35.71	100.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	0.30	100.00	100.00	0.00
5 min	2.00	0.20	66.67	100.00	0.00
1 h	2.00	0.20	71.43	100.00	0.00
3 h	1.90	0.19	70.37	100.00	0.32
6 h	1.00	0.10	38.46	100.00	0.00
12 h	1.00	0.10	35.71	100.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00

Table 4B Motility score of negative control and positive control group on *O. viverrini* NEJs

Negative control (RPMI-1640)					
Time	$\bar{x}$	**MI**	**MR**	**SI**	**S.D.**
0 min	3.00	0.30	100.00	100.00	0.00
5 min	3.00	0.30	100.00	100.00	0.00
1 h	2.80	0.28	100.00	100.00	0.42
3 h	2.70	0.27	100.00	100.00	0.48
6 h	2.60	0.26	100.00	100.00	0.52
12 h	2.80	0.28	100.00	100.00	0.42
24 h	0.60	0.06	100.00	60.00	0.52
Positive control (PZQ) 2 mg/ml					
Time	$\bar{x}$	**MI**	**MR**	**SI**	**S.D.**
0 min	3.00	0.30	100.00	100.00	0.00
5 min	1.60	0.16	53.33	100.00	0.52
1 h	1.50	0.15	53.57	100.00	0.53
3 h	1.40	0.14	51.85	100.00	0.52
6 h	1.40	0.14	53.85	100.00	0.52
12 h	1.10	0.11	39.29	100.00	0.32
24 h	0.00	0.00	0.00	0.00	0.00

Table 5B Motility score of *A. sativum* treated groups on *O. viverrini* adult worms.

Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.17	9.33	50.00	100.00	0.25
1 h	1.21	9.67	56.86	100.00	0.31
3 h	0.50	4.00	23.52	50.00	0.40
6 h	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			30 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.17	9.33	50.00	100.00	0.18
1 h	1.00	8.00	47.06	100.00	0.00
3 h	0.38	3.00	17.64	37.50	0.28
6 h	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.17	9.33	50.00	100.00	0.18
1 h	1.00	8.00	47.06	100.00	0.00
3 h	0.33	2.67	15.68	25.00	0.31
6 h	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00

Table 6B Motility score of *T. laurifolia* treated groups on *O. viverrini* adult worms.

Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.13	9.00	48.21	100.00	0.17
1 h	1.13	9.00	52.94	100.00	0.17
3 h	1.08	8.67	50.97	100.00	0.15
6 h	1.04	8.33	60.98	100.00	0.12
12 h	0.50	4.00	40.00	50.00	0.53
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			30 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.21	9.67	51.79	100.00	0.25
1 h	1.38	11.00	64.71	100.00	0.45
3 h	1.38	11.00	64.69	100.00	0.38
6 h	1.04	8.33	60.98	100.00	0.21
12 h	0.50	4.00	40.00	50.00	0.53
24 h	0.00	0.00	0.00	0.00	0.00
Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.17	9.33	50.00	100.00	0.18
1 h	1.08	8.67	50.98	100.00	0.15
3 h	1.04	8.33	49.01	100.00	0.12
6 h	1.08	8.67	63.41	100.00	0.15
12 h	0.50	4.00	40.00	50.00	0.53
24 h	0.00	0.00	0.00	0.00	0.00

Table 7B Motility score of *E. longifolia* treated groups on *O. viverrini* adult worms.

Concentration			20 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	2.04	16.33	87.50	100.00	0.42
1 h	2.83	22.67	133.33	100.00	0.25
3 h	2.67	21.33	125.47	100.00	0.00
6 h	2.50	20.00	146.34	100.00	0.56
12 h	1.81	14.50	145.00	100.00	0.65
24 h	0.25	2.00	25.00	25.00	0.46
Concentration			30 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.75	14.00	75.00	100.00	0.30
1 h	2.46	19.67	115.69	100.00	0.40
3 h	2.38	19.00	111.74	100.00	0.42
6 h	2.46	19.67	143.90	100.00	0.35
12 h	1.50	12.00	120.00	100.00	0.53
24 h	0.25	2.00	25.00	25.00	0.46
Concentration			40 mg/ml		
Time	$\bar{x}$	MI	MR	SI	S.D.
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.46	11.67	62.50	100.00	0.25
1 h	2.13	17.00	100.00	100.00	0.80
3 h	2.29	18.33	107.82	100.00	0.33
6 h	2.54	20.33	148.78	100.00	0.25
12 h	1.38	11.00	110.00	100.00	0.44
24 h	0.25	2.00	25.00	25.00	0.46

Table 8B Motility score of negative control and positive control group on *O. viverrini* adult worms

Negative control (RPMI-1640)					
Time	$\bar{x}$	**MI**	**MR**	**SI**	**S.D.**
0 min	3.00	24.00	100.00	100.00	0.00
30 min	2.33	18.67	100.00	100.00	0.40
1 h	2.13	17.00	100.00	100.00	0.17
3 h	2.13	17.00	100.00	100.00	0.30
6 h	1.71	13.67	100.00	100.00	0.28
12 h	1.25	10.00	100.00	100.00	0.27
24 h	1.00	8.00	100.00	100.00	0.00
Positive control (PZQ) 20 mg/ml					
Time	$\bar{x}$	**MI**	**MR**	**SI**	**S.D.**
0 min	3.00	24.00	100.00	100.00	0.00
30 min	1.21	9.67	51.79	100.00	0.25
1 h	1.08	8.67	50.98	100.00	0.15
3 h	0.92	7.33	43.13	100.00	0.24
6 h	0.67	5.33	39.02	75.00	0.25
12 h	0.44	3.50	35.00	25.00	0.42
24 h	0.00	0.00	0.00	0.00	0.00

Table 9B The CTWF fluorescence intensity of *A. sativum* groups treated on *O. viverrini* adult worms.

	Area	S.D. area	Mean	IntDen	S.D intDent	RawIntDen	CTWF
Negative control	9981747.40	204737.66	40.95	139231109.10	2304431.87	7689038.00	143983788.9
Positive control (PZQ)	7899963.78	173713.31	64.42	175907587.14	291690.35	9714496.50	187160492.4
As 20 mg/ml	11619746.49	63649.21	403.08	1580221901.31	4001936.29	87267754.50	1747422800
As 30 mg/ml	15114893.62	129782.42	372.19	1880680597.56	11301928.72	103860586.00	2078240194
As 40 mg/ml	10279701.22	151818.29	481.79	1681697120.87	977631.94	92871723.50	1861959005
Background	774784.97	18886.06	1.11	863259.39	39961.62	47673.50	-

Table 10B The CTWF fluorescence intensity of *T. laurifolia* groups treated on *O. viverrini* adult worms.

	Area	S.D. area	Mean	IntDen	S.D intDent	RawIntDen	CTWF
Negative control	9981747.40	204737.66	40.95	139231109.10	2304431.87	7689038.00	143983788.9
Positive control (PZQ)	7899963.78	173713.31	64.42	175907587.14	291690.35	9714496.50	187160492.4
TL 20 mg/ml	12196206.43	82496.86	105.83	437797673.16	2838478.35	24177376.50	474120033.9
TL 30 mg/ml	11433915.80	117669.74	192.93	714487867.81	962292.62	39457592.50	783202102.5
TL 40 mg/ml	9473734.72	331549.53	114.23	360218660.03	5175074.13	19893075.50	390729846.8
Background	774784.97	18886.06	1.11	863259.39	39961.62	47673.50	-

Table 11B The CTWF fluorescence intensity of *E. longifolia* groups treated on *O. viverrini* adult worms.

	Area	S.D. area	Mean	IntDen	S.D intDent	RawIntDen	CTWF
Negative control	9981747.40	204737.66	40.95	139231109.10	2304431.87	7689038.00	143983788.9
Positive control (PZQ)	7899963.78	173713.31	64.42	175907587.14	291690.35	9714496.50	187160492.4
EL 20 mg/ml	12010955.18	268988.67	55.17	223544490.72	1888900.20	12345244.50	235648358.6
EL 30 mg/ml	13096985.06	314929.80	75.75	335950294.25	1664405.80	18552855.00	359658586.4
EL 40 mg/ml	14430765.05	60179.30	148.57	724329850.61	376082.22	40001116.00	790827581.3
Background	774784.97	18886.06	1.11	863259.39	39961.62	47673.50	-

Table 12B The concentration of NO generation in *A. sativum* groups treated on *O. viverrini* adult worms.

Time	Negative control (RPMI)	Positive control (PZQ 20 mg/ml)	As 20 mg/ml	As 30 mg/ml	As 40 mg/ml
30 min	1.454	1.514	1.490	1.490	1.478
1 h	1.502	1.550	1.503	1.502	1.478
3 h	1.526	1.563	1.527	1.526	1.526
6 h	1.526	1.575	1.575	1.526	1.526
12 h	1.575	1.575	1.575	1.577	1.671
24 h	1.599	1.599	1.599	1.647	1.719
$\bar{x}$	1.530	1.563	1.545	1.545	1.567
S.D.	0.051	0.028	0.044	0.058	0.103

Table 13B The concentration of NO generation in *T. laurifolia* treated groups on *O. viverrini* adult worms.

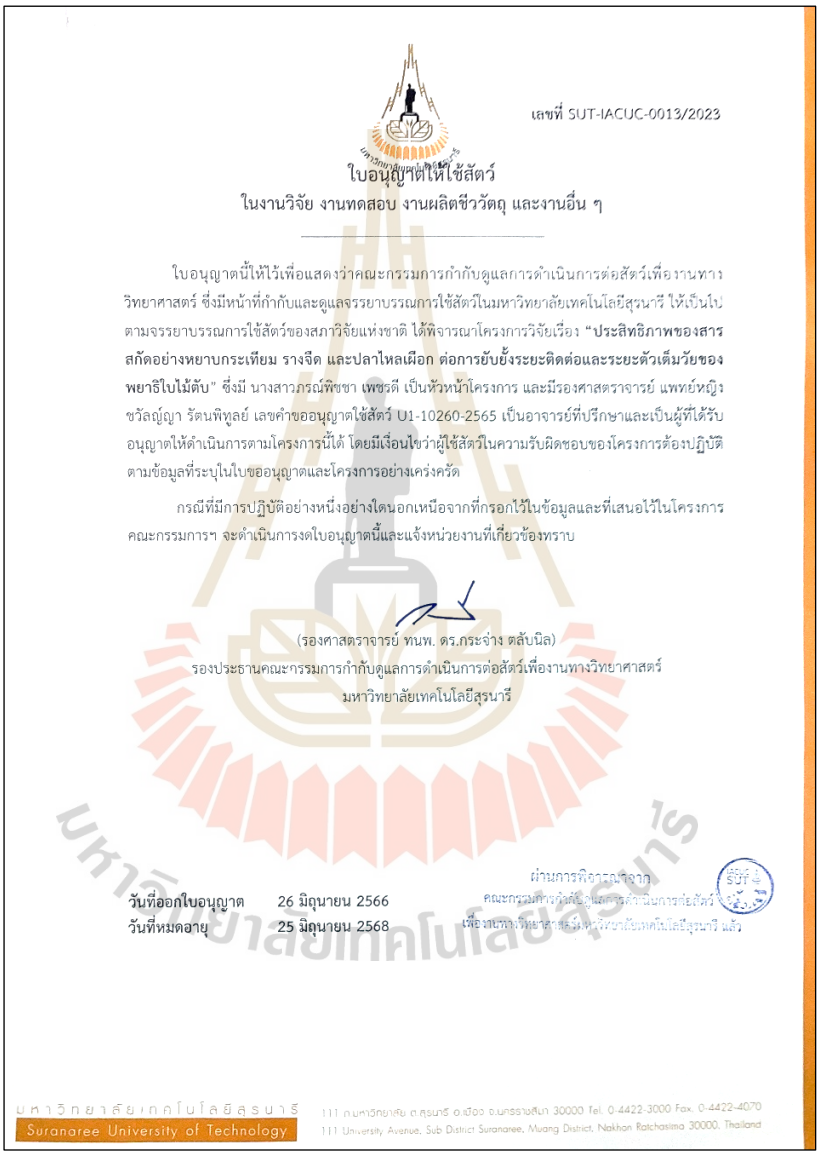
Time	Negative control	Positive control (PZQ)	TL 20 mg/ml	TL 30 mg/ml	TL 40 mg/ml
30 min	1.454	1.514	1.490	1.611	1.538
1 h	1.502	1.550	1.490	1.611	1.623
3 h	1.526	1.563	1.502	1.635	1.635
6 h	1.526	1.575	1.538	1.647	1.635
12 h	1.575	1.575	1.623	1.683	1.647
24 h	1.599	1.599	1.635	1.695	1.647
$\bar{x}$	1.530	1.563	1.545	1.647	1.621
S.D.	0.051	0.028	0.044	0.036	0.041

Table 14B The concentration of NO generation in *E. longifolia* groups treated on *O. viverrini* adult worms.

Time	Negative control	Positive control (PZQ)	EL 20 mg/ml	EL 30 mg/ml	EL 40 mg/ml
30 min	1.454	1.514	1.454	1.430	1.611
1 h	1.502	1.550	1.430	1.466	1.647
3 h	1.526	1.563	1.502	1.659	1.659
6 h	1.526	1.575	1.611	1.659	1.695
12 h	1.575	1.575	1.647	1.647	1.767
24 h	1.599	1.599	1.611	1.695	1.779
$\bar{x}$	1.530	1.563	1.545	1.593	1.693
S.D.	0.051	0.028	0.044	0.113	0.068

APPENDIX C

RESEARCH ETHICS DOCUMENTS



เลขที่ SUT-IACUC-0013/2023

ใบอนุญาตให้ใช้สัตว์
ในงานวิจัย งานทดสอบ งานผลิตชีววัตถุ และงานอื่น ๆ

ใบอนุญาตนี้ให้ไว้เพื่อแสดงว่าคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์ ซึ่งมีหน้าที่กำกับและดูแลจรรยาบรรณการใช้สัตว์ในมหาวิทยาลัยเทคโนโลยีสุรนารี ให้เป็นไปตามจรรยาบรรณการใช้สัตว์ของสภาวิจัยแห่งชาติ ได้พิจารณาโครงการวิจัยเรื่อง **"ประสิทธิภาพของสารสกัดอย่างหยาบกระเทียม รางจืด และปลาไหลเผือก ต่อการยับยั้งระยะติดต่อและระยะตัวเต็มวัยของพยาธิใบไม้ตับ"** ซึ่งมี นางสาวภรณ์พิชชา เพชรดี เป็นหัวหน้าโครงการ และมีรองศาสตราจารย์ แพทย์หญิงชวัลญ์ญา รัตนพิทูลย์ เลขคำขออนุญาตใช้สัตว์ U1-10260-2565 เป็นอาจารย์ที่ปรึกษาและเป็นผู้ที่ได้รับอนุญาตให้ดำเนินการตามโครงการนี้ได้ โดยมีเงื่อนไขว่าผู้ใช้สัตว์ในความรับผิดชอบของโครงการต้องปฏิบัติตามข้อมูลที่ระบุในใบขออนุญาตและโครงการอย่างเคร่งครัด

กรณีที่มีการปฏิบัติอย่างหนึ่งอย่างใดนอกเหนือจากที่กรอกไว้ในข้อมูลและที่เสนอไว้ในโครงการ คณะกรรมการฯ จะดำเนินการงดใบอนุญาตนี้และแจ้งหน่วยงานที่เกี่ยวข้องทราบ

(รองศาสตราจารย์ ทนพ. ดร.กระจ่าง ตลับนิล)
รองประธานคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผ่านการพิจารณาจาก
คณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์
เพื่องานทางวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว

วันที่ออกใบอนุญาต 26 มิถุนายน 2566
วันที่หมดอายุ 25 มิถุนายน 2568

มหาวิทยาลัยเทคโนโลยีสุรนารี
Suranaree University of Technology

111 ถ.มหาวิทยาลัย ต.สุรนารี อ.เมือง จ.นครราชสีมา 30000 Tel. 0-4422-3000 Fax. 0-4422-4070
111 University Avenue, Sub District Suranaree, Muang District, Nakhon Ratchasima 30000, Thailand

Figure 1C The animal ethics protocols were approved by the Animal Care and Use Committee of the Institute of Research and Development, Suranaree University of Technology.

เลขที่รับรอง SUT-IBC-008/2023

ใบรับรองการดำเนินงานด้านความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี

ชื่อโครงการ	: ประสิทธิภาพของสารสกัดอย่างหยาบกระเทียม รางจืด และปลาไหลเผือก ต่อการยับยั้งระยะติดต่อและระยะตัวเต็มวัยของพยาธิใบไม้ตับ
รหัสโครงการ	: IBC-66-08
หัวหน้าโครงการ	: นางสาวภรณ์พิชชา เพชรดี
อาจารย์ที่ปรึกษา	: รองศาสตราจารย์ แพทย์หญิง ชวัลญ์ญา รัตนพิทูลย์
สังกัด	: สำนักวิชาแพทยศาสตร์
ประเภทงานวิจัย	: งานวิจัยประเภทที่ 2
ระดับห้องปฏิบัติการ	: Biosafety Level 1 (BSL 1)
รายงานความก้าวหน้า	: ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี

ข้อเสนอโครงการวิจัยและเอกสารประกอบของข้อเสนอโครงการวิจัยนี้ ได้รับการพิจารณาจากคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยเทคโนโลยีสุรนารีแล้ว คณะกรรมการฯ ลงความเห็นว่า ข้อเสนอโครงการวิจัยที่จะดำเนินการมีความสอดคล้องกับแนวทางปฏิบัติเพื่อความปลอดภัยทางชีวภาพ และพระราชบัญญัติเชื้อโรคและพิษจากสัตว์ พ.ศ. 2558 จึงเห็นสมควรให้ดำเนินการวิจัยตามข้อเสนอการวิจัยนี้ได้

กรณีที่มีการปฏิบัติอย่างหนึ่งอย่างใดนอกเหนือจากที่กรอกไว้ในข้อมูลและที่เสนอไว้ในโครงการ คณะกรรมการฯ จะดำเนินการงดใบรับรองนี้ และแจ้งมายังคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มทส. หรือหน่วยงานที่เกี่ยวข้องทราบ

ลงชื่อ.....
(รองศาสตราจารย์ ดร.ระพี อูทเคอ)
ประธานคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี

วันที่ออกใบรับรอง 20 มิถุนายน 2566
วันที่ใบรับรองหมดอายุ 19 มิถุนายน 2567

มหาวิทยาลัยเทคโนโลยีสุรนารี
Suranaree University of Technology
111 ถ.มหาวิทยาลัย ต.สุรนารี อ.เมือง จ.นครราชสีมา 30000 Tel. 0-4422-3000 Fax. 0-4422-4070
111 University Avenue, Sub-District Suranaree, Muang District, Nakhon Ratchasima 30000, Thailand

Figure 2C The biosafety and ethics protocols were approved by the Biosafety and Ethics Committee of the Institute of Research and Development, Suranaree University of Technology.

สำนักงานการวิจัยแห่งชาติ
สถาบันพัฒนาการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
ขอมอบประกาศนียบัตรฉบับนี้ไว้เพื่อแสดงว่า
นางสาวภรณ์พิชชา เพชรดี
เลขที่คำขอรับใบอนุญาตใช้สัตว์ U1-09993-2565
ได้เข้ารับการอบรมและผ่านการทดสอบความรู้
หลักสูตร "ผู้ขอรับใบอนุญาตใช้สัตว์เพื่องานทางวิทยาศาสตร์" ครั้งที่ ๒-๒๕๖๔
วันที่ ๑๘ - ๑๙ สิงหาคม ๒๕๖๔
(ดร.วิภารัตน์ ดีอ่อง)
ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ

Figure 3C Certificate of completion for permission to use animals for scientific purposes from the National Research Council of Thailand No.: U1-09993-2565.

สถาบันวิจัยและพัฒนา
มหาวิทยาลัยเทคโนโลยีสุรนารี
ขอมอบเกียรติบัตรฉบับนี้ไว้เพื่อแสดงว่า
ภรณ์พิชชา เพชรดี
ได้สอบผ่านการฝึกอบรมหลักสูตร
"ความปลอดภัยทางชีวภาพ (Biosafety)
และการรักษาความปลอดภัยทางชีวภาพ (Biosecurity)" รุ่นที่ 3
(เลขทะเบียนรับรองหลักสูตรจากกรมวิทยาศาสตร์การแพทย์ที่ สธ 0621.06/3211)
ให้ไว้ ณ วันที่ 4 เมษายน 2566
หมดอายุ วันที่ 3 เมษายน 2569
รองศาสตราจารย์ ดร.ระพี ภูทเคอ
ผู้อำนวยการสถาบันวิจัยและพัฒนา
มหาวิทยาลัยเทคโนโลยีสุรนารี

Figure 4C Certificate of completion for biosafety and biosecurity course.

APPENDIX D

RESEARCH DISTIBUTIONS

1. Poster presentation in titled Efficacy of *Allium sativum* crude extract against the human liver fluke, *Opisthorchis viverrini*' at The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023
2. Oral presentation in titled Effects of garlic crude extract (*Allium sativum* Linn.) against *Strongyloides stercoralis* larva at the 2nd international virtual conference on science and technology (SUT-IVCST 2021) hosted by Suranaree University of Technology, Thailand on 6th August 2021.

'

KKU
KHON KAEN UNIVERSITY
60 years of creativity and development for society

ICP 2023

CERTIFICATE OF APPRECIATION

Presented to

Phornphitcha Pechdee

For sharing your valuable knowledge as the poster presentation during

"The 3rd International Conference on Parasitology (ICP2023)"

Organized by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand
9th- 10th November 2023

Assoc. Prof. Charnchai Panthongviriyakul
President
Khon Kaen University

Prof. Thidarut Boonmars
Head of Department of Parasitology
Faculty of medicine, Khon Kaen University

Figure 1D Certificate of appreciation for poster presentation titled "Efficacy of *Allium sativum* crude extract against the human liver fluke, *Opisthorchis viverrine"* at The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023

Figure 2D Certificate of appreciation for attending The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023

IVCST 2021

Certificate of Participation

This is to certify that

Phornphitcha Pechdee

from Suranaree University of Technology

has participated and presented a paper titled

Effects of garlic crude extract (Allium sativum Linn.) against nematode Strongyloides stercoralis larva

at The 2nd International Virtual Conference on Science and Technology (SUT-IVCST 2021)
hosted by Suranaree University of Technology, Thailand
hold on 6th August, 2021.

Associate Professor Flt. Lt. Dr. Kontorn Chamniprasart
SUT-IVCST 2021 Conference Chair

At Suranaree University of Technology

Figure 3D Certificate of participation for oral presentation titled Effects of garlic crude extract (*Allium sativum* Linn.) against *Strongyloides stercoralis* larva at the 2nd international virtual conference on science and technology (SUT-IVCST 2021) hosted by Suranaree University of Technology, Thailand on 6th August 2021.

Figure 4D Certificate of best presentation award for oral presentation titled Effects of garlic crude extract (*Allium sativum* Linn.) against *Strongyloides stercoralis* larva at the 2nd international virtual conference on science and technology (SUT-IVCST 2021) hosted by Suranaree University of Technology, Thailand on 6th August 2021.

APPENDIX E

RESEARCH ARTICLE ACHIEVEMENT

APPENDIX 1E Publications

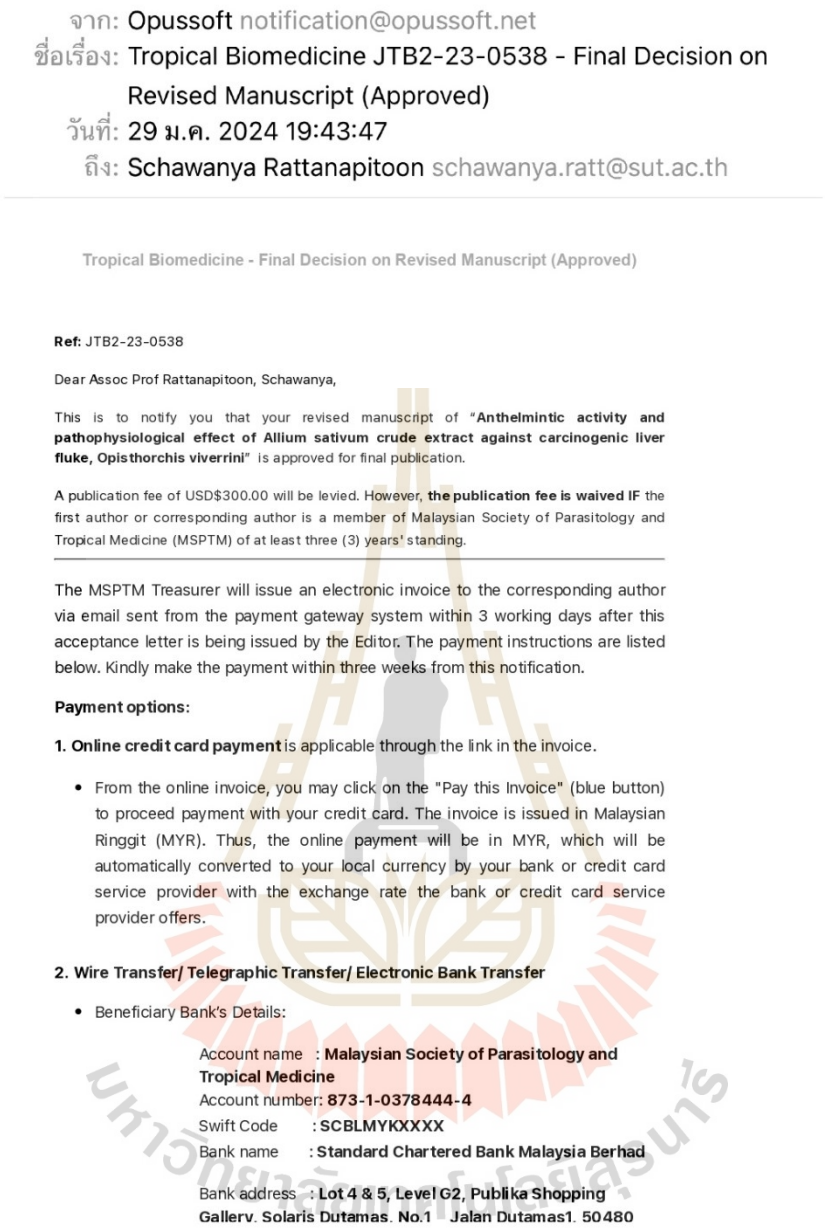
1. Pechdee, P., Boonsuya, A., Arunsan, P., Thanchonnang, C., La, N., Rattanapitoon, N. K., Laha, T., Nuansing, W., & Rattanapitoon, S. K. (2024). Anthelmintic activity and pathophysiological effect of *Allium sativum* crude extract against carcinogenic liver fluke, *Opisthorchis viverrini*. *Tropical Biomedicine,* **approved for publication.**
2. Boonsuya, A., Pechdee P., Arunsan P., Thanchonnang, C. , Phinsiri, C., Rattanapitoon, N. K., & Rattanapitoon, S. K. (2024). Papaya Seeds Extract Combined Lauryl Glucoside Against Human Parasites Contaminated in Fresh Vegetables. *The Natural Products Journal,* **approved for publication.**
3. Boonsuya, A., Arunsan, P., Pechdee, P., La, N., Thanchonnang, C., Rattanapitoon, N.K., & Rattanapitoon, S.K. (2024). Diagnosis of the carcinogenic liver fluke, *Opisthorchis viverrini*: comparison of two coprological methods versus the automatic feces analyzer. *Tropical Biomedicine,* **approved for publication.**
4. Sangkam,W., Arunsan, P., Pechdee, P., Boonsuya, A., Thanchonnang, C., Rattanapitoon, N.K., & Rattanapitoon, S.K. (2024). Anthelmintic activity andpathophysiological effect of anthelmintic drugs against carcinogenic liver fluke,*Opisthorchis viverrini. Tropical Biomedicine,* **approved for publication.**
5. Boonsuya, A., Chitpitaklert, P., Pechdee, P., Srithongklang, W., Thanchonnang, C., La, N., Gordon, C. N., Rattanapitoon, N. K., Arunsan, P., & Rattanapitoon, S. K. (2023). Oral parasitic protozoan *Entamoeba gingivalis* in periodontal disease patients, northeastern Thailand. *Tropical Biomedicine, 40*(4), 471-477. https://doi.org/10.47665/tb.40.4.013

6. Chitpitaklert, P., Boonsuya, A., Pechdee, P., Thanchonnang, C., La, N., Rattanapitoon, N. K., Arunsan, P., & Rattanapitoon, S. K. (2023). Molecular detection of oral *Trichomonas tenax* in periodontal disease patients by polymerase chain reaction -based 18S rRNA gene. *Tropical Biomedicine, 40*(3), 307-312. https://doi.org/10.47665/tb.40.3.006
7. La, N., Leng, M., Arunsan, P., Pechdee, P., Boonsuya, A., Thanchonnang, C., Rattanapitoon, N. K., & Rattanapitoon, S. K. (2023). Molecular identification of *Opisthorchis viverrini* among the northeastern Cambodian population by internal transcribed spacer 2 based polymerase chain reaction. *Tropical Biomedicine, 40*(4), 383-391. https://doi.org/10.47665/tb.40.4.002
8. La, N., Leng, M., Rattanapitoon, K., Pechdee, P., Boonsuya, A., Arunsan, P., & Rattanapitoon, S. K. (2022). Intestinal parasitic infections and risk factors among the population in Cambodia. *Tropical Biomedicine, 39*(4), 539-546. https://doi.org/10.47665/tb.39.4.009

APPENDIX 2E Patents and copyrights

Table 1E The copyrights and patents to this research

No.	Content	Years	ID
1.	คู่มือสมุนไพรไทยต้านปรสิต (Thai Herbs of Anti-Parasite)	2021	Copyrights ID : ว .044597
2.	Bioactive intergradient of *Allium sativum* crude extract for against liver fluke *Opisthorchis viverrini*	2024	In the process of applying for a patent.
3.	Biomedical Evaluation of *Allium sativum* Extract Protected Copper Nanoparticles Against parasite	2024	In the process of applying for a patent.



จาก: Opussoft notification@opussoft.net
ชื่อเรื่อง: Tropical Biomedicine JTB2-23-0538 - Final Decision on Revised Manuscript (Approved)
วันที่: 29 ม.ค. 2024 19:43:47
ถึง: Schawanya Rattanapitoon schawanya.ratt@sut.ac.th

Tropical Biomedicine - Final Decision on Revised Manuscript (Approved)

Ref: JTB2-23-0538

Dear Assoc Prof Rattanapitoon, Schawanya,

This is to notify you that your revised manuscript of "**Anthelmintic activity and pathophysiological effect of Allium sativum crude extract against carcinogenic liver fluke, Opisthorchis viverrini**" is approved for final publication.

A publication fee of USD$300.00 will be levied. However, **the publication fee is waived IF** the first author or corresponding author is a member of Malaysian Society of Parasitology and Tropical Medicine (MSPTM) of at least three (3) years' standing.

The MSPTM Treasurer will issue an electronic invoice to the corresponding author via email sent from the payment gateway system within 3 working days after this acceptance letter is being issued by the Editor. The payment instructions are listed below. Kindly make the payment within three weeks from this notification.

Payment options:

1. Online credit card payment is applicable through the link in the invoice.

- From the online invoice, you may click on the "Pay this Invoice" (blue button) to proceed payment with your credit card. The invoice is issued in Malaysian Ringgit (MYR). Thus, the online payment will be in MYR, which will be automatically converted to your local currency by your bank or credit card service provider with the exchange rate the bank or credit card service provider offers.

2. Wire Transfer/ Telegraphic Transfer/ Electronic Bank Transfer

- Beneficiary Bank's Details:

Account name : **Malaysian Society of Parasitology and Tropical Medicine**
Account number: **873-1-0378444-4**
Swift Code : **SCBLMYKXXXX**
Bank name : **Standard Chartered Bank Malaysia Berhad**
Bank address : **Lot 4 & 5, Level G2, Publika Shopping Gallery, Solaris Dutamas, No.1 Jalan Dutamas1, 50480**

Figure 1E Acceptance email from the Tropical Biomedicine Journal for the manuscript titled "Anthelmintic activity and pathophysiological effect of *Allium sativum* crude extract against the carcinogenic liver fluke, *Opisthorchis viverrini*," for publication.

Gmail Schawanya Rattanapitoon <schawanya.ratt@g.sut.ac.th>

Preliminary Acceptance | BMS-NPJ-2024-156

The Natural Products Journal <admin@bentham.manuscriptpoint.com> จ. 8 ก.ค. 08:13
ตอบกลับ: The Natural Products Journal <npj@benthamscience.net>
ถึง: <schawanya.ratt@g.sut.ac.th>
สำเนา: <ambreenirshad@benthamscience.net>

Reference#: BMS-NPJ-2024-156

Submission Title: Papaya Seeds Extract Combined Lauryl Glucoside Against Human Parasites Contaminated in Fresh Vegetables

Dear Dr. Schawanya Rattanapitoon,

On behalf of Bentham Science, I am pleased to inform you that your article titled **"Papaya Seeds Extract Combined Lauryl Glucoside Against Human Parasites Contaminated in Fresh Vegetables"**, with Reference No. "BMS-NPJ-2024-156" has been found generally acceptable for publication, following an independent peer review.

Certain aspects of your submission relating to the quality of language, plagiarism/ similarity, figures and use of AI tools (if any) may require additional scrutiny by our Quality Assurance Department before final acceptance and publication.

Attached is your digitally signed copyright letter for your records.

We appreciate your valuable contribution to "**The Natural Products Journal**" and look forward to successful collaboration in the future.

With warm regards,

Editorial Office
The Natural Products Journal
Bentham Science Publishers

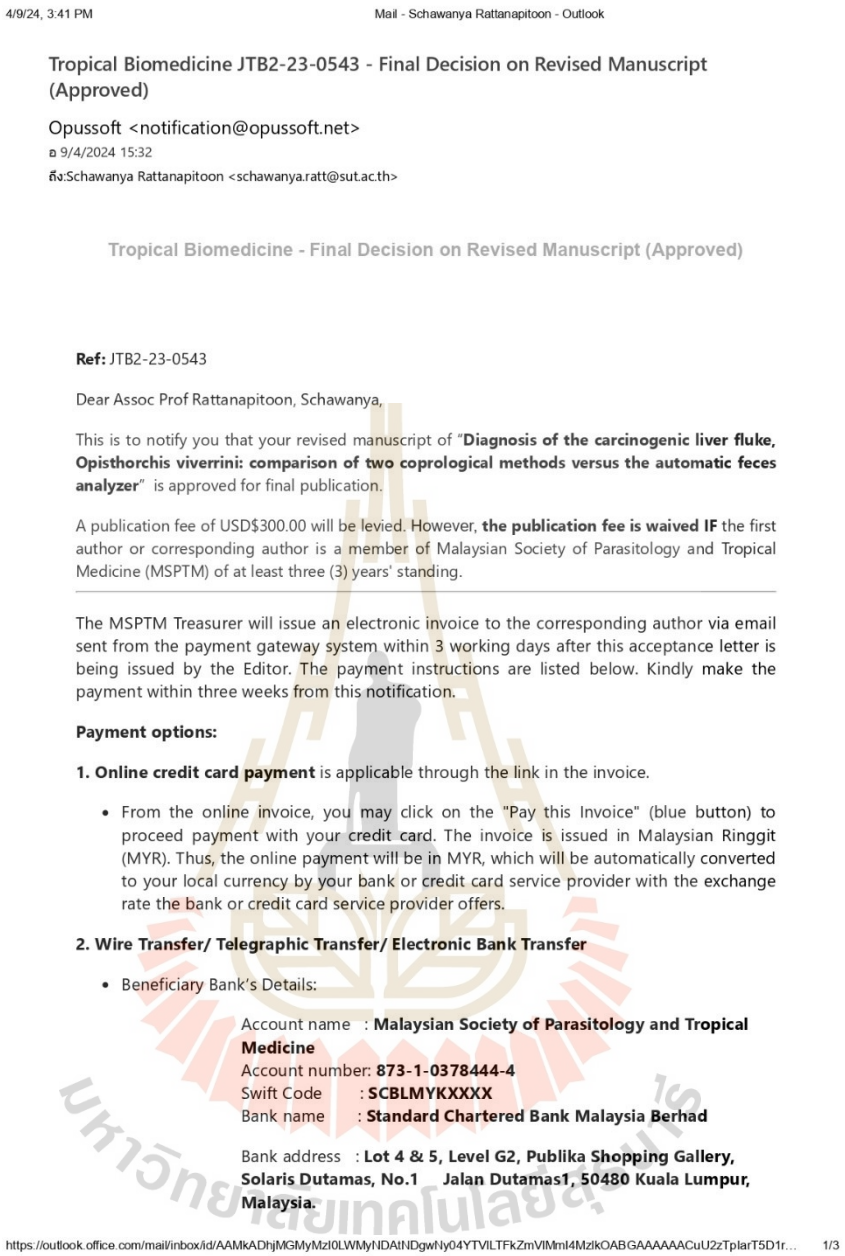
For complaints contact: complaint@benthamscience.net
Contribute to our Newsletter: Send us updates about your research to newsletter@benthamscience.net.
Sign up to receive updates from Bentham Science.

Attachments:
copyright-letter-The Natural Products journal_17 April 2024.pdf copyright_form.pdf

To unsubscribe from MPS and stop receiving emails further. **Please Click Here**

Powered by Bentham Manuscript Processing System

Figure 2E Acceptance email from The Natural Products Journal for the manuscript titled “Papaya Seeds Extract Combined Lauryl Glucoside Against Human Parasites Contaminated in Fresh Vegetables,” for publication.



4/9/24, 3:41 PM Mail - Schawanya Rattanapitoon - Outlook

Tropical Biomedicine JTB2-23-0543 - Final Decision on Revised Manuscript (Approved)

Opussoft <notification@opussoft.net>
ถ 9/4/2024 15:32
ถึง:Schawanya Rattanapitoon <schawanya.ratt@sut.ac.th>

Tropical Biomedicine - Final Decision on Revised Manuscript (Approved)

Ref: JTB2-23-0543

Dear Assoc Prof Rattanapitoon, Schawanya,

This is to notify you that your revised manuscript of "**Diagnosis of the carcinogenic liver fluke, Opisthorchis viverrini: comparison of two coprological methods versus the automatic feces analyzer**" is approved for final publication.

A publication fee of USD$300.00 will be levied. However, **the publication fee is waived IF** the first author or corresponding author is a member of Malaysian Society of Parasitology and Tropical Medicine (MSPTM) of at least three (3) years' standing.

The MSPTM Treasurer will issue an electronic invoice to the corresponding author via email sent from the payment gateway system within 3 working days after this acceptance letter is being issued by the Editor. The payment instructions are listed below. Kindly make the payment within three weeks from this notification.

Payment options:

1. Online credit card payment is applicable through the link in the invoice.

- From the online invoice, you may click on the "Pay this Invoice" (blue button) to proceed payment with your credit card. The invoice is issued in Malaysian Ringgit (MYR). Thus, the online payment will be in MYR, which will be automatically converted to your local currency by your bank or credit card service provider with the exchange rate the bank or credit card service provider offers.

2. Wire Transfer/ Telegraphic Transfer/ Electronic Bank Transfer

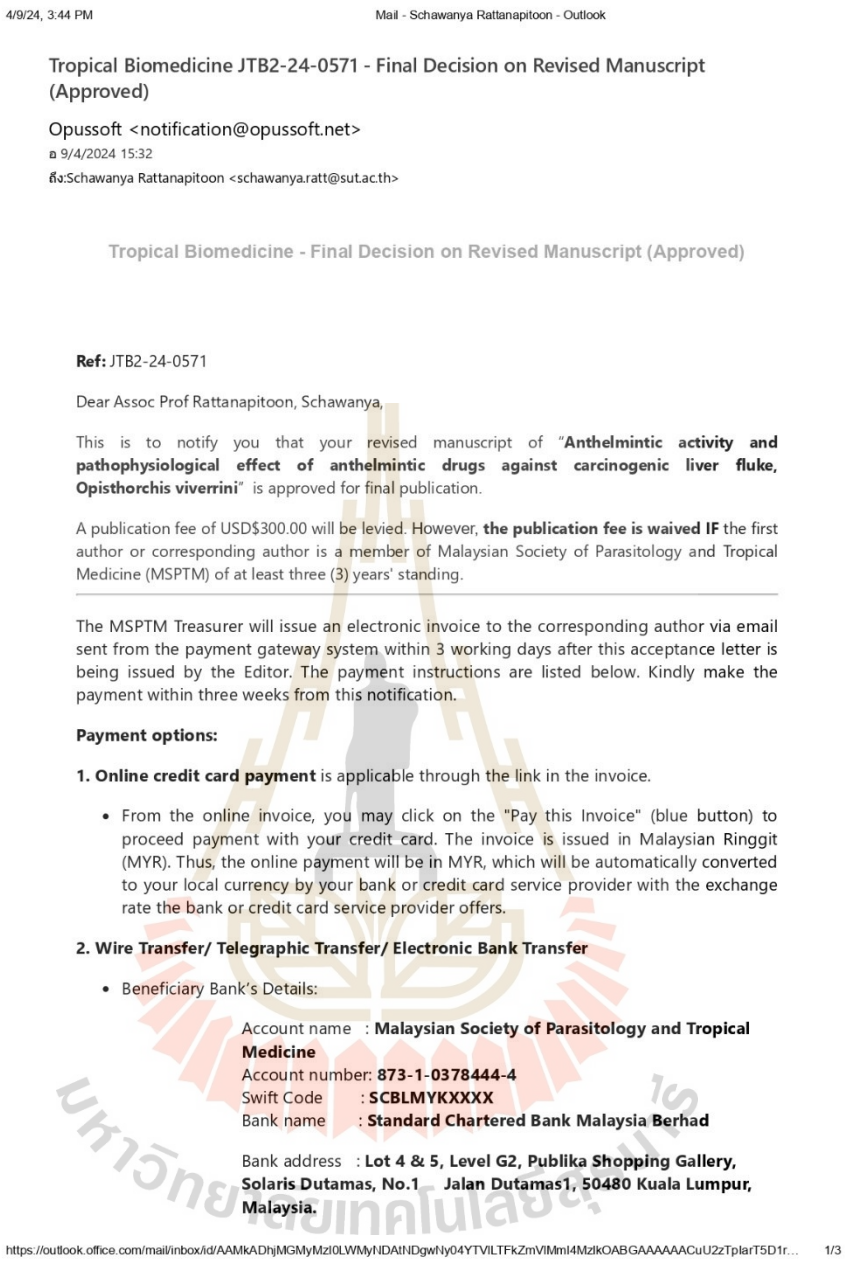
- Beneficiary Bank's Details:

Account name : **Malaysian Society of Parasitology and Tropical Medicine**
Account number: **873-1-0378444-4**
Swift Code : **SCBLMYKXXXX**
Bank name : **Standard Chartered Bank Malaysia Berhad**

Bank address : **Lot 4 & 5, Level G2, Publika Shopping Gallery, Solaris Dutamas, No.1 Jalan Dutamas1, 50480 Kuala Lumpur, Malaysia.**

https://outlook.office.com/mail/inbox/id/AAMkADhjMGMyMzI0LWMyNDAtNDgwNy04YTVlLTFkZmVlMml4MzlkOABGAAAAAACuU2zTplarT5D1r... 1/3

Figure 3E Acceptance email from the Tropical Biomedicine Journal for the manuscript titled "Diagnosis of the carcinogenic liver fluke, *Opisthorchis viverrini*: comparison of two coprological methods versus the automatic feces analyzer" for publication.



4/9/24, 3:44 PM Mail - Schawanya Rattanapitoon - Outlook

Tropical Biomedicine JTB2-24-0571 - Final Decision on Revised Manuscript (Approved)

Opussoft <notification@opussoft.net>
9/4/2024 15:32
ถึง:Schawanya Rattanapitoon <schawanya.ratt@sut.ac.th>

Tropical Biomedicine - Final Decision on Revised Manuscript (Approved)

Ref: JTB2-24-0571

Dear Assoc Prof Rattanapitoon, Schawanya,

This is to notify you that your revised manuscript of "**Anthelmintic activity and pathophysiological effect of anthelmintic drugs against carcinogenic liver fluke, Opisthorchis viverrini**" is approved for final publication.

A publication fee of USD$300.00 will be levied. However, **the publication fee is waived IF** the first author or corresponding author is a member of Malaysian Society of Parasitology and Tropical Medicine (MSPTM) of at least three (3) years' standing.

The MSPTM Treasurer will issue an electronic invoice to the corresponding author via email sent from the payment gateway system within 3 working days after this acceptance letter is being issued by the Editor. The payment instructions are listed below. Kindly make the payment within three weeks from this notification.

Payment options:

1. Online credit card payment is applicable through the link in the invoice.

- From the online invoice, you may click on the "Pay this Invoice" (blue button) to proceed payment with your credit card. The invoice is issued in Malaysian Ringgit (MYR). Thus, the online payment will be in MYR, which will be automatically converted to your local currency by your bank or credit card service provider with the exchange rate the bank or credit card service provider offers.

2. Wire Transfer/ Telegraphic Transfer/ Electronic Bank Transfer

- Beneficiary Bank's Details:

Account name : **Malaysian Society of Parasitology and Tropical Medicine**
Account number: **873-1-0378444-4**
Swift Code : **SCBLMYKXXXX**
Bank name : **Standard Chartered Bank Malaysia Berhad**

Bank address : **Lot 4 & 5, Level G2, Publika Shopping Gallery, Solaris Dutamas, No.1 Jalan Dutamas1, 50480 Kuala Lumpur, Malaysia.**

https://outlook.office.com/mail/inbox/id/AAMkADhjMGMyMzI0LWMyNDAtNDgwNy04YTVlLTFkZmVlMmI4MzlkOABGAAAAAACuU2zTplarT5D1r... 1/3

Figure 4E Acceptance email from the Tropical Biomedicine Journal for the manuscript titled "Anthelmintic activity and pathophysiological effect of anthelmintic drugs against carcinogenic liver fluke, *Opisthorchis viverrini.*" for publication.

Tropical Biomedicine 40(4): 471-477 (2023)
https://doi.org/10.47665/tb.40.4.013

RESEARCH ARTICLE

Oral parasitic protozoan *Entamoeba gingivalis* in periodontal disease patients, northeastern Thailand

Boonsuya, A.[1], Chitpitaklert, P.[1,2], Pechdee, P.[1,3], Srithongklang, W.[1], Thanchonnang, C.[1], La, N.[1], Gordon, C.N.[4], Rattanapitoon, N.K.[1,5], Arunsan, P.[1,3], Rattanapitoon, S.K.[1,6*]

[1]Parasitic Disease Research Center, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[2]Oral Health Center, Suranaree University of Technology Hospital, Nakhon Ratchasima 30000, Thailand
[3]Institution of Research and Development, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[4]Department of Medical Sciences, Faculty of Allied Health Sciences, Burapha University, Chonburi 20131, Thailand
[5]FMC Medical Center, Nakhon Ratchasima 30000, Thailand
[6]Department of Family Medicine and Community Medicine, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*Corresponding author: schawanya.ratt@sut.ac.th

ARTICLE HISTORY

Received: 21 August 2023
Revised: 3 October 2023
Accepted: 4 October 2023
Published: 15 January 2024

ABSTRACT

Entamoeba gingivalis is present in the oral cavity of humans and is associated with periodontal disease. Consequently, this study aimed to comprehensively investigate the *E. gingivalis* infection and the associated risk factors among individuals suffering from periodontal conditions. A cross-sectional descriptive study was carried out within a cohort of periodontal patients. Dental plaque specimens were meticulously collected and subsequently subjected to thorough examination using the polymerase chain reaction (PCR)-based technique targeting the small subunit ribosomal RNA (SrRNA) gene of the organism. The occurrence of risk factors for *E. gingivalis* infection was analyzed by the chi-square test and binary logistic regression. Out of the 230 participants, 60 were clinically diagnosed with periodontitis, while 170 were afflicted with gingivitis. Out of the 230 patients, 25 (10.9%) tested positive for *E. gingivalis* infections. An in-depth analysis unveiled that a significant majority of infections were recorded within subgroups characterized by a marital status (15.45%), manifestation of periodontitis (25.00%), and concomitant presence of underlying disease (20.83%). Furthermore, the high risk factor associated with *E. gingivalis* infection was the female (OR_{adj} = 13.65, 95% CI = 1.08-173.21), followed by periodontitis (OR_{adj} = 3.30, 95% CI = 1.21-9.00), respectively. The study employs a molecular diagnostic approach to screen for *E. gingivalis* enrichment within a subset of periodontal patients with advancing disease. The findings emphasize the necessity for further research to elucidate the pathogenesis of *E. gingivalis* and advocate for vigilant surveillance within a substantial population of periodontal patients.

Keywords: *Entamoeba gingivalis*; polymerase chain reaction (PCR); small subunit ribosomal RNA (SrRNA) gene; Thailand; periodontal disease.

INTRODUCTION

Entamoeba gingivalis, an amoebic protozoan, inhabits the oral cavity of individuals exhibiting inadequate oral hygiene practices. It is detected within dental plaques on gingival and tooth surfaces, interdental spaces, and carious lesions (Alhammza Abbass *et al.*, 2020). While the amoeba's trophozoite form could potentially be transmitted, the infective stage exclusively spreads through direct droplet exposure or intimate contact such as kissing (Bonner *et al.*, 2018; Mielnik-Blaszczak *et al.*, 2018). *E. gingivalis* scavenges dental plaques within the oral cavity, yet the accuracy of its impact on oral hygiene remains inconsistent (Smith & Barrett, 1915; Craig, 1916). Prior investigations have reported *E. gingivalis* as an opportunistic pathogen that aggravates periodontitis within the complex molecular milieu shaped by periodontal disease (Ponce de León *et al.*, 2001). Acting synergistically with symbiotic bacteria (*Porphyromona gingivalis, Treponema denticola,* and *Tannerella forsythia*), *E. gingivalis* contributes to the onset of periodontal disease in immunocompromised hosts (Chen *et al.*, 2001; Socransky & Haffajee, 2005; Dubar *et al.*, 2020). Moreover, studies employing progressive molecular methodologies have explored the prevalence of *E. gingivalis* in both healthy individuals and those affected by oral cavity diseases, effectively capturing the genetic variability of the organism (Badri *et al.*, 2021). The occurrence of *E. gingivalis* prevalence has been examined across different countries, with the highest rates documented in Jordan (87%) and comparatively lower rates observed in Portugal (3%). In Thailand, a previous small-scale study in Suphanburi Province revealed that 9 out of 95 participants (9.5%) had tested positive for *E. gingivalis* infection in dental plaque samples. The infection rates were 9.7% for males and 9.4% for females (Siriba *et al.*, 2016). Among diverse diagnostic techniques, molecular approaches (53%) and other methodologies (36%) have exhibited the highest combined prevalence (Badri *et al.*, 2021). Furthermore, research efforts have documented *E. gingivalis*

Published by Malaysian Society of Parasitology and Tropical Medicine.

Figure 5E Research article titled 'Oral parasitic protozoan *Entamoeba gingivalis* in periodontal disease patients, northeastern Thailand' has been published in the Tropical Biomedicine Journal.

Tropical Biomedicine 40(3): 307-312 (2023)
https://doi.org/10.47665/tb.40.3.006

RESEARCH ARTICLE

Molecular detection of oral *Trichomonas tenax* in periodontal disease patients by polymerase chain reaction -based 18S rRNA gene

Chitpitaklert, P.[1,2,3], Boonsuya, A.[1,3], Pechdee, P.[1,3,4], Thanchonnang, C.[1,3], LA, N.[1,3], Rattanapitoon, N.K.[1,5], Arunsan, P.[1,4], Rattanapitoon, S.K.[1,6*]

[1]Parasitic Disease Research Center, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[2]Oral Health Center, Suranaree University of Technology Hospital, Nakhon Ratchasima 30000, Thailand
[3]Translational Medicine Program, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[4]Institution of Research and Development, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand.
[5]FMC Medical Center, Nakhon Ratchasima 30000, Thailand
[6]Department of Family Medicine and Community Medicine, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*Corresponding author: schawanya.ratt@sut.ac.th

ARTICLE HISTORY

Received: 3 April 2023
Revised: 26 June 2023
Accepted: 26 June 2023
Published: 30 September 2023

ABSTRACT

Trichomonas tenax, an oral flagellated protozoon found in humans, potentially associated with the inflammation of periodontal tissues and decreased immunity that causes the tissue damage and tooth loss from chronic infection. Currently, there is a lack of data regarding the prevalence of *T. tenax* infection in Thailand. Therefore, this study aimed to measure prevalence of *T. tenax* in periodontal disease patients by using polymerase chain reaction (PCR) to amplify the 18S ribosomal RNA (18S rRNA) gene and to determine the factors associated with the presence of this protozoan. A cross-sectional descriptive study was conducted among 230 patients with periodontal disease, who visited the oral health center of Suranaree University of Technology Hospital, Thailand from 2021 to 2022. Dental plaque specimens were collected and examined to identify the presence of *T. tenax* using the PCR-based 18S rRNA gene. The occurrence of factors associated with *T. tenax* infection was analyzed by the chi-square test and binary logistic regression. The prevalence of *T. tenax* infection was 13.48% (31/230), in patients, including 96.77% (30/31) and 3.23% (1/31) in periodontitis and gingivitis patients, respectively. The presence of *T. tenax* was associated with periodontal disease ($p<0.001$) and the Periodontal Screening and Record (PSR) index ($p=0.001$). The significant risk factors for *T. tenax* infection were periodontitis ($OR_{adj}=239.89$, 95% CI=23.801-2417.746), no-underlying disease ($OR_{adj}=0.31$, 95% CI=0.099–0.942), and male sex ($OR_{adj}=0.25$, 95% CI=0.062-0.981). Dentists should be concerned about this oral protozoan in periodontitis patients. Furthermore, epidemiologic studies of *T. tenax* are still needed to investigate the mechanism of pathogenesis from *T. tenax* infection.

Keywords: Oral protozoa; *Trichomonas tenax*; periodontal disease; polymerase chain reaction (PCR); Thailand.

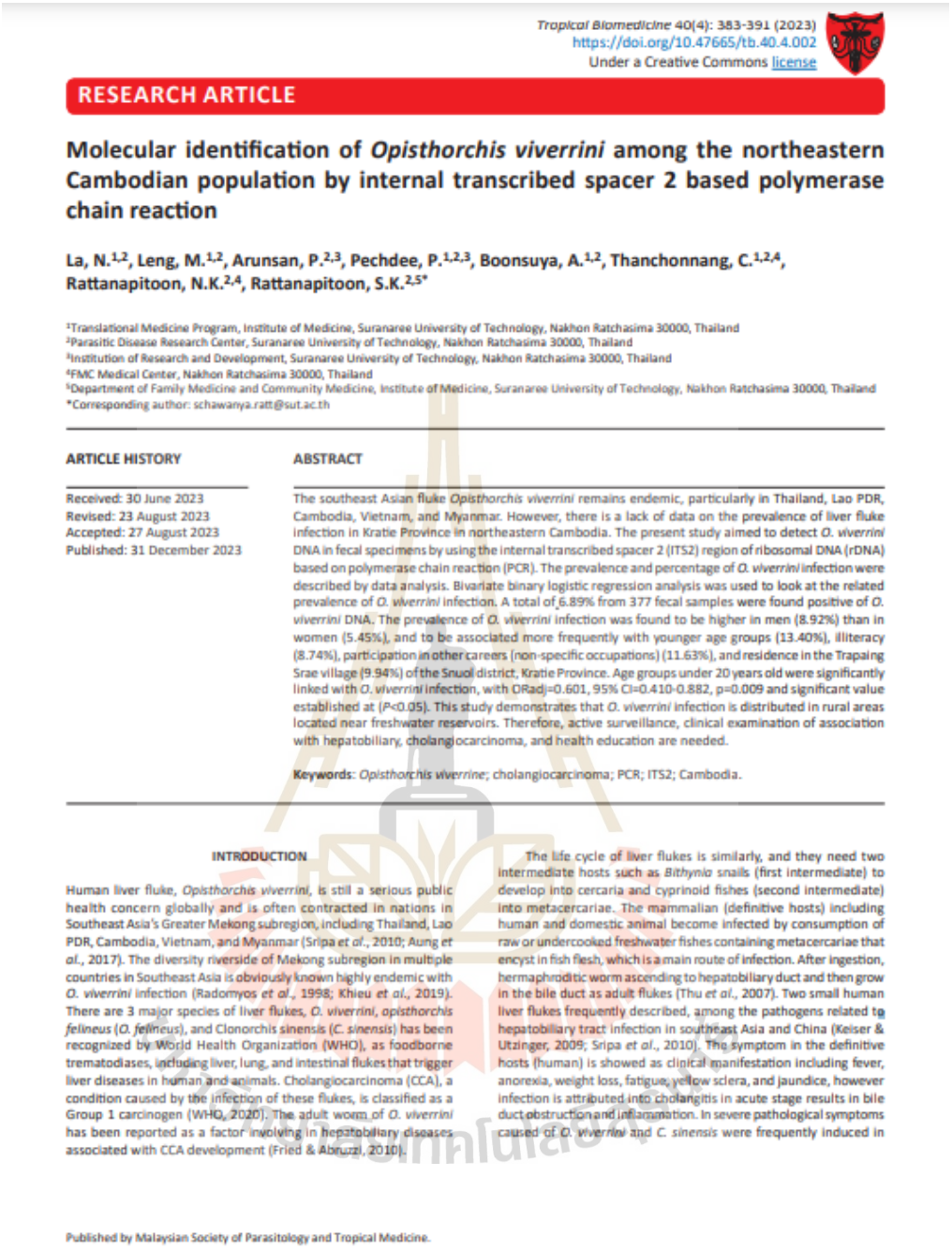
INTRODUCTION

Trichomonas tenax is a motile-flagellated protozoan is one of the risk factors for inflammation of periodontal tissues. It was first considered to be a commensal protozoan in the oral cavity and nasopharyngeal cavity (Hamadto *et al.*, 2014) until the 1940s, when Dobell (1939) and Wenrich (1944) described the oral flagellate with the name *T. tenax* (Honigberg & Lee, 1959). It may be found in the intraoral cavity, such as in dental plaque, calculus, saliva of periodontal disease patients (Ribeiro *et al.*, 2015), and in the extraoral cavity, such as in the respiratory tract, lung, maxillary sinus in compromised patients, other organs, and tissue (Mallat *et al.*, 2004; Marty *et al.*, 2017). For several decades, studies in oral protozoans have been of interest, and some authors have reported that *T. tenax* has a potential pathogenic role and is a coinfection pathogen in various infections (Socransky & Haffajee, 1992; Ribeiro *et al.*, 2015; Dybicz *et al.*, 2018). The occurrence of *T. tenax* has been observed in the oral cavity of patients with pulmonary disease and rheumatoid arthritis as well as in immunosuppressive patients. The functions of the immune system are impaired due to the main disease (Kikuta *et al.*, 1997; Marty *et al.*, 2017). Moreover, immunosuppressive patients can develop opportunistic parasitic disease (Dybicz *et al.*, 2018). In various studies, *T. tenax* has recently been reported to damage mammalian epithelial cells, and it behaves similarly to and is closely related *Trichomonas vaginalis*, and pathogenic *Trichomonas* species of the genitourinary tract, thus satisfying the requirements to be considered a parasite (Ribeiro *et al.*, 2015).

Periodontal disease is a chronic illness in humans that is characterized by inflammation and the loss of both soft and hard tissue supporting the teeth, as shown in Figure 1. In addition, periodontal disease associated to worsening systemic disorders such as diabetes, atherosclerosis, and cardiovascular diseases

Published by Malaysian Society of Parasitology and Tropical Medicine.

Figure 6E Research article titled "Molecular detection of oral *Trichomonas tenax* in periodontal disease patients by polymerase chain reaction based 18S rRNA gene." has been published in the Tropical Biomedicine Journal.



Tropical Biomedicine 40(4): 383-391 (2023)
https://doi.org/10.47665/tb.40.4.002

RESEARCH ARTICLE

Molecular identification of *Opisthorchis viverrini* among the northeastern Cambodian population by internal transcribed spacer 2 based polymerase chain reaction

La, N.[1,2], Leng, M.[1,2], Arunsan, P.[2,3], Pechdee, P.[1,2,3], Boonsuya, A.[1,2], Thanchonnang, C.[1,2,4], Rattanapitoon, N.K.[2,4], Rattanapitoon, S.K.[2,5]*

[1]Translational Medicine Program, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[2]Parasitic Disease Research Center, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[3]Institution of Research and Development, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[4]FMC Medical Center, Nakhon Ratchasima 30000, Thailand
[5]Department of Family Medicine and Community Medicine, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*Corresponding author: schawanya.ratt@sut.ac.th

ARTICLE HISTORY

Received: 30 June 2023
Revised: 23 August 2023
Accepted: 27 August 2023
Published: 31 December 2023

ABSTRACT

The southeast Asian fluke *Opisthorchis viverrini* remains endemic, particularly in Thailand, Lao PDR, Cambodia, Vietnam, and Myanmar. However, there is a lack of data on the prevalence of liver fluke infection in Kratie Province in northeastern Cambodia. The present study aimed to detect *O. viverrini* DNA in fecal specimens by using the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA) based on polymerase chain reaction (PCR). The prevalence and percentage of *O. viverrini* infection were described by data analysis. Bivariate binary logistic regression analysis was used to look at the related prevalence of *O. viverrini* infection. A total of 6.89% from 377 fecal samples were found positive of *O. viverrini* DNA. The prevalence of *O. viverrini* infection was found to be higher in men (8.92%) than in women (5.45%), and to be associated more frequently with younger age groups (13.40%), illiteracy (8.74%), participation in other careers (non-specific occupations) (11.63%), and residence in the Trapaing Srae village (9.94%) of the Snuol district, Kratie Province. Age groups under 20 years old were significantly linked with *O. viverrini* infection, with ORadj=0.601, 95% CI=0.410-0.882, p=0.009 and significant value established at (P<0.05). This study demonstrates that *O. viverrini* infection is distributed in rural areas located near freshwater reservoirs. Therefore, active surveillance, clinical examination of association with hepatobiliary, cholangiocarcinoma, and health education are needed.

Keywords: *Opisthorchis viverrine*; cholangiocarcinoma; PCR; ITS2; Cambodia.

INTRODUCTION

Human liver fluke, *Opisthorchis viverrini*, is still a serious public health concern globally and is often contracted in nations in Southeast Asia's Greater Mekong subregion, including Thailand, Lao PDR, Cambodia, Vietnam, and Myanmar (Sripa *et al.*, 2010; Aung *et al.*, 2017). The diversity riverside of Mekong subregion in multiple countries in Southeast Asia is obviously known highly endemic with *O. viverrini* infection (Radomyos *et al.*, 1998; Khieu *et al.*, 2019). There are 3 major species of liver flukes, *O. viverrini*, *opisthorchis felineus* (*O. felineus*), and Clonorchis sinensis (*C. sinensis*) has been recognized by World Health Organization (WHO), as foodborne trematodiases, including liver, lung, and intestinal flukes that trigger liver diseases in human and animals. Cholangiocarcinoma (CCA), a condition caused by the infection of these flukes, is classified as a Group 1 carcinogen (WHO, 2020). The adult worm of *O. viverrini* has been reported as a factor involving in hepatobiliary diseases associated with CCA development (Fried & Abruzzi, 2010).

The life cycle of liver flukes is similarly, and they need two intermediate hosts such as *Bithynia* snails (first intermediate) to develop into cercaria and cyprinoid fishes (second intermediate) into metacercariae. The mammalian (definitive hosts) including human and domestic animal become infected by consumption of raw or undercooked freshwater fishes containing metacercariae that encyst in fish flesh, which is a main route of infection. After ingestion, hermaphroditic worm ascending to hepatobiliary duct and then grow in the bile duct as adult flukes (Thu *et al.*, 2007). Two small human liver flukes frequently described, among the pathogens related to hepatobiliary tract infection in southeast Asia and China (Keiser & Utzinger, 2009; Sripa *et al.*, 2010). The symptom in the definitive hosts (human) is showed as clinical manifestation including fever, anorexia, weight loss, fatigue, yellow sclera, and jaundice, however infection is attributed into cholangitis in acute stage results in bile duct obstruction and inflammation. In severe pathological symptoms caused of *O. viverrini* and *C. sinensis* were frequently induced in

Published by Malaysian Society of Parasitology and Tropical Medicine.

Figure 7E Research article titled "Molecular identification of *Opisthorchis viverrini* among the northeastern Cambodian population by internal transcribed spacer 2 based polymerase chain reaction." has been published in the Tropical Biomedicine Journal.

Tropical Biomedicine 39(4): 539-546 (2022)
https://doi.org/10.47665/tb.39.4.009

RESEARCH ARTICLE

Intestinal parasitic infections and risk factors among the population in Cambodia

La, N.[1], Leng, M.[1], Rattanapitoon, N.K.[1], Pechdee, P.[1,2], Boonsuya, A.[1], Arunsan, P.[1,2], Rattanapitoon, S.K.[1,3*]

[1]Parasitic Disease Research Center, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[2]Institution of Research and Development, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[3]Department of Family Medicine and Community Medicine, Institute of Medicine, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*Corresponding author: schawanya.ratt@g.sut.ac.th

ARTICLE HISTORY

Received: 13 July 2022
Revised: 10 November 2022
Accepted: 11 November 2022
Published: 31 December 2022

ABSTRACT

Many species of helminths and protozoa caused intestinal parasitic infections (IPIs). It belongs to neglected tropical diseases (NTDs) and remains a major public health problem in several Southeast Asian countries. The present study aimed to investigate the prevalence of IPIs and associated risk factors among the population in Kratie Province in northeastern Cambodia and Phnom Penh is the capital that locates in southern Cambodia. Fecal specimens (*n*=366) were collected in 10 villages in Kratie Province and Phnom Penh from 2019 to 2021. They were processed using the formalin ethyl-acetate concentration technique (FECT) to investigate parasites at egg and cyst stages and then examined under a light microscope. The results revealed that the prevalence of IPIs among the population in Kratie Province (*n*=317) and Phnom Penh (*n*=49) was 16.12% (*n*=59); of Kratie Province (*n*=50, 13.66%) and Phnom Penh (*n*=9, 2.46%), 12.02% (*n*=44) were helminths and 4.10% (*n*=15) were protozoa. The parasitic infection rate was higher in males (9.02%) than in females (7.10%) and more likely to be due to helminths (7.38%) than protozoa (1.64%). Prevalence of *Opisthorchis viverrini* was the highest (5.74%), followed by those of *Entamoeba coli* (4.10%), hookworm (3.83%), *Ascaris lumbricoides* (1.10%), *Hymenolepis nana* (1.09%), *Taenia* spp. (0.54%), *Trichuris trichiura* (0.55%), and *Enterobius vermicularis* (0.27%), respectively. Moreover, *O. viverrini* infection was the most common infection in the >20-year age group in Kratie Province. In addition, the bivariate and multivariate analyses showed that the association between gender. Gender was a significant risk factor positively associated with *O. viverrini* and hookworm infections (OR_{adj}=0.318, 95% CI=0.122-0.8270, *P*=0.019 and OR_{adj}=0.085, 95% CI=0.017-0.436, *P*=0.003, respectively). In conclusion, the IPIs were highly prevalent, especially *O. viverrini* and hookworm infections, among the population in Cambodia. These IPIs impact the public health burden but can be prevented by education regarding good sanitary practices in this community.

Keywords: Intestinal parasitic infections (IPIs); formalin ethyl-acetate concentration technique (FECT); Cambodia; prevalence; Kratie Province and Phnom Penh.

INTRODUCTION

Intestinal parasitic infections (IPIs) are a serious public health problem in several countries throughout the world. IPIs are highly prevalent in Southeast Asia (Dunn *et al.*, 2016), including Thailand, Cambodia, Lao PDR, and Myanmar (Han *et al.*, 2019). Neglected helminth and protozoa infections have affected almost 200 million people who live in poverty in countries belonging to the Association of Southeast Asian Nations (ASEAN) (Hotez *et al.*, 2015). The main cause of intestinal parasite transmission is poor hygiene conditions, such as a lack of clean water and sanitary toilets (Ziegelbauer *et al.*, 2012; Echazu *et al.*, 2015). More than 10 million people suffer from parasitic infections, including infections of liver and intestinal flukes, in Southeast Asia (Furst *et al.*, 2012). In Myanmar, the prevalence of intestinal helminth infection is approximately 11.3 million people (Hotez *et al.*, 2015; WHO, 2018). The primary socioeconomic factors related to the high prevalence of IPIs include poor food hygiene, unsanitary housing, congested living conditions, agricultural conditions, insufficient water resources, animal living near the reservoirs, and poor healthcare services (Echazu *et al.*, 2015; Hotez *et al.*, 2015; Dunn *et al.*, 2016;). Nevertheless, these parasitic infections are classified as neglected tropical diseases. The most patients are unawareness of symptoms until progression and illness presentation, leading to individual, economic, and society burdens due to chronic infection or reinfection. Consequently, this problem has persisted. Approximately 9.3 million cases of liver fluke infection have been reported in ASEAN countries (Hotez *et al.*, 2015). A previous study reported on the prevalence of intestinal helminth infections among villages in Kratie Province, northeastern Cambodia, using fecal specimens and the Kato-Katz thick smear technique. The highest prevalence of *Opisthorchis viverrini* infections occurred in Roka Kandal A (10.4%), followed by Talous (5.9%); in both these

Figure 8E Research article titled "Intestinal parasitic infections and risk factors among the population in Cambodia." has been published in the Tropical Biomedicine Journal.

ทะเบียนข้อมูลเลขที่ ว.044597

คำขอแจ้งข้อมูลเลขที่ 388412

หนังสือแสดงการแจ้งข้อมูลลิขสิทธิ์

ออกให้เพื่อแสดงว่า

สำนักงานพัฒนาการวิจัยการเกษตร (องค์การมหาชน)

มหาวิทยาลัยเทคโนโลยีสุรนารี

ได้แจ้งข้อมูลลิขสิทธิ์ไว้ต่อกรมทรัพย์สินทางปัญญา

เมื่อวันที่ 23 เดือน พฤศจิกายน พ.ศ. 2563

ประเภทงาน วรรณกรรม ลักษณะงาน งานนิพนธ์

ชื่อผลงาน คู่มือสมุนไพรไทยต้านปรสิต

ออกให้ ณ วันที่ 2 เดือน ธันวาคม พ.ศ. 2563

ลงชื่อ.....

(นางธนิฏฐา โชติดิลก)

นักวิชาการพาณิชย์ชำนาญการพิเศษ

ปฏิบัติราชการแทนผู้อำนวยการกองลิขสิทธิ์

หมายเหตุ เอกสารนี้มิได้รับรองความเป็นเจ้าของลิขสิทธิ์

Signed by DIP-CA

Figure 9E The copyright pertaining to this research, titled "คู่มือสมุนไพรไทยต้านปรสิต (Thai Herbs of Anti-Parasite)," has Copyright ID: ว .0445972021.

CURRICULUM VITAE

Name: Miss Phornphitcha Pechdee

Date of Birth: 07th March 1989

Place of Birth: Roi-Et, Thailand

Grant: External grants and scholarships for graduate students, One Research One Graduate (OROG)

Education

- 2017: Graduated with a Master of Science (Parasitology), Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
- 2012: Graduated with a Bachelor of Science (Biology), Department Biology, Faculty of Science, Mahasarakham University, Mahasarakham Thailand.

Poster presentation

Poster presentation in titled Efficacy of *Allium sativum* crude extract against the human liver fluke, *Opisthorchis viverrini*' at The 3rd International Conference on Parasitology 2023 (ICP2023). Oganiaed by Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand on 9th -10th November 2023

Publication

Pechdee, P., Boonsuya, A., Arunsan, P., Thanchonnang, C., La, N., Rattanapitoon, N. K., Laha, T., Nuansing, W., & Rattanapitoon, S. K. (2024). Anthelmintic activity and pathophysiological effect of *Allium sativum* crude extract against carcinogenic liver fluke, *Opisthorchis viverrini*. *Tropical Biomedicine,* **approved for publication.**

Pechdee, P., Boonsuya, A., Arunsan, P., Thanchonnang, C., La, N., Rattanapitoon, N. K., Laha, T., & Rattanapitoon, S. K. (2024). Efficacy of crude extracts of *Thunbergia laurifolia* and *Eurycoma longifolia* against human liver fluke, *Opisthorchis viverrini*, **manuscript submitted**.

Pechdee, P., Boonsuya, A., Arunsan, P., Thanchonnang, C., La, N., Rattanapitoon, N., Pholyiam P., Punnasirimangmee K., & Rattanapitoon S. (2024). Effect of Allium sativum, *Thunbergia laurifolia*, and *Eurycoma longifolia* crude extracts on the minute intestinal fluke, *Haplorchis taichui*, **manuscript submitted**