ฤทธิ์การก่อกลายพันธุ์และต้านการก่อกลายพันธุ์ในสิ่งสกัดยาสามัญประจำบ้านแผนโบราณบางตำรับโดยวิธีเอมส์

นางสาวปรียากมล มีอยู่เต็ม

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EFFECTS OF SELECTED THAI ANCIENT REMEDIES EXTRACTS ON MUTAGENICITY AND ANTIMUTAGENICITY USING AMES TEST

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ปรียากมล มีอยู่เต็ม : ฤทธิ์การก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ในสิ่งสกัดยาสามัญประจำบ้านแผนโบราณบางตำรับโดยวิธีเอมส์ (Effect of selected Thai ancient remedies extracts on mutagenicity and antimutagenicity using Ames test) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. นิจศิริ เรืองรังษี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. ชนิดา พลานุเวช, 72 หน้า.

ฤทธิ์การก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ของสิ่งสกัดเอทานอลและน้ำของยาสามัญประจำบ้านแผนโบราณ 9 ตำรับได้แก่ ยาจันทลีลา ยาประสะจันทน์แดง ยาเขียวหอม ยาตรีหอม ยาอัมฤควาที ยาประสะมะแว้ง ยาวิสัมพยาใหญ่ ยาธรณีสัณฑฆาต และยาหอมทิพโอสถ การศึกษาฤทธิ์ก่อกลายพันธุ์ของสิ่งสกัดในสภาวะที่ไม่มีการกระตุ้นด้วยเอนไซม์ ด้วยวิธีการทดลองเอมส์ ใช้ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 ผลการศึกษาพบว่า สิ่งสกัดเอทานอลและน้ำส่วนใหญ่ของยาสามัญประจำบ้านแผนโบราณไม่แสดงฤทธิ์ก่อกลายพันธุ์ โดยมีเพียงแต่สิ่งสกัดเอทานอลของยาตรีหอมที่แสดงฤทธิ์ก่อกลายพันธุ์ โดยแสดงค่า Mutagenic Index 3.64 และ 2.21 ในสายพันธุ์ *S. typhimurium* TA98 และ *S. typhimurium* TA100 ตามลำดับ และจากการศึกษาฤทธิ์ก่อกลายพันธุ์ของสิ่งสกัดเมื่อทำปฏิกิริยากับไนไตรทในสภาวะที่ไม่มีการกระตุ้นด้วยเอนไซม์พบว่าสิ่งสกัดส่วนใหญ่มีฤทธิ์ก่อกลายพันธุ์ สิ่งสกัดเอทานอลและน้ำของยาธรณีสัณฑฆาตแสดงฤทธิ์ก่อกลายพันธุ์สูงทั้งในสายพันธุ์ TA98 และ TA100 นอกจากนี้การศึกษาฤทธิ์ต้านการก่อกลายพันธุ์ของสิ่งสกัดของยาสามัญประจำบ้านแผนโบราณต่อผลิตภัณฑ์ที่เกิดจากปฏิกิริยาของ 1-อมิโนพัยรีนทำปฏิกิริยากับไนไตรทในสภาวะที่ไม่มีการกระตุ้นด้วยเอนไซม์ จากผลการศึกษาสายพันธุ์ TA98 พบว่าสิ่งสกัดเอทานอลของยาวิสัมพญาใหญ่ (10 มิลลิกรัมต่อจานเลี้ยงเชื้อ) มีฤทธิ์ต้านก่อกลายพันธุ์สูงสุด 155% ในขณะที่สายพันธุ์ TA100 สิ่งสกัดเอทานอลของยาจันทลีลา (15 มิลลิกรัมต่อจานเลี้ยงเชื้อ) มีฤทธิ์ต้านก่อกลายพันธุ์สูงสุด 107%.

สาขาวิชา : วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต ปรียากมล มีอยู่เต็ม

ปีการศึกษา : 2553 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก

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PREEYAKAMOL MEEYUTEM: EFFECT OF SELECTED THAI ANCIENT REMEDIES EXTRACTS. ON MUTAGENICITY AND ANTIMUTAGENICITY USING AMES TEST ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D. CO - ADVISOR: CHANIDA PALANUVEJ, Ph.D., 72 pp.

The extracts of selected Thai household ancient remedies namely, Chantaleela, Prasachandang, Keawhom, Treehom, Ummalukkawatee, Prasamawaeng, Wisampayayai, Thoraneesantakat and Homtip-osot were determined the mutagenicity and antimutagenicity effects in the absence of metabolic activation using *Salmonella typhimurium* TA98 and TA100. It was found that most ethanolic and water extracts of selected Thai ancient remedies in treating without nitrite were not directly mutagenic, except that the ethanolic extract of Treehom exhibited mutagenicity. The mutagenic index of ethanolic Treehom extract was 3.64 and 2.21 on *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100 respectively. However, after treating with nitrite, all extracts showed the mutagenicity against both strains. It was demonstrated that the ethanolic and water extracts of Thoraneesantakat showed the highest mutagenic index on TA98 and TA100. Furthermore ethanolic extracts seemed to be more mutagenic than water extracts. The antimutagenicity of Thai ancient remedies extracts against the product of the reaction mixture of 1-aminopyrene-nitrite model in the absence of metabolic activation. The results showed ethanolic of Wisampayayai remedy (10mg/plate) showed the highest antimutagenicity on TA98 (155%) and ethanolic of Chantaleela remedy (15mg/plate) showed the highest antimutagenicity on TA100 (107%).

Field of Study: Public health sciences Student's Signature Preeyakamol Meeyutem

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
DMSO	=	Dimethyl sulfoxide
g	=	Gram
hr.	=	Hour
hrs.	=	Hours
kg	=	Kilogram
L	=	Liter
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
μg	=	Microgram
μl	=	Microliter
N	=	Normality
mM	=	Millimolar
HCl	=	Hydrochloric acid
His^+	=	Histidine prototrophy

CHAPTER I

INTRODUCTION

Background and Significance of the Study

Thai ancient remedies have been widely used in Thailand for a long time until the present [1]. Although toxicity has less reported after a long history use of Thai ancient remedies, research of mutagenicity is still needed to be investigated. This study aims to assess the mutagenicity and antimutagenicity of ethanol and water extracts from the selected Thai household ancient remedies namely, Chantaleela, Prasachandang, Keaw-hom, Tree-hom, Ummalukkawatee, Prasamawaeng, Wisampayayai, Thoraneesantakat and Homtip-osot which consisted in the list of Herbal Medicinal Products of the National List of Essential Drugs [2, 3].

In recent years, interest in the relationship between diet (food/herb) and cancer has increased and there have been numerous surveys of the occurrence of mutagens and carcinogens in food. Many types of mutagen are present in our foods. Some of them occur naturally, the others can be produced during preparation of foods for consumption [4].

Mutagenic precursors in dietary ingredients may also be important factors causing cancer [5]. Various foods produced in Thailand have been shown to produce a direct-acting on mutagenicity after nitrite treatment [6, 7]. Salted/smoked and pickled/preserved foods rich in salt, nitrites and preformed nitroso compounds were associated with an increased risk of gastric cancer [8, 9]. Recently a number of laboratories have reported that fruit and herb extracts contain antimutagenic compounds [10-12] including flavonoids, phenolic, beta-carotene, vitamins C and E, dietary fiber, SH-containing amino acids and peptides. Thai ancient remedies are made up of many herbs in combination and there's been a lack of information about their mutagenic and antimutagenic potential.

The Ames test is a very sensitive and simple procedure. This test uses various strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involving in histidine synthesis, so that they require histidine for growth. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium [13-17].

This research assessed the selected Thai ancient remedies extracts for the direct and nitrite–induced mutagenic potential as well as the antimutagenic property against mutagen derived from nitrite tread aminopyrene. *S. typhimurium* strains TA98 and TA100 were used for Ames assay. The information in this study served for consumer protection and has been associated to reduce a rick of cancer.

Objectives of this study

1. To study the mutagenicity of selected Thai ancient remedies treated with and without sodium nitrite using Ames test.
2. To study the antimutagenicity of selected Thai ancient remedies against mutagens from the nitrite treatment of 1-aminopyrene.

Benefit of the study

1. The study provides information regarding the mutagenicity and antimutagenicity of selected nine formulae of Thai ancient remedies extracts.
2. The information of this study is served for consumer protection.

CHAPTER II

LITERATURE REVIEWS

Thai ancient remedies

Thai traditional medicine is the accumulated knowledge and experiences of the indigenous people that is used to maintain health, as well as to prevent and treat illnesses in primary health care. It was revealed in Paad-Sard-Song-Khro scripture [18].

In the present time, Thai household ancient remedies have been notified in the list of Health Medicinal Products of the National List of Essential Drugs A.D. 2006 [2, 3]. The Ministry of Public Health accepted that remedies have effectiveness in treating of the ailments. This research was investigated for nine remedies such as Chan-ta-lee-la, Pra-sa-chan-dang, Keaw-hom, Tree-hom, Um-ma-luk-ka-wa-tee, Pra-sa-ma-waeng, Wi-sam-pa-ya-yai, Tho-ra-nee-san-ta-kat and Hom-tip-o-sot as followed as Table 1 to Table 9.

Chantaleela remedy (ตำรับยาจันทลีลา)

Chantaleela remedy has a property for relief of fever [2, 3, 18-20].

Table 1 Ingredients of Chantaleela remedy

Scientific name	Thai name	Part used	Weight ratio
Angelica duhurica Benth.	โกฐสอ	Rhizome	4
Atractylodes lyrata Sieb.	โกฐเขมา	Rhizome	4
Artemisia vulgaris L.	โกฐจุฬาลัมพา	Herb	4
Myristica fragrans L.	จันทน์เทศ	Heartwood	4
Dracaena loureiri Gagnep.	จันทน์แดง	Heartwood	4
Gymnopetalum cochinchinense (Lour.) (Kurz.)	กระดอม	Fruit	4
Tinospora crispa (L.) / *Ilex umbellulata* Loes.	บอระเพ็ด	Root	4
Eurycoma longifolia Jack.	ปลาไหลเผือก	Root	4
Borneol	พิมเสน	Crystalized compound	1

Prasachandang remedy (ตำรับยาประสะจันทน์แดง)

Prasachandang remedy has a property for relief of fever [2, 3, 18-20].

Table 2 Ingredients of Prasachandang remedy

Scientific name	Thai name	Part used	Weight ratio
Simplocos racemosa Roxb.	เหมือดคน	Root	4
Bouea burmanica Griff.	มะปรางหวาน	Root	4
Citrus aurantifolia (Chrism. & Panz.) Swing.	มะนาว	Root	4
Kaempferia galanga L.	เปราะหอม	Rhizome	4
Conioselinum univitatum Turczaninow.	โกฐหัวบัว	Rhizome	4
Myristica fragrans L.	จันทน์เทศ	Seed	4
Caesalpinia sappan L.	ฝางเสน	Heartwood	4
Nelumbo nucifera Gaertn.	บัวหลวง	Stamen	1
Mesua ferrea L.	บุนนาค	Flower	1
Mammea siamensis Kosterm.	สารภี	Flower	1

Keawhom remedy (ตำรับยาเขียวหอม)

Keawhom remedy has a property for relief of fever [2, 3, 18-20].

Table 3 Ingredients of Keawhom remedy

Scientific name	Thai name	Part used	Weight ratio
Pogostemon cabin Beasth.	พิมเสน	Leaf	1
Limnophila rugosa Merr.	ผักกระโฉม	Leaf	1
Areca catechu L.	หมากผู้	Leaf	1
Cordyline fruticosa Goeppert.	หมากเมีย	Leaf	1
Eupatorium stoechadosum Hance.	สันพร้าหอม	Leaf	1
Vetiveria zizanioides Stapf.	แฝกหอม	Root	1
Myristica fragrans L.	จันทน์เทศ	Seed	1

Table 3 Ingredients of Keawhom remedy (Cont.)

Scientific name	Thai name	Part used	Weight ratio
Dracaena loureiri Gagnep.	จันทน์แดง	Heartwood	1
Angiopteris evecta Hoffm.	ว่านกีบแรด	Rhizome	1
Globba malaccensis Ridl.	ว่านร่อนทอง	Rhizome	1
Dryopteris syrmatica O. Kze.	เนระพูสี	Rhizome	1
Sophora exigua Craib.	พิษนาศน์	Root	1
Alsophila latebrosa Hook.	มหาสดำ	Heartwood	1
Aristolochia sp.	ไคร้เครือ	Root	1
Mimusops elengi L.	พิกุล	Flower	1
Mesua ferrea L.	บุนนาค	Flower	1
Mammea siamensis Kosterm.	สารภี	Flower	1
Nelumbo nucifera Gaertn.	บัวหลวง	Stamen	1

Homtip-osot remedy (ตำรับยาหอมทิพโอสถ)

Hom-tip-o-sot remedy has a property for relief nausea and dizziness [2, 3, 18-20].

Table 4 Ingredients of Homtip-osot remedy

Scientific name	Thai name	Part used	Weight ratio
Mimusops elengi L.	พิกุล	Flower	4
Mesua ferrea L.	บุนนาค	Flower	4
Mammea siamensis Kosterm.	สารภี	Flower	4
Jusminum sambac Lour.	มะลิ	Flower	4
Nelumbo nucifera Gaertn.	บัวหลวง	Stamen	4
Cananga odorata (Lam.) Hooker f. & Thoms.	กระดังงา	Flower	4
Michelia champaca L.	จำปา	Flower	4
Nymphaea lotus L.	บัวจงกลนี	Flower	4

Table 4 Ingredients of Homtip-osot remedy (Cont.)

Scientific name	Thai name	Part used	Weight ratio
Cyperus esculentus L.	แห้วไทย	Corm	4
Trapa bicornis Osb. var. *cochin-chinensis* Gliick ex Steenis.	กระจับ	Endosperm	4
Caesalpinia sappan L.	ฝาง	Heartwood	4
Dracaena loureiri Gagnep.	จันทน์แดง	Heartwood	4
Diospyros decandra Lour.	จันทน์ขาว	Heartwood	4
Myristica fragrans L.	จันทน์เทศ	Fruit	4
Aquilaria agallocha Roxb.	กฤษณา	Wood	4
Alyxia reinwardtii Blume.	ชะลูด	Bark	4
Cinnamomum iners Blume.	อบเชย	Bark	4
Cinnamomum bejolghota Ham.	สมุลแว้ง	Bark	4
Thuja orientalis L.	สนเทศ	Leaf	4
Acorus calamus L.	ว่านน้ำ	Rhizome	4
Boesenbergia rotunda (L.) Mansf.	กระชาย	Rhizome	4
Kaempferia galanga L.	เปราะหอม	Rhizome	4
Bixa orellana L.	คำไทย	Flower	4
Glycyrrhiza glabra L.	ชะเอมเทศ	Root	4
Cocculus laurifolius DC.	สุรามฤต	Stem	4
Cinnamomum siamense Craib	ข่าต้น	Bark	4
Myristica fragrans L.	จันทน์เทศ	Seed	4
Myristica fragrans L.	จันทน์เทศ	Aril	4
Angelica sylvestris L.	โกฐสอ	Root	2
Atractylodes lyrata Sieb. et Zucc.	โกฐเขมา	Rhizome	2
Conioselinum univitatum Turczaninow.	โกฐหัวบัว	Root	2
Livisticum officinale Koch.	โกฐเชียง	Root	2
Artemisia vulgaris L.	โกฐจุฬาลัมพา	Herb	2
Saussurea lappa C.B.Clarke.	โกฐกระดูก	Root	2

Table 4 Ingredients of Homtip-osot remedy (Cont.)

Scientific name	Thai name	Part used	Weight ratio
Picrorhiza kurroa Benth.	โกฐก้านพร้าว	Root	2
Terminilia chebula Retz.	โกฐพุงปลา	Gall	2
Nardostachys jatamansi DC.	โกฐชฎามังสี	Herb	2
Nigella sativa L.	เทียนดำ	Seed	1
Lepidium sativum L.	เทียนแดง	Seed	1
Cuminum cyminum L.	เทียนขาว	Fruit	1
Foeniculum vulgare Mill. var. dulce Alef.	เทียนข้าวเปลือก	Fruit	1
Anethum graveolens L.	เทียนตาตั๊กแตน	Fruit	1
Petroselinum crispum (Mill.) Numan.	เทียนเยาวพาณี	Fruit	1
Plantago ovata Forskall.	เทียนเกล็ดหอย	Seed	1
Pimpinlla anisum L.	เทียนสัตตบุษย์	Fruit	1
Carum carvi L.	เทียนตากบ	Fruit	1
Cinnamomum camphora (L.) J.S. Presl	การบูร	Crystalized compound	1
Borneol	พิมเสน	Crystalized compound	2

Ummalukkawatee remedy (ตำรับยาอำมฤควาที)

Ummalukkawatee remedy has a property to reduce phlegm and cough [2, 3, 18-20].

Table 5 Ingredients of Ummalukkawatee remedy

Scientific name	Thai name	Part used	Weight ratio
Aristolochia sp.	ไคร้เครือ	Root	7
Terminilia chebula Retz.	โกฐพุงปลา	Gall	7
Cuminum cyminum L.	เทียนขาว	Fruit	7
Coriandrum sativum L.	ผักชีลา	Fruit	7
Phyllanthus emblica L.	มะขามป้อม	Fruit	7
Terminalia bellirica (Gaertn.) Roxb.	สมอพิเภก	Fruit	7
Glycyrrhiza glabra L.	ชะเอมเทศ	Root	43

Prasamawaeng remedy (ตำรับยาประสะมะแว้ง)

Prasamawaeng remedy has properties to reduce phlegm and cough [2, 3, 18-20].

Table 6 Ingredients of Prasamawaeng remedy

Scientific name	Thai name	Part used	Weight ratio
Alum	สารส้ม	Crystalized compound	1
Curcuma zedoaria Rose.	ขมิ้นอ้อย	Rhizome	3
Caesalpinia bonducella Fleming.	สวาด	Leaf	4
Vernonia elliptica DC.	ตานหม่อน	Leaf	4
Ocimum sanctum L.	กะเพรา	Leaf	4
Solanum indicum L.	มะแว้งต้น	Fruit	8
Solanum trilobatum L.	มะแว้งเครือ	Fruit	8

Wisam-payayai remedy (ตำรับยาวิสัมพยาใหญ่)

Wisam-payayai remedy has a property for relief of gastric hyperacidity and excess gas [2, 3, 18-20].

Table 7 Ingredients of Wisam-payayai remedy

Scientific name	Thai name	Part used	Weight ratio
Coriandrum sativum L.	ผักชีลา	Fruit	8
Myristica fragrans L.	จันทน์เทศ	Seed	8
Myristica fragrans L.	จันทน์เทศ	Aril	8
Amomum krervanh Pierre.	กระวาน	Fruit	2
Syzygium aromaticum (L.) Merr. & Perry.	กานพลู	Flower	2
Angelica sylvester L.	โกฐสอ	Root	2
Atractylodes lyrata Sieb. et Zucc.	โกฐเขมา	Rhizome	2
Conioselinum univitatum Turczaninow.	โกฐหัวบัว	Root	2
Livisticum officinale Koch.	โกฐเชียง	Root	2
Artemisia vulgaris L.	โกฐจุฬาลัมพา	Herb	2
Cinnamomum iners Blume.	อบเชย	Bark	2
Cinnamomum bejolghota Ham.	สมุลแว้ง	Bark	2
Terminalia sp.	สมอเทศ	Fruit	2
Terminalia chebula Retz.	สมอไทย	Fruit	2
Aristolochia sp.	ไคร้เครือ	Root	2
Acorus calamus L.	ว่านน้ำ	Root	2
Tinospora tuberculata Beumee.	บอระเพ็ด	Stem	2
Zingiber officinale Rose.	ขิงแห้ง	Rhizome	2
Clerodendrum petasites S. Moore	พญารากขาว	Root	2
Piper longum L.	ดีปลี	Fruit	56

Treehom remedy (ตำรับยาตรีหอม)

Treehom remedy has been used as laxative in clinical practice [2, 3, 18-20].

Table 8 Ingredients of Treehom remedy

Scientific name	Thai name	Part used	Weight ratio
Terminalia sp.	สมอเทศ	Fruit	4
Phyllanthus emblica L.	มะขามป้อม	Fruit	4
Terminalia bellirica (Gaertn.) Roxb.	สมอพิเภก	Fruit	4
Coriandrum sativum L.	ผักชี	Fruit	4
Aristolochia sp.	ไคร้เครือ	Root	1
Angelica sylvester L.	โกฐสอ	Root	1
Glycyrrhiza glabra L.	ชะเอมเทศ	Root	1
Borax	ประสารทอง	Pure compound	1
Trigonella foenum-graceum L.	ซัด	Seed	1
Terminalia chebula Retz.	สมอไทย	Fruit	22
Rheum officinale Baill.	โกฐน้ำเต้า	Rhizome	22

Thoraneesantakat remedy (ตำรับยาธรณีสัณฑฆาต)

Thoraneesantakat remedy has been used as laxative in clinical practice [2, 3, 18-20].

Table 9 Ingredients of Thoraneesantakat remedy

Scientific name	Thai name	Part used	Weight ratio
Myristica fragrans L.	จันทน์เทศ	Seed	1
Myristica fragrans L.	จันทน์เทศ	Aril	1
Amomum krervanh Pierre.	กระวาน	Fruit	1
Syzygium aromaticum (L.) Merr. & Perry.	กานพลู	Flower	1
Nigella sativa L.	เทียนดำ	Seed	1

Table 9 Ingredients of Thoraneesantakat remedy (Cont.)

Scientific name	Thai name	Part used	Weight ratio
Cuminium cyminum L.	เทียนขาว	Seed	1
Gloriosa superba L.	ดองดึง	Tuber	1
Amorphophallus campanulatus Blume., A.rex Hook.f.	บุก	Tuber	1
Dioscorea hispida Dennst He.	กลอย	Tuber	1
Colocasia gigantea Hook.f.	กระดาดขาว	Corm	1
Alocasia indica var. *metallica* Schott.	หัวกระดาดแดง	Corm	1
Amomun villosum Lour., *Amomun xanthioides* Wall.	เร่ว	Fruit	1
Zingiber officinale Rose.	ขิง	Rhizome	1
Glycyrrhiza glabra L. var. typica Regel.	ชะเอมเทศ	Root	1
Plumbago indica L.	เจตมูลเพลิงแดง	Root	1
Saussurea lappa C.B.Clarke.	โกฐกระดูก	Root	1
Atractylodes lyrata Sieb. et Zucc.	โกฐเขมา	Rhizome	1
Rheum officinale Baill.	โกฐน้ำเต้า	Rhizome	1
Iresine herbstii Hook f.	ผักแพวแดง	Herb	2
Phyllanthus emblica L.	มะขามป้อม	Fruit	2
Terminalia chebula Retz.	สมอไทย	Fruit	6
Ferula assa-foetida L.	มหาหิงคุ์	Oleo resin	6
Cinnamomum camphora (L.) J.S. Presl	การบูร	Crystalized compound	6
Garcinia hanburyi Hook f.	รงทอง	Gum resin	4
Aloe vera (Linn.) Burm. f.	ยาดำ	Water extract	20
Piper nigrum L.	พริกไทย	Seed	96

Ames test [13-17]

The Ames test is a biological assay to assess the mutagenicity potential of chemical compounds [14]. A positive test indicates that the chemical might act as a carcinogen. As cancer is often linked to DNA damage, the test serves as a quick assay to estimate the carcinogenic potential of a compound prior to the animal model testing.

This simple, indirect assay for potential carcinogens has been developed since the early 1970s by Bruce Ames and his group at the University of California, Berkeley. The assay is based upon the reversion of mutations in the histidine operon in the bacterium *Salmonella Typhimurium*. The operon encodes enzymes required for the biosynthesis of the amino acid histidine. Strains with mutations in the operon are histidine auxotroph. They are unable to grow without added histidine. Revertants that restore the His^+ phenotype will grow on minimal medium plates without histidine. This provides a simple, sensitive selection for revertants of His mutants.

Metabolic activation systems

Some carcinogenic chemicals, such as aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active form. In human and lower animals, the cytochrome-base P450 metabolic oxidative system, which is present mainly in the liver and to lesser extent in the lung and kidneys, is capable of metabolizing a large number of these chemical to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added to the petri plate together with the test chemical and the bacteria. For this intention, a rodent metabolic activation system was introduced into the test system [21-25]. The metabolic activation system usually consists of a 9000xg supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactor for NADPH-supported oxidation (S-9 mix) [26]. To increase the level of metabolizing enzymes, the animals are pretreated with the mixed-function oxidase inducer Aroclor 1254. Other inducers, such as Phenobarbital and β-naphthoflavone, can also be used.

The metabolic activation system can also consist of a reductive enzyme system for classes of chemicals containing azo and diazo bonds. Reduction of chemicals substances can occur in mammals, including humans, by anaerobic intestinal microflora, and very likely by mammalian reductases in the intestinal wall or in the liver. Two types of reductive *in vitro* metabolic activation system have generally been used, those based on a liver homogenate supplemented with FMN [27-28] and those that are base on rat intestinal microflora preparations [29-30].

The mutagenicity test (preincubation method) using *Salmonella typhimurium*

Some mutagen, such as, dimethylnitrosamine and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described [31] in which carcinogenic were found to be mutagenic. The mutagen and bacteria are incubated for 20-30 min at 37˚C and then added the top agar. The assay has been also used to detect the mutagenicity of 10 carcinogenic nitrosamines [32] and several carcinogenic alkaloids [33]. The mutagenic activity of aflatoxin B1, benzidine, benzo[α]pyrene and methyl methane sulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay [34]. The increased activity is assigned to the fact that the test compound and bacteria are incubated at higher concentration in the preincubation assay than in the standard plate incorporation test [35].

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. However, many laboratories use it routinely because of the increased sensitivity towards some compounds [36].

The *Salmonella* mutagenic assay (Ames test)

Bacterial mutaqenicity assays, especially the Ames test (*Salmonella typhimurium his*$^{-}$ reversion assay) have been used world-wide in experimentation laboratories. Their applications are motivated by several intentions, the identification of genotoxic hazards; the quantitation and regulation of health risks resulting from environment chemical detection and the explain of the biochemical mechanisms of mutagenesis. The potential of this method for used as a bioassay for the evolution of

safe, useful chemicals raised many questions about the extent to which this kind of approach should be used in a program aimed at cancer prevention.

The *Salmonella* histidine reverse mutation assay is based on the use of several selected histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased frequency in the presence of a mutagen. The test detects a wide variety of mutagens, including many that require an exogenous metabolic activation system. The test is used as a screen for mutagenic activity of complex mixtures and body fluids. At present, the most commonly used *Salmonella* strains are TA 1535, TA1537, TA1538, TA98 and TA100. The number and type of strains used depend upon the availability and type of sample, the point of the study, and previous knowledge respecting the test material. In addition to having a mutation that impart other specific characteristics to the tester strain, one mutation (*rfa*) leads to a defective lipopolysaccharide coat; another is a deletion of genes involved in the synthesis of the vitamin biotin (*bio*) and in the extraction repair of DNA damage (*uvr* B). The *rfa* mutation increases the permeability of the strains to large molecules, thereby increasing the mutagenicity and/or toxic effects of these chemicals. The *uvr* B mutation leads to a reduced level of error-free repair of some types of DNA damage and thereby enhances the strains sensitivity to certain chemical and physical mutagens. Strain TA100 is derived from TA 1535 by the introduction of the plasmid pKM 101, which increases the sensitivity of mutagen detection by enhancing error-prone DNA repair. The presence of this plasmid makes TA 100 respond to some frameshift mutagensas well as base-pair substitution mutagens, strain TA98 is derived from TA 1538 by the introduction of plasmid pKM101. All tester strains should be maintained and stored according to published methods [21-22]. They should be analyzed on a frequent and rational basis for each characteristic that could affect the test. For example, strain identification could incorporate the following: histidine and biotin requirement, UV sensitivity (presence of the *uvr* B deletion), crystal violet sensitivity (presence of the *rfa* mutation), ampicillin and for tetracycline resistance (presence of the appropriate plasmid) spontaneous reversion frequency, and reversion characteristics to various positive controls.

Three of the most important *his*$^-$ alleles found in the Ames tester strains are listed below, along with typical strains bearing the allele; the nature of the mutation in the target gene; and the most common pathway for its reversion:

- *hisD3052* ; TA 1538 , TA98 : -1 frameshift; ΔGpC frameshift in (GC)4 run

- *hisG46* ; TA 1535 , TA 100 : missense; base-substition at G:C base-pair

- *hisG428*; TA 102, TA 104, TA2659: ochre; base-substitution at A:T base pair

Each Ames test strain evaluates mutagenic activity at a specific (reversion) purpose sequence. In the case of the frameshift allele *hisD3052* revertants bearing many different sequence changes (spanning a region of more than 50bp) can be recovered: of course, each such event restores the correct reading frame. Multiple classes of revertants of the base-substitution alleles can also recovered, including transitions, transversion, and some extragenenic suppressor mutations.

The experimental basis for their current assessment of the value of the test as useful predictive tools: [37]

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weaknesses of the test at this stage in its development.
2. Current applications of the test method to problems that were not approachable using conventional animal test methods.
3. Some of the environmental chemicals that have already been pinpointed as potential carcinogens by the test and the current status of carcinogenicity tests of these chemicals in animals.
4. The proof that the correlation between carcinogenicity and mutagenicity in the *Salmonella* test reflected more than a useful coincidence and appropriated into a compelling collection of evidence supporting a central role for somatic mutation in the initiation of human cancer.

Spontaneous control values

Each tester strain has a characteristic spontaneous mutant frequency. There is usually some day-to-day and laboratory-to-laborotory variation in the number of spontaneous revertant colonies. Selection of solvent may also affect the spontaneous mutant frequency [38]. Table 10 shows a range of spontaneous histidine revertant control values per plate with and without metabolic activation. The values obtained in the presence of a metabolic activation system includes both rat and hamster liver S-9. The spontaneous values presented for S-9 were from 10% S-9 in the S-9 mix. Some of the strains (e.g., TA97, TA102, TA104) are highly sensitive to S-9 concentrations and their spontaneous reversion values will increase with the S-9 concentration.

Table 10 Spontaneous revertant control values [14].

Strain	Number of revertants	
	Without S-9	With S-9
TA97	75-200	100-200
TA98	20-50	20-50
TA100	75-200	75-200
TA102	100-300	200-400
TA104	200-300	300-400
TA1535	5-20	5-20
TA1537	5-20	5-20
TA1538	5-20	5-20

Nitrite induced mutagens

Nitrate and nitrite occurred in the diet from numerous distinct sources [5, 7, 39]. Vegetables and herbs are major sources of nitrate. Nitrates single are not toxic, however they become converted to nitrite when such foods are stored at room temperature. The salts of nitrate and nitrite are often used as a nutrition additive for preservation due to antimicrobial properties, particularly inhibition of the growth of *Clostridium botulinum* and also their ability to give a pleasing color and taste [5, 40-41]. It is proposed that nitrate involves in formation of carcinogen *N-nitroso* compounds *via* two distinct phase of gastric carcinogenesis. Firstly, after invasion and absorption of nitrate in stomach, nitrate is secreted in the saliva in concentrated form. Oral bacteria can then reduce nitrate to nitrite [42] (Figure 1). In the second phase, nitrite is converted in the stomach to nitrous acid or nitrosating agents and reacts with certain substrates (amines, amides or other precursors in foods) to form carcinogenic *N-nitroso* compounds [43]. Recent hypothesis for the development of gastric cancer suggest that exposure in the stomach to direct-acting genotoxic *N-nitroso* compounds, form endogenously, may be involed. Nitrosamine can form in the gastric juice of the human stomach. This is normally referred to as endogenous nitrosation. Many foods contain amines that can react with nitrosating agents in the acidic stomach to form nitrosamines. Synthesis of the compounds from nitrite and amines or amides has been demonstrated in vitro simulated gastric conditions and in vivo in animals [44-45].

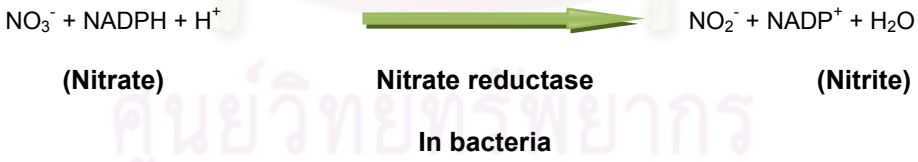

Figure 1 The conversion of nitrate to nitrite by nitrifying bacteria.

Various foods produced in Asia were reported on their direct-acting mutagenicity after nitrite treatment. Kimchi, sun-dried fish and squid, soy sauces, fish sauces, bean and shrimp pastes produced in Korea, the Philippines and Thailand showed direct-acting mutagenicity after nitrite treatment [6, 7]. It has been indicated that salted/smoked and pickled/preserved foods rich in salt, nitrites and preformed nitroso compounds were associated with an increased risk of gastric cancer [8]. Additionally, the extracts of raw and pickled vegetables and fruits, namely garlic,

cabbage, shallot, mushroom, cucumber, ginger, Chinese mustard, bamboo shoot and mango exhibited direct acting mutagenicity on *Salmonella Typhimurium* assays with nitrite in the absence of metabolic activation [46].

Antimutagenic study using 1-Aminopyrene

1-Aminopyrene is a derivative of 1-nitropyrene in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. 1-nitropyrene is generally a product of incomplete combustion and is the predominant nitro-polycyclic hydrocarbon (nitro-PAH) emitted in diesel exhaust, exhaust of kerosene heaters, petroleum gas burners and some of food products. This is a result of incomplete combustion or pyrolysis of fat in meat produced pyrene and NO_2 from burning of cooking gas during barbecuing [47-49]. This is the most primary route of potential human exposure to 1-nitropyrene is inhalation.

1-Aminopyrene has been known to be non-mutagenic when it is tested without metabolic activation [50]. However it was demonstrated that aminopyrene treated with nitrite at pH 3.0 and 37 ^{0}C showed mutagenicity on *Salmonella typhimurium* TA98 and TA100 without metabolic activation [51]. The result agreed with the work of Kangsadalampai and Suharittamrong [6] which showed that nitrite treated with 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene toward *Salmonella typhimurium* both strains, TA98 (frameshift mutation) and TA100 (base-pair substitution mutation) in the absence of metabolic activation. The mutations appear to be due to the presence of nitroreductase [52] and O-acetyltransferase [14, 53-54] which are the two activating systems presented in bacterial cells for nitrite treated aminopyrene (supposed to be 1-nitropyrene). Such enzymes metabolize 1-nitropyrene to be arylhydroxylamine, which is active to interact with DNA. Evidence has been shown that 1-nitropyrene induced tumors in experimental animals [55-57]. Thus, the mutagenicity of 1-aminopyrene and nitrite in acid conditions has been established as a model for antimutagenicity studies of some chemicals during stomach digestion [58].

CHAPTER III

MATERIALS AND METHODS

Chemicals

1. Magnesium sulfate ($MgSO_4 7H_2O$) (Ajax Finechem Pty Ltd, Australia)
2. Cirtric acid monohydrate (BDH Prolabo chemicals, England)
3. Potassium phosphate dibasic(anhydrous) (Ajax Finechem Pty Ltd, Australia)
4. Sodium ammonium phosphate Tetrahydrate (Fluka Chemika, Switzerland)
5. Bacto agar, 40%glucose (Merck, Damstadt, Germany)
6. Oxoid nutrient broth No.2 (Himedia Laboratories. Pvt. Ltd., India)
7. Sodium chloride (NaCl) (Mallinckrodt® Laboratory Chemicals, USA.)
8. L-histidine HCl (Fluka Chemika, Switzerland)
9. Biotin (Sigma Chamical, St Louis, USA.)
10. Sodium dihydrogen phosphate (NaH_2PO_4) (Sigma Chamical, St Louis, U.S.A.)
11. Disodium hydrogen phosphate dehydrate (BDH Prolabo chemicals, England)
12. Potassium chloride (KCl) (Ajax Finechem Pty Ltd, Australia)
13. Sodium nitrite (Ajax Finechem Pty Ltd, Australia)
14. Ammonium sulfamate (Fluka Chemika, Switzerland)
15. Conc. Hydrochloric acid (Mallinckrodt® Laboratory Chemicals, USA.)
16. Acetonitrile (J. T. Baker, Phillipsburg, USA.)
17. 1-Aminopyrenr (Aldrich, St. Louis, USA.)

Instrumentations

1. Filter paper grade 4 (Whatman, Kent, United Kingdom)
2. Antibiotic assay Disc, 6 mm (Whatman Kent United Kingdom)
3. Spectrophotometer (T60, PG Instruments Ltd., United Kingdom)
4. Rotary Evaporation (Buchi R210, Switzerland)
5. Autoclave (ALP Co., Ltd., Japan)
6. Hot air oven (WTB binder No.4940006, Germany)
7. Incubator (Memmert, Germany)
8. Lyophilizer (Labconco, Missouri, USA.)

Sample preparation

The characteristics of nine selected Thai ancient remedies used in this study are shown in Table 11 All selected Thai ancient remedies were exhaustively extracted with ethanol and water respectively. The ethanol extract was evaporated *in vacuo.* The marc was dried and then exhaustively extracted with boiling water. The water extract was dried using lyophilizer.

Table 11 Selected Thai ancient remedies in this study.

Remedy	Thai name	Property
Chantaleela	จันทลีลา	Relief of fever
Prasachandang	ประสะจันทน์แดง	Relief of fever
Keawhom	เขียวหอม	Relief of fever
Homtip-osot	หอมทิพโอสถ	Relief dizziness
Ummalukkawatee	อัมฤควาที	Reduce cough
Prasamawaeng	ประสะมะแว้ง	Reduce cough
Wisam-payayai	วิสัมพยาใหญ่	Relief of excess gas
Treehom	ตรีหอม	Relief of laxative
Thoraneesantakat	ธรณีสัณฑฆาต	Relief of laxative

Bacterial tester strain [3, 5]

Salmonella typhimurium tester strains used in this study were dependent strains TA98 and TA100 which are able to detect frameshift mutation and base-pair substitution respectively. These strains are kindly provided by Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Cultures are stored at -70^{0}C. Each culture is inoculated in Oxoid nutrient broth No.2 overnight at 37 °C in a shaking water bath before used.

Nutrient agar preparation

Preparation of minimal agar plates

Minimal agar containing 1.5% Bacto agar was autoclaved at 121°C 20 minutes and then mixed with 2% sterile glucose and Vogel-Bonner medium E stock salt solution (VB salt) (see in appendix B). Approximately 30 ml of molten agar was poured into the sterile Petri dish. It was left until solidify and stored at 37°C in the incubator for 48 hours before using.

Preparation of top agar

Top agar containing 0.6% Bacto agar and 0.5% sodium chloride was autoclaved at 121°C 20 minutes. 10% (v/v) of a sterile solution of 0.5 mM histidine and biotin were added to the molten top agar, then, was maintained at 45°C in the water bath.

Spontaneous control values [38, 59]

Each tester strain has a characteristic spontaneous mutant frequency. There was uaually some day-to-day and laboratory-to-laboratory variation in the number of spontaneous revertant colonies. Choice of solvent may also affect the spontaneous revertant frequency. Spontaneous control values are used for calculated mutagenic index (MI), by compared with the test plates.

Mutagenicity of Thai ancient remedies extracts without nitrite treatment

Ethanol extracts [12, 13, 16, 60-62]

An aliquot (0, 25, 50, 100 and 200 µl) of selected Thai ancient remedy extract (200 mg/ml in DMSO) was added to the tube containing DMSO to obtain the final volume of 200 µl. Add 650 µl of 0.2N HCl to acidify the reaction mixture to pH 3.0 – 3.5 and add 250 µl of DMSO to the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37^{o}C for 4 hr. Stop reaction for 1 min in an ice bath. DMSO (250 µl) was added to the tube, mixed well, and the whole was allowed to stand for 10 min in an ice bath before using the Ames test. Each sample was assayed using triplicate plate. It was shown in Figure 1.

Water extracts [12, 13, 16, 60-62]

An aliquot (0, 25, 50, 100 and 200 µl) of selected Thai ancient remedy extract (200 mg/ml in distilled water) was added to the tube containing distill water to obtain the final volume of 200 µl. Add 650 µl of 0.2N HCl to acidify the reaction mixture to pH 3.0 – 3.5 and add 250 µl of distill water to the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37^{o}C for 4 hr. Stop reaction for 1 min in an ice bath. Water (250 µl) was added to the tube, mixed well, and the whole was allowed to stand for 10 min in an ice bath before using the Ames test. Each sample was assayed using triplicate plate. It was shown in Figure 1.

Mutagenicity of Thai ancient remedies extracts with nitrite treatment

Ethanol extracts [12, 13, 16, 60-62]

An aliquot (0, 25, 50, 100 and 200 µl) of selected Thai ancient remedy extract (200 mg/ml in DMSO) was added to the tube containing DMSO to obtain the final volume of 200ml. Add 650 of 0.2N HCl to acidify the reaction mixture to pH 3.0 – 3.5 and add 250 µl of $NaNO_2$ to the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37^{o}C for 4 hr. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Then, 250 µl ammonium sulfamate was added to the reaction mixture and then the reaction tube will be immersed in an ice bath for 10 min. The mixture was determined for it mutagenicity using the Ames test protocol. Each sample was assayed using triplicate plate.

Water extracts [12, 13, 16, 60-62]

An aliquot (0, 25, 50, 100 and 200 µl) of selected Thai ancient remedy extract (200 mg/ml in distilled water) was added to the tube containing of distill water to obtain the final volume of 200ml. Add 650 of 0.2N HCl to acidify the reaction mixture to pH 3.0 – 3.5 and add 250 µl of $NaNO_2$ to the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37 ^{o}C for 4 hr. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Then, 250 µl ammonium sulfamate was added to the reaction mixture and then the reaction tube will be immersed in an ice bath for 10 min. The mixture was determined for it mutagenicity using the Ames test protocol. Each sample was assayed using triplicate plate.

Ames test protocol for mutagenicity [12-16]

Mix 100 µl of preparation sample with 500 µl of 0.5M phosphate buffer (pH 7.4), add 100 µl of each tester strain (*Salmonella typhimurium* TA 98 and TA 100) and incubate at 37 ^{o}C in shaking water bath for 20 min. After incubation, add 2 ml of top agar containing 0.5 mM L-histidine and 0.5mM biotin, mix well and pour onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37^{o}C in darkness for 48 hr, then count number of His^{+} revertant colonies. Each sample was assayed using triplicate plate.

Standard direct mutagens [63-67] [52-56]

Ten microlitres (for testing on *Salmonella typhimurium* TA 98) or 20 µl (for testing on *Salmonella typhimurium* TA 100) of 1-aminopyrine (0.075 mg/ml) in a tube fitted with a plastic stopper was mixed with 550 µl of 0.2N hydrochloric acid (sufficient to acidify the reaction mixture to pH3-3.5) then 250µl of 2 M sodium nitrite was added to the reaction mixture. The reaction tube was shaken at 37^{o}C for 4 h and the reaction was stopped by placing the tube in an ice bath for 1 min. Two hundred and fifty microlitres of 2M ammonium sulfamate was added to the tube mix well, and the whole was allowed to stand for 10 min in an ice bath. It was shown in Figure 2.

Effect of the extracts of Thai ancient remedies on the standard mutagen

Ethanol extracts

Twenty five microlitres of the mixture above was mixed with 500 µl of 0.5M phosphate buffer (pH 7.4), add 100 µl of each tester strain (*Salmonella typhimurium* TA98 and TA100). An aliquot (0, 25, 50 and 75 µl) of Thai ancient remedies extract (200 mg/ml in DMSO) was added and the final volume was adjusted to 700 µl with DMSO. The mixture was incubated at 37^{o}C in a shaking water bath for 20 min. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5mM D-biotin was added, mix well and pour onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37^{o}C in darkness for 48 hr, then count number of His^{+} revertant colonies. Each sample was assayed using triplicate plate.

Water extracts

Twenty five microlitres of the mixture above was mixed with 500 µl of 0.5M phosphate buffer (pH 7.4), add 100 µl of each tester strain (*Salmonella typhimurium* TA98 and TA100). An aliquot (0, 25, 50 and 75 µl) of Thai ancient remedies extract (200 mg/ml in distilled water) was added and the final volume was adjusted to 700 µl with distilled water. The mixture was incubated at 37^{o}C in a shaking water bath for 20 min. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5mM D-biotin was added, mix well and pour onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37^{o}C in darkness for 48 hr, then count number of *His*$^{+}$ revertant colonies. Each sample was assayed using triplicate plate.

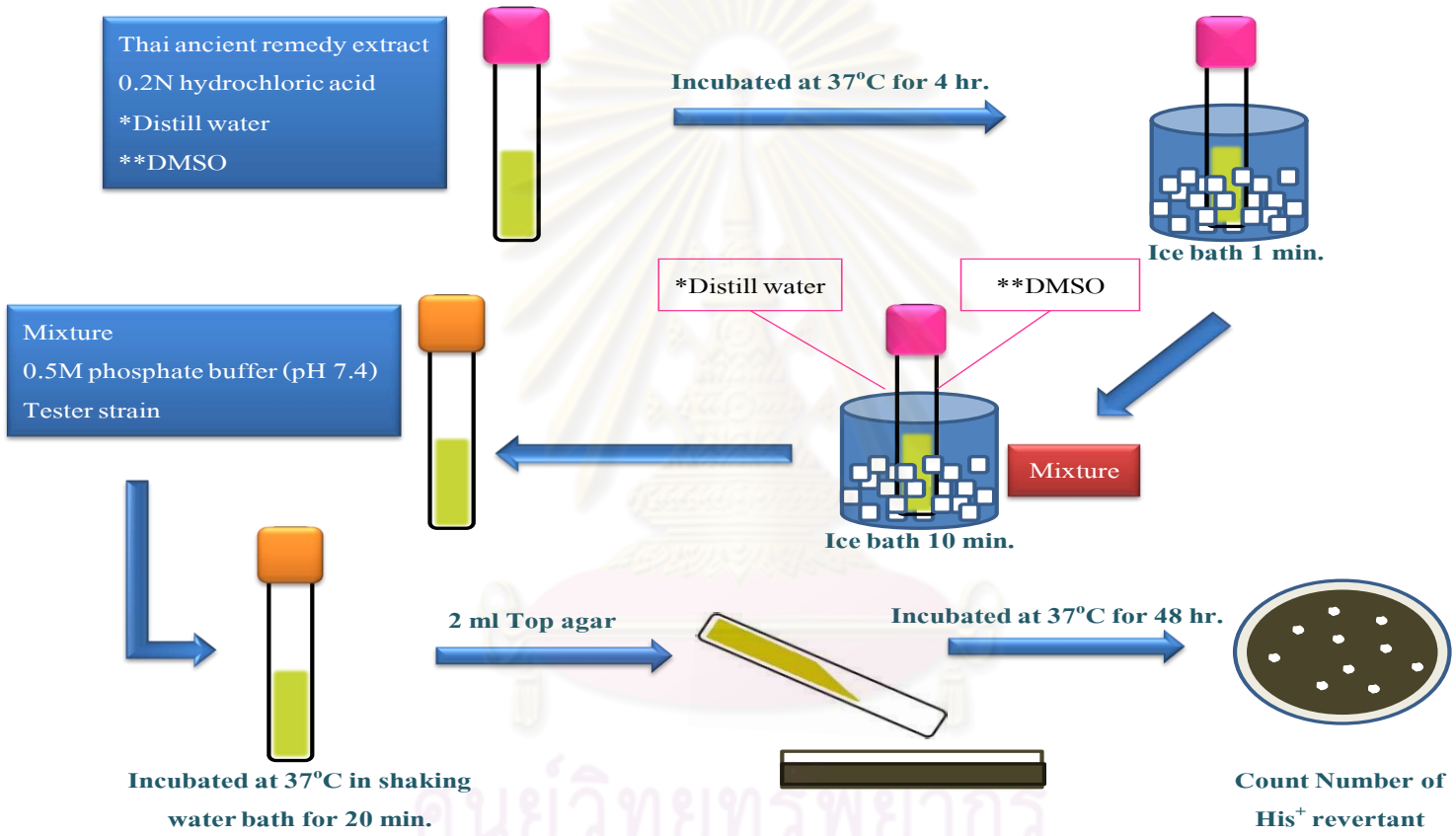

Figure 2 Step to determine the mutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

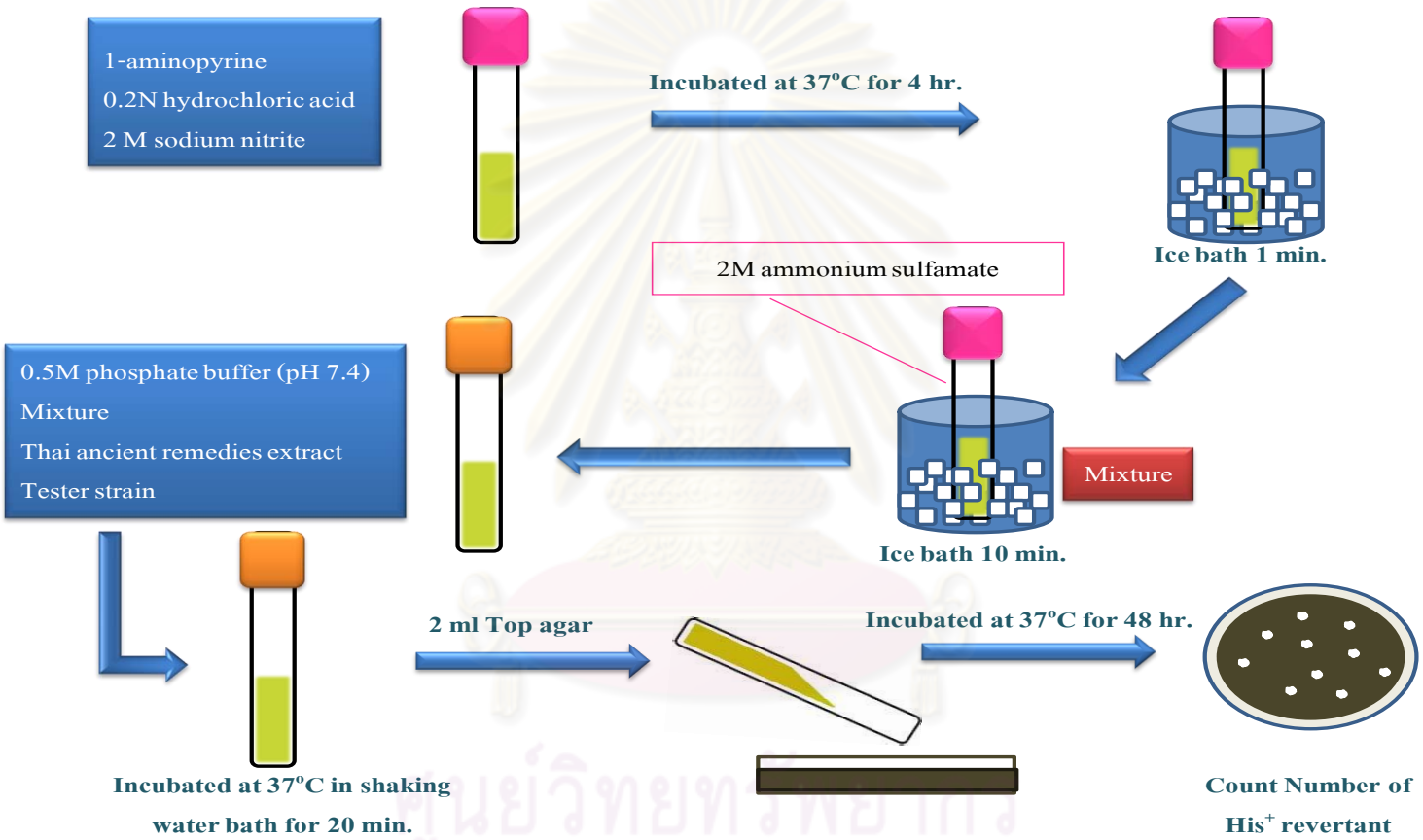

Figure 3 Step to determine the antimutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

Data analysis

Ames test

Mutagenicity index and a percentage of modification are calculated as suggested by calomme as following [68]

Mutagenicity index:

$$MI = \frac{\text{Average(N)}}{\text{Average (S)}}$$ *(Equation 1)*

N = a number of histidine revertants per plate of the sample

S = a number of spontaneous revertants per plate of the negative control (the tube without Thai ancient remedy extract)

The mutagenicity of sample is determined by number of histidine revertants with at least one concentration higher than 2 times of spontaneous revertants.

***A percentage of modification*:**

$$\% \text{ modification} = 100 \times \left(\frac{A - B}{A - C}\right)$$ *(Equation 2)*

A = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP)

B = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP) in the present of selected Thai ancient remedy extract

C = a number of spontaneous revertants (negative control)

From the equation 1, the inhibition of mutagenicity may be classified into four levels as shown in table 12A

Table 12A Criteria of evaluation as the inhibition of mutagenicity

% modification	inhibition
more than 60%	strongly inhibition
41 - 60%	moderately inhibition
21 - 40%	weakly inhibition
0 - 20%	negligible inhibition

From the equation 2, the enhancement of mutagenicity may be classified into four levels as shown in table 12B

Table 12B Criteria of evaluation as the enhancement of mutagenicity

% modification	enhancement
0 to - 20%	negligible enhancement
-40 to - 21%	weakly enhancement
-60 to - 41%	moderately enhancement
more than -60%	strongly enhancement

CHAPTER IV

RESULTS

Sample preparation

Nine selected of Thai ancient remedies for instance, Chantaleela, Prasa-chandang, Keaw-hom, Tree-hom, Ummalukkawatee, Prasa-mawaeng, Wisam-payayai, Thoraneesantakat and Homtip-osot were successively extracted with ethanol and water respectively. Then, they were explained for their mutagenic and antimutagenic effect by Ames test.

The physical characteristic of each extract in item of color is described in Table13 and the percent yields of each dried extract of Thai ancient remedy extracts are shown in Table 14.

Table 13 The color of Thai ancient remedies extracts.

Remedy	Ethanol extract	Water extract
Chantaleela	Brown	Brown
Homtip-osot	Yellow	Yellow
Keaw-hom	Brown	Brown
Prasachandang	Red	Red
Prasamawang	Yellow	Yellow
Treehom	Brown	Brown
Thoraneesantakad	Brown	Brown
Ummalukkavatee	Yellow	Yellow
Wisampayayai	Yellow	Yellow

Table 14 Percent yield of Thai ancient remedy extracts.

Remedy	Yield (%)	
	Ethanol extract	Water extract
Chantaleela	13.8	12.5
Homtip-osot	29.9	12.1
Keaw-hom	17.3	16.8
Prasachandang	28.2	12.9
Prasamawang	23.1	12.8
Treehom	35.5	14.6
Ummalukkavatee	22.1	24.7
Thoraneesantakad	29.1	13.9
Wisampayayai	15.6	12.9

Mutagenicity of Thai ancient remedies extracts in Ames test

Mutagenicity of Thai ancient remedies extracts without nitrite treatment

Figure 4A and 4B showed mutagenic Index obtained from each concentration of Thai ancient remedies extracts toward S. typhimurium TA98 and TA 100 respectively. Most of them were not directly mutagenic except the ethanolic extract of Treehom which exhibited mutagenicity. The mutagenic indies of ethanolic Treehom extracts were 3.64 and 2.21 on TA98 and TA100 respectively.

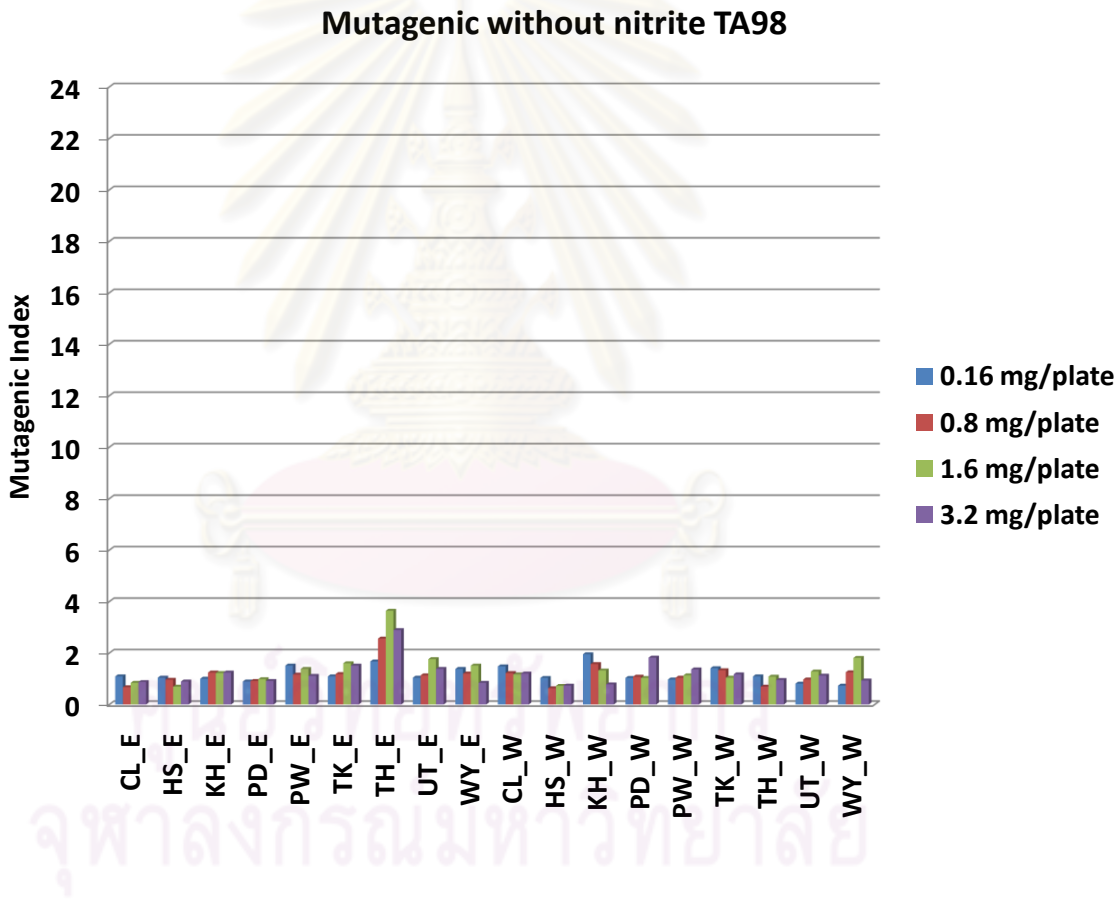

Figure4A: Mutagenic index without nitrite effect among selected Thai ancient remedies extracts on *S. typhimurium* strains TA98. Each value represents as the mutagenic index (MI).Abbreviations including E: ethanol extract, W: water extract, CL: Chantaleela, HS: Hoptiposot, KH: Keawhom, PD: Prasachandang, PW: Prasamawarng, TK: Thoraneesantakat, TH: Treehom, UT: Ummalukkawatee, WY: Wisampayayai.

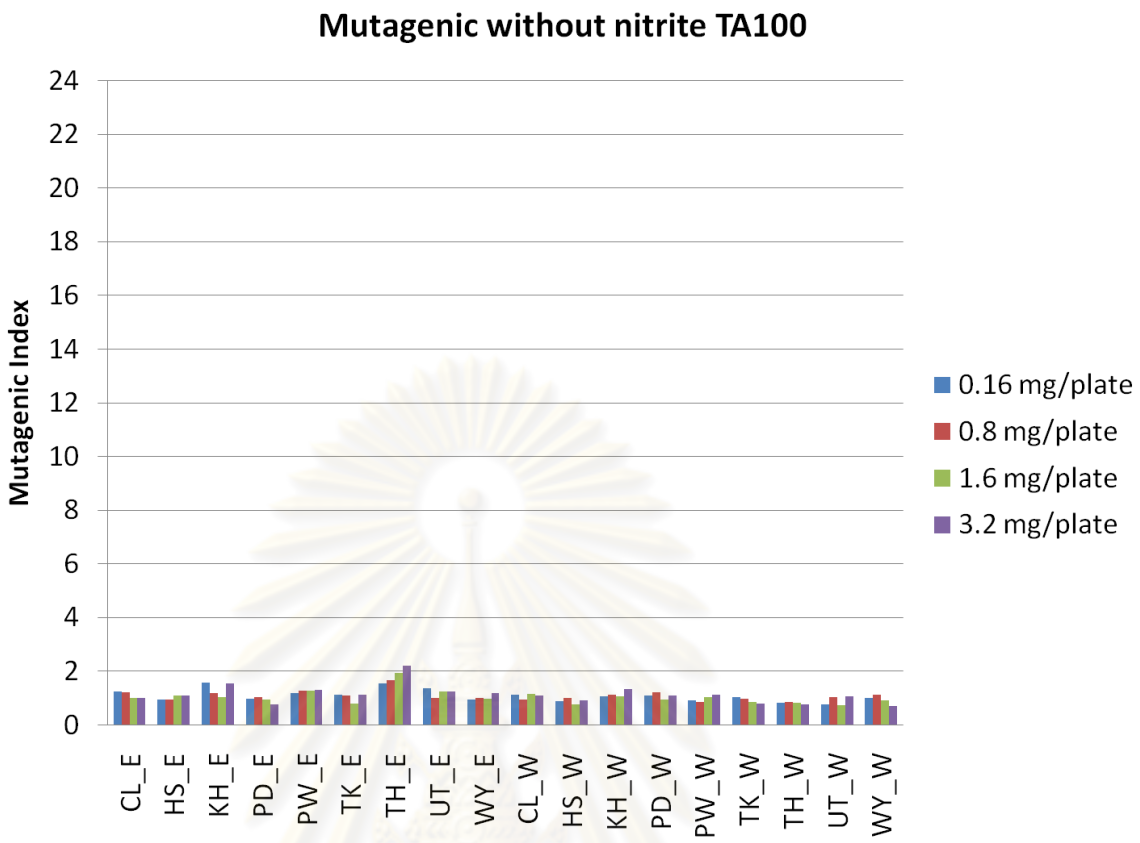

Figure4B: Mutagenic index without nitrite effect among selected Thai ancient remedies extracts on *S. typhimurium* strains TA100. Each value represents as the mutagenic index (MI). Abbreviations including E: ethanol extract, W: water extract, CL: Chantaleela, HS: Hoptiposot, KH: Keawhom, PD: Prasachandang, PW: Prasamawarng, TK: Thoraneesantakat, TH: Treehom, UT: Ummalukkawatee, WY: Wisampayayai.

Mutagenicity of Thai ancient remedies extracts with nitrite treatment

Figure 5A and 5B showed mutagenic Index obtained from each concentration of Thai ancient remedies extracts toward S. typhimurium TA98 and TA 100 respectively. All extracts showed the mutagenicity against both strains. It was demonstrated that the ethanolic and water extracts of Thoraneesantakat showed the highest mutagenic index of 23.8 and 13.8 on TA98 and TA100 respectively.

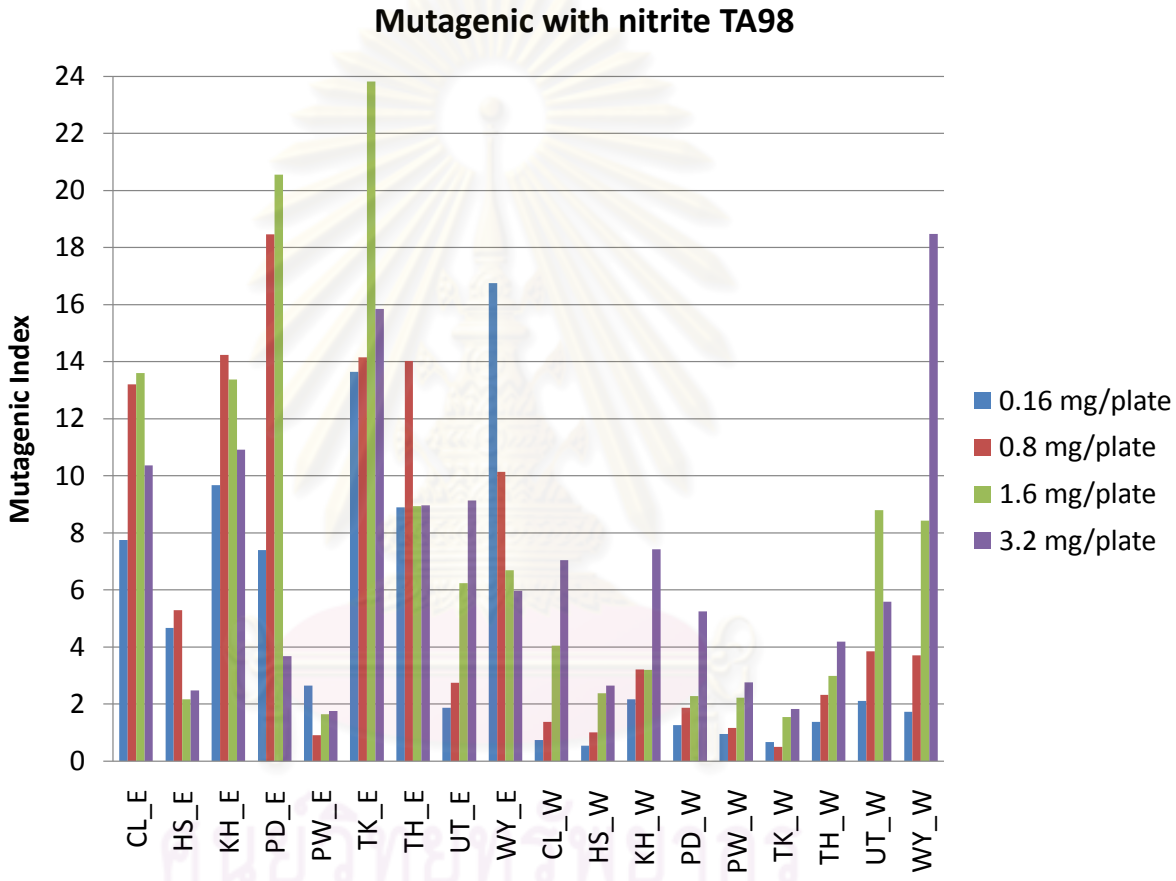

Figure5A: Mutagenic index with nitrite effect among selected Thai ancient remedies extracts on *S. typhimurium* strains TA98. Each value represents as the mutagenic index (MI) Abbreviations including E: ethanol extract, W: water extract, CL: Chantaleela, HS: Hoptiposot, KH: Keawhom, PD: Prasachandang, PW: Prasamawarng, TK: Thoraneesantakat, TH: Treehom, UT: Ummalukkawatee, WY: Wisampayayai.

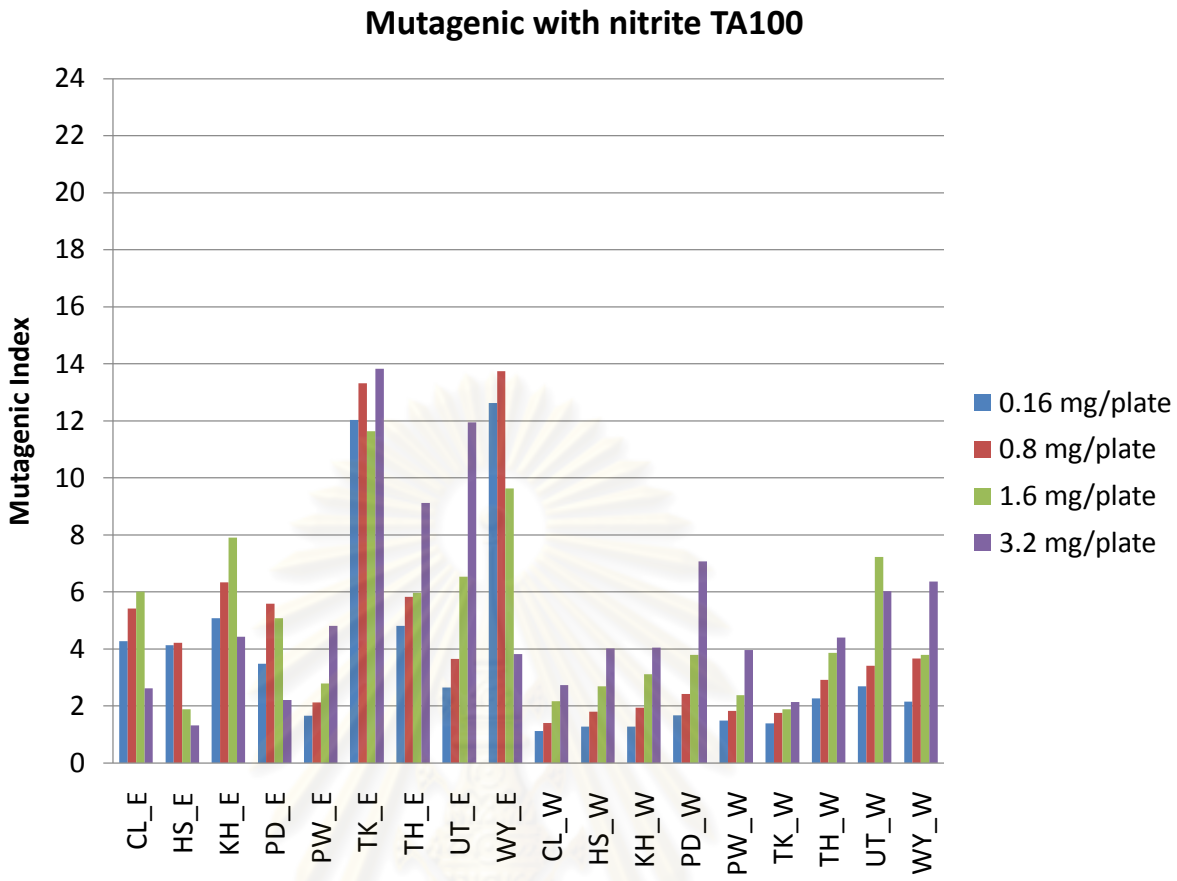

Figure5B: Mutagenic index without nitrite effect among selected Thai ancient remedies extracts on *S. typhimurium* strains TA100. Each value represents as the mutagenic index (MI) Abbreviations including E: ethanol extract, W: water extract, CL: Chantaleela, HS: Hoptiposot, KH: Keawhom, PD: Prasachandang, PW: Prasamawarng, TK: Thoraneesantakat, TH: Treehom, UT: Ummalukkawatee, WY: Wisampayayai.

Antimutagenicity of Thai ancient remedies extracts in Ames test

Modification effect of Thai ancient remedies extracts on the mutagenicity of sodium nitrite treated 1-aminopyrene-nitrite expressed as percent modification of number of revertants of Salmonella typhimurium strain TA98 and TA100 without metabolic activation is shown in Table 15 and 16, respectively.

Ethanol extracts of all kinds of Thai ancient remedies inhibited the mutagenicity of the product of the reaction mixture of 1-aminopyrene nitrite model in the absence of metabolic activation on *Salmonella typhimurium* TA98.They ranged from weakly (21-40%) to strongly active (more than 60%). In addition, it was found that on *Salmonella typhimurium* TA100 except for Keaw-hom, Prasa-chandang and Prasa-mawaeng, their ranged from (-40 to -21%) to strongly active (more than -60%). The results showed ethanolic of Wisampayayai remedy (10mg/plate) showed the highest antimutagenicity on TA98 (155%) and ethanolic of Chantaleela remedy (15mg/plate) showed the highest antimutagenicity on TA100 (107%).

Most of water extracts showed negligible to strongly inhibition effects on both tester strains. *Salmonella typhimurium* TA98 was found that it was enhanced from Chantaleela and Thoraneesantakat. For *Salmonella typhimurium* TA100 was found that it was enhanced from Homtip-osot, Thoraneesantakat and Wisampayayai. They ranged from (-40 to -21%) to strongly active (more than -60%). The results shown water extract of Ummaluk-kawatee remedy (15mg/plate) showed the highest antimutagenicity on TA98 (72%) and water extract of Treehom remedy (15mg/plate) showed the highest antimutagenicity on TA100 (66%).

Table 15 Modification effect of the Thai ancient remedies extracts on the mutagenicity of sodium nitrite treated 1-aminopyrene-nitrite expressed as percent modification of number of revertants of *Salmonella typhimurium* strain TA98 without metabolic activation

Sample	Amount of extracts (mg/plate)	Ethanol extract			Water extract		
		Number.of revertants/ plate[a]	% Modification		Number.of revertants/ plate	% Modification	
			Inh	Enh		Inh	Enh
Chantaleela							
-Negative control	0	14±1			27±13		
-Positive control	0	408±18			925±50		
	5	155±26	**64 (s)**	-	1227±188	-	**30 (w)**
	10	94±7	**80 (s)**	-	1290±366	-	**37 (w)**
	15	79±6	**83 (s)**	-	1183±472	-	**25 (w)**
Hoptip-osot							
-Negative control	0	14±1			27±13		
-Positive control	0	408±18			925±50		
	5	187±31	**56 (m)**	-	815±154	**16 (n)**	-
	10	85±7	**82 (s)**	-	482±102	**53 (m)**	-
	15	51±5	**91 (s)**	-	316±36	**71 (s)**	-
Keaw-hom							
-Negative control	0	14±1			27±13		
-Positive control	0	408±18			925±50		
	5	201±21	**53 (m)**	-	672±24	**32 (w)**	-
	10	118±5	**74 (s)**	-	780±112	**20 (n)**	-
	15	85±6	**82 (s)**	-	879±134	**9 (n)**	-
Prasa-chandang							
-Negative control	0	14±1			27±13		
-Positive control	0	408±18			925±50		
	5	115±25	**74 (s)**	-	938±100	**2 (n)**	-
	10	32±16	**95 (s)**	-	1213±328	-	**28 (w)**
	15	225±38	**47 (w)**	-	865±179	**10 (n)**	-

Table 15 Modification effect of the Thai ancient remedies extracts on the mutagenicity of sodium nitrite treated 1-aminopyrene-nitrite expressed as percent modification of number of revertants of *Salmonella typhimurium* strain TA98 without metabolic activation (Cont.)

Sample	Amount of extracts (mg/plate)	Ethanol extract			Water extract		
		Number.of revertants/ plate[a]	% Modification		Number.of revertants/ plate	% Modification	
			Inh	Enh		Inh	Enh
Prasa-mawarng							
-Negative control	0	45±15			21±5		
-Positive control	0	695±51			963±117		
	5	310±63	**25 (w)**	**-**	880±47	**9 (n)**	-
	10	270±23	**35 (w)**	**-**	736±167	**25 (w)**	-
	15	245±13	**41 (m)**	**-**	590±115	**41 (m)**	-
Thoraneesantakat							
-Negative control	0	45±15			21±5		
-Positive control	0	695±51			963±117		
	5	74±18	**94 (s)**	-	907±42	**6 (n)**	-
	10	50±14	**99 (s)**	-	660±223	**32 (w)**	-
	15	53±1	**99 (s)**	-	977±91	**-**	**1 (n)**
Treehom							
-Negative control	0	45±15			21±5		
-Positive control	0	695±51			963±117		
	5	149±25	**80 (s)**	-	460±138	**54 (m)**	-
	10	102±14	**89 (s)**	-	565±103	**42 (m)**	-
	15	57±8	**98 (s)**	-	496±81	**50 (m)**	-
Ummaluk-kawatee							
-Negative control	0	45±15			21±5		
-Positive control	0	695±51			963±117		
	5	128±22	**84 (s)**	-	405±82	**60 (s)**	-
	10	132±27	**83 (s)**	-	289±32	**72 (s)**	-
	15	81±10	**93 (s)**	-	288±56	**72 (s)**	-
Wisampayayai							
-Negative control	0	45±15			21±5		
-Positive control	0	695±51			963±117		
	5	35±102	**102 (s)**	-	411±79	**59 (s)**	-
	10	15±106	**106 (s)**	-	370±71	**63 (s)**	-
	15	8±107	**107 (s)**	-	341±35	**66 (s)**	-

[a] mean±SD of His^+ revertants per plate of independent experiment (N = 3). Antimutagenic potential: (n) = negligible, (w) = weak, (m) = moderate, (s) = strong, Inh = Inhibition, Enh = Enhancement

Table 16 Modification effect of the Thai ancient remedies extracts on the mutagenicity of sodium nitrite treated 1-aminopyrene-nitrite expressed as percent modification of number of revertants of *Salmonella typhimurium* strain TA100 without metabolic activation

sample	Amount of extracts (mg/plate)	Ethanol extract			Water extract		
		No.of revertants/ plate[a]	% Modification		Number.of revertants/ plate	% Modification	
			Inh	Enh		Inh	Enh
Chantaleela							
-Negative control	0	146±23			130±16		
-Positive control	0	204±19			473±31		
	5	149±43	**95 (s)**	-	373±84	**29 (w)**	**-**
	10	114±34	**155 (s)**	-	472±51	**0 (n)**	**-**
	15	173±14	**53 (m)**	-	468±46	**1 (n)**	**-**
Hoptip-osot							
-Negative control	0	146±23			130±16		
-Positive control	0	204±19			473±31		
	5	199±27	**8 (n)**	-	525±54	**-**	**15 (n)**
	10	152±21	**90 (s)**	-	521±16	**-**	**14 (n)**
	15	118±15	**148 (s)**	-	389±43	**24 (w)**	**-**
Keaw-hom							
-Negative control	0	146±23			130±16		
-Positive control	0	204±19			473±31		
	5	225±21	**-**	**36** (w)	424±21	**14 (n)**	**-**
	10	154±42	**86 (s)**	-	344±43	**38 (w)**	**-**
	15	135±27	**118 (s)**	-	363±24	**32 (w)**	**-**
Prasa-chandang							
-Negative control	0	146±23			130±16		
-Positive control	0	204±19			473±31		
	5	132±47	**124 (s)**	-	317±46	**46 (m)**	**-**
	10	152±9	**90 (s)**	-	363±61	**32 (w)**	**-**
	15	256±59	**-**	**89 (s)**	384±71	**26 (w)**	**-**

Table 16 Modification effect of the Thai ancient remedies extracts on the mutagenicity of sodium nitrite treated 1-aminopyrene-nitrite expressed as percent modification of number of revertants of *Salmonella typhimurium* strain TA100 without metabolic activation (Cont.)

sample	Amount of extracts (mg/plate)	Ethanol extract			Water extract		
		No.of revertants /plate[a]	% Modification		No.of revertants /plate[a]	% Modification	
			Inh	Enh		Inh	Enh
Prasa-mawarng							
-Negative control	0	140±21			141±21		
-Positive control	0	321±9			498±61		
	5	296±30	-	**159 (s)**	373±56	**29 (w)**	-
	10	275±65	-	**122 (s)**	412±54	**18 (n)**	-
	15	252±33	-	**83 (s)**	416±90	**17 (n)**	-
Thoraneesantakat							
-Negative control	0	140±21			141±21		
-Positive control	0	321±9			498±61		
	5	195±43	**70 (s)**	-	569±49	-	**20 (n)**
	10	126±40	**108 (s)**	-	349±38	**42** (m)	-
	15	143±6	**98 (s)**	-	582±39	-	**23 (w)**
Treehom							
-Negative control	0	140±21			141±21		
-Positive control	0	321±9			498±61		
	5	194±11	**70 (s)**	-	436±71	**17 (n)**	-
	10	165±10	**86 (s)**	-	448±32	**14 (n)**	-
	15	153±17	**93 (s)**	-	264±34	**66 (s)**	-
Ummaluk-kawatee							
-Negative control	0	140±21			141±21		
-Positive control	0	321±9			498±61		
	5	201±22	**66 (s)**	-	311±45	**52 (m)**	-
	10	198±24	**68 (s)**	-	267±36	**65 (s)**	-
	15	178±7	**79 (s)**	-	286±45	**59 (m)**	-
Wisampayayai							
-Negative control	0	140±21			141±21		
-Positive control	0	321±9			498±61		
	5	125±9	**108 (s)**	-	838±121	-	**95 (s)**
	10	80±14	**133 (s)**	-	802±85	-	**85 (s)**
	15	105±7	**120 (s)**	-	536±168	-	**11 (n)**

[a] mean±SD of His^+ revertants per plate of independent experiment (N=3). Antimutagenic potential: (n) = negligible, (w) = weak, (m) = moderate, (s) = strong, Inh = Inhibition, Enh = Enhancement

CHAPTER V

DISCUSSION AND CONCLUSION

The mutagenic and antimutagenic potential of ethanol and water extracts of selected Thai ancient remedies, namely Chantaleela, Prasachandang, Keaw-hom, Tree-hom, Ummalukkawatee, Prasamawaeng, Wisampayayai, Thoraneesantakat and Homtip-osot, were studied using Ames test toward *S. typhimurium* TA98 and TA100 under acidic condition (pH 3.0-3.5) without metabolic activation. All of the plants are commonly used as therapeutic agents for treating a variety of human diseases. Mutagenicity trial has been used by different laboratories all over the world to study the mutagenicity of complex biological mixtures including medicinal herbs [69-71].

Mutagenicity of Thai ancient remedies extracts

Most of water and ethanol extracts from the Thai ancient remedies in this study were not mutagenic on both *S.typhimurium* TA98 and TA100 in the absence of metabolic activating system except the ethanol extract of Treehom remedies (1.6 and 3.2 mg/plate) on TA98 and TA100 respectively. The results were in accordance with the experiment of Horn that the water extracts of three plant species, *Vitex montevidensis*, *Gochnatia cordata* and *G. polymorpha* were not mutagenic on framesshift mutations (TA98) and base-pair substiution (TA100) in the absence of metabolic activating system [72]. However, when the Thai ancient remedies extracts were treated with nitrite, sample of the ethanol and water extracts were mutagenic on both strains TA98 and TA100. The previous experiment of Higashimoto demonstrated that the methanol and water extracts of medicinal plant treated with sodium nitrite exhibited the mutagenicity [73]. Tongyonk also reported that ethanol and water extracts of Ya-ris-si-duang-mahakal remedy had mutagenic activity when treated with nitrite [74]. The finding that selected Thai ancient remedies showed genotoxicity by Ames test after treating with nitrite implied that some chemical component in the remedies could react with nitrite under acidic condition to form *N-nitroso* typed mutagenic compounds. The results in this study were in accordance with the experiment of Kangsadalampai that the ethanolic and hexane extracts of Chantaleela, Prasama-waeng, Keaw-hom and Treehom, treated with sodium nitrite, exhibited the mutagenicity. Therefore co-administration of the remedies with nitrite-

containing food should be avoided. Nevertheless the mutagenic assay in this study was performed in the absence of metabolic activation. Hepatic metabolizing enzymes could modulate biotransformation of chemicals and affect on increasing or decreasing of chemical toxicity *in vivo*. In the condition with rat liver enzyme (S9 mix) in Ames system, the mutagenicity of medicinal herbs and remedies might to either diminished or enhanced [75, 76].

Modifying effect of the extracts of Thai ancient remedies on the mutagenicity of the reaction product of 1- aminoprrene-nitrite model

The reaction between nitrite and dietary amines and amides under the stomach pH could lead to the formation of nitrosated products which possible to develop gastric cancer in human [77]. It is well known that ingredients in diet including herbs, fruits and seeds may exert anticarcinogenic and antimutagenic activities [78-80]. The competence of the extracts of Thai ancient remedies to inhibit mutagenic reaction induced by the product of the reaction mixture of 1- aminopyrene nitrite model on *S.typhimurium* TA98 and TA100 was demonstrated. The result indicated that most of the extracts of selected Thai ancient remedies exhibited antimutagenicity from negligible to strongly effect through *S. typhimurium* TA98 and TA100. Only the ethanol extract of Keaw-hom, Prasa-chandang and Prasa-mawarng showed the enhancement effect with TA100. The water extracts of Chantaleela and Thoraneesantakat showed the enhancement effect with TA 98 and Homtip-osot, Thoraneesantakat and Wisampayayai showed the enhancement effect with TA100. Ethanol extracts of Wisampayayai remedy and Chantaleela remedy showed the highest antimutagenic activity on TA98 and TA100 respectively at high concentration. The results that ethanol extracts of Thai ancient remedies were highest antimutagenic against the reaction product of 1-aminopyrene treated with nitrite model was in accordance with the experiment of Wongwattanasatheun and Botting who reported that the extracts derived from low polar solvent caused high inhibition to mutagenicity than the extracts were derived from high polar solvents [17, 81].Loh *et al* performed Ames test for antimutagenic assay of aqueous and methanol extracts from *Euphorbia hirta* and found that the extracts exhibited strong antimutagenic activity only in the presence of S9 mix. The antimutagenic property of the extracts was related to the ability to modulate the metabolising enzymes, either by preventing the metabolic activation of the mutagen or by altering the enzymatic activity in the detoxification pathway of the mutagen leading to induce the disposal of the mutagen.

In addition, it was also possible that antimutagenic metabolites were generated *via* the extracts biotransformation by the metabolizing enzymes [82].

In conclusion, as well as other modern medicines, Thai ancient remedies should be concerned for nitrosation induced mutagenicity. Furture studies were required to develop clearer understanding of the mutagenic activity regarding to Thai ancient remedies especially in the presence of metabolic activation. The studies should also be performed using eukaryotic system, for example the Somatic Mutation and Recombination test on *Drosophilia Melanogaster* [83]. SMART is non-mammalian *in vivo* model representing metabolism similar to that found in mammalian cell and has been used in genotoxicity and antigenotoxicity detection of chemical substances as well as herbal extracts [84]. Ames in combination with SMART tests can provide more reliable evidences on the mutagenic and antimutagenic potential of Thai ancient remedies.

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APPENDICES

APPENDIX A

1. Preparation of stock solution and media (Maron and Ames, 1983)

Voget Bonner media E stock salt solution (VB salt)

Use: Minimal agar

Ingredient	Per liter
Warm distilled water (45˚C)	670 ml
Magnesium sulfate ($MgSO_4 7H_2O$)	10 g
Cirtric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	175 g

Add salts in the order indicated to warm water and allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Filter the solutions and then autoclave at 121˚C for 15 min.

Minimal glucose agar plate

Use: Mutagenicity assay

Ingredient	Per liter
Bacto agar	15 g
Distilled water	930 ml
VB salt	20 ml
40%glucose	50 ml

Add agar to distilled water in glass bottle. Autoclave at 121˚C for 15 min. When the solution has cooled slightly, add sterile VB salt and sterile 40% glucose. Mix and pour 30 ml into each sterile petri plant. Minimal glucose agar plates were kept in incubator at 37˚C before using. (the VB salts and 40% glucose should be autoclaved separately)

Oxoid nutrient broth No.2

Use: Growing culture

Dissolve 2.5 g of nutrient broth No.2 in 100 ml distilled water. Transfer 12 ml of nutrient broth for each 50 ml Erlenmeyer flask (covered with sterile gauze). Autoclave at 121˚C for 15 min.

Top agar

Use: Mutagenicity assay

Ingredient	100	ml
Bacto agar	0.6	g
Sodium chloride (NaCl)	0.5	g
Distilled water	100	ml

Dissolve ingredients in distilled water. Store in a glass bottle. Autoclave for 15 min at 121˚C and then add 10 ml of 0.5 mM L-histidine/biotin solution and mixed thoroughly by swirling.

0.1 M L-histidine HCl stock

Use: Fortification of minimal agar plate

Ingredient	10	ml
L-histidine HCl	0.2096	g
Distilled water	10	ml

Dissolve 0.2096 g of L-histidine HCl (MW 209.63) in 10 ml distilled water. Autoclave at 121˚C for 15 min. Store in glass bottle 4˚C.

1 mM L-histidine HCl stock

Use: Fortification of minimal agar plate

Ingredient	100	ml
0.1 M L-histidine HCl	1	ml
Distilled water	99	ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml distilled water. Autoclave at 121˚C for 15 min.

1 mM biotin stock

Use: Fortification of minimal agar plate

Ingredient	100	ml
Biotin	24.43	mg
Distilled water	100	ml

Dissolve biotin (WM 244.3) in distilled H_2O. Warm it until dissolve completely. Autoclave at 121˚C for 15 min.

0.5 mM L-histidine / biotin solution.

Use: Mutagenicity assay (add 10 ml to 100 ml of Top agar)

Ingredient	200 ml
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

Mix ang autoclave at 121˚C for 15 min.

1 M potassium chloride (KCl)

Use: Na_3PO_4-KCl buffer for mutagenicity assay

Ingredient	100 ml
Potassium chloride	7.456 g
Distilled water	100 ml

Mix and autoclave at 121˚C for 15 min.

0.5 M sodium phosphate (Na_3PO_4) pH 7.4

Use: Na_3PO_4-KCl buffer for mutagenicity assay

Ingredient

0.5 M Sodium dihydrogen phosphate (NaH_2PO_4) (MW 120)

(30 g / 500 ml)

0.5 M Disodium hydrogen phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$) (MW 177.99)

(44.5 g / 500 ml)

Dissolve 44.5 g disodium hydrogen phosphate dehydrate in 300 ml of distilled water. Add 0.5 M sodium dihydrogen phosphate until to pH 7.4, and then adjust volume to 500 ml. Autoclave at 121◦C for 15 min.

Na_3PO_4 – KCl buffer

Use: Mutagenicity assay

Ingredient	330 ml
0.5 M sodium phosphate pH 7.4	100 ml
1 M potassium chloride (KCl)	16.5 ml
Distilled water	213.5 ml

Autoclave at 121˚C for 15 min.

2. Recipes for Some Reagents and Test Chemicals

2 M sodium nitrite

Use: Nitrosation

Ingredient	10	ml
Sodium nitrite	1.38	g
Distilled water to	10	ml

Mix and autoclave at 121˚C for 15 min.

2 M ammonium sulfamate

Use: Reaction mixture

Ingredient	10	ml
Ammonium sulfamate	2.28	g
Distilled water to	10	ml

Dissolve ammonium sulfamate in distilled water and adjust volume. Autoclave for 15 min at 121˚C.

0.2 N Hydrochloric acid

Use: Reaction mixture

Ingredient	100	ml
Conc. Hydrochloric acid	1.66	g
Distilled water	98.34	ml

Dissolve conc. hydrochloric acid in distilled water. Store in sterile glass tube or bottle with screw caps.

0.3 mg/ml amonopyrene

Use: standard

Ingredient	1	ml
Aminopyrene	3	mg
Acetonitrile	1	ml

Dissolve aminopyrene in acetonitrile.Store in sterile vial with screw caps in the freezer. Preparation of this solution must use sterile technique.

Ingredient	1	ml
3 mg/ml aminopyrene	0.1	ml
Acetonitrile	0.9	ml

Dissolve 3 mg/ml aminopyrene in acetonitrile. Store in sterile vial with screw caps in freezer. There preparation must be used sterile technique.

8 mg/ml ampicillin solution

Ingredient	10	ml
Ampicillin sodium	80	mg
Distilled water	10	ml

0.1% crystal violet

Ingredient	10	ml
Crystal violet	10	mg
Distilled water to	10	ml

2.5 and 2.6 : Store at 4◦C in glass bottle with screw cap.

3. Procedure for Reisolation and Growing Culture

Tester strains, TA 98 and TA 100 are grown in Oxoid nutrient broth NO.2 and incubated overnight in a 37°C shaking water bath. The growth period should not exceeded 16 h. these culture are re-isolated by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 of 1 mM biotin. The plates are incubated at 37°C for 48 h. After incubation, the 4 single colonies per strain TA 98 and TA 100 are pick and grown in Oxiod nutrient broth No.2 overnight at 37°C in shaking water bath. Each culture is confirmed genotype of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 40 µl of the culture aseptically into sterile cryotubes. The tubes then transfer to a -80°C freezer.

4. Confirmimg Genotype of Tester strains

The broth cultures of TA 98 and TA 100 are used to confirm genetype in the following ways.

Histidne requirement

The his^+ character of the strains confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin.

Procedure:

Plate A: no histidine and biotin

Plate B: 0.1 ml of 1 mM bioti

Plate C: 0.3 ml of 0.1 M His-Hel

Plate D: 0.3 ml of 0.1 M His-HCI + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strains. Each plate is applied on the surface with 0.1 ml of 1 mM biotin or no application (plate B. C. D. A, respectively) Made a single streak of each strains across these plates. Four strains could be tested on the same plate Incubated at 37°C for 24 h. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

R-factor

The R-factor strains (TA 97. TA 98. TA 100 and TA 102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure: For each- tester strain. Add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.1 M histidine - HCI followed by adding 20 ml of molten top agarcontaining 0.5 mM. Mixed and poured on a minimal glucose agar plant. Rotated the plate to distribute the mixtures and allowed several minutes for agar to become firm. R- factor an *rfa* mutation (see the next section) are performed in the same plant by dividing the plate into 2 areas one for R-factor and the other for *rfa* mutation. For R-factor. commercial ampicillin disc or filter paper disc containing 8 mg/ml ampicllin is applied on the surface of the agar by using sterile forceps. The dish. is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 h. The absence of the clear zones of inhibition around the disc indicates resistance to ampicllin.

***rfa* Mutation**

Strains having the deep rough (*rfa*) character should be tested for crystal violet Sensitivity

Procedure: Pipetted 0.1 % solution of crystal violet to the sterile filter paper disc (1/4 inch) and transferred the disc to plates. seeded with bacteria (the procedure is similar to R-factor). Incubated at 37°C for 48 h. The clear zone appeared around the disc indicated the presence of the *rfa* mutation that permitted crystal violet to enter and kill bacteria.

5. Spontaneous Reverion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenic experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotropic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants form one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: 0.2 ml of sterile distilled water (solvent in the experiment) is added to cap tube. Add 0.5 ml of Na_3P0_4-KCl buffer pH 7.4 in the absence of metabolic activation, 0.1 ml of fresh overnight culture of TA 98 or TA 100, followed plates and left it to become harden. Incubated at 37 QC for 48 h. and the his + reveratant colonies were counted.

6. The Response to Standard carcinogen.

Standard carcinogens or positive carcinogen are used routinely in mutagenicity experiment to confirm the reversion property and specificity of each strain. The standard mutagen which used in these experiments is aminopyrene in the absence of metabolic activation. Tester strain which highly response to positive mutagens must be collected.

Procedure: The procedure is as described in spontaneous reversion except aminopyrene (0.06, and 0.12 µl per plate for TA 98 and TA 100, respectively) are used instead of sterile distilled water in absence S-9 mix, respectively. The characteristic of stock culture for TA 98 and TA 100 as the source of bacteria for mutagenicity is

1) Contained R-factor (pKM 101) and *rfa* mutation
2) His^+ requirement
3) Low spontaneous reversion
4) Highly response to standard carcinogen

After the characteristics of the culture was tested, the mutagenicity test was started.

APPENDIX B

Data of mutagenic test

Table 17 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation

Remedy extracts	Amount of extracts (mg/plate)	Number of His^+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Chantaleela	Spontaneous[c]	15±4	29±5		
	0.16	16±2	225±24	1.09	7.75
	0.8	10±3	383±17	0.67	13.2
	1.6	13±2	394±39	0.84	13.6
	3.2	13±3	300±44	0.87	10.4
Homtip-osot	Spontaneous[c]	15±4	29±5		
	0.16	16±6	135±21	1.04	4.66
	0.8	14±2	153±17	0.96	5.29
	1.6	10±4	63±16	0.69	2.17
	3.2	13±8	72±11	0.89	2.47
Keaw-hom	Spontaneous[c]	15±4	29±5		
	0.16	15±2	280±33	1	9.67
	0.8	19±5	413±89	1.24	14.2
	1.6	18±2	388±54	1.22	13.4
	3.2	19±3	317±28	1.24	10.9
Prasa-chandang	Spontaneous[c]	15±4	29±5		
	0.16	13±6	215±44	0.89	7.4
	0.8	14±1	536±41	0.91	18.5
	1.6	15±4	596±94	0.98	20.6
	3.2	14±3	107±35	0.91	3.68

Table 17 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His^+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Prasa-mawarng	Spontaneous[c]	15±2	24±2		
	0.16	23±9	63±25	1.51	2.68
	0.8	17±4	22±18	1.16	0.9
	1.6	21±1	39±11	1.38	1.64
	3.2	17±3	42±16	1.11	1.75
Thoraneesantakat	Spontaneous[c]	15±2	24±2		
	0.16	16±8	327±48	1.09	13.6
	0.8	18±7	340±31	1.18	14.2
	1.6	24±4	57267	1.6	23.8
	3.2	23±9	380±57	1.51	15.9
Treehom	Spontaneous[c]	15±2	24±2		
	0.16	25±10	213±43	1.67	8.89
	0.8	38±4	337±37	2.56	14
	1.6	55±5	214±56	3.64	8.93
	3.2	43±6	215±18	2.89	8.96
Ummalukkawatee	Spontaneous[c]	15±2	24±2		
	0.16	16±2	45±6	1.04	1.86
	0.8	17±1	66±61	1.13	2.74
	1.6	26±13	150±20	1.76	6.24
	3.2	21±5	219±35	1.38	9.13

Table 17 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His^+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Wisampayayai	Spontaneous[c]	15±2	24±2		
	0.16	21±3	402±70	1.38	16.8
	0.8	18±3	243±110	1.2	10.1
	1.6	23±2	160±41	1.51	6.68
	3.2	13±6	143±66	0.84	5.97

[a] mean±SD of His^+ revertants per plate of independent experiment (N=3) of each concentration of sample

[b] Mutagenic Index (MI) is calculated from the average value of a number of histidine revertants/plate of the ethanol extract of thai ancient remedies divided by that of spontaneous revertants.

[c] Spontaneous mutation

Table 18 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Chantaleela	Spontaneous[c]	97±12	136±36		
	0.16	121±12	581±30	1.25	4.27
	0.8	118±22	737±50	1.22	5.42
	1.6	98±16	817±126	1.01	6.01
	3.2	97±15	355±79	1	2.61
Hoptiposot	Spontaneous[c]	97±12	136±36		
	0.16	91±16	561±63	0.94	4.13
	0.8	92±12	572±89	0.95	4.21
	1.6	108±21	255±47	1.11	1.88
	3.2	105±28	179±39	1.09	1.32
Keawhom	Spontaneous[c]	97±12	136±36		
	0.16	153±4	689±64	1.57	5.07
	0.8	116±26	860±25	1.2	6.33
	1.6	101±27	1074±77	1.04	7.9
	3.2	150±20	602±29	1.55	4.43
Prasachandang	Spontaneous[c]	97±12	136±36		
	0.16	94±12	473±42	0.97	3.48
	0.8	100±9	759±81	1.03	5.58
	1.6	93±7	691±135	0.96	5.08
	3.2	74±4	300±21	0.76	2.2

Table 18 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Prasamawarng	Spontaneous[c]	119±20	127±112		
	0.16	141±32	185±27	1.19	1.65
	0.8	151±26	238±26	1.27	2.12
	1.6	151±31	312±36	1.27	2.75
	3.2	156±19	538±4	1.31	4.81
Thoraneesantakat,	Spontaneous[c]	119±20	127±112		
	0.16	133±24	1348±23	1.12	12
	0.8	131±14	1492±61	1.1	13.3
	1.6	96±15	1303±74	0.8	11.6
	3.2	136±31	1549±63	1.14	13.83
Treehom	Spontaneous[c]	119±20	127±112		
	0.16	185±21	538±60	1.56	4.81
	0.8	197±11	653±68	1.66	5.83
	1.6	232±38	668±42	1.95	5.93
	3.2	263±31	1021±28	2.21	9.12
Ummalukkawatee	Spontaneous[c]	119±20	127±112		
	0.16	164±6	297±20	1.38	2.65
	0.8	119±11	409±25	1	3.65
	1.6	150±11	732±14	1.26	6.53
	3.2	149±16	1338±96	1.25	11.9

Table 18 Mutagenicity of the ethanol extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Wisampayayai	Spontaneous[c]	119±20	127±112		
	0.16	114±37	1414±53	0.96	12.6
	0.8	119±21	1539±11	1	13.7
	1.6	116±18	1085±83	0.97	9.68
	3.2	140±13	427±133	1.18	3.82

[a] mean±SD of His^+ revertants per plate of independent experiment (N=3) of each concentration of sample

[b] Mutagenic Index (MI) is calculated from the average value of a number of histidine revertants/plate of the ethanol extract of thai ancient remedies divided by that of spontaneous revertants.

[c] Spontaneous mutation

Table 19 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Chantaleela	Spontaneous[c]	20±2	28±18		
	0.16	30±8	21±9	1.48	0.74
	0.8	24±9	38±7	1.22	1.37
	1.6	23±8	113±17	1.17	4.05
	3.2	24±4	197±5	1.2	7.04
Hoptiposot	Spontaneous[c]				
	0.16	21±7	15±1	1.03	0.54
	0.8	13±3	28±5	0.63	1.01
	1.6	14±5	67±10	0.72	2.38
	3.2	15±4	74±15	0.73	2.65
Keawhom	Spontaneous[c]				
	0.16	39±17	61±46	1.95	2.17
	0.8	31±5	90±35	1.57	3.21
	1.6	26±8	90±6	1.32	3.2
	3.2	16±4	208±36	0.78	7.42
Prasachandang	Spontaneous[c]				
	0.16	26±6	35±7	1.3	1.26
	0.8	22±3	52±2	1.08	1.87
	1.6	21±10	64±4	1.03	2.27
	3.2	36±5	147±28	1.82	5.24

Table 19 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Prasamawarng	Spontaneous[c]	25±2	21±7		
	0.16	24±10	20±7	0.97	0.95
	0.8	26±9	24±5	1.04	1.16
	1.6	33±4	47±25	1.31	2.22
	3.2	34±6	58±18	1.36	2.76
Thoraneesantakat,	Spontaneous[c]				
	0.16	35±15	14±6	1.41	0.67
	0.8	33±12	10±8	1.33	0.49
	1.6	26±4	32±12	1.04	1.54
	3.2	29±10	38±13	1.17	1.83
Treehom	Spontaneous[c]				
	0.16	27±8	29±6	1.07	1.37
	0.8	17±6	49±8	0.69	2.32
	1.6	27±13	63±22	1.08	9.98
	3.2	24±1	88±13	0.95	4.1
Ummalukkawatee	Spontaneous[c]				
	0.16	20±5	44±5	0.81	2.1
	0.8	24±4	81±26	0.97	3.84
	1.6	32±4	185±92	1.28	8.79
	3.2	28±1	117±23	1.12	5.59

Table 19 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA98 (frameshift mutation) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Wisampayayai	Spontaneous[c]				
	0.16	18±3	36±22	0.73	1.73
	0.8	31±7	78±11	1.25	3.7
	1.6	45±49	177±80	1.81	8.43
	3.2	23±11	388±221	0.93	18.48

[a] mean±SD of His^+ revertants per plate of independent experiment (N=3) of each concentration of sample

[b] Mutagenic Index (MI) is calculated from the average value of a number of histidine revertants/plate of the ethanol extract of thai ancient remedies divided by that of spontaneous revertants.

[c] Spontaneous mutation

Table 20 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Chantaleela	Spontaneous[c]	128±28		143±18	
	0.16	145±10	160±17	1.13	1.12
	0.8	122±12	200±65	0.95	1.4
	1.6	148±6	310±8	1.16	2.17
	3.2	142±10	390±78	1.11	2.73
Hoptiposot	Spontaneous[c]				
	0.16	116±6	181±23	0.9	1.27
	0.8	131±7	258±6	1.02	1.8
	1.6	97±12	385±27	0.76	2.69
	3.2	116±7	573±55	0.91	4.01
Keawhom	Spontaneous[c]				
	0.16	139±9	181±51	1.08	1.27
	0.8	146±3	276±60	1.14	1.93
	1.6	137±16	445±27	1.07	3.11
	3.2	172±13	578±45	1.34	4.04
Prasachandang	Spontaneous[c]				
	0.16	139±15	239±45	1.09	1.67
	0.8	155±17	346±7	1.21	2.42
	1.6	123±5	542±45	0.96	3.79
	3.2	141±7	1012±45	1.1	7.07

Table 20 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Prasamawarng	Spontaneous[c]	186±19		120±12	
	0.16	169±45	178±23	0.91	1.49
	0.8	160±52	219±8	0.86	1.83
	1.6	193±48	286±10	1.04	2.83
	3.2	209±26	476±29	1.13	3.96
Thoraneesantakat,	Spontaneous[c]				
	0.16	191±9	167±24	1.03	1.38
	0.8	181±30	211±18	0.97	1.76
	1.6	159±14	225±18	0.85	1.88
	3.2	148±16	256±48	0.79	2.14
Treehom	Spontaneous[c]				
	0.16	156±12	272±32	0.84	2.26
	0.8	157±21	349±33	0.85	2.91
	1.6	155±33	464±60	0.83	3.86
	3.2	141±56	527±79	0.76	4.39
Ummalukkawatee	Spontaneous[c]				
	0.16	144±52	323±27	0.77	2.69
	0.8	192±13	409±31	1.03	3.41
	1.6	138±20	866±87	0.74	7.22
	3.2	198±14	722±11	1.07	6.02

Table 20 Mutagenicity of the water extracts of selected Thai ancient remedies in acid solution pH3.0-3.5 on *Samonella typhimurium* TA100 (base-pair substitution) without metabolic activation (Cont.)

Remedy extracts	Amount of extracts (mg/plate)	Number of His+ / plate[a]		Mutagenicity Index (MI)[b]	
		Without nitrite	With nitrite	Without nitrite	With nitrite
Wisampayayai	Spontaneous[c]				
	0.16	188±60	258±54	1.05	2.15
	0.8	209±26	440±202	1.12	3.66
	1.6	171±20	455±83	0.92	3.79
	3.2	132±33	763±50	0.71	6.36

[a] mean±SD of His^+ revertants per plate of independent experiment (N=3) of each concentration of sample

[b] Mutagenic Index (MI) is calculated from the average value of a number of histidine revertants/plate of the ethanol extract of thai ancient remedies divided by that of spontaneous revertants.

[c] Spontaneous mutation

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