GENOTOXIC EVALUATION OF *AEGLE MARMELOS* EXTRACTS IN AMINO ACID DEPENDENT STRAINS OF *SALMONELLA TYPHIMURIUM* AND *SACCHAROMYCES CEREVISIAE*

Miss Arunrat Saeou

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การประเมินความเป็นพิษต่อสารพันธุกรรมของสิ่งสกัดจากมะตูมต่อเชื้อ
ซาลโมเนลลา ไทฟิมิวเรียม และแซกคาโรไมซีส ซีรีวิซิอี สายพันธุ์ที่ต้องพึ่งกรดอะมิโน

นางสาวอรุณรัตน์ แซ่อู้

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	GENOTOXIC EVALUATION OF *AEGLE MARMELOS* EXTRACTS IN AMINO ACID DEPENDENT STRAINS OF *SALMONELLA TYPHIMURIUM* AND *SACCHAROMYCES CEREVISIAE*
By	Miss Arunrat Saeou
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Chanida Palanuvej, Ph.D.
Thesis Co-Advisor	Somchit Palakas, D.Sc.

Accepted by the Faculty of College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the College of Public Health Sciences

(Associate Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

.....Thesis Advisor

(Assistant Professor Chanida Palanuvej, Ph.D.)

.....Thesis Co-Advisor

(Somchit Palakas, D.Sc.)

.....Examiner

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

.....External Examiner

(Assistant Professor Suchada Jongrungruangchok, Ph.D.)

อรุณรัตน์ แซ่อู้ : การประเมินความเป็นพิษต่อสารพันธุกรรมของสิ่งสกัดจากมะตูมต่อเชื้อซาลโมเนลลา ไทฟิมิวเรียม และแซกคาโรไมซีส ซีรีวิซิอี สายพันธุ์ที่ต้องพึ่งกรดอะมิโน (GENOTOXIC EVALUATION OF *AEGLE MARMELOS* EXTRACTS IN AMINO ACID DEPENDENT STRAINS OF *SALMONELLA TYPHIMURIUM* AND *SACCHAROMYCES CEREVISIAE*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.สมจิตต์ ปาละกาศ{, 173 หน้า.

รากมะตูมใช้เป็นส่วนประกอบสำคัญของตำรับยาแผนไทยซึ่งมีฤทธิ์ลดไข้ ในประเทศไทยมีการใช้ผลมะตูมเป็นส่วนประกอบของตำรายาและยังนิยมใช้เป็นส่วนประกอบของอาหาร การศึกษาในครั้งนี้จึงได้มีการประเมินความปลอดภัยและประสิทธิศักย์ของสิ่งสกัดด้วยแอลกอฮอล์และสิ่งสกัดน้ำแยกส่วนของรากและผลมะตูมด้วยวิธีการทดสอบทั้งในเซลล์โพรคาริโอตและยูคาริโอต การประเมินฤทธิ์ก่อกลายพันธุ์และต้านการก่อกลายพันธุ์ต่อเชื้อซาลโมเนลลา ไทฟิมิวเรียม สายพันธุ์ TA98 และ TA100 ใช้เทคนิค pre-incubation ด้วยวิธีการทดสอบเอมส์ ภายใต้สภาวะกรด ในระบบที่ไม่มีการกระตุ้นด้วยเอนไซม์ พบว่าสิ่งสกัดส่วนใหญ่ไม่มีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อซาลโมเนลลา ไทฟิมิวเรียม ทั้งสองสายพันธุ์ยกเว้นสิ่งสกัดน้ำแยกส่วนของรากมะตูม อย่างไรก็ตามสิ่งสกัดจะแสดงฤทธิ์ก่อกลายพันธุ์ต่อเชื้อซาลโมเนลลา ไทฟิมิวเรียม สายพันธุ์ TA98 และ TA100 หลังทำปฏิกริยากับไนไตรท (ปฏิกริยาไนโตรเซชัน) นอกจากนี้ยังพบว่าสิ่งสกัดด้วยแอลกอฮอล์จากรากและผลของมะตูมมีฤทธิ์ต้านการก่อกลายพันธุ์ที่เหนี่ยวนำโดยปฏิกริยาของไนไตรทกับอะมิโนไพรีนต่อเชื้อทั้งสองสายพันธุ์ การประเมินความเป็นพิษต่อสารพันธุกรรมในเซลล์ยูคาริโอต ศึกษาในแซกคาโรไมซีส ซีรีวิซิอี สายพันธุ์ D7 พบว่าสิ่งสกัดด้วยแอลกอฮอล์ของรากและผลมะตูมแสดงฤทธิ์แรงกว่าสิ่งสกัดน้ำแยกส่วนโดยเพิ่มอัตราการเกิดโคโลนีจากยีนคอนเวอร์ชันและครอสซิ่งโอเวอร์อย่างมีนัยสำคัญ และเพิ่มอัตราการเกิดโคโลนีสี ในการทดสอบสิ่งสกัดร่วมกับสารก่อกลายพันธุ์ Ethyl methanesulfonate (EMS) พบว่าสิ่งสกัดจากผลมะตูมเสริมฤทธิ์การเหนี่ยวนำการเกิดโคโลนีจากยีนคอนเวอร์ชัน ครอสซิ่งโอเวอร์ และการเกิดโคโลนีสี ในทางกลับกันสิ่งสกัดจากรากมะตูมแสดงฤทธิ์ต้านและเสริมฤทธิ์การเหนี่ยวนำการเกิดโคโลนีจากยีนคอนเวอร์ชันและครอสซิ่งโอเวอร์ ขณะที่แสดงฤทธิ์ต้านการเกิดโคโลนีสีจาก EMS ซึ่งการเสริมฤทธิ์ของสิ่งสกัดอาจเกิดจากการเกิดการทำปฏิกริยาร่วมกันระหว่างสิ่งสกัดและ EMS

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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ARUNRAT SAEOU: GENOTOXIC EVALUATION OF *AEGLE MARMELOS* EXTRACTS IN AMINO ACID DEPENDENT STRAINS OF *SALMONELLA TYPHIMURIUM* AND *SACCHAROMYCES CEREVISIAE*. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: SOMCHIT PALAKAS, D.Sc.{, 173 pp.

The root of *Aegle marmelos* (L.) Correa ex Roxb. has been used as essential ingredient of some traditional Thai remedies which have a property for relief fever. The fruit of *A. marmelos* has been used in traditional medicine and also used as a popular herbal dietary source in Thailand. In this study, the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* were assessed for their safety and efficacy in *in vitro* model with both prokaryotic and eukaryotic cells. The mutagenic and anti-mutagenic activities of the root and the fruit extracts were evaluated by pre-incubation method of Ames test toward *Salmonella typhimurium* strains TA98 and TA100 in an acidic condition (pH 3-3.5) without enzyme activating system. Most of the extracts were not directly mutagenic except the fractionated water extract from the root of *A. marmelos* which exhibited mutagenicity on both strains. However, all of the extracts showed mutagenic effect on both strains after they were treated with sodium nitrite (nitrosation). In addition, this study showed that only the ethanolic extracts from the root and the fruit showed inhibitory effect against mutagenicity induced by nitrite-treated 1-aminopyrene on both strains. All of the extracts were also performed the genotoxic potential in yeast-based genotoxic assay using *Saccharomyces cerevisiae* strain D7. It was found that the ethanolic extracts from the root and the fruit were more effective than their fractionated water extracts which significantly increased the frequency of convertants and revertants, including the increased frequency of total aberrant colonies. In the treatments of the extracts in combination with EMS, it was found that most of all doses of the extracts from the fruit exhibited significant enhancement of EMS-induced the frequency of convertants, revertants, and total aberrant colonies. On the other hand, the extracts from the root showed significant either inhibitory or enhancement effect of EMS-induced the convertants and revertants frequencies, and inhibited the frequency of total aberrant colonies. The enhancement effect of the extracts might possibly be due to a synergistic interaction between the extracts and EMS in inducing the genotoxic effect in the combined treatment.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

Ade	Adenine
℃	Degree Celsius
et al.	*et alia* (and others)
hr	Hour
His^+	Histidine independent
HCl	Hydrochloric acid
l	Liter
Ilv	Isoleucine
Ilv^+	Isoleucine prototrophy
m	Meter
min	Minute
mm	Millimeter
mg	Milligram
ml	Milliliter
M	Molar

LIST OF ABBREVIATIONS

mM	Millimolar
µm	Micrometer
µl	Microliter
N	Normality
pH	Potential of hydrogen ion
rpm	Revolution per minute
SD	Standard deviation
Trp	Tryptophan
Trp^+	Tryptophan prototrophy
UV	Ultraviolet
VII	Seven
V	Five
v/v	Volume in a volume
XV	Fifteen
EMS	Ethyl methanesulfonate

CHAPTER I

INTRODUCTION

Background and rationale

Human health effects are generally as having multifactorial origin such as different genetics, environment, socioeconomic factors, lifestyle, occupational exposures and others [1]. Dietary intake of some foods may also be important factors that can increase the incidence of human diseases, especially cancers. Several types of mutagens can be formed during preparation of different food types or high dietary intake of some foods whereas some mutagenic substances may occur naturally which are associated with an increased risk of common diseases [2, 3]. On the other hand, the dietary used of some food ingredients is consistently associated with protection of human diseases [4]. In Thailand, many herbal medicines have been used since historical times for prevention and treatment of various health ailments both in folk medicine and traditional medicines [5]. The different parts of herbal plants are valued to use extensively in herbal drugs or remedies which consist of raw or processed ingredients from herbal plants. Moreover, some herbal medicines are not only used for management of various diseases but also widely used as valuable food ingredients in the commercial markets which has been had a great acceptance by the population [6]. Therefore, the efficacy and safety of these medicinal plants are invaluable information to support the usage of safe medicinal plants for consumers.

Aegle Marmelos (L.) Correa ex Roxb. is commonly known as bael which is an indigenous medicinal plant from the family Rutaceae. In Thailand, *A. marmelos* is an important medicinal plant in traditional Thai medicines, which is called in native language as "Ma-tum". *A. marmelos* is a slow-growing of medium size, aromatic tree [7]. The fruits are ovoid shape and contain numerous seeds. The fruits have been used in traditional medicine as an anti-proliferative activity [8], antimicrobial activity [7, 9, 10], anti-diabetic activity [7, 10, 11], and anti-inflammatory activity [7, 8]. The roots also showed anti-diarrhoeal activity [12], anti-pyretic, analgesic, and antiproliferative activity [13]. The root of *A. marmelos* is one of the important ingredients of indigenous drugs of India, *Dasmula* (ten roots) recipe, and some Thai ancient remedies [13, 14]. The decoction of the root and root bark are useful in treatment intermittent fever, hypo-chondriasis, and palpitation of the heart [14]. The fruits are also used in the treatment of dyspepsia, dysentery, and diarrhea. Moreover, the fruits are one of popular herbal dietary source, especially for health-conscious consumers which are used as herbal drinks, syrups, tea or desserts [15].

The bacterial reverse mutation assay, Ames test, is used world-wide as a screening method for determination of mutagenic potential of compounds in prokaryotic cells such as histidine dependent auxotrophic mutants of *Salmonella typhimurium*. Another test using yeast *Saccharomyces cerevisiae* is a well-established tool which based on eukaryotic cells for detection the genotoxic effects of several compounds with a simple and sensitivity equal or higher to Ames test [16].

In previous study, the ethanolic and fractionated water extracts from the root of *A. marmelos* which is one of the plant species in Ben-Cha-Moon-Yai remedy possessed mutagenic activity toward *Salmonella typhimurium* strain TA98 and TA100. In addition, ethanolic extract from the root of *A. marmelos* also exhibited strong inhibitory effect against nitrite-treated 1-aminopyrene in Ames test [13].

The purpose of this study was to investigate the fruit extracts from *A. marmelos* for direct and nitrite-induced mutagenic activity and anti-mutagenic activity induced by nitrite-treated 1- aminopyrene by performing Ames test using *S. typhimurium* strains TA 98 and TA 100. Furthermore, the fruit extracts were used to investigate the genetic effects on the frequency of mitotic crossing-over, mitotic gene conversion, and reverse mutation by direct treatment and co-treatment with EMS-induced mutagenesis in strain D7 of *S. cerevisiae*. Furthermore, both ethanolic and fractionated water extracts from the root of *A. marmelos* from previous studies were reevaluated the mutagenicity using the diploid yeast *S. cerevisiae* strain D7.

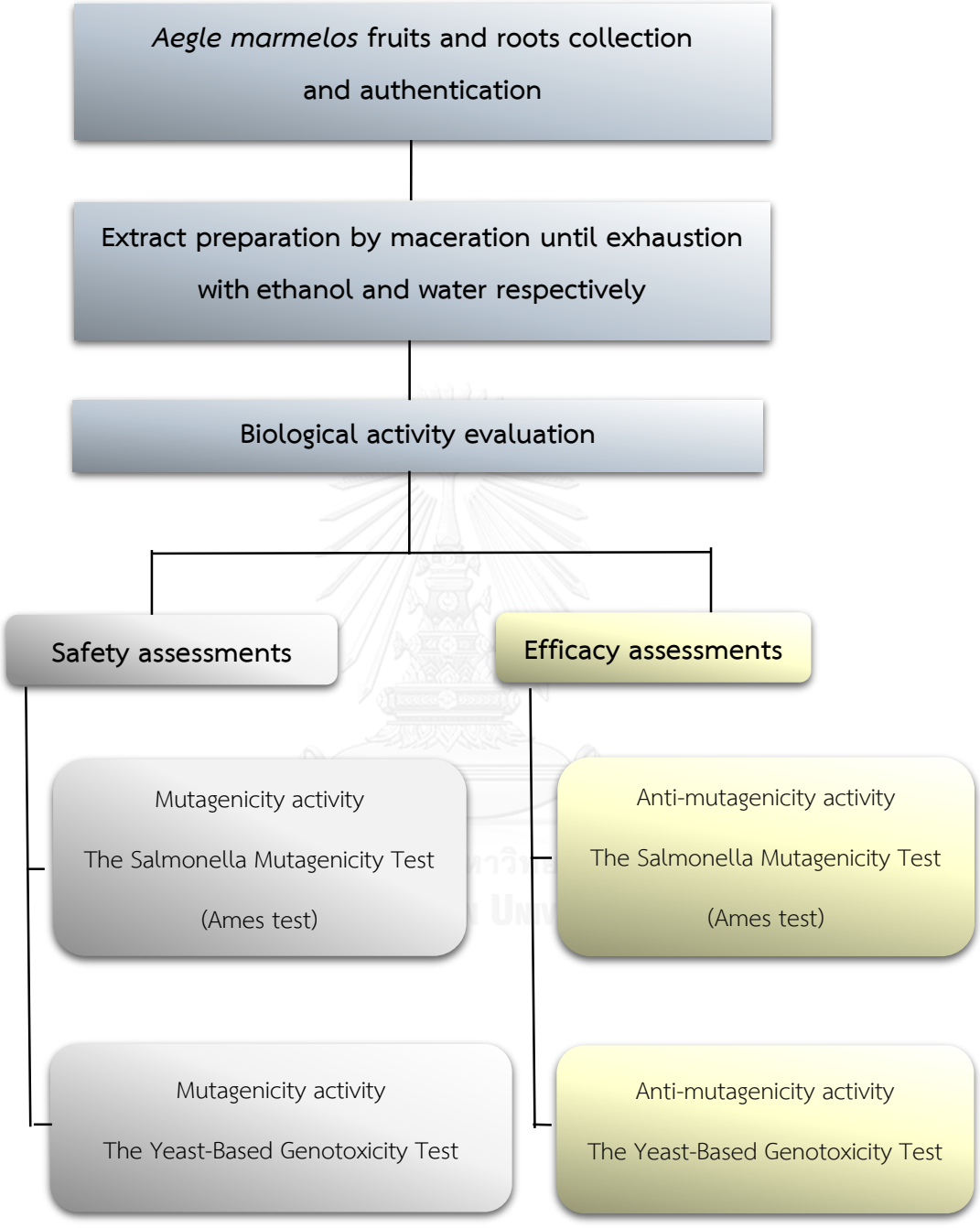
Objectives of the study

1. To determine the mutagenic activity of the fruit and the root extracts of *A. marmelos* with and without nitrosation using Ames test.
2. To determine anti-mutagenic activity of the fruit and the root extracts of *A. marmelos* on mutagenicity induced by nitrite-treated 1-aminopyrene using Ames test.
3. To evaluate the genotoxic effects of the extracts from the fruit and the root of *A. marmelos* on the induction of mitotic crossing-over, mitotic gene conversion, and reverse mutation using strain D7 of *Saccharomyces cerevisiae*.
4. To evaluate the genotoxic effects of the extracts from the fruit and the root of *A. marmelos* with ethyl methanesulfamate (EMS) in inducing mitotic crossing-over, mitotic gene conversion, and reverse mutation using strain D7 of *Saccharomyces cerevisiae*.

Benefits of the study

1. This research provides the information regarding the mutagenicity and genotoxicity of extracts from the fruit and the root of *A. marmelos.*
2. This research provides the *in vitro* models for genotoxic evaluation of medicinal plants.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

Rutaceae family

Rutaceae family is one of a great economic importance family plant, commonly known as citrus family, which includes the orange, lemon, grapefruit and lime. The Rutaceae family belongs to the order *Sapindales*, consists of approximately 155 genera and 1,600 species widely distributes mainly in subtropical and tropical regions. Most species in this family are plants with glands containing aromatic volatile oils which are visible at the surface of inflorescences, young branchlets, leaves, fruits, or cotyledons in seed [17].

Botanical description

"Trees, shrubs, or herbs, sometimes scandent or xeromorphic, armed or unarmed; stems, leaves, flowers, and fruits usually punctate glandular, the glands containing aromatic volatile oils. Leaves alternate or rarely opposite, 1-3 foliolate or simple, persistent or deciduous; petioles sometimes winged; stipules absent. Flowers bisexual and/or unisexual, regular or rarely irregular, hypogynous, usually 3-5-merous, solitary and axillary or in various axillary or terminal, often cymose inflorescences; plants dioecious, monoecious, or polygamous; sepals rarely absent, free or connate, usually imbricate; petals free, rarely connate or absent; stamens as many as the petals and in 1 whorl, or twice as many to numer-ous and in 2 whorls, the outer whorl often shorter than the inner or occasionally reduced to staminodes, the filaments

free or connate, often conspicuously dilated or rarely appendaged basally, the anthers 2(-4)-thecate, versatile, often glandu-lar tipped, introrse, longitudinally dehiscent; intrastaminal disc annular, cupular, or cushionlike, nectariferous, rarely obscure or absent; gynoecium of (1-)2-5 (-several) free or variously connate carpels, occasionally rudimentary or absent, the ovary sessile to stipitate, (1-)2-5(-several)-loculed, the ovules 1-2 or more per locule, usually anatropous and epitropous, rarely apotropous, placentation axile or rarely parietal, the styles basal, more or less lateral, or terminal, free, connivent or connate, persistent or deciduous, the stigmas free or connate, simple or lobed. Fruit of (1-)2-5(-several) follicles or drupes, or a berry, capsule, samara, or schizocarp; seeds 1 or 2 to several per locule, sessile or funiculate, the endosperm present or absent, the embryo relatively large, straight or curved, the cotyledons plano-convex, sometimes convolute, rarely plicate, the radicle superior" [17].

Aegle marmelos (L.) Correa ex Roxb.

Aegle marmelos (L.) Corr., commonly known as "bael" and belonging to Rutaceae family. It is an indigenous plant and commonly found growing in India and most of countries of Southeast Asia including Thailand [6]. Bael is slow growing and is the only plant belonging to the genus *Aegle.* Bael is medium size, subtropical tree, about 12 to 15 m in height with short trunk, thick, flaking bark, and spiny branches. The alternate leaves, borne singly or in group, are made up of 3 to 5 oval, pointed, shallowly toothed leaflets. The fruits are either round, pyriform, oval, or oblong in shape. The fruit pulp is made of a hard central core with 8 to 16 triangular segments, resinous, highly aromatic and sweet to taste. The seeds are flat and oblong in shape which are embedded in the pulp, about 10 to 15 in number. Each seed is enclosed in a sac of adhesive, transparent mucilage that solidifies on drying. [7, 15]

Figure 1. *Aegle marmelos* (L.) Corr.; (1) Leaves (2) Flowers (3) Fruits (4) Dried fruits [18]

Chemical constituents

Many types of chemical constituents, isolated from various parts of *Aegle marmelos*, have been reported such as volatile compounds, coumarins, tannins, and flavonoid glycosides. The occurrence of some chemical constituents in this plant were shown in Table 1.

Table 1. A large number of chemical constituents isolated from the various parts of *Aegle marmelos*

Part	Chemical constituents
Root	marmesin, lupeol, skimmianine, auroptene, marmin, umbelliferone, coumarins, alkaloids, skimming, psoralen [19]
Bark	skimmianine, fagarine , marmin [7]
Leaf	skimmianine, aegelin, lupeol, cineole, citral, citronellal, cuminaldehyde, eugenol, marmesinine, rutin, flavone, glycoside, o-isopentenyl, halfordiol, marmeline, cuminaldehyde phenylethyl cinnamamides [7, 20]
Fruit	citronellal, marmelosin, luvangetin, aurapten, alloimperatorin, psoralen, marmelide, tannins, hexanal, isoamyl acetate, limonene, citronella, citral, cuminaldehyde, hexadecane, caryophyllene oxide, pulegone, cineole, verbenone, carvone, carvyl acetate, humulene oxide, hexadecanoic acid, o-isopentenyl halfordinol, p-cymene, marmelin, o-mtheyl halfordinol, aegeline, aegelenine, , furocoumarins [7, 15]
Seed	citral, cumin aldehyde, a-d-phellandrene, d-limonene, cineol, citronellal, p-cyrnene [20]

Medicinal uses

The different parts of bael plant are used to cure a variety of disorders in different Indian traditional system of medicines, Ayurvedic, such as for treatment of healing of wounds, high blood pressure, swollen joints, diarrhea and dysentery. Moreover, *A. marmelos* has been used for the management of diabetes mellitus in Ayurvedic, Unani and Siddha systems of medicine in India, SriLanka and Bangladesh [7].

The roots are sweet and are used to cure urinary troubles, fever, abdominal pain, and cardiac malfunction by making the decoction. *A. marmelos* root is one of the essential ingredients of some traditional Thai medicines such as Tree-Sin-Thu-Rot, Ben-Cha-Moon-Yai and Tot-Sa-Moon-Yai remedies, each of which has a property for relief fever [21]. In India, the root is an ingredient of the drug, *dashamula*, which is used in the treatment of fever, colitis, flatulence, diarrhea, and dysentery [22]. The leaves are used as an astringent, digestive and febrifuge and are useful in the treatment of inflammation, fever, and opthalmia. In the Ayurvedic system of medicine, bael fruits are used as an excellent remedy to cure diarrhea. The unripe fruits are astringent, sour and bitter and aid in digestion and alleviate stomach irritation. The ripe fruits are used to prevent sub-acute and chronic dysentery. The fruit pulp acts as a mild stimulant to the intestinal mucus membrane and stops diarrhea which it is prepared as sweet drink for the patients who have just recovered from bacillary dysentery [7, 22].

Dietary agent and nutritive value of bael fruit

The bael fruits have been used as a dietary source with different forms in each country which are rich in water, fibers, and carbohydrates and also contain essential vitamins and minerals (Table 2). In India, the ripe fruit is prepared “bael sherbet” which is a popular drink whereas the semi ripe fruits are used in making jam. Moreover, marmalade and syrups are made from the fruit pulp and consumed with Indian bread. In Indonesia, the ripe fruit pulp is consumed for breakfast by scooped dressed with palm sugar. In Thailand, the dried fruits are either cut into pieces and packed in bags or powdered and packed as tea bags. In addition, the preserved fruit in syrup is used as an ingredient for preparing cakes or as dessert [15].

Table 2. Nutritional value from the fruit of *A. marmelos* (% per 100 g wet pulp fruit without seeds) [15].

Components	Value
Water (%)	64.2
Fiber, total dietary (%)	31.8
Protein (%)	1.8
Carbohydrate (%)	31.8
Fat (%)	0.3
Vitamins	
Vitamin A (mg/100g)	0.055
Vitamin B2 (mg/100g)	1.2
Vitamin C, total ascorbic acid (mg/100g)	8.00
Thiamin (mg/100g)	0.13
Riboflavin (mg/100g)	0.03
β carotene (mg/100g)	55.00
Minerals	
Calcium (mg/100g)	85.0
Phosphorus (mg/100g)	31.8
Iron (mg/100g)	0.6
Potassium (mg/100g)	600
Copper (mg/100g)	0.21
Energy (kcal/g)	137

Pharmacological activities

Various crude extracts from different parts of this plants have been studied for their biological activities. The methanolic and aqueous extracts of *A. marmelos* was studied by Rani and Khullar (2004) and showed the antimicrobial effects against multi-drug resistant *Salmonella typhi* strains B 330 and MTCC 531 causing enteric diseases. The minimum inhibitory concentration (MIC) of the methanolic extract against *S. typhi* B330 and *S.typhi* M531 was >256 and 256 µg/ml, respectively [23]. Previous studies reported that the aqueous extract from the leaves of *A. marmelos* and its essential oil possessed the antibacterial and antifungal activities [9, 24]. Similarly, Khanna *et al.* (1991), also demonstrated that the essential oil from the leaves of *A. marmelos* exhibited the activity against *Xanthomonas vesicatoria*, *Escherichia coli*, *Aeromonas* sp., and *Pseudomonas salanacearum* [25]. In experimental models, hydroalcoholic extract of *A. marmelos* leaves was evaluated for anticancer effect by Jagetia *et al.* (2005) in animal model of Ehrlich ascites carcinoma. This study proposed that the extract showed anticancer effect and administration of the extract at 400 mg/kg has shown the greatest anti-tumor effect [26]. In addition, the ethanolic extract from the root exhibited the activity against *Staphylococcus aureus*, *Escheichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* [10].

The anti-diarrheal effect of the hot aqueous extract (decoction) from the pulp of dried unripe fruit are studied by Brijesh *et al.* (2009) and showed that the decoction was ineffective on six bacterial strains whereas showed cytotoxic activity against rotavirus and *Giardia* and reduced the bacterial invasion and adherence of HEp-2 cells [14].

Moreover, the water extract from the fruit of *A. marmelos* demonstrated the antioxidant activity toward in various *in vitro* models such as DPPH radical scavenging, PCL, and FRAP assays. The water extract from the fruit was also possessed high quantities of total phenolic compound contents [2, 27].

The ethanolic extract of *A. marmelos* stem bark was evaluated the anticancer potential by using the MTT assays using tumor cell lines, sea urchin eggs, brine shrimp lethality, and hemolysis. The extract showed a positive result in brine shrimp assay, presenting a LD_{50} of 17.5 ± 2.0 µg/ml. The extract was also strongly active in sea urchin eggs assay, inhibiting the 1^{st} cleavage, 3^{rd} cleavage and the blastulae with an IC_{50} of 50.5, 22.7, and 13.2 µg/ml, respectively. The results of the extract from the hemolytic assay presented EC_{50} values of 395.8 µg/ml. The extract exhibited the activity in all assay, indicating the presence of cytotoxic substances which might be toxic to normal and tumor cells [28].

A few chemical constituents, isolated from various parts of *A. marmelos* have been studied for their biological activity. Marmelide and the fruit extract possessed antiviral effects against the human coxsackieviruses B1–B6 in the 96 h plaque inhibition assay. On the other hand, marmelide and *A. marmelos* extract did not showed any toxicity to vero cells suggesting that the extract and this pure compound of *A. marmelos* did not possess any toxicity on normal cells and was possibly safe for human use [29]. Moreover, lupeol, a triterpene, has been isolated from *A. marmelos* possessing anti-inflammatory activity by the reduction of paw swelling in rats [30].

It is also shown to possess antineoplastic effects on various human neoplastic cell lines [31-33].

Nitrite induced mutagens

Nitrates and nitrites are nitrogen-oxygen chemical units that take place naturally in environments as a part of the nitrogen cycle. Nitrates are present in numerous different sources of food, but the important dietary intake of nitrate are vegetables, fruit and drinking water which is reduced to nitrite by microflora (Figure 2.) in the oral cavity and gastrointestinal tract [34]. Nitrates and nitrites are usually used as a preservative in the form of sodium and potassium salt [35]. Sodium and potassium nitrates are used in the manufacturing processes of meat products, particularly cured meats such as hams, hot dogs, bacon, and salted fishes [36]. Curing of meat using nitrate and nitrite can inhibit the spoiling microorganisms growth and its toxin production, especially *Clostridium botulinum* and give the meat product a desirable bright red color [37]. The exposure to nitrates and nitrites at high level has been reported to have adverse health effects, causing infantile methemoglobinemia (blue baby syndrome) and gastrointestinal cancer through the formation of carcinogenic *N-nitroso* compounds. Under the acidic condition of the human intestinal tract, nitrite can react with amines, amides, and other precursors in foods to form nitrosamines which can increase the risks of cancers in the oral cavity, esophagus, stomach, and urinary bladder [38].

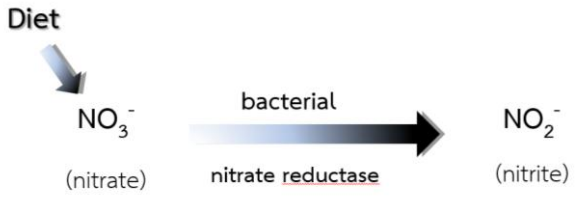


Figure 2. The reduction of nitrate to nitrite by microorganism

Several Thai and Asian food products, for example, broiled chicken, fried pork, kimchis, sun-dried seafood, pickled vegetables, soy sauces, and shrimp pastes were reported their direct-acting mutagenic effect after nitrite treatment [39, 40]. The incidence of gastric cancer was related to the high dietary intakes of smoked, salt and salt-preserved foods which are rich in salt, nitrite and preformed nitroso compounds. Similarly, Chen *et al.* (1992), indicated that gastric cancer mortality in southern China has been associated with the high consumption of salted fermented fish products [41]. Furthermore, the raw and pickled vegetables and fruits extracts, namely mango, shallot, mushroom, bamboo shoot, cabbage, cucumber, ginger, garlic, and Chinese mustard showed direct-acting mutagenicity after nitrite treatment in *Salmonella* assay [42]. Consequently, the consumption of several nitrosable mutagen precursors in foods might be concerned with the causation of human cancer, especially in the stomach.

Nitrite-treated 1-aminopyrene model

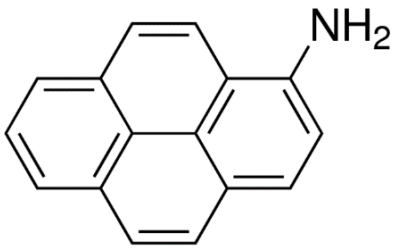


Figure 3. Structure of 1-aminopyrene

Molecular formula: $C_{16}H_{11}N$

Molecular weight: 217.27 g/mol

Description:

1-Aminopyrene (1-AP) is a metabolite of 1-nitropyrene (1-NP) which is reduced by nitro-reeductases in the living systems. The anaerobic intestinal microflora are able to convert 1-nitropyrene to 1- aminopyrene [43]. 1-Nitropyrene is the predominant nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) , which is one of the most important classes of environmental pollutants. Some of which are highly effective as bacterial and mammalian cell mutagens and as carcinogens in animals that it may have an effect on human health [44]. Nitro-PAHs formed by reactions of nitrogen oxides with PAHs as a result of diesel and gasoline exhaust, petroleum gas burns and kerosene heater emissions, food preparation and contamination, industrial process emissions and other combustion processes. Inhalation of these complex mixtures is the major route of human exposure to 1-nitropyrene which has the capacity to develop animal and human cancers [45].

1-Aminopyrene was known to be mutagenic in mutagenicity assay against *Salmonella typhimurium* when it was tested in the presence of metabolic activation [46]. It was found to be direct-acting mutagen after being treated with sodium nitrite in acid condition. In previous studies, Kangsadalampai *et al.* (1996) showed the mutagenic results of 1-aminopyrene after treatment with sodium nitrite at 37°C for 4 hours in acid solution pH 3.0-3.5 which exhibited strong mutagenic effect on *Salmonella typhimurium* strains TA98 (frameshift mutation) and TA100 (base-pair substitution) without metabolic activation [47]. Similarly, Kato *et al.* (1991) also indicated that nitrite-treated 1-aminopyrene at 37°C and pH 3.0 showed mutagenic effect on *Salmonella typhimurium* strains TA98 and TA100 [48]. Nitroreduction of 1-nitropyrene in mammalian cells is catalyzed by cytosolic aldehyde oxidase, xanthine oxidase, microsomal NADPH cytochrome P450 reductase, or DT-diaphorase through reduction of the nitro group to form 1-nitrosopyrene, N-hydroxy-1-aminopyrene, and 1-aminopyrene [49]. In addition, 1-nitropyrene is metabolized by nitro-reductase presented in bacterial cells to be arylhydroxylamine which can react with DNA and subsequently lead to mutagenesis [50]. Therefore, the mutagenicity of nitrite-treated 1-aminopyrene in acid condition has been used as a model for anti-mutagenicity study of certain chemical substances or complex mixtures concerning the phenomenon occurred during stomach ingestion.

The bacterial reverse mutation assay (Ames test)

The bacterial reverse mutation assay, also known as the *Salmonella* test or Ames test, is used as an preliminary screening to determine the mutagenic potential of several chemical compounds as well as complex mixtures including drugs that can produce genetic damage [51]. The evaluation of induced-mutation activity in the Ames test can be used amino acid requiring standard strains of *Salmonella typhimurium* or *Escherichia coli* [52]. Each of the bacterial tester strains used in the Ames test carries a deficient gene that inhibits them to synthesize an essential amino acid. Therefore, these strains unable to grow and form colonies in the absence of supplementation with required amino acid [52]. The mutagenic induction at these preexisting mutations site induced by mutagenic chemicals can restore the functional ability to synthesize required essential amino acid of the bacteria. These newly mutated cells can grow in the absence of the essential amino acid required by the bacterial tester strains and form colonies. Consequently, these mutations which cause a regaining normal function of bacterial tester strains are called “reverse mutation’’ and the test can refer to as a “reversion assay” [51, 52].

Several different strains of *Salmonella typhimurium* are used to detect mutagenic activity by their ability to revert from histidine dependence (auxotrophy) to histidine independence (prototrophy), which can grow on the minimal medium without histidine in the presence of mutagenic compounds. Some of which contained single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases (frameshift mutants) [51]. This test may utilize several

strains of the *Salmonella typhimurium*, prokaryotic cells, which are different from mammalian cells in disability to metabolize variety of chemical mutagens *via* cytochromes P450. Therefore, certain mutagens are directly active in the system while others require an exogenous source of metabolic activation such as rodent liver enzymes (S9 fraction) added to the test system to promote metabolic conversion of the tested chemicals [53].

The several methods for detecting pure compounds and complex mixtures with the *Salmonella* mutagenicity test were described previously [54]. In 1966, the spot test procedure was proposed by Ames and Whitfield [55] that was an uncomplicated way to the initial rapid screening of a variety of chemical compounds. The spot test is an initially qualitative test and has certain some limitations although it is used for the screening the mutagenic potential of several compounds. Therefore, the plate incorporation procedure which is more sensitive and quantitative than the spot test was developed by Ames *et al.* for validating the test using hundreds of compounds [51]. The procedure is composed of the test compound, the buffer or S9 fraction, and the bacterial tester strain to 2 ml of top agar containing biotin and a trace amount of histidine which is mixed and poured onto a glucose minimal agar plate. Positive and negative controls are also included in each assay. All plates are incubated at 37°C for 48 hours, and counted the histidine revertant colonies [56].

The pre-incubation method

Some mutagens are not detected or are weakly detected in the standard plate incorporation assay and should be assayed using a modification of the plate incorporation procedure. This modification has been developed to increase the sensitivity of the test and permit the evaluation of mutagenic potential in a variety of chemical compounds. [56]. The most commonly used modification procedure is the pre-incubation assay first described by Yahagi *et al*. in which the carcinogenic azo dyes and derivatives were found to be mutagenic on *Salmonella typhimurium* TA98 and TA100 [57].

The plate incorporation and pre-incubation procedures have been used to determine the mutagenic effects of aflatoxin B_1, benzidine, benzo[*a*]pyrene, and methyl methanesulfonate in which the pre-incubation assay is equal or greater sensitivity than the plate incorporation assay [58]. The increased activity of the pre-incubation assay is the causes of incubation of the test compound, S9 fraction and bacteria at higher concentrations than in the standard plate incorporation test [59].

In the pre-incubation assay, the bacterial tester strains are incubated with the tested chemical for a short portion of time (20 to 30 min) in a small volume (0.5 ml) of either buffer or S9 fraction before plating on glucose agar minimal medium (GM agar) supplemented with a trace amount of histidine. This procedure is more sensitive than the plate incorporation assay because the bacterial tester strains may have a better chance to react with the mutagenic metabolites in the small volume

of pre-incubation mixture, and the effective concentration of S9 fraction in the pre-incubation volume is higher than on the plate [51, 57].

The *Salmonella* tester strains

The genotypes of the *Salmonella* tester strains commonly used for mutagenicity testing are showed in Table 3. Each tester strain is histidine dependent and contains a different type of mutation in the histidine operon. Supplementary mutations that greatly increase the bacterial tester strains more sensitive to a wide variety of substances are listed below.

Table 3. Genotype of the most commonly used *Salmonella* tester strains [51].

Strain	Amino acid marker		Other relevant mutations			
	Histidine mutation	Reversion event	LPS defect	Biotin requirement	DNA-repair	Plasmid
TA100	*hisG46*	Base-pair substitution	*rfa*	*bio-*	*uvrB*	pKM101
TA1535	*hisG46*	Base-pair substitution	*rfa*	*bio-*	*uvrB*	No plasmid
TA98	*hisD3052*	Frameshift	*rfa*	*bio-*	*uvrB*	pKM101
TA1538	*hisD3052*	Frameshift	*rfa*	*bio-*	*uvrB*	No plasmid
TA1537	*hisC3076*	Frameshift	*rfa*	*bio-*	*uvrB*	No plasmid
TA97	*hisD6610*	Frameshift	*rfa*	*bio-*	*uvrB*	pKM101
TA102	*hisG428*	Base-pair substitution	*rfa*	*bio-*	-	pKM101, pAQ1

There is deletion mutation through the *uvrB-bio* genes in all of standard tester strains, except TA102. This mutation was constructed primarily that require an intact excision repair system. The *uvrB* mutation causes highly increase sensitivity to detect the effects of several chemical mutagens by a deletion of a gene coding for the DNA excision repair system. The deletion excising the *uvrB* gene extends through the *bio* gene, resulting in the biotin requirement for growth of each tester strain [51, 56].

The *rfa* mutation in all bacterial tester strains accumulates permeability to bulky chemicals that normally do not pass through the bacterial cell wall which result from the partial losing of lipolysaccharide (LPS) barrier that coats the surface of the bacteria [56].

The R-factor plasmid, pKM101, is commonly present in these organisms which can increase chemical and spontaneous mutagenesis by enhancing an error-prone recombinational DNA repair pathway [60]. The plasmid provides ampicillin resistance, which is an appropriate marker to detect the presence of the plasmid.

Insertion of the mutation *hisG428* on the multicopy plasmid pAQ1 which was introduced in strain TA102 with the aim of amplifying the number of target sites. To enhance the ability of this strain to detect DNA cross-linking agents, the *uvrB* gene was retained making the bacterium DNA repair proficient [51].

Spontaneous reversion

Spontaneous mutant frequency of the tester strains to histidine independence is measured and expressed as the number of spontaneous histidine revertant colonies per plate [56]. The number of spontaneous histidine revertant colonies from one experiment or one plate may be different from another as a result of the variability in each laboratory or each day of experiments. The spontaneous reversion of each bacterial tester strain is referred to historical values, which is a characteristic range of spontaneous histidine revertant colonies for the tester strain. Table 4 presents the acceptable ranges of spontaneous histidine revertant (negative solvent) control values per plate with and without metabolic activation [51, 56].

Table 4. Spontaneous revertant control values [51]

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

Yeasts

Yeasts are single-celled organisms classified in the kingdom fungi, a diverse group of eukaryotic microorganisms. Yeasts are widely spread in nature about 1,500 species, which are classified into the *Ascomycotes* and the *Basidiomycetes*. Budding yeasts, referred to “true fungi”, are members of the phylum *Ascomycestes* and are separated into order *Saccharomycetales*.

Yeasts are the most important organisms in the world because of their essential roles in ecosystem function and having both positive and negative effects on human activities. Natural strains of the yeast have been commonly found in a wide dispersion of natural habitats, for example, plant leaves and flowers, soil, marine and other aqueous environments. They are also symbiotically or as parasites natural inhabitants of man and animals. On the other hand, some species is normal inhabitant of humans which also causes a variety of diseases such as vaginitis, thrush, and diaper rash [61].

Taxonomy of Saccharomyces cerevisiae

Kingdom: *Fungi*

Phylum: *Ascomycota*

Class: *Saccharomycetes*

Order: *Sacharomycetales*

Family: *Saccharomycetaceae*

Genus: *Saccharomyces*

Species: *S.cerevisiae*

The yeast *Saccharomyces cerevisiae* is an unicellular and uninuclear eukaryotic organism belonging to the *Ascomycetes* in the fungi kingdom [62]. *Saccharomyces cerevisiae*, commonly known as Baker's yeast or Brewer's yeast, is the most well-known commercially yeast in fermentation and food. This organism has an extensive history for centuries as a fermenter of alcoholic beverages behind the ability to convert sugars into alcohol. It is also used as a leavening agent in the baking process which results in the spongy-like texture of bakery products such as breads and cakes [61].

Yeast, *Saccharomyces cerevisiae* is one of the most eukaryotic model organisms which is used for investigate molecular genetics as well as biological studies because of its important advantages which are easy cultivation, inexpensive, and short generation times. Moreover, its cellular activities such as replication, recombination, cell division and metabolism are generally conserved between yeast and many other higher eukaryotes, including humans [61, 63].

Life cycle of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae can grow vegetatively as either haploid (1n) or diploid cells (2n). Each strain of yeast cell shows various characteristic sizes with the different phase of growth. Typically, the diploid form is ellipsoid-shaped with a diameter of 5-6 μm and the haploid form is 4 μm diameter spheroids [61]. *S. cerevisiae* cells can undergo both asexual and sexual reproduction to produce new cells which depend on the environmental condition. Asexual reproduction of yeast cells is a process known as budding, in which a new daughter cell grows in size and eventually separates from its parent cell [64]. The buds of haploid cells are adjoining each other called radial budding, while the buds of diploid cells are located in opposite poles or axial budding. (figure 4.)

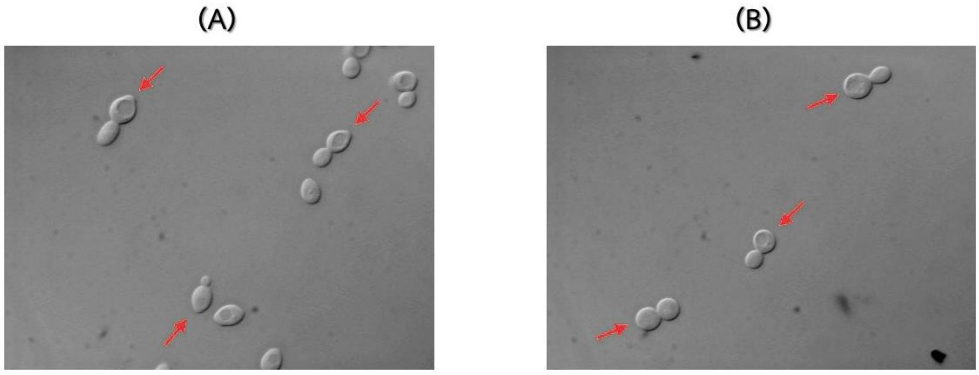


Figure 4. Budding pattern and morphology of diploid and haploid cells:

(A) Diploid, lemon-shape, axial budding (B) haploid, egg-shape, radial budding [61].

Sexual reproduction, the yeast cells will undergo a meiotic process called sporulation to produce four progeny haploid spores, which enclosed as ascospore within a sac-like structure called an ascus upon facing stressful conditions. These four haploid cells can be either of two mating type, two a spores and two α (alpha) spores. When the environmental stressfully conditions get better, these spores are released from the ascus. Both mating types can also reproduce vegetatively as stable haploid cells. The transition from haploid to diploid cells can occur *via* the fusion of two haploid cells of opposite mating type to forming a diploid zygote, results in a/α diploids. The diploid zygote can undergo meiosis and form four haploid spores then these haploids go through the sexual reproduction cycle once again [61, 64]. It is shown in Figure 5.

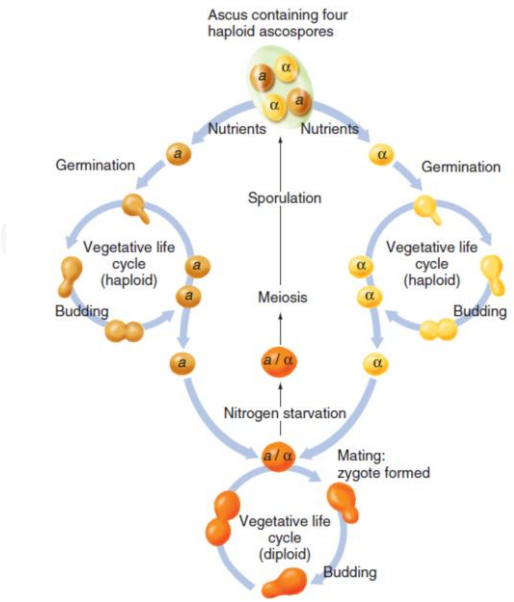


Figure 5. Life cycle of *Saccharomyces cerevisiae* [65]

Yeast-based genotoxicity assay

Genotoxicity refers to the processes which can cause changing in the structure, information content, or segregation of DNA. A number of physical and chemical agents may be involved in the evidence of mutation by causing a wide variety of possible damages to genetic materials such as DNA adduct formation, DNA strand-breaks, chromosomal breakage and sister chromatid exchange. The assessment of the genotoxic property of substances can be observed both in prokaryotic cells, eukaryotic cells, and mammalian systems which the most commonly used *in vitro* method on prokaryotic organisms is bacterial reverse mutation assay or the Ames test [5]. Although the assay using prokaryotic cells is mainly used to investigate the mutagenic activities of complex mixtures, the prokaryotic cells do not respond to a number of genotoxic compounds [66-68].

A yeast-based assay is one of the most frequently used method that combine the advantages of eukaryotic cells for assessing the genotoxic potential of chemical compounds and complex mixtures. The test using diploid yeast *S. cerevisiae* is a simplest and rapid method for examining many kinds of genotoxic effects of mutagens and carcinogens that cannot be detected in prokaryotic cells [68]. The diploid yeast *S. cerevisiae* is a convenient model eukaryotic organism which has several advantages as a test organism to study the induction of reciprocal and non-reciprocal type of mitotic recombination, forward mutation and reverse mutation [69]. A number of different strains of *S. cerevisiae* can be utilized to detect the production of a variety of genetic events by chemical mutagens such as the detection of chemical-induced

mitotic recombination from strains D3 and D4 and mitotic crossing-over from strains D5 [70]. The yeast *S. cerevisiae* strain D7 is one of the first yeast test system which does not only lead to the induction of classical types of mutations, point mutations, chromosomal aberrations, chromosomal deletion or loss of an entire chromosome, but also mitotic recombination [71]. When the tested compound mutates DNA of diploid yeast cells, the phenotypic changes at separate genomic sites are produced that these changes are easy to observe the phenotypes of diploid yeast cells by the color of colonies and the growth on selective media.

Mitotic recombination in diploid yeast *Saccharomyces cerevisiae*

The induction of mitotic recombination in diploid yeast cells with physical and chemical mutagens can be divided into two categories (1) reciprocal recombination (2) non-reciprocal recombination. Reciprocal recombination or crossing-over is a recombination event which involves the exchange of segments of chromatids between homologous chromosomes, resulting in reciprocal products. The process of unidirectional transfer of a DNA sequence, between non-sister chromatids of homologous chromosomes, is called non-reciprocal recombination or mitotic gene conversion [72]. Mitotic recombination can be observed in heteroallelic diploids with a specific nutritional requirement. A heteroallelic diploid (Figure 6.) carries two different inactive alleles of the same gene in each pair of homologous chromosome with the mutational defects in different sites [62, 73]. Both reciprocal and non-reciprocal recombination have been reported the effects by many of chemical mutagens and irradiation in a number of organisms such as *Escherichia coli* [74], *Drosophila melanogaster* [73], and

the mouse [75]. Furthermore, it was also detected in several fungi, including *Aspergillus nidulans* [5], *Neurospora crassa* [76], and *Saccharomyces cerevisiae* [71, 73].

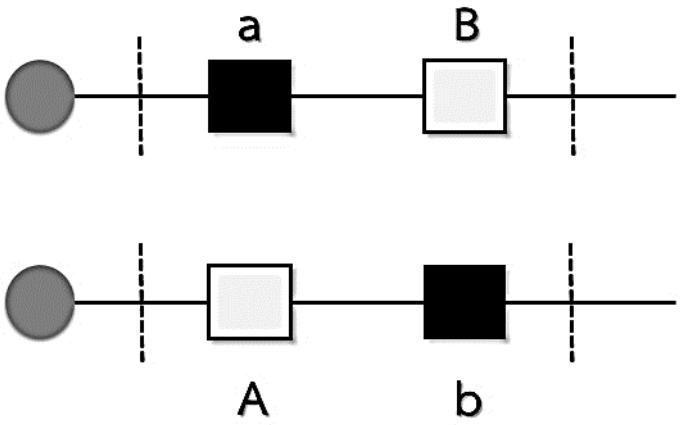


Figure 6. Heteroallelism. The open circles represent centromeres and the solid lines represent chromosomes. The boundaries of a gene are delineated by the dashed lines. DNA sequences of wild-type are indicated by open boxes and mutant sequences by closed boxes. In the heteroallelic case, there are two different mutant sites (a and b), one in each of a pair of homologous loci.

Mitotic crossing-over

Mitotic crossing-over in diploid yeast cells is a genetic event which is easily recognized by the formation of "twin spots" in suitable strains [62]. The genetic characteristic of a diploid organism which used to monitor mitotic crossing-over, is based on the heterozygous condition of two linked genes located on the same chromosome arm which consists of a dominant and a recessive allele in each one [77]. The induction of this mechanism can be examined by the production of homozygosity for recessive linked markers and led to formation of twin spots [78]. During mitotic growth, heterozygotes can segregate recombinants for heterozygous markers which can produce the phenotypically expression of recessive genes [73]. The mitotic crossing-over between the proximal recessive marker and the centromere will lead to a 50% chance in the segregation of recessive alleles to formation of two daughter cells. One of the daughter cells is homozygous for the proximal recessive marker, and the other is homozygous for the distal recessive marker [77].

A strain of *S. cerevisiae*, D5 has been used for screening the induction of mitotic crossing-over on selective media. Strain D5 is diploid yeast cell which carries a pair of phenotypically distinguishable and recessive alleles in the *ade2* locus. The defective mutants at *ade2* locus causes a requirement of adenine for growth and the formation of the red colonies. One allele, *ade2-40*, which causes an absolute block of purine biosynthesis, is adenine-requiring and forms deep red colonies. The other allele, *ade2-119*, causes a leaky requirement for adenine, and the formation of a pink colonies on low adenine medium. The two alleles, *ade2-40* and *ade2-119*, complement each

other which heteroallelic diploid forms white colonies and does not exhibit an adenine requirement. A crossing-over event will lead to the segregation of two reciprocal products, homoallelic for *ade2-40* and homoallelic for *ade2-119*. The chemical-induced mutagenesis causes the appearance of a red and a pink sectors colonies, twin-spotted colonies, on limited adenine medium [62, 69, 77]. Furthermore, the appearance of all other phenotypically distinguishable colored colony types, such as sectored red/pink, red/white, and pink/white or wholly red and wholly pink colonies, may result from mitotic recombination, lethal sectoring or aneuploidy [79].

The expression of adenine biosynthetic mutations in yeast *S. cerevisiae* is based on the formation of a red colony color in diploid yeast cells carrying mutation in the *ADE2* gene [80]. The *ADE2* yeast gene on chromosome XV encodes AIR-carboxylase which catalyzes Phosphoribosylaminoimidazole (AIR) to phosphoribosylaminoimidazole carboxylate (CAIR) in the biosynthesis pathway of adenine [81]. Consequently, the defect in the pathway of adenine biosynthesis results in adenine auxotrophy and causes the accumulation of AIR in the cells which the aerobically grown colonies will oxidize AIR to develop into the red pigment [80, 82].

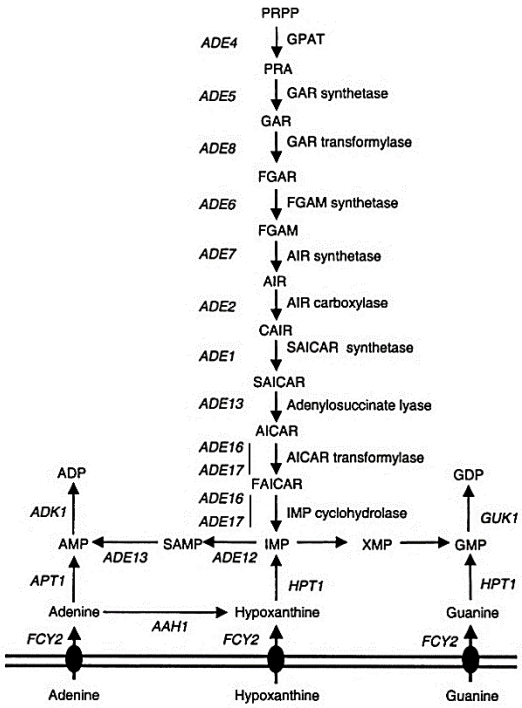


Figure 7. Scheme for the biosynthesis pathway of adenine [81].

Mitotic gene conversion

Mitotic gene conversion in diploid yeast cell can conveniently be observed in heteroallelic diploids requiring nutritional supplement caused by the pair of heteroalleles [62]. The restoration of a wild-type genotype will be resulted from the mutational reversion transfer of the intact region of a mutant allele to replace the defective mutational site in the other mutant allele. The formation of intact and dominant wild-type allele eliminates the originally required nutrient. Consequently, the occurrence of this process can be detected on a synthetic medium free of the originally required growth factor which the convertant cells will grow on the indicator media. However, different strains of *S. cerevisiae* have been used to detect mitotic gene conversion on a several type of mutagens. The most generally used strain for

detection mitotic gene conversion is D4 which is heteroallelic at *ade2-1/ade2-2* and *trp5-12/trp5-27*. The induction of mitotic gene conversion can be detected by plating the cells on adenine-free medium selects convertants at *ade2* locus, and plating on tryptophan-free medium selects convertants at *trp5* locus [69].

Reverse mutation

Reverse mutation experiment is a simple technic for assessing the production of mutation induced by chemical agents in yeast cells. The induction of reverse mutation is based on the restoration of function which a full or a partial restoration causes a reversal of original defect in a gene coding for a required enzyme. The reversion of mutant cells can be detected by plating mutagenized cells on a selective medium lacking nutritional supplement required by original cells. However, the disadvantage of a reverse mutation system is that it usually requires specific genetic alterations for the restoration and the induction of mutation system is prone to some mutagen specificity. Accordingly, a reverse mutation system can be demonstrated the mutation induced by some type of mutagens [62, 69].

Although the reverse mutation is a particular test for detection of the mutagenicity of some chemical mutagens, the alleles at the gene locus *LIV1* in mutant strain of the yeast *S. cerevisiae* are reverted by various allele-specific and locus-specific suppressors [71, 83]. The *S. cerevisiae LIV1* gene, encoding threonine dehydratase which catalyzes the formation of α-ketobutyrate from threonine in the first step of L-isoleucine biosynthesis pathway. (Figure 8.) The mutant strains at the *LIV1* gene lack

threonine dehydratase activity in isoleucine biosynthesis pathway and require isoleucine for growth [73]. Therefore, the induction of reverse mutation induced by chemical mutagens can be detected on isoleucine-free medium. The occurrence of this mechanism in both of homologues would allow the formation of colonies on synthetic medium lacking isoleucine [79].

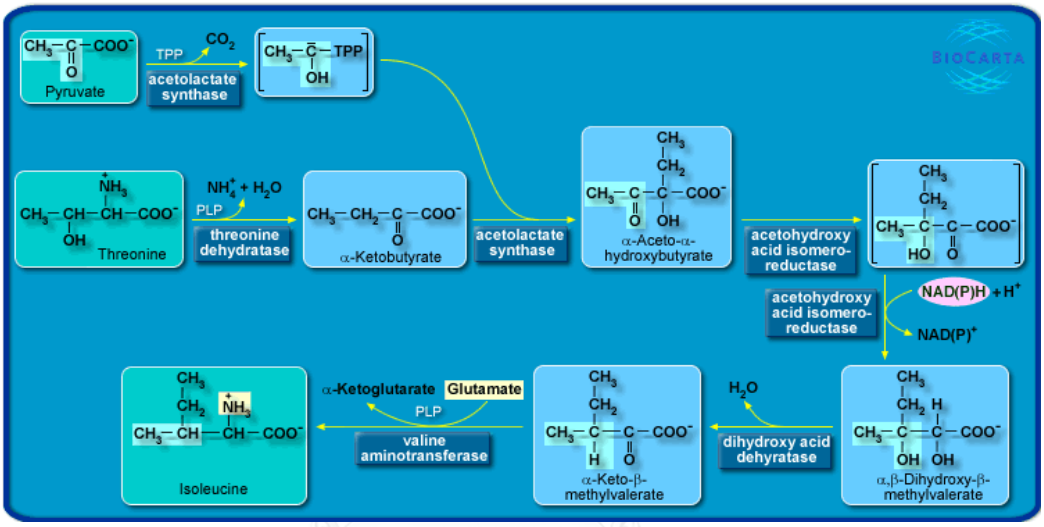


Figure 8. Scheme for the biosynthesis pathway of isoleucine

***Saccharomyces cerevisiae* D7**

Mutagens are preferentially induced a certain type of genetic alteration. The organism, capable for testing of different genetic modifications, is therefore, the model of choice. A diploid yeast *S. cerevisiae* is a choice of model organism which is capable for detecting wide spectrum of genetic alterations, especially strain D7 of *S. cerevisiae*. It is an alternative strain which was constructed to detect more than one mitotic recombinational events in different chromosomes, including induction of mitotic crossing over, mitotic gene conversion and reverse mutation [62, 71]. The relevant genotype of the diploid strain D7 is:

$$\frac{a}{\alpha}\ \frac{ade2-40}{ade2-119}\ \frac{trp5-12}{trp5-27}\ \frac{ilv1-92}{ilv1-92}$$

This strain is heteroallelic at *ade2* site, a pair of complementing colony color mutant alleles (*ade2-40* and *ade2-119)* on chromosome XV, which allows for detection of mitotic crossing-over. The *ade2-40* causes an adenine auxotrophy and forming deep red colony color while the *ade2-119* causes a weak requirement for adenine and a light pink pigmentation of colonies [63]. Both alleles are recessive and complement each other which make D7 an adenine prototroph and produce white colonies [84]. Reciprocal mitotic crossing-over at the *ade2* locus can be recognized by the formation of red and pink colonies (twin-spotted colonies) on nutrient-rich media. However, the expression of the other types of colored colonies can be occur which may be caused by any of other genetic effects. The D7 strain also carries the non-complementing alleles on chromosome VII, *trp5-12* and *trp5-27*, which requires tryptophan

supplementation for growth, in termed tryptophan auxotrophy. Restoration of a functional allele by mitotic gene conversion gives rise to tryptophan prototrophs that the cells are able to grow on synthetic media lacking tryptophan [73]. In addition, the D7 strain carries the homoallelic condition of *ilv1-92* locus: *ilv1-92/ilv1-92* on chromosome V which allows for the detection of reverse mutation of isoleucine requirement [63].

Direct mutagen for yeast-based genotoxicity assay

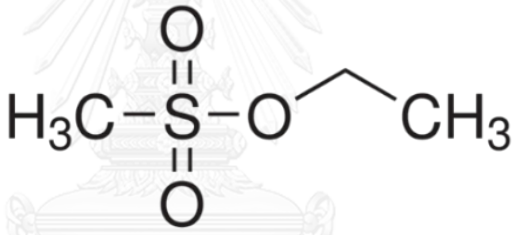


Figure 9. Chemical structure of Ethyl methanesulfonate

Scientific Name: Ethyl methanesulfonate (EMS)

Molecular formula: $C_3H_8O_3S$

Molecular weight: 124.15882 g/mol

Description:

Ethyl methanesulfonate (EMS) is a monofunctional alkylating agent which is able to transfer an alkyl group, for example, methyl group (CH_3-), ethyl group (CH_3CH_2-) and others into nucleotides. Its mechanism results in miscoding of DNA and induce mutagenesis in gene or chromosome.

Ethyl methanesulfonate is one of the most effective chemical mutagen which commonly used for mutagenesis studies. The mechanism of action of EMS affects structure and pairing properties of nitrogenous bases of DNA (Figure 10.). Alkylation of alkyl group at the number 6 position of guanine, forming O^6-ethylguanine, which can pair with thymine (T) instead of cytosine (C). Its mechanism leads primarily G/C- to -A/T transitions [85]. EMS has been widely used as a mutagen to induce a variety of genetic effects in a number of microorganisms including mammals. Moreover, EMS has been found to be mutagenic in a large number of organisms such as *Escherichia coli and Salmonella typhimurium* [86], *Drosophila melanogaster* [87], *Arabidopsis thaliana* [88, 89].

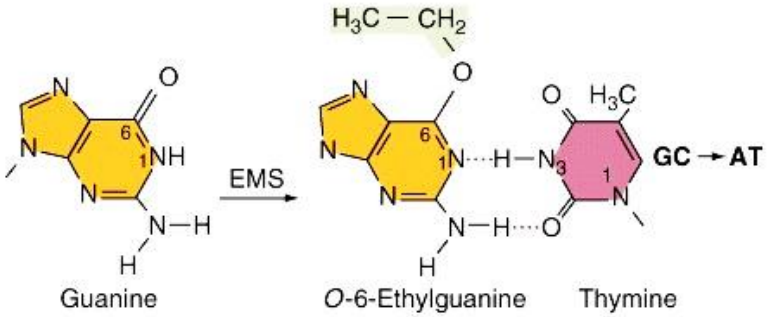


Figure 10. Mutagenesis by ethyl methanesulfonate

CHAPTER III

MATERIALS AND METHODS

Chemicals

1. Acetonitrile (Merck, Germany)
2. Adenine hemisulfate (Sigma-Aldrich, St. Louis, U.S.A.)
3. Agar-Agar (Merck, Darmstadt, Germany)
4. Ammonium sulfamate (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
5. Ampicillin (T.P. Drug Laboratories (1996) Co., Ltd., Thailand)
6. 1-aminopyrene (Sigma-Aldrich, St. Louis, U.S.A.)
7. Citric acid monohydrate (BDH prolabo, UK)
8. Crystal violet indicator (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
9. D-biotin (Sigma-Aldrich, St. Louis, U.S.A.)
10. D-glucose monohydrate (Ajax Finechem Pty Ltd.)
11. Difco ^{TM} yeast nitrogen base w/o amino acids (Becton, Dickinson and company)
12. Dimethylsulfoxide (DMSO) (RCI Labscan Limitted)
13. Di-potassium hydrogen orthophosphate (Ajax Finechem Pty Ltd.)
14. Ethanol (Merck, Germany)

15. Ethyl methane sulfamate (Sigma-Aldrich, St. Louis, U.S.A.)
16. Hydrochloric acid (RCI Labscan Limitted)
17. L-histidine monohydrochloride monohydrate (Sigma-Aldrich, St. Louis, U.S.A.)
18. L-isoleucine (Sigma-Aldrich, St. Louis, U.S.A.)
19. L-tryptophan (Sigma-Aldrich, St. Louis, U.S.A.)
20. Magnesium sulfate (Ajax Finechem Pty Ltd.)
21. Oxiod nutrient broth No.2 (HiMedia Laboratories Pvt. Ltd., Mumbai, India)
22. Peptone (HiMedia Laboratories Pvt. Ltd., Mumbai, India)
23. Sodium ammonium hydrogen phosphate tetrahydrate (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
24. Sodium Chloride (QReC, Auckland, New zealand)
25. Sodium hydroxide (NaCl) (QReC, Auckland, New Zealand)
26. Sodium nitrite (Ajax Finechem Pty Ltd.)
27. Yeast extract powder (HiMedia Laboratories Pvt. Ltd., Mumbai, India)

Materials

1. Cuvette (Barloworld Scientific Ltd., Staffordshrine, United kingdom)
2. Filter paper whatman No.4 (Whatman, England)
3. Sterile syringe filter 25 mm Dia., 0.2 µm Pore size (Whatman, England)
4. Sterile syringe filter 25 mm Dia., 0.45 µm Pore size (Whatman, England)
5. Syringe 5 ml (Becton Dickinson, Singapore)

Instruments and Equipments

1. Autoclave (ALP Co., Ltd, Japan)
2. Centrifuge (Scientific industries INC., Bohemia, New York, U.S.A.)
3. Centrifuge (MSE Mistral 3000, SANYO Gallenkamp PLC, United Kingdom)
4. Hemocytometer and cover slip (Improved Neubauer, Boeco, Germany)
5. Lamina Hood (Astec SC 1200 AC, Bioquell UK Ltd., United Kingdom)
6. Microscope (Carl Zeiss, Germany)
7. Rotary vacuum evaporator (Büchi R210, Switzerland)
8. Spectrophotometer (Shimadzu-w 1800, shimadzu Corp., Japan)
9. Tally Counter
10. Ultrasonic sonicator (Analytical Lab Science Co. Ltd., Thailand)
11. Water bath (Brinkmann, USA)
12. Ultra-pure water purification NW20VF (Heal Force, China)

Sample Preparation

Plant materials

Fruits of *A. marmelos* were collected from local market in Bangkok, Thailand. The roots were collected from the provinces of Chiang Rai, Tak, Surin and Nakhon ratchasima, Thailand. All samples were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi and kept in the closed container under ambient condition at College of Public Health Sciences, Chulalongkorn University, Thailand.

Preparation of extraction

The fruits and the roots were shade-dried and ground to powders. The powders of each part were continuously macerated with 95% ethanol and water until exhaustion, respectively. The ethanolic extracts were filtered through Whatman No.4 (pore size 20-25 μm) and evaporated by vacuum rotary evaporator, whereas the fractionated water extracts were lyophilized to dryness. The extract yields were weighed, recorded and stored at -20°C until used. The ethanolic extracts from the fruit and the root were dissolved in dimethyl sulfoxide (DMSO) and filtered through syringe filter (pore size 0.2 μm), whereas the fractionated water extracts from the fruit and the root were dissolved in distilled water and filtered through syringe filter (pore size 0.45 μm) for the Ames test and yeast-based genotoxicity assay.

Bacterial reverse mutation test (Ames test)

Bacterial Tester Strain

Salmonella typhimurium tester strains used in this study were histidine dependent strain (His^-) for frame-shift mutation, TA98 (*hisD3052, bio, uvrB-bio, rfa* and pKM101) and strain for base-pair substitution mutation, TA100 (*hisG46, bio, uvrB-bio, rfa* and pKM101) which have been kindly provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. Both tester strains from frozen stock culture were grown in an Oxiod nutrient broth No.2 and incubated overnight in a shaking water bath at 37°C before used. The mutant strains were confirmed for the genotypes of histidine/biotin dependence, *rfa* marker, *uvrB* deletion gene mutations and presence of plasmid pKM101 before used in mutagenic and anti-mutagenic assay of Ames test (Appendix A).

Mutagenicity of extracts

Mutagenic activity assay without nitrite treatment

The pre-incubation method of Ames test was employed to determine the mutagenic effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* on both *S. typhimurium* strains TA 98 and TA100 without enzyme activating system. The ethanolic extract was dissolved in dimethyl sulfoxide (DMSO) whereas the fractionated water extract was dissolved in distilled water to initial concentration of 25, 50, 100, and 200 mg/ml, respectively. For mutagenicity testing, 200 µl of each solution was added to the tube containing 10 µl of 0.2N HCl to acidify the reaction mixture to pH 3-3.5 and adjusted the final volume to 1000 µl with 250 µl of dimethyl sulfoxide or distilled water. Each reaction tube was shaken at 37°C for 4 hr then placed in an ice bath for 1 min to stop the reaction. Finally, add 250 µl of dimethyl sulfoxide or distilled water and allow the tube to stand in an ice bath for 10 min. Then, 100 µl of this extract mixture was mixed with 100 µl of bacterial suspension and 500 µl of 0.2 M phosphate buffer pH (7.4) and then incubated at 37°C in shaking water bath for 20 min. After incubation, 2 ml of molten top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin was added to the tube, mixed well and poured onto a minimal glucose agar plate. The final concentration of the extracts were 0.4, 0.8, 1.6 and 3.2 mg/plate. All plates were incubated at 37°C for 48 hr and the number of his^+ revertant colonies on each plate were counted. Dimethyl sulfoxide (DMSO) or distilled water was used as a negative control to determine the spontaneous reversion activity. Each concentration of all extracts was done in sixtuplets.

Mutagenic activity assay with nitrite treatment

The ethanolic extract and fractionated water extracts from the root and the fruit of *A. marmelos* were dissolved in dimethyl sulfoxide (DMSO) or distilled water to initial concentration of 25, 50, 100, and 200 mg/ml, respectively. A volume of 200 µl of each extract was added to the tube containing 550 µl of 0.2N HCl to acidify the reaction mixture to pH 3-3.5 and adjusted the final volume to 1000 µl with 250 µl of 2M sodium nitrite. Each reaction tube was shaken at 37°C for 4 hr then placed in an ice bath for 1 min to stop the reaction. Finally, 250 µl of 2M ammonium sulfamate was added to the reaction mixture to decompose the residue nitrite and allowed the reaction tube to stand in an ice bath for 10 min. A volume of 100 µl of nitrite treated the extracts from *A. marmelos* was determined for their mutagenicity by Ames test using on *S. typhimurium* TA98 and TA100 as described above. Each concentration of all extracts was done in sixtuplets. It was shown in Figure 11.

Standard direct mutagens

1-Aminopyrene (AP) treated with nitrite in acid solution was used as a positive mutagen since it gave direct-acting mutagenicity in the condition similar to that occurred during stomach digestion [47, 90]. Briefly, 10 µl (tested on *S. typhimurium* TA98 of the Ames test) or 20 µl (tested on *S. typhimurium* TA100 of the Ames test) of 1-aminopyrene (0.0375 mg/ml) was mixed with 740 µl or 730 µl of 0.2N HCl to acidify the reaction mixture to pH 3-3.5 and 250 µl of 2M sodium nitrite was added to obtain the final volume of 1000 µl. The reaction tube was shaken at 37°C for 4 hr and the reaction was stopped by placing the tube in an ice bath for 1 min to stop the reaction. Finally, 250 µl of 2M ammonium sulfamate was added to the tube and the whole was allowed to stand in an ice bath for 10 min before it was used as the positive standard control. Then, a volume of 100 µl of this mixture (nitrite treated 1-aminopyrene) was determined for its mutagenicity by Ames test using on *S. typhimurium* TA98 and TA100.

Anti-mutagenicity of extracts

The anti-mutagenic effect of all extracts against 1-aminopyrene treated with sodium nitrite was determined by the pre-incubation method of Ames test similar to the mutagenic testing. For anti-mutagenicity testing, 40 µl (tested on *S. typhimurium* TA98 of the Ames test) or 80 µl (tested on *S. typhimurium* TA100 of the Ames test) of 0.0375 mg/ml 1-aminopyrene was transferred into the sterile test tube. Then, 710 µl or 670 µl of 0.2N HCl and 250 µl of 2M sodium nitrite was added to obtain to total volume at 1000 µl. The mixtures were shaken at 37°C for 4 hr. Later on, the test tubes were placed in an ice bath for 1 min to stop the reaction mixture. Two-hundred fifty microliter of 2M ammonium sulfamate was added to the test tube, mixed well, and allowed to reaction tube to stand in an ice bath for 10 min. A volume of 25 µl of the mutagen (nitrite-treated 1-aminopyrene) was tested for anti-mutagenicity. Various concentrations of each extract (25, 50, and 75 µl) were transferred into sterile test tube containing 25 µl of the mutagen (nitrite-treated 1-aminopyrene). This mixture was adjusted with DMSO (for ethanolic extract) or with distilled water (for fractionated water extract) to the final volume of 100 µl. The mixture (100 µl) was mixed with 500 µl of 0.2 M phosphate buffer (pH 7.4), 100 µl bacterial suspension and it was incubated at 37°C in shaking water bath for 20 min. After incubation, 2 ml of molten top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin was added, mixed well and poured onto a minimal glucose agar plate. The final concentration of the extracts were 5, 10 and 15 mg/plate. All plates were incubated at 37°C for 48 hr and the number of his^+ revertant colonies on each plate were counted. Each concentration of all extracts was done in sixtuplets. It was shown in Figure 12.

Interpretation and reporting data

The results were reported as mean histidine (His^+) revertant colonies per plate ± SD for each experiment and the control.

The mutagenic index (MI) was calculated from the number of revertant colonies of the sample treatment divided by the number of spontaneous revertant colonies (the tube without the extract). A compound was classified as positive mutagenic effect if the results satisfied two criteria: (1) the number of induced revertant colonies increase in a dose response relationship manner (2) at least two doses were higher than spontaneous revertants and at least one dose give rise to twice over the spontaneous revertant (MI > 2) [51].

The percentage modification (either increase or decrease on mutagenicity of standard direct mutagens) was calculated by the following formula:

$$\% \text{ Modification} = \frac{(A - B)}{(A - C)} \times 100$$

Where A is the number of histidine revertants colonies per plate induced by nitrite-treated 1-Aminopyrene, B is the number of histidine revertants colonies per plate induced by nitrite-treated 1-Aminopyrene in the presence of each extract and C is the number of spontaneous histidine revertants colonies per plate. In terms of anti-mutagenicity activity, it was classified as following:

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

In terms of increase mutagenicity, it was classified as following:

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

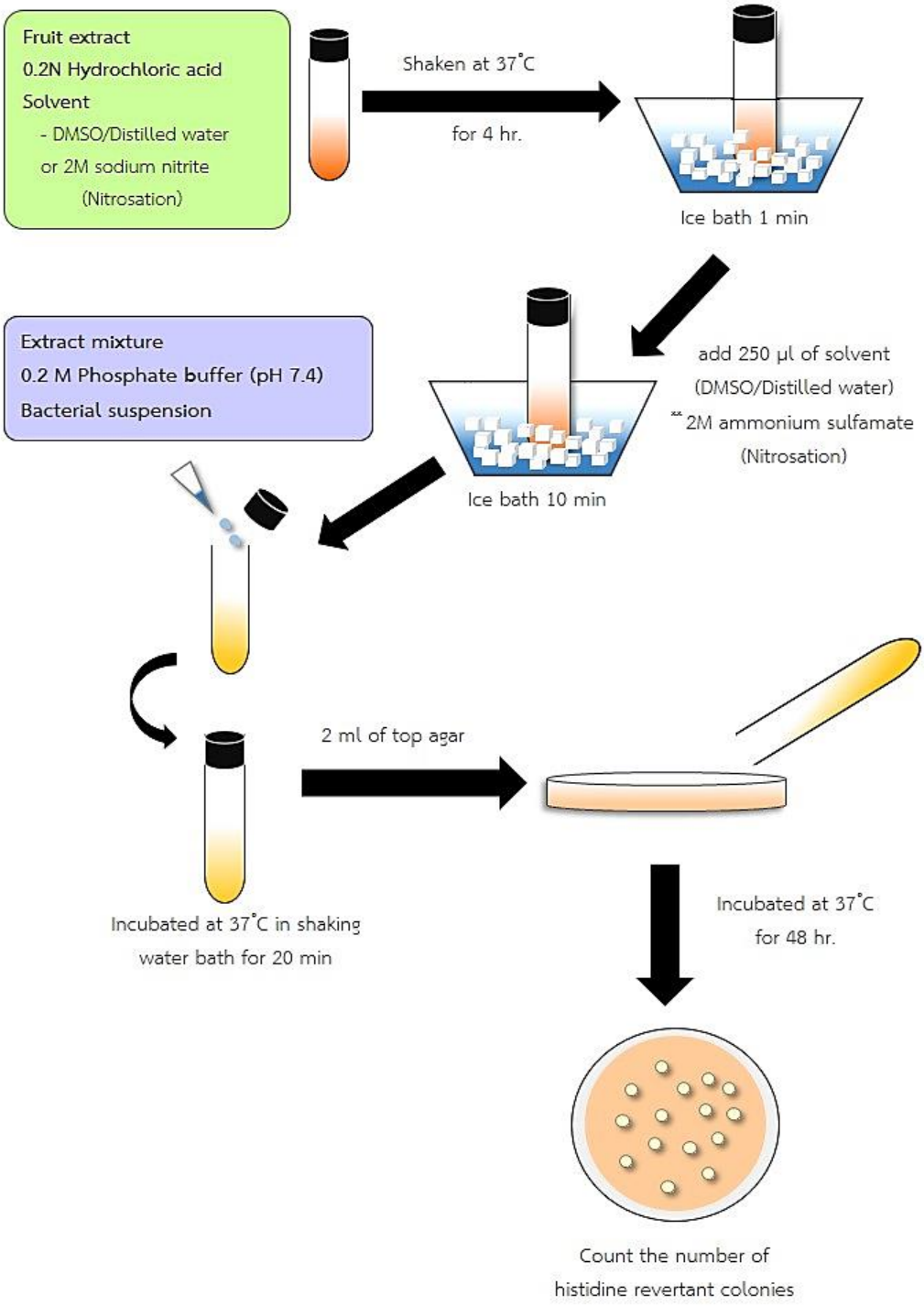


Figure 11. Steps to determine the mutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation

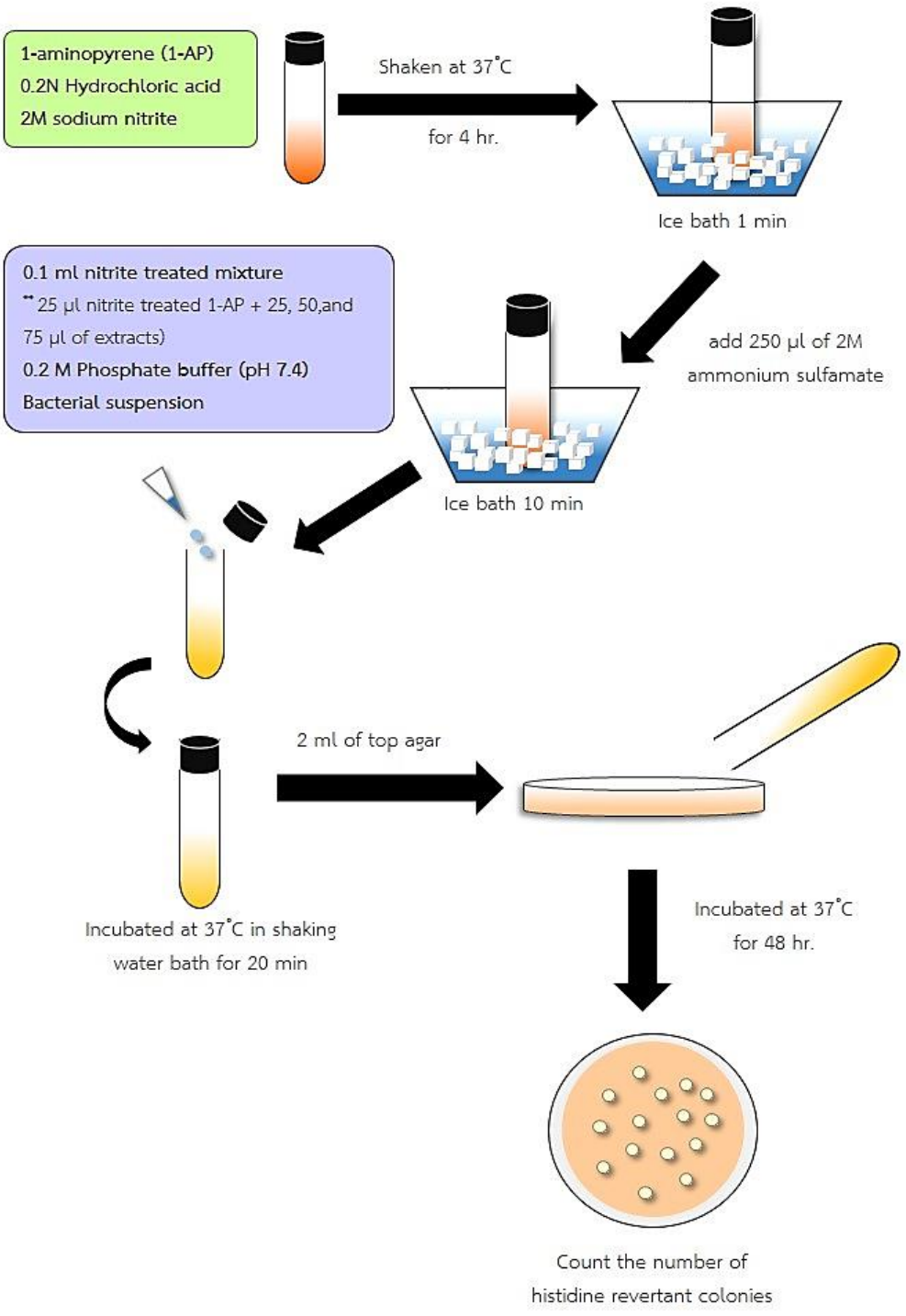


Figure 12. Steps to determine the anti-mutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation

Genotoxic assay in *Saccharomyces cerevisiae*

Yeast strain and media

The diploid yeast strain *Saccharomyces cerevisiae* D7 (MATa/α *ade2-40/ade2-119 trp5-12/trp5-27 ilv1-92/ilv1-92*) was used for the simultaneous detection of induced mitotic gene conversion, mitotic crossing-over, and reverse mutations. The yeast *Saccharomyces cerevisiae* D7 has been kindly provided by Dr. Somchit Palakas, Kasetsart University Nuclear Technology Research Center (KU-NTRC), Bangkok, Thailand. The lyophilized yeast cells were inoculated into 50 ml of liquid YEPD medium consisted of 1% yeast extract, 2% peptone and 2% D-glucose. The yeast cells were grown on YEPD agar and kept at 4°C until used. The stock cultures on YEPD agar were sub-cultured every month.

Before used in all experiments, the number of survivors were determined on complete medium containing 0.67% Difco Yeast Nitrogen Base without amino acids, 2% D-glucose, 2% Difco Bacto-agar, and supplemented with adenine sulfate (5 mg/l), isoleucine (60 mg/l), and tryptophan (10 mg/l). Tryptophan and isoleucine were omitted from the complete medium for selection of Trp^+ convertants, and Ilv^+ revertants.

Isolation of non-revertant D7 cells

Yeast was transferred from YEPD agar into 50 ml Erlenmeyer flasks containing 10 ml of liquid YEPD medium. The culture was incubated overnight in a shaker incubator at 30°C (150 rpm). After incubation, the cells were harvested by centrifugation 10 min at 5000 rpm, and washed twice with 0.1 M phosphate buffer, pH 7.0. Then, the cells were re-suspended in 0.1 M phosphate buffer for preparation appropriate cell suspension as determined by hemocytometer counts. The dilutions of cells were plated onto a synthetic medium lacking isoleucine (about 2×10^6 cells per plate), synthetic medium lacking tryptophan (about 2×10^5 cells per plate) and approximately 200 cells per plate on complete medium. The plates were incubated at 30°C for 72 hr and the formation of yeast colonies on each agar medium were observed before used in genotoxic assay.

Preparation of extract solution

The ethanolic and fractionated water extracts were dissolved in dimethyl sulfoxide (DMSO) or distilled water to initial concentration of 12.5, 25, 50, 100 mg/ml, respectively for used in genotoxic assay. The final concentration of test compound were 0.25, 0.5, 1, and 2 mg/ml. The final concentration of DMSO of the test compound and the control were 2% and 1% v/v, respectively.

Treatments with extracts

For the yeast-based genotoxicity assays, the procedure adapted from previously reported method [72, 84] was used. Yeast cells from YEPD agar were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YEPD liquid medium, and grown at 30°C (150 rpm) in a shaker until they reached stationary phase (16 hr). The cells were harvested by centrifugation at 3600 rpm for 10 min in a MSE Mistral 3000 centrifuge, then washed twice with 0.1 M phosphate buffer, pH 7.0. The collected cells were re-suspended in 0.1 M phosphate buffer then diluted and counted in hemocytometer. In most experiment, the cell density of stationary phase cells (2×10^8 cells/ml) was prepared.

One hundred microliters of each concentration of the test compound was added in a screw-cap tube and mixed well with 3.9 ml of phosphate buffer (0.1 M, pH 7.0). Solvent (DMSO or distilled water) was used as control. The cell suspensions (1 ml) were treated in a screw-cap tube together with this mixture to final volume of 5 ml and incubated in a shaker for 3 hr at 30°C. At the end of each treatment, the treated cells were harvested by centrifugation, washed twice and then re-suspended in 1 ml of cold-phosphate buffer (0.1 M, pH 7.0) and counted in hemocytometer. Appropriate cell suspension were spread onto: (a) petri dish containing synthetic complete medium lacking isoleucine (about 2×10^6 cells per plate); (b) petri dish containing synthetic complete medium lacking tryptophan (about 2×10^5 cells per plate); (c) petri dish containing complete medium (about 200 cells per plate). All plates were incubated at room temperature. The number of survivors

in the petri dishes were counted after 3 days of incubation whereas the convertant colonies and total aberrant colonies were counted after 7 days of incubation. Isoleucine revertant colonies were counted after 14 days of incubation. All experiments were carried out in sixtuplets.

Co-treatments of extracts and ethyl methanesulfonate (EMS)

The preparation of cell suspension (2×10^8 cells/ml) for co-treatments of all extracts with EMS was similar to the treatments of extracts alone. For each treatment, the cell suspensions (1 ml) were treated in a screw-cap tube containing 100 µl of each concentration of the test compound, 100 µl of 10% ethyl methanesulfonate (EMS) and mixed with 3.8 ml of phosphate buffer (0.1 M, pH 7.0) to final volume of 5 ml. Ethyl methanesulfonate (EMS) (10% v/v dissolved in sterile distilled water) was used as a direct standard mutagen (positive control) and phosphate buffer was used as negative control. The final concentration of EMS was 1% v/v. The condition and method in the co-treatments of the test compound and mutagen were similar to the treatments of extracts alone.

Quantification of survival colonies, total aberrant colonies, convertants, and revertants

The cytotoxicity of the experiments were determined by the number of survival colonies as well as the survival calculated fraction as the percentage of survivors in control experiment. The total aberrant colonies were reported as the percentage of total aberrant colonies per survival colonies. The frequencies of Trp^+ mitotic gene convertants and Ilv^+ gene revertants were expressed as mean convertants per 10^5 viable cells ± SD and mean revertants per 10^6 viable cells ± SD, respectively. The number of survival colonies and the frequencies of convertants and reveratants of treated groups were compared to the control group by ANOVA with a Dunnett multiple comparisons test.

The percentage of the inhibition of the extracts (in combination with ethyl methanesulfonate) was calculated by the following formula:

$$\text{Percent of the inhibition (\%)} = \left(1 - \frac{X1}{X2}\right) \times 100$$

Where X1 and X2 are the number of yeast colonies in the presence of ethyl methanesulfonate (EMS) with and without extract, respectively [91].

CHAPTER IV

RESULTS

The root of *A. marmelos* has been used as an important ingredient of some Thai ancient remedies, while the fruit has been used as both herbal medicine and food ingredients, particularly herbal drinks in Thailand. In this study, the root and the fruit of *A. marmelos* were continuously extracted with 95% ethanol and water, respectively. Both extracts were illustrated for their safeness and anti-mutagenic effect against nitrite-treated 1-aminopyrene in Ames test. They were also elucidated for their genotoxic effects against *Saccharomyces cerevisiae* strain D7.

The percent yields of the extracts from the root and the fruit of *A. marmelos* were shown in Table 5. The percent yields of the fruit extracts were higher than the root extracts, which the fractionated water extract from the fruit exhibited the highest percentage at 23.51%.

Table 5. Percent yield of the root and the fruit extracts of *A. marmelos*

Sample	Percentage of yield (%)		Total yield (%)
	Ethanolic extract	Fractionated water extract	
A. marmelos root	**8.53**	5.92	14.45
A. marmelos fruit	21.13	**23.51**	44.64

Mutagenicity of the extracts from the root and the fruit of *A. marmelos* in Ames test

Mutagenicity of the extracts from the root and the fruit without nitrite treatment

The effects of the root and the fruit of *A. marmelos* against *S. typhimurium* strain TA98 (detecting frameshift mutagens) and TA100 (detecting base-pair substitution mutagens) were tested in a range of concentration (0.4-3.2 mg/plate) by pre-incubation method of Ames test in acidic condition (pH 3-3.5) without enzyme activating system.

The results of mutagenic assay, the mutagenic index (MI) values of the root and the fruit extracts toward *S. typhimurium* TA98 and TA100 were presented in figure 13-14, respectively. Most of the extracts were not directly mutagenic except the fractioned water extract from the root of *A. marmelos* which showed highest mutagenicity on both strains. The mutagenic indices of the fractionated water extract from the root were 2.95 and 22.72 at 1.6 mg/plate and at 3.2 mg/plate for strain TA98, respectively and 6.45 at 3.2 mg/plate for strain TA100.

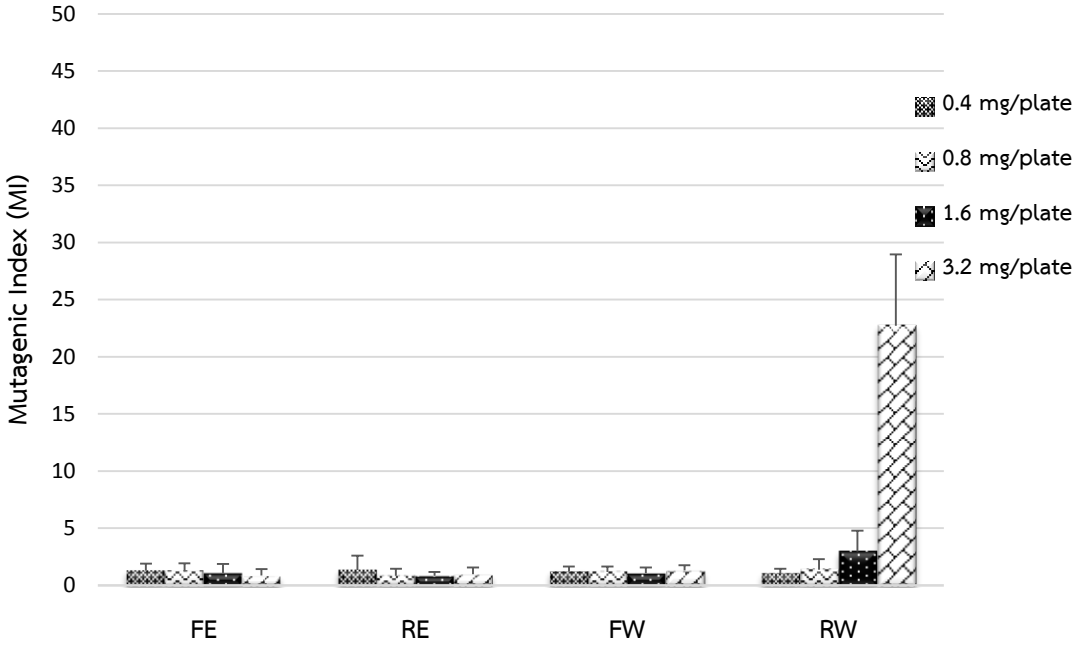


Figure 13. The mutagenic index of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* without nitrite treated on *S. typhimurium* strain TA98. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit, RW: fractionated water extract from the root.

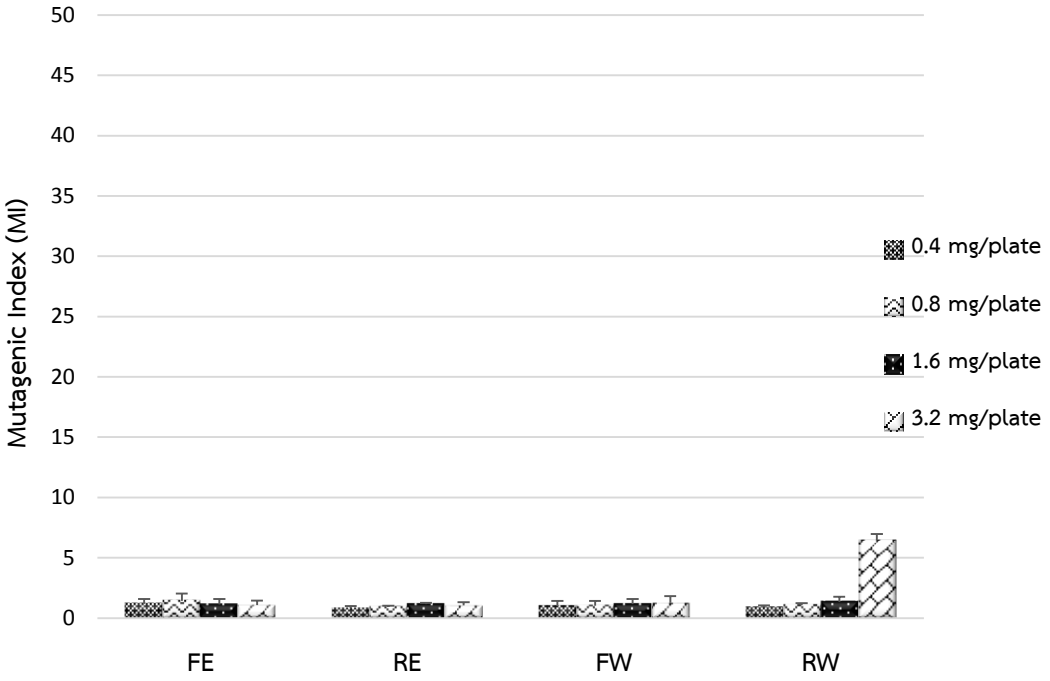


Figure 14. The mutagenic index of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* without nitrite treated on *S. typhimurium* strain TA100. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit, RW: fractionated water extract from the root.

Mutagenicity of the extracts from the root and the fruit with nitrite treatment

Figure 15 and 16 presented the mutagenic index (MI) values of the root and the fruit extracts of *A. marmelos* toward *S. typhimurium* strains TA98 and TA100, respectively. Both ethanolic and fractionated water extracts from the root and the fruit at all range of concentration showed indirect mutagenic effect induced by nitrosation (sodium nitrite-treated 1-aminopyrene) under acidic condition without metabolic activation on both strains. Most of the extracts illustrated the mutagenic index in strain TA98 higher than strain TA100. It was demonstrated that the ethanolic extract from the fruit of *A. marmelos* showed highest positive response of mutagenicity against TA98 with dose-response relationship (MI = 34.49).

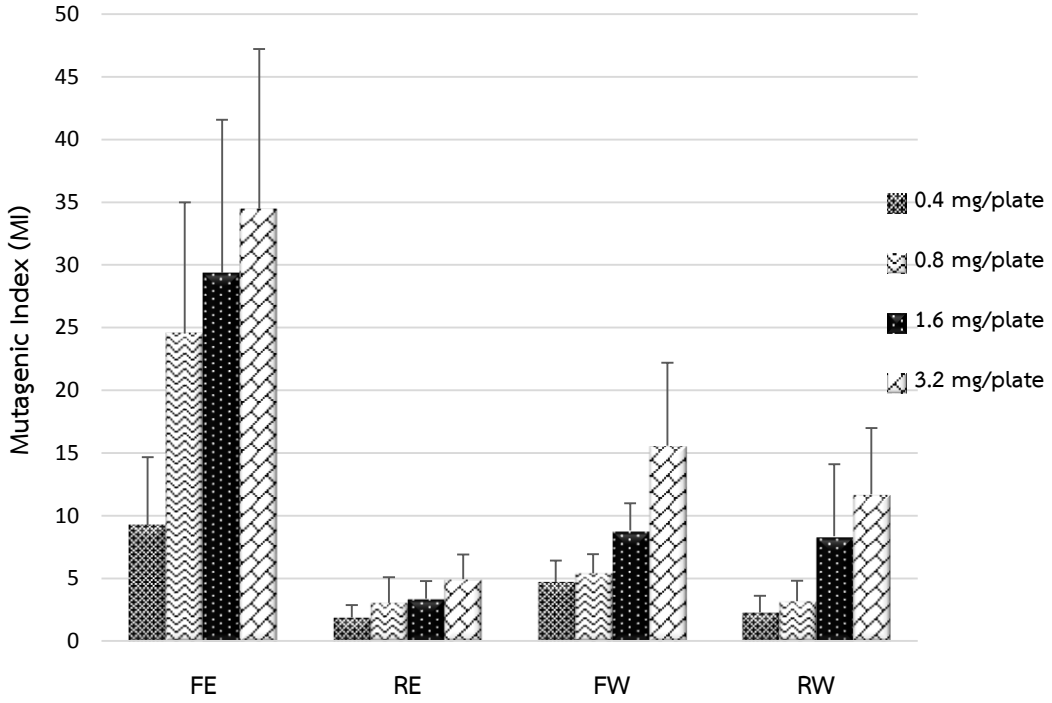


Figure 15. The mutagenic index of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* with nitrite treated on *S. typhimurium* strain TA98. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit, RW: fractionated water extract from the root.

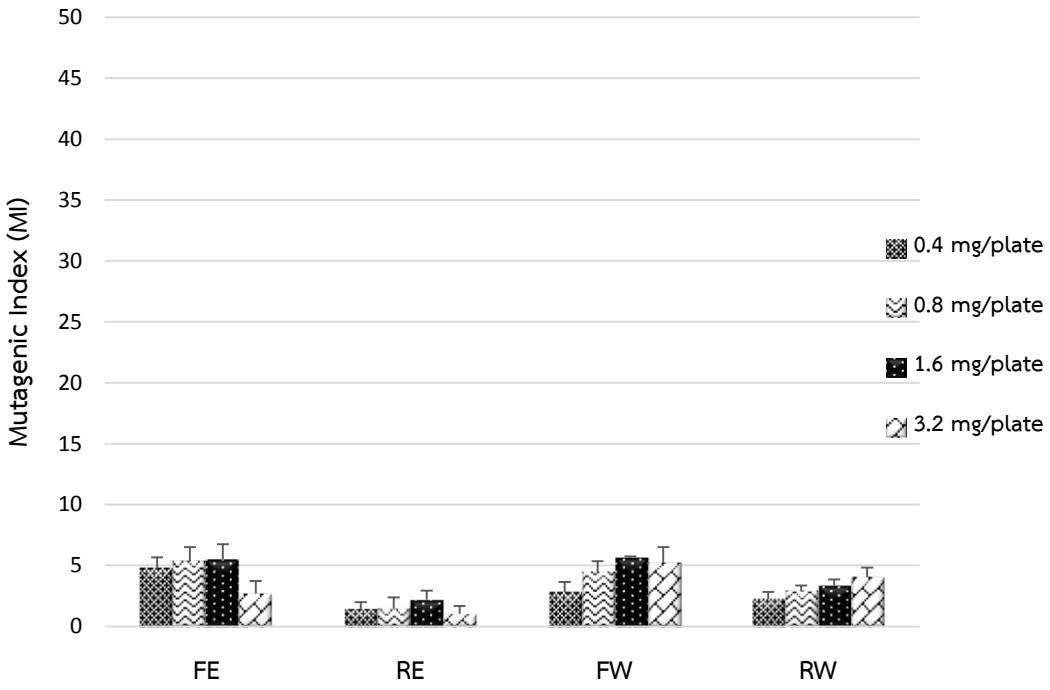


Figure 16. The mutagenic index of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* with nitrite treated on *S. typhimurium* strain TA100. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit, RW: fractionated water extract from the root.

Anti-mutagenicity of the extracts from the root and the fruit of *A. marmelos*

The anti-mutagenic assay determined inhibition of mutation induced by sodium nitrite-treated 1-aminopyrene in the absence of metabolic activation toward *Salmonella typhimurium* strains TA98 and TA100 (Figure 17-18). The effects expressed as percent modification of revertant colonies of bacterial tester strains per plate with the range from negligible (0-20%) to strong (>60%) inhibition.

Most ethanolic extracts from the root and the fruit of *A. marmelos* expressed strong inhibitory effect (>60%) against nitrite-treated 1-aminopyrene model on both strains of *S. typhimurium* in the manner of concentration dependence. At the dose of 15 mg/plate, the ethanolic extract from the root showed strong anti-mutagenic effects of 97.02% and 101.73% on TA98 and TA100, respectively, as well as the ethanolic extract from the fruit showed strong anti-mutagenicity on TA98 (86.71 %) and on TA100 (113.86 %).

In addition, no anti-mutagenic potential of the fractionated water extract from the root of *A. marmelos* on both strains. However, the fractionated water extract from the fruit exhibited weak effect (21.46%) at the concentration of 15 mg/plate on *S. typhimurium* TA98 and negligible effect (5.02%) at the concentration of 5 mg/plate on *S. typhimurium* TA100. Moreover, both strains of *S. typhimurium* were found to be enhanced from the fractionated water extract with negligible enhancement effect (0 to -20%).

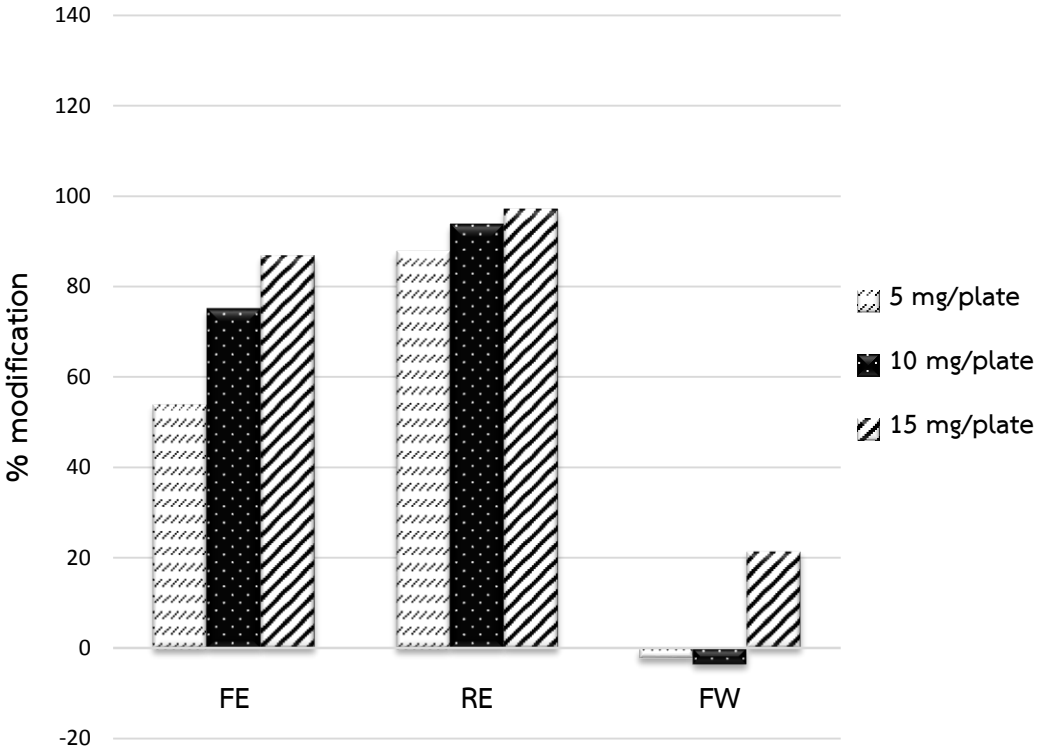


Figure 17. Modification effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *S. typhimurium* strain TA98. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit; no modification observed from fractionated water extract from the root.

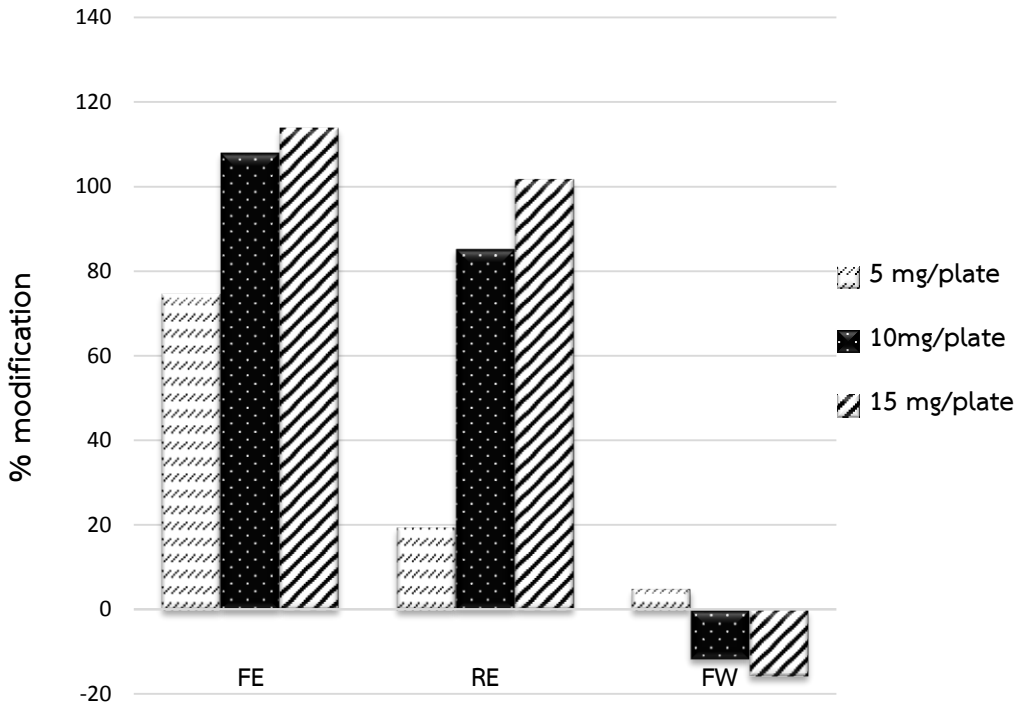


Figure 18. Modification effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *S. typhimurium* strain TA100. Abbreviations including FE: ethanolic extract from the fruit, RE: ethanolic extract from the root, FW: fractionated water extract from the fruit; no modification observed from fractionated water extract from the root.

Genotoxic assay in *Saccharomyces cerevisiae*

Cytotoxic effect of the extracts alone and in combination with EMS in diploid yeast cells (strain D7)

With the aim to investigate the genotoxic potential of the extracts from the root and the fruit of *A. marmelos*, the diploid yeast strain D7 was assayed with the range of concentration extracts alone (0.25-2 mg/ml) and in combination with ethyl methanesulfonate (1% EMS dissolved in distilled water) for detection of the frequencies of Trp^+ gene convertants at the *trp5* locus, Ilv^+ revertants at the *ilv1* locus, twin-spotted colonies and other colony color types involving the *ade2* locus.

Tables 6 and 7 showed the induction of cytotoxicity from the number of surviving yeast cells culture on complete agar medium containing the ethanolic and fractionated water extracts from the root and the fruit, negative control medium, and positive control medium. The cytotoxicity was determined by the alteration of surviving yeast cells counts in each treatment in comparison with the negative control (solvent) for a portion of time (3 hr). The results showed that the percentages of surviving yeast cells on all treatments were higher than 50%. The data indicated that all extracts were not toxic for this testing.

Table 6. The percentage of surviving yeast cells of *S. cerevisiae* strain D7 on complete agar medium containing the extracts from *A. marmelos*

Sample concentration	Yeast surviving fraction (% of control)			
	RE	**FE**	**RW**	**FW**
Negative control	100	100	100	100
0.25 mg/ml	97.6 ± 5.35	103.07 ± 5.10	100.14 ± 4.39	109.02 ± 6.78
0.5 mg/ml	111.38 ± 3.50	101.26 ± 4.58	99.58 ± 4.95	107.87 ± 6.94
1 mg/ml	91.17 ± 4.20	98.19 ± 4.09	100 ± 4.07	113.44 ± 5.29
2 mg/ml	93.86 ± 2.83	98.92 ± 3.33	101.82 ± 3.28	109.98 ± 6.46

The results presented percentage of surviving yeast cells from experiment performed in sixtuplets and compared to negative control. The data showed no significant difference compared to the control by ANOVA with a Dunnett multiple comparisons test ($^{*}p<0.05$).

Negative control: 1% v/v DMSO (for the ethanolic extract), distilled water (for the fractionated water extract) Abbreviations including RE: ethanolic extract from the root, FE: ethanolic extract from the fruit, RW: fractionated water extract from the root, FW: fractionated water extract from the fruit

Table 7. The percentage of surviving yeast cells of *S. cerevisiae* strain D7 on complete medium containing the extracts from *A. marmelos* and ethyl methanesulfonate (EMS)

Sample concentration	Yeast surviving fraction (% of control)			
	RE	**FE**	**RW**	**FW**
Negative control	100	100	100	100
Positive control	93.65 ± 6.06	97.12 ±4.97	94.16 ± 2.13	96.61 ± 4.21
0.25 mg/ml	96.44 ± 1.23	100 ± 7.55	97.81 ±3.53	99.41 ± 5.80
0.5 mg/ml	96.28 ± 2.17	99.57 ± 2.82	98.1 ± 3.18	96.61 ± 2.41
1 mg/ml	96.28 ± 5.18	98.85 ± 5.48	99.42 ± 6.64	99.56 ± 4.89
2 mg/ml	109.35 ± 1.55	119.14 ± 4.54	119.98 ± 4.57	99.85 ± 4.87

The results presented percentage of surviving yeast cells from experiment performed in sixtuplets and compared to negative control. The data showed no significant difference compared to the control by ANOVA with a Dunnett multiple comparisons test ($^{*}p<0.05$).

Negative control: Phosphate buffer (pH 7.0).

Positive control: Ethyl methanesulfonate (1% EMS dissolved in distilled water)

Abbreviations including RE: ethanolic extract from the root, FE: ethanolic extract from the fruit, RW: fractionated water extract from the root, FW: fractionated water extract from the fruit

Induction of Trp^+ gene convertants and Ilv^+ revertants colonies by the treatment of the extracts

The experimental data of the induction of gene conversion at the *trp5* locus and reversion of the *ilv1* locus induced by the treatment of diploid yeast cells with the extracts from the root and the fruit of *A. marmelos* (0.25-2 mg/ml) was summarized in Tables 8 and 9. The results showed that the ethanolic extracts were more effective in inducing gene conversion at the range of concentrations tested than the fractionated water extracts by increasing the frequency of Trp^+ convertants in comparison with the negative control.

The ethanolic extract from the root significantly ($p<0.05$) increased the frequency of convertants at the *trp5* locus. It also significantly ($p<0.05$) induced 13.83 ± 2.79 and 15.5 ± 4.09 revertants at 1 mg/ml and 2 mg/ml, respectively in comparison with the negative control (Figure 19).

The ethanolic extract from the fruit showed a significant ($p<0.05$) genotoxic effect by inducing the frequency of convertants at all concentrations tested in a dose-dependent manner, but it did not significantly induce the frequency of revertants at the *ilv1* locus in all the range of concentrations applied. The extract induced 30.5 ± 2.26 and 31.83 ± 2.32 convertants at 1 mg/ml and 2 mg/ml, respectively (Figure 20).

The fractionated water extract from the root significantly (p<0.05) induced the frequency of revertants at the highest concentration (2 mg/ml) compared to the negative control by induced 16.5 ± 2.95 revertant colonies, whereas the frequency of convertants did not differ significantly (p>0.05) from the negative control in all range of concentrations applied (Figure 21).

The fractionated water extract from the fruit at the highest concentration tested (2 mg/ml) did not significantly induce either the frequency of convertants or revertants. The extract significantly (p<0.05) induced the frequency of convertants (8.17 ± 2.14) at the lowest concentration (0.25 mg/ml) and significantly induced the frequency of revertants at the concentrations of 0.25-1 mg/ml when compared to the negative control (Figure 22).

Table 8. Effect of the ethanolic extracts from the root and the fruit of *A. marmelos* on the diploid yeast *S. cerevisiae* strain D7

Sample	Concentration (mg/ml)	Convertants per 10^5 cells	% of control	Revertants per 10^6 cells	% of control
The ethanolic extract from the root					
control	0	20.67 ± 1.86	100	9.67 ± 2.66	100
	0.25	23.67 ± 1.97*	114.52 ± 9.51	11.17 ± 2.56	115.52 ± 26.51
	0.5	24.67 ± 2.73*	119.35 ± 13.22	11.5 ± 3.39	118.97 ± 35.08
	1	22.5 ± 1.22	108.87 ± 5.93	13.83 ± 2.79*	143.10 ± 28.83
	2	24.5 ± 2.07*	118.55 ± 10.03	15.5 ± 4.09*	160.34 ± 42.27
The ethanolic extract from the fruit					
control	0	24 ± 1.41	100	14.17 ± 2.14	100
	0.25	29.17 ± 1.94*	112.53 ± 8.09	14.5 ± 2.5	102.35 ± 17.72
	0.5	29.33 ± 1.37*	122.22 ± 5.69	14.5 ± 4.39	102.35 ± 23.94
	1	30.5 ± 2.26*	127.08 ± 9.41	16.67 ± 2.58	117.65 ± 18.23
	2	31.83 ± 2.32*	132.64 ± 9.65	16.67 ± 2.34	117.65 ± 16.50

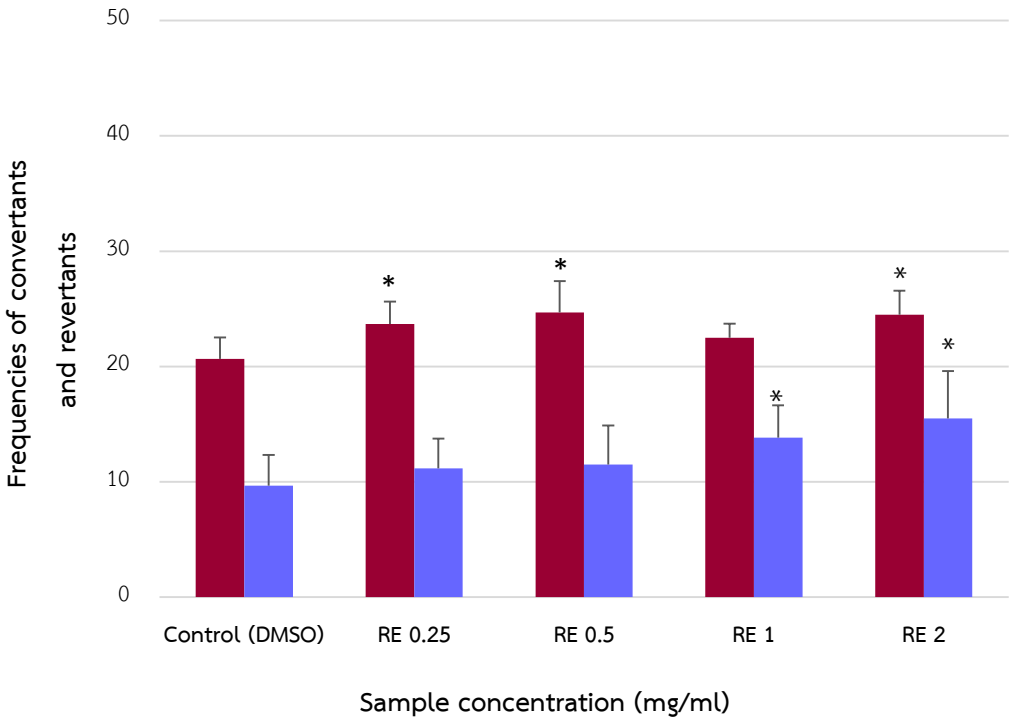
Each value presented was means ± SD from experiment performed in sixtuplets on synthetic medium lacking tryptophan and synthetic medium lacking isoleucine. For each experiment, the significance of differences between treatments and negative control was determined by ANOVA with a Dunnett multiple comparisons test ($^*p < 0.05$).

Table 9. Effect of the fractionated water extracts from the root and the fruit of *A. marmelos* on the diploid yeast *S. cerevisiae* strain D7

Sample	Concentration (mg/ml)	Convertants per 10^5 cells	% of control	Revertants per 10^6 cells	% of control
The fractionated water extract from the root					
control	0	16.67 ± 0.82	100	11.17 ± 2.04	100
	0.25	17.83 ± 2.14	103.03 ± 46.95	13.33 ± 2.58	119.40 ± 23.12
	0.5	18 ± 2.28	109.09 ± 25.71	13.33 ± 2.50	119.40 ± 22.42
	1	19 ± 2.68	121.21 ± 29.69	14.5 ± 2.88	129.85 ± 25.80
	2	18.17 ± 3.25	121.21 ± 51.00	16.5 ± 2.95*	147.76 ± 26.41
The fractionated water extract from the fruit					
control	0	5.17 ± 1.17	100	6.17± 1.17	100
	0.25	8.17 ± 2.14*	158.06 ± 41.36	9.33 ± 1.37*	151.35 ± 22.16
	0.5	7.67 ± 2.50	148.39 ± 48.45	9.17 ± 1.60*	148.65 ± 25.98
	1	7 ± 3.10	135.48 ± 59.97	9.83 ± 1.47*	159.46 ± 23.87
	2	6 ± 1.90	116.13 ± 36.72	7.83 ± 1.17	127.03 ± 18.96

Each value presented was means ± SD from experiment performed in sixtuplets on synthetic medium lacking tryptophan and synthetic medium lacking isoleucine. For each experiment, the significance of differences between treatments and negative control was determined by ANOVA with a Dunnett multiple comparisons test ($^{*}p < 0.05$).

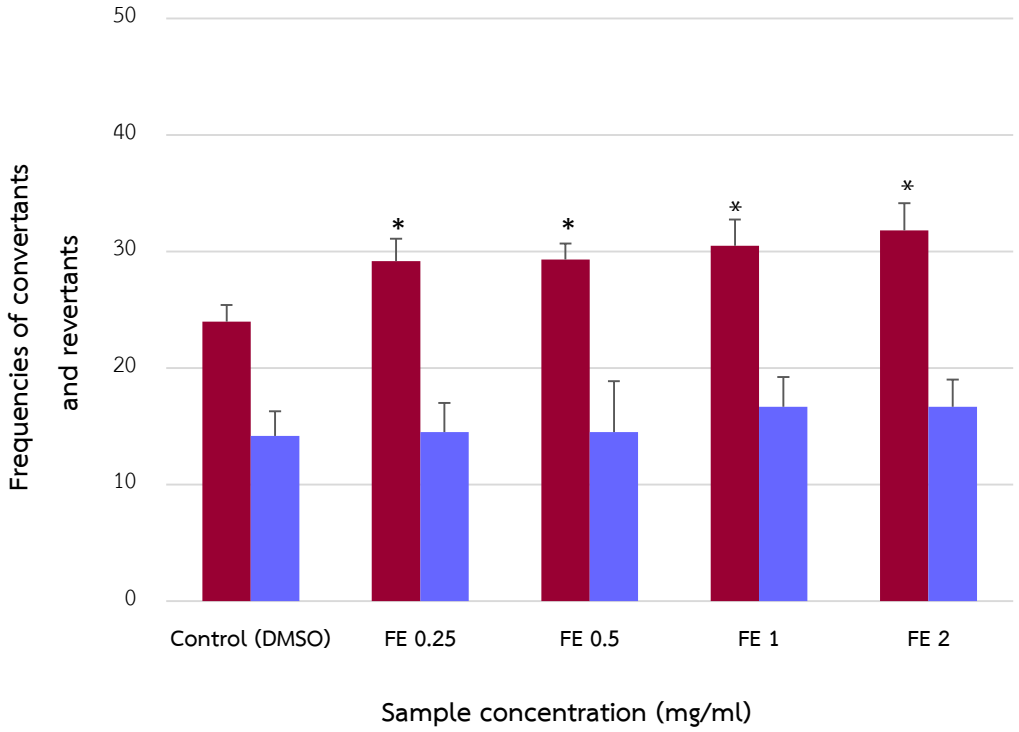
Figure 19. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the ethanolic extract from the root of *A. marmelos*



*$p<0.05$, significant difference compared to the negative control (DMSO); N=6 for each experiment

- The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)
- The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

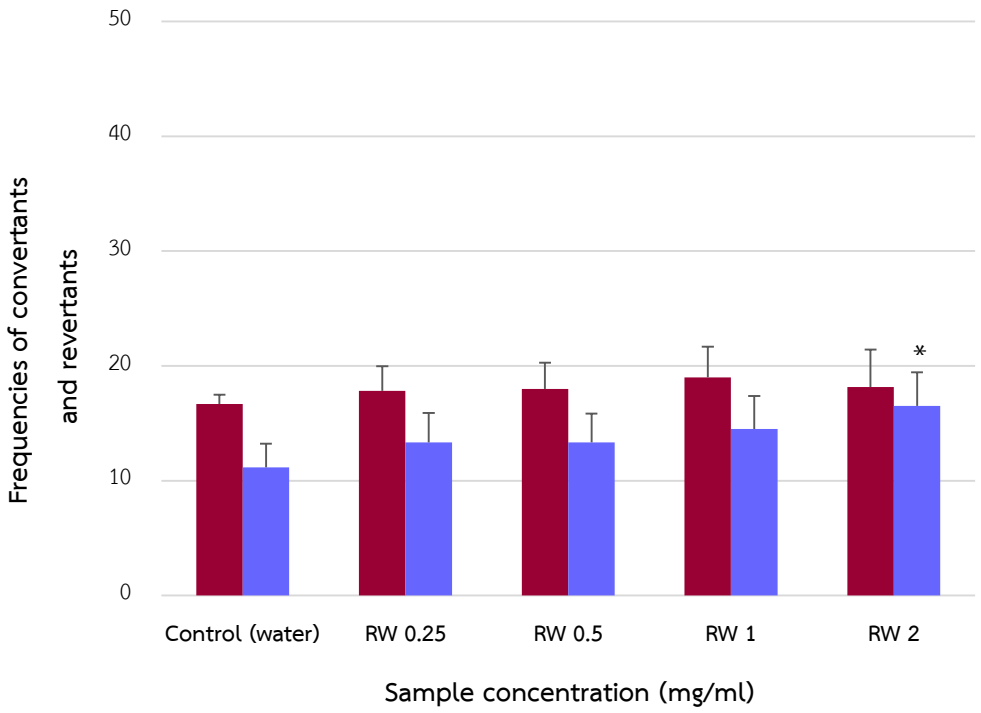
Figure 20. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the ethanolic extract from the fruit of *A. marmelos*



*p<0.05, significant difference compared to the negative control (DMSO); N=6 for each experiment

- The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)
- The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Figure 21. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the fractionated water extract from the root of *A. marmelos*

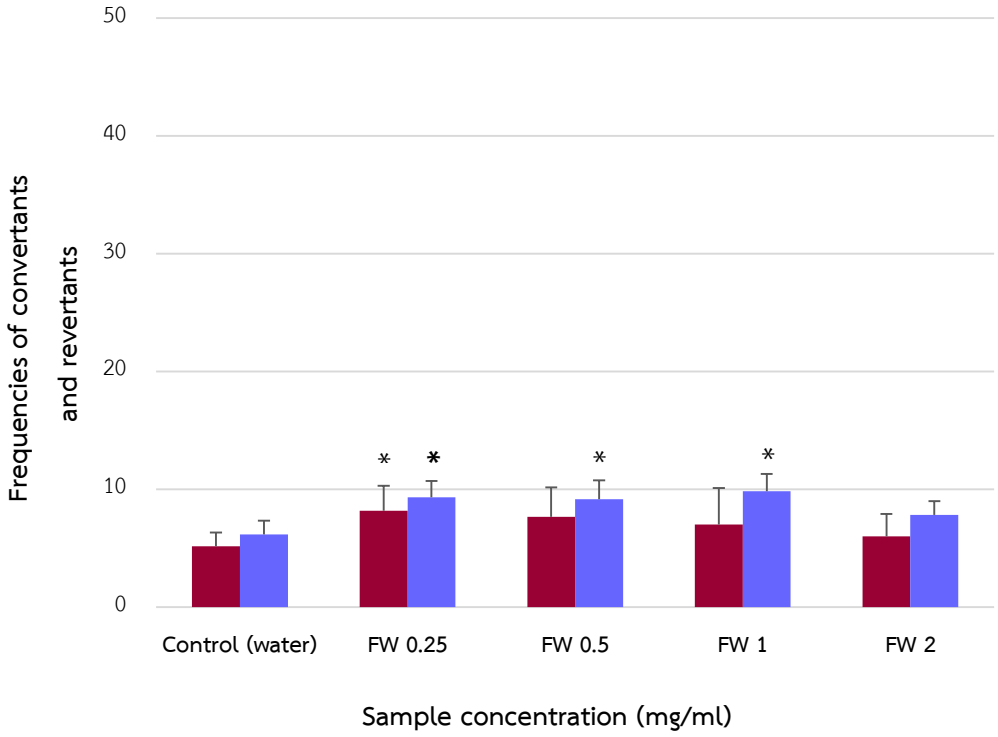


*$p<0.05$, significant difference compared to the negative control (distilled water); N=6 for each experiment

■ The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

■ The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Figure 22. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the fractionated water extract from the fruit of *A. marmelos*



*$p<0.05$, significant difference compared to the negative control (distilled water); N=6 for each experiment

■ The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

■ The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Induction of mitotic recombination and total aberrants involving the *ade2* locus by the extracts

The genotoxic effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* in diploid yeast cells was assayed in various concentrations (0.25-1 mg/ml) on the induction of mitotic recombination and a variety of genetic events such as point mutation, mitotic gene conversion and aneuploidy involving the *ade2* locus.

The incidence of mitotic recombination due to crossing-over and several genetic events induced by the root and the fruit extracts was visualized by red and pink sectored colonies and other aberrant types on the complete medium that were expressed as the percentage of total aberrant colonies per surviving yeast cells in each experiment. On the other hand, the induction of altered colonies caused by the genotoxic effect of extracts were also scored among convertant colonies on synthetic lacking tryptophan and the percentage of total aberrant colonies was calculated from the number of convertants and total aberrant colonies (Table 10). This study was observed that only the ethanolic extracts from the root and the fruit of *A. marmelos* induced the frequency of the altered colonies among Trp^+ convertants at all doses except 2 mg/ml. The highest percentage of the total aberrant colonies induced by the ethanolic extracts from the root and the fruit was 3.70% and 4.37%, respectively at 1 mg/ml.

Table 10. Induction of *ade2* altered colonies among Trp^+ convertant colonies by the extracts in diploid *S. cerevisiae* strain D7

Sample	Concentration (mg/ml)	ade2 altered colonies among	
		Synthetic lacking tryptophan	
		All cells on synthetic m. without tryptophan	**%**
RE	control	2/124	1.61
	0.25	4/142	2.82
	0.5	4/148	2.70
	1	5/135	3.70
	2	1/147	0.68
FE	control	2/144	1.39
	0.25	3/175	1.71
	0.5	3/176	1.70
	1	8/183	4.37
	2	2/191	1.75
RW	control	ND	ND
	0.25	ND	ND
	0.5	ND	ND
	1	ND	ND
	2	ND	ND
FW	control	ND	ND
	0.25	ND	ND
	0.5	ND	ND
	1	ND	ND
	2	ND	ND

The data represented the total value of the number of altered colonies and survival colonies on synthetic medium lacking tryptophan in sixtuplets.

ND = no aberrant colonies were detected, N=6 for each experiment

Induction of Trp^+ gene convertants and Ilv^+ revertants by the combination of the extracts and ethyl methanesulfonate (EMS)

The effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* (0.25-2 mg/ml) on EMS-induced the frequencies of convertants at the *trp5* locus, and revertants at the *ilv1* locus in diploid yeast cells was summarized in Tables 11-12.

After co-treatment of the ethanolic and fractionated water extracts from the root of *A. marmelos* with EMS, the results indicated that the ethanolic extract from the root of *A. marmelos* at the doses of 0.25-1 mg/ml exhibited a significant (p<0.05) genotoxic effect by decreasing the frequency of EMS-induced revertants. It produced a maximum reduction of EMS-induced revertants by induced 36.67 ± 2.94 revertant colonies (21.14% inhibition) at 0.25 mg/ml compared to 1% EMS. On the contrary, the highest concentration of the extract (2 mg/ml) did not show the genotoxic effect in inducing the frequency of revertants after co-treated with EMS because the frequency of revertants did not significant (p<0.05) from the positive control (1% EMS). The fractionated water extract from the root of *A. marmelos* significantly (p<0.05) decreased the frequency of EMS-induced convertants at 0.25 mg/ml by induced 7.17 ± 0.98 convertant colonies (46.56% inhibition) compared to 1% EMS.

On the other hand, in combination with EMS, most extracts enhanced the frequency of convertants at the *trp5* locus and revertants at the *ilv1* locus induced by EMS. Figure 23 showed the genotoxic effect of the ethanolic extract from the root of *A. marmelos* combined with EMS in diploid yeast cells. The data indicated that the extract exhibited significant ($p<0.05$) enhancement of EMS-induced the frequency of the convertants at all doses applied in comparison with the positive control (1% EMS). The percent enhancement was 47.8-98.5% and the highest enhancement induced 22.83 ± 4.07 and 22.83 ± 2.93 convertant colonies at 0.5 mg/ml and 2 mg/ml, respectively.

The genotoxic effect of the ethanolic extract from the fruit of *A. marmelos* combined with EMS in diploid yeast cells was presented in Figure 24. The experimental data demonstrated that the ethanolic extract from the fruit showed significant ($p<0.05$) enhancement of EMS-induced the frequency of convertants and revertants at all range of doses applied in comparison with the positive control (1% EMS). The percent enhancement was 18.83-79.95% and produced a maximum enhancement of EMS-induced convertants by induced 19.83 ± 2.23 (69.92% enhancement) and 21.00 ± 2.83 (79.95% enhancement) convertants at 1 mg/ml and 2 mg/ml, respectively. The extract exhibited significant ($p<0.05$) enhancement of EMS-induced revertants in a dose-dependent manner, induced 27.83 ± 1.94 revertant colonies at 2 mg/ml (21% enhancement).

The fractionated water extract from the root of *A. marmelos* at the dose of 1 mg/ml and 2 mg/ml were slightly genotoxic after combination with EMS (Figure 25). The fractionated water extract from *A. marmelos* root exhibited significant (p<0.05) enhancement of EMS-induced the frequency of revertants by induced 31.67 ± 3.33 (36.69% enhancement) and 28.50 ± 1.38 (23% enhancement) revertant colonies at 1 mg/ml and 2 mg/ml, respectively compared to 1% EMS.

As shown in Figure 26, the genotxic effect of the fractionated water extract from the fruit of *A. marmelos* was lower than the other extracts. The experimental data demonstrated that almost all doses of the extract were no genotoxic effects after co-treated with EMS either inhibition or enhancement of the frequency of convertants and revertants. Excepting the extract at the dose of 1 mg/ml, it showed significant (p<0.05) enhancement of EMS-induced the frequency of convertants by induced 22.00 ± 5.14 (31.97% enhancement) convertant colonies compared to 1% EMS.

Table 11. Effect of the ethanolic extracts from the root and the fruit of *A. marmelos* on EMS-induced the genotoxic effect in the diploid yeast *S. cerevisiae* strain D7

Co-treatment	Sample conc. (mg/ml)	Convertants per 10^5 cells	Revertants per 10^6 cells	Inhibition (%)	
				Conversion	Reversion
The ethanolic extract from the root					
Negative Control	0	7.83 ± 1.83	3.33 ± 1.63	-	-
Positive Control	0	11.5 ± 2.66 (100)	46.5 ± 2.59 (100)	-	-
	0.25	18.83 ± 1.47* (163.77 ± 12.80)	36.67 ± 2.94* (78.85 ± 6.33)	63.74 (En)	21.14 (In)
	0.5	22.83 ± 4.07* (198.55 ± 35.39)	39.5 ± 2.26* (84.95 ± 4.86)	98.52 (En)	15.05 (In)
	1	17 ± 2.83* (147.83 ± 24.60)	39.5 ± 1.76* (84.95 ± 3.79)	47.83 (En)	15.05 (In)
	2	22.83 ± 2.93* (198.55 ± 25.45)	48.83 ± 2.48 (105.02 ± 5.34)	98.52 (En)	5.01 (En)
The ethanolic extract from the fruit					
Negative Control	0	4 ± 1.79	2.83 ± 0.75	-	-
Positive Control	0	11.67 ± 2.34 (100)	23 ± 3.74 (100)	-	-
	0.25	16.17 ± 2.48* (138.57 ± 21.29)	27.33 ± 1.63* (118.84 ± 7.10)	38.56 (En)	18.83 (En)
	0.5	15.67 ± 2.42* (134.29 ± 20.76)	27.33 ± 2.34* (118.80 ± 10.17)	34.28 (En)	18.83 (En)
	1	19.83 ± 2.23* (170 ± 19.10)	27.67 ± 2.34* (120.29 ± 10.17)	69.92 (En)	20.30 (En)
	2	21 ± 2.83* (180 ± 24.24)	27.83 ± 1.94* (121.01 ± 8.44)	79.95 (En)	21.00 (En)

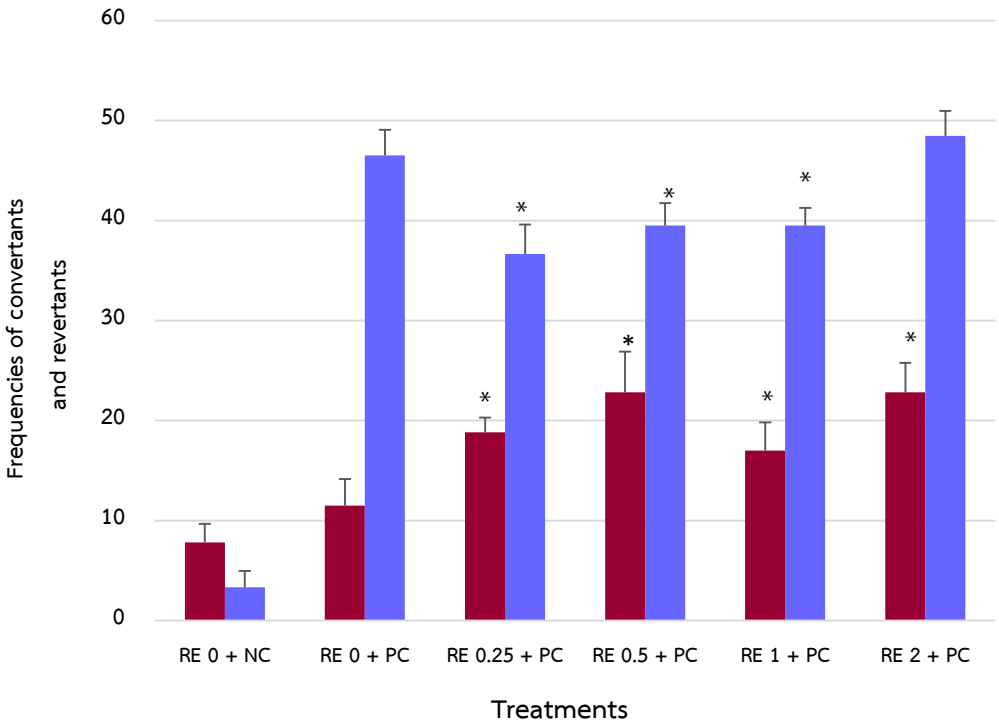
The results presented means ± SD from experiment performed in sixtuplets. Figure in brackets represented the percentage of survivors in the control experiment. For each experiment, the significance of differences between treatments and positive control was determined by ANOVA with a Dunnett multiple comparisons test ($^{*}p < 0.05$). En: enhancement, In: inhibition

Table 12. Effect of the fractionated water extracts from the root and the fruit of *A. marmelos* on EMS-induced the genotoxic effect in the diploid yeast *S. cerevisiae* strain D7

Co-treatment	Sample conc. (mg/ml)	Convertants per 10^5 cells	Revertants per 10^6 cells	Inhibition (%)	
				Conversion	Reversion
The fractionated water extract from the root					
Negative Control	0	5.67 ± 1.86	3 ± 0.89	-	-
Positive Control	0	13.17 ± 3.19 (100)	23.17 ± 3.82 (100)	-	-
	0.25	7.17 ± 0.98* (54.43 ± 7.47)	27.33 ± 2.94 (117.99 ± 12.71)	45.56 (In)	17.95 (En)
	0.5	11.17 ± 1.47 (84.41 ± 11.17)	27.33 ± 3.33 (117.99 ± 14.36)	15.19 (In)	17.95 (En)
	1	15.5 ± 3.08 (117.72 ± 23.41)	31.67 ± 3.33* (136.69 ± 14.36)	17.69 (En)	36.69 (En)
	2	15.83 ± 1.72 (120.25 ± 13.08)	28.5 ± 1.38* (123.02 ± 5.95)	20.20 (En)	23.00 (En)
The fractionated water extract from the fruit					
Negative Control	0	5 ± 1.22	4.5 ± 1.05	-	-
Positive Control	0	16.67 ± 4.27 (100)	26.5 ± 1.87 (100)	-	-
	0.25	14.67 ± 2.07 (88 ± 12.39)	25.17 ± 2.79 (94.97 ± 10.52)	12.00 (In)	5.02 (In)
	0.5	18 ± 2.83 (108 ± 16.97)	25.5 ± 1.52 (96.23 ± 5.72)	7.98 (En)	3.77 (In)
	1	22 ± 5.14* (132 ± 30.83)	25.17 ± 1.72 (94.97 ± 6.50)	31.97 (En)	5.02 (In)
	2	19.67 ± 2.5 (118 ± 15.02)	27.5 ± 1.87 (103.77 ± 7.06)	18.00 (En)	3.77 (In)

The results presented means ± SD from experiment performed in sixtuplets. Figure in brackets represented the percentage of survivors in the control experiment. For each experiment, the significance of differences between treatments and positive control was determined by ANOVA with a Dunnett multiple comparisons test ($^*p < 0.05$). En: enhancement, In: inhibition

Figure 23. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the ethanolic extract from the root of *A. marmelos* and ethyl methanesulfonate (EMS)

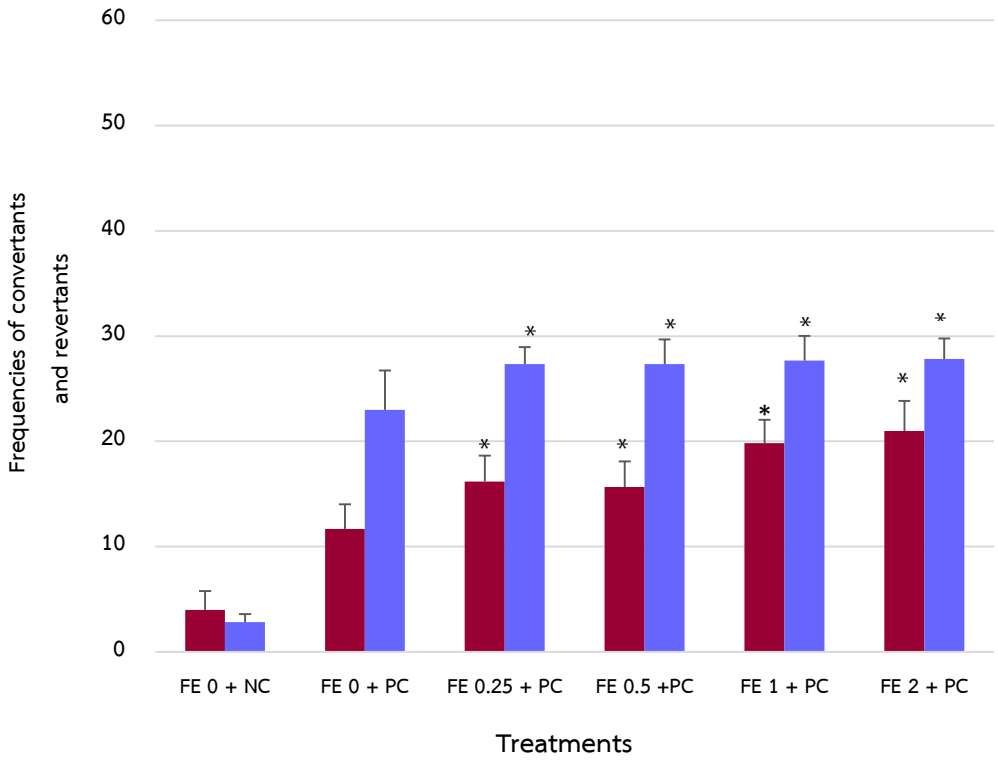


*p<0.05, significant difference compared to the positive control (1% EMS); N=6 for each experiment

■ The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

■ The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Figure 24. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the ethanolic extract from the fruit of *A. marmelos* and ethyl methanesulfonate (EMS)

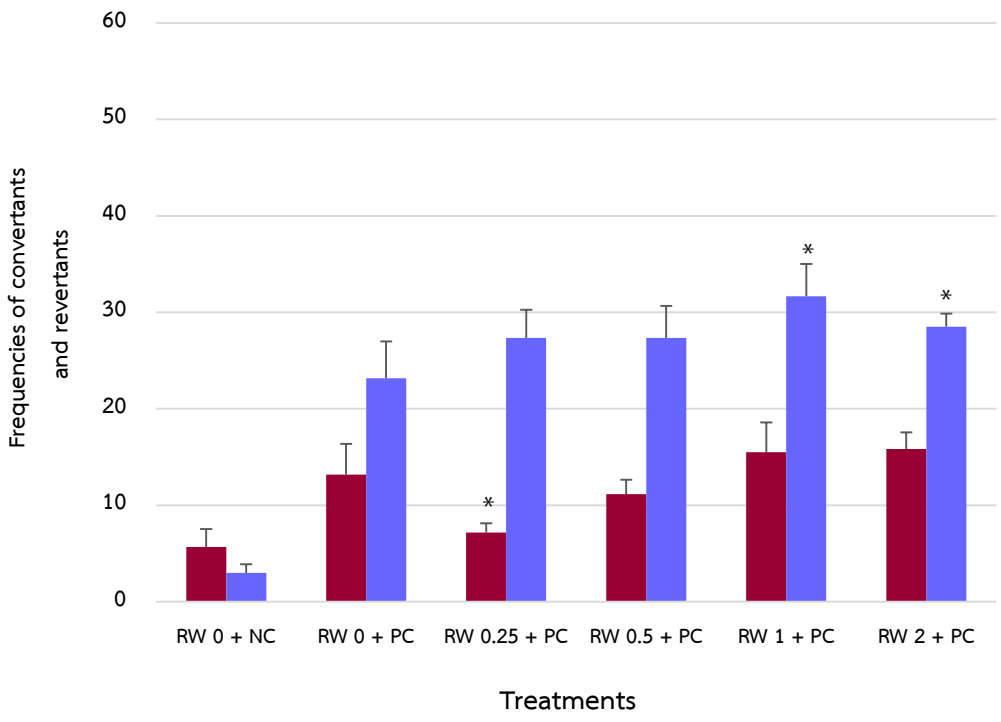


*p<0.05, significant difference compared to the positive control (1% EMS); N=6 for each experiment

■ The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

■ The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Figure 25. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the fractionated water extract from the root of *A. marmelos* and ethyl methanesulfonate (EMS)

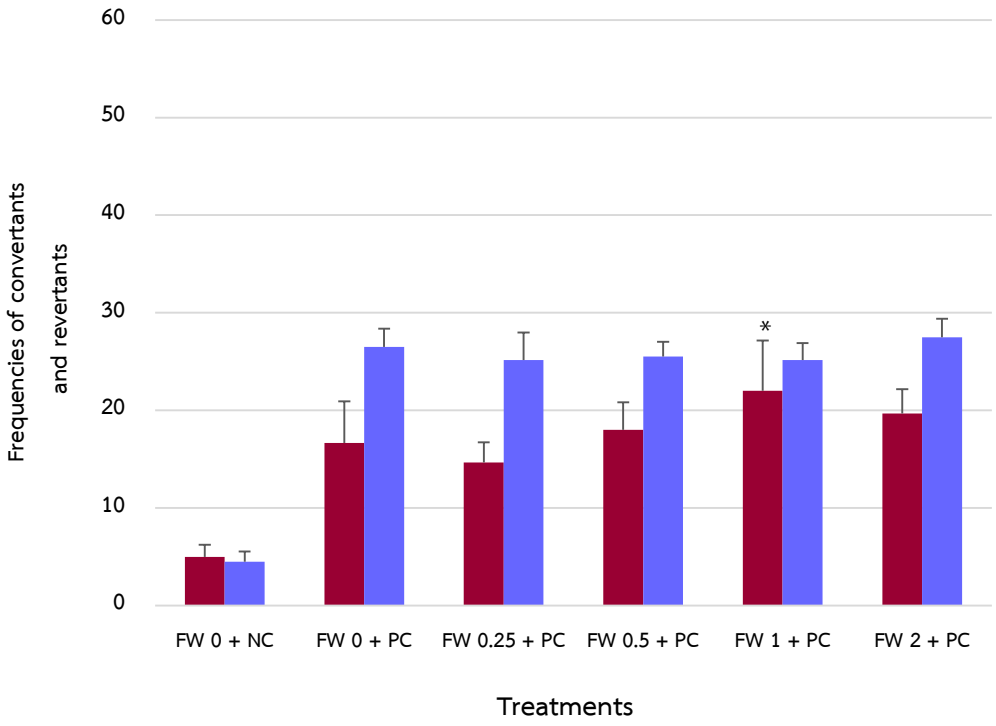


*p<0.05, significant difference compared to the positive control (1% EMS); N=6 for each experiment

The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Figure 26. Frequencies of gene conversion and reversion after treatment of *S. cerevisiae* D7 with the fractionated water extract from the fruit of *A. marmelos* and ethyl methanesulfonate (EMS)



*p<0.05, significant difference compared to the positive control (1% EMS); N=6 for each experiment

■ The number of convertants per 10^5 cells (plating yeast cell densities were 2×10^5 cells per plate in the selection for convertants)

■ The number of revertants per 10^6 cells (plating yeast cell densities were 2×10^6 cells per plate in the selection for revertants)

Induction of mitotic recombination and total aberrants involving the *ade2* locus by the treatments of the extracts in combination with ethyl methanesulfonate

The mitotic recombination and a variety of genetic events such as point mutation, mitotic gene conversion and aneuploidy involving the *ade2* locus induced by the treatments of EMS with the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* in various concentrations (0.25-1 mg/ml) were assayed in diploid yeast *S. cerevisiae*.

The effect of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* in combination with ethyl methanesulfonate (EMS) was tested by plating the treated cells on complete medium, which the incidence of the altered colonies among surviving yeast cells was calculated and expressed as the percentage of total aberrant colonies. The ability of *A. marmelos* extracts to decrease the number of red and pink altered colonies as well as other aberrant types indicated the anti-genotoxic potential of the extracts induced by EMS in diploid yeast cells. The response of diploid yeast cells to the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* was shown in table 13.

After treatment with EMS, the reduction of EMS-induced the total aberrant colonies was detected in both fractions from the root. The highest percentage of the total aberrant colonies induced by the ethanolic extract from the root was 0.85% at 2 mg/ml, whereas the fractionated water extract was 0.59% at 1 mg/ml. However, the fractionated water extract from the fruit was no evidence of anti-genotoxic effect in some range of doses, while the ethanolic extract from the fruit showed enhancement effect after the treatment combined with EMS. The highest percentage of the total aberrant colonies induced by the ethanolic extract from the fruit was 2.04% at 2 mg/ml.

Table 13. Induction of *ade2* altered colonies among surviving yeast cells by the extracts combined with EMS in diploid *S. cerevisiae* strain D7

Sample	Concentration (mg/ml)	ade2 altered colonies among	
		Complete medium	
		All cells on complete m.	%
RE	Negative control	ND	ND
	Positive control	7/605	1.16
	0.25	5/623	0.8
	0.5	3/622	0.48
	1	3/622	0.48
	2	5/585	0.85
FE	Negative control	ND	ND
	Positive control	8/675	1.19
	0.25	10/695	1.44
	0.5	14/692	2.02
	1	8/687	1.16
	2	15/685	2.04
RW	Negative control	ND	ND
	Positive control	7/645	1.09
	0.25	6/670	0.9
	0.5	2/672	0.3
	1	4/681	0.59
	2	3/681	0.44
FW	Negative control	ND	ND
	Positive control	7/656	1.07
	0.25	8/675	1.19
	0.5	7/656	1.07
	1	7/676	1.04
	2	8/678	1.18

The data represented the total value of the number of altered colonies and survival colonies on complete medium in sixtuplets.

ND = no aberrant colonies were detected, N=6 for each experiment

CHAPTER V

DISCUSSION AND CONCLUSION

The usage of traditional medicines and herbal products have been increasing greatly for solving the human health care problems in general consumers which have confidence in the safety and efficacy of herbal medicines and its products. The belief of safety and efficacy of herbal medicines may result from their long historical usage and the ingredients of herbal medicines were obtained from either raw or processed herbal plant materials or its crude extracts in the term of natural ingredients. For these reasons, these herbal medicines were claimed to be safe or no side effects on human body which may not be true in some herbal medicines. However, only a few herbal medicines have scientific information to support their therapeutic benefits and toxicity for the usage and consumer protections.

As a result of increasingly interest in traditional medicines and herbal products, the information of safety and efficacy evaluation of herbal plants are very important for public health systems. The assessment of genotoxic potential of herbal plants is a simple way to examine the potential hazard of herbal products used in daily life which is necessarily evaluated by the use of multiple test system, *in vitro* and *in vivo* models [5]. Consequently, the root and the fruit of *A. marmelos* which have been used as traditional medicines and as ingredients of some foods and drinks were studied for genotoxic potential *in vitro* models in both prokaryotic and eukaryotic cells.

In this study, the extraction of the root and the fruit of *A. marmelos* were done by maceration with ethanol and followed by water. It was found that ethanol was a better solvent for extraction of the root while water was a better solvent for extraction of the fruit. The results in the present study were dissimilar to the study of Varughese and Tripathi (2013) which demonstrated that the percentage yield of the ethanolic extract from the fruit of *A. marmelos* was better than water (aqueous) extract. The different percent yields of each extract might be due to variation in the solubility of various types of chemical constituents of herbal crude drugs in different solvent used and extraction methods. Moreover, the different sources of herbal crude drugs or the period of gathering herbal products might influence on the quantity of their chemical constituents [8].

Mutagenicity of the extracts from the root and the fruit in Ames test

A number of herbal plants have been commonly used as traditional medicines and as dietary agents in daily life which provide the basis to study the mutagenic potential of these herbal plants and its mixtures in different labolatories. The purpose of this study was to evaluate the possible risk of the ethanolic and fractionated water extracts from the root and the fruit *in vitro* model, the bacterial reverse mutation assay or the Ames test. This assay is frequently used as *in vitro* method to investigate the genotoxic activity of herbal products toward *S. typhimurium* strains TA98 and TA100 which contain point mutations leading to histidine auxotrophs (his^-) and can revert to histidine prototrophs (his^+) by mutagenic induction of several mutagens. Each tester

strain is deleted a gene coding for DNA excision repair system (*uvrB* mutation). The tester strains also contain *rfa* mutation conferring gathering permeability to large molecules and presence of *pKM101*. All tester strains can restore to wild-type by the presence of particular mutagens [51].

In this study, the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* were studied for the mutagenic activity with and without nitrosation using pre-incubation method of Ames test under acidic condition (pH 3.0-3.5) in the absence of enzyme activating system against *S. typhimurium* strains TA98 for frameshift mutations and TA100 for base-pair substitution mutations. The mutagenic results on both strains showed that most extracts exhibited non-mutagenic activity except the fractionated water extract from the root of *A. marmelos*. The experimental data led to hypothesis that the mutagenicity of the extract may be due to its chemical constituents. It indicated the mutagenic potential of this part of herbal plant to support the usage and development of herbal products as traditional Thai medicines.

The relation between high dietary intake of nitrate and the mortality from human cancers, especially gastric cancer in various countries has been reported [92-94]. In the stomach, nitrite can react with several components in food and form direct-acting genotoxin under acidic conditions similar to gastric juice of human [92]. In conclusion, the human stomach is the most high-risk for mutagenic compound synthesis in the diet when gastric juice catalyses nitrosation reactions.

In this study, the ethanolic and fractionated water extracts from the root and the fruit showed mutagenic effect against *S. typhimurium* strains TA98 and TA100 after being treated with sodium nitrite. The mutagenic results implied that all of extracts contained some precursors which could react with nitrite to produce direct-acting mutagenic products causing frameshift mutation on TA98 and base-pair substitution mutation on TA100 in the acidic condition of the human stomach. The study of Wakabayashi *et al*. in 1985 found that Chinese cabbage was mutagenic to *S. typhimurium* strain TA100 and its mutagen precursor, Indole-3-acetonitrile, showed direct-acting mutagenicity on the treatment with nitrite against TA98 and TA100 by the pre-incubation method of Ames test [95]. Similarly, Higashimoto *et al*. (1993) reported that the hot water and methanol extracts of three types of spices (black pepper seeds, coriander, and caraway) showed mutagenic activity on strain TA100 after interacted with nitrite in the absence of metabolic activation [96]. Moreover, this data agreed with the previous study of Wongwattanasathien *et al*. (2010) who found that most fractions of flower extracts except the water extract of *Hibiscus rosa-sinensis*, dichloromethane extracts of *Syzygium malaccense, Hibiscus rosa-sinensis, Plumeria obtuse* and methanol extract of *Syzygium malaccense* were mutagenic against both bacterial tester strains after nitrite treatment in acidic solution [97]. In conclusion, the consumption of these parts of *A. marmelos* as traditional medicines and as some ingredients in food should avoid nitrates and nitrites containing food products which have a chance to form carcinogens in the stomach.

Anti-mutagenicity of the extracts from the root and the fruit in Ames test

The screening of anti-mutagenic effect of herbal extract is very important that can indicate the possible anti-carcinogen of herbal extract. 1-Aminopyrene is a metabolite of 1-nitropyrene which is reduced by anaerobic bacteria in gastrointestinal tract. The intermediate derivatives from reduction processes can react with DNA to form DNA covalent adducts and produce mutagenicity [50]. Previous study found that 1-aminopyrene is direct-acting mutagen of diesel engine particulate in mutagenicity assay of the Ames test [98].

The anti-mutagenic potential of the ethanolic and fractionated water extract of *A. marmelos* against the product of the mutagenic reaction induced by nitrite-treated 1-aminopyrene in the acidic condition (pH 3-3.5) was exhibited in the pre-incubation method of Ames test on both tester strains of *S. typhimurium* TA98 and TA100. The extract of *A. marmelos* which was able to inhibit the mutagenic response of 1-aminopyrene nitrite-induced mutagenicity, revealed that they exerted the inhibitory effect on base-pair substitution and frame-shift type mutations.

The results can be implied that most of the extracts showed anti-mutagenic activity with the range from negligible (0-20%) to strong (>60%) inhibitory effects against both tester strains of *S. typhimurium*. In this study, crude drug was primarily extracted with ethanol by maceration until exhaustion. Then the marc of crude drug was continuously extracted with water. The results of this study presented that the ethanolic extract from the root and the fruit of *A. marmelos* was more effective than the fractionated water extract from both parts of crude drug which might be due to

various types of chemical constituents in each extract fraction. The results were in agreement with the study of Meeyutem *et.al* (2012) that the ethanolic extracts of nine Thai ancient remedies were more potent in mutagenic and anti-mutagenic activities than the fractionated water extracts [3]. The previous studied of Wongwattanasathien *et al*. (2010) suggested that the polarity of solvents related to the anti-mutagenic potential of the crude drug which the extracts with low polar solvent caused higher anti-mutagenicity than the extracts with high polar solvent [97].

However, the results showed that the fractionated water extract from the fruit of *A. marmelos* could either inhibit or enhance mutagenicity induced by nitrite-treated 1-aminopyrene with the range from negligible to weak effect toward both tester strains of *Salmonella typhimurium*. On the contrary, the study of Kruawan *et al.* (2006), reported that the water extract from the fruit of *A. marmelos* showed moderate anti-mutagenicity on *S. typhimurium* strain TA100 [2]. Similarly, Natarajan *et al.* (2012) also demonstrated that the acetone and water extracts from the fruit of *A. marmelos* showed anti-mutagenic effect against reactive oxygen species induced by hydrogen peroxide in isolated human peripheral blood lymphocytes using comet assay [99]. Furthermore, the anti-mutagenic effect of water-methanolic extract of ripe *A. marmelos* fruit was also demonstrated by Supriya *et al.* (2011) that the extract significantly prevented chromosomal aberrations in mouse bone marrow in a dose dependent manner [100].

The overall results of this study revealed that some chemical constituents in the extracts from the root and the fruit of *A. marmelos* were capable to be co-mutagen which could be reacted with the direct-acting mutagen produced by nitrite-treated 1-aminopyrene in the acidic condition. They were able to exhibit co-mutagenic potential both mutagenic inhibition and enhancement effect against prokaryotic cells.

Genotoxicity and anti-genotoxicity of the root and the fruit extracts in diploid yeast *Saccharomyces cerevisiae* strain D7

The safety and efficacy of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* were assessed against the Ames test which was based on the identification of several compound capability on inducing gene mutation in bacterial cells. All extracts were also assessed their genotoxic effects in eukaryotic model system using yeast-based genotoxicity assay.

Yeast-based genotoxicity assay is one of the most widely *vitro* methods used for the estimation of genotoxic activity of herbal plant products which has been recommended by the Organization for Economic Co-operation and Development (OECD, 2012). This assay is performed with dipiod yeast *S. cerevisiae* strain D7 that several mutagens can more easily be detected than prokaryotic model organism. Diploid yeast *S. cerevisiae* is utilized to detect reverse mutation, including reciprocal and non-reciprocal recombination [69]. The phenotypic changes of diploid yeast cells are produced when the mutagenic compounds mutate their DNA which are observed

by the color of colonies as well as the growth on selective media. The color of colonies is based on the induction of homozygosity of recessive linked genes and utilized for the detection of reciprocal recombination (crossing-over) [77]. The growth on selective media is used for the determination of non-reciprocal recombination (gene conversion) by mutagenic compound-induced the loss of heterozygosity of diploid yeast cells [101]. The other mechanism results in reverse mutation and the growth of colonies are counted on a medium depleted in the essential amino acid in the presence of mutagenic compounds.

Both ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* at all range of concentrations tested were evaluated genotoxic effect of the extracts alone and the combined treatment with ethyl methanesulfonate (EMS) in yeast-based genotoxicity assay using *S. cerevisiae* on the induction of mitotic gene conversion, reverse mutation including the appearance of total aberrant colonies due to mitotic crossing-over and other genetic events either on complete medium and two types of synthetic medium.

The results in the present study showed that the percentages of survival colonies on the treatments of the ethanolic extract and fractionated water extract did not significantly difference from the control. It was demonstrated that both extract fractions were not cytotoxic on diploid yeast cells. In the treatments of extracts, the genotoxic effect of the ethanolic extracts from the root and the fruit were more effective than their fractionated water extracts on inducing the frequencies of convertants, revertants, and total aberrant colonies. It may be due to the difference

of chemical constituents in each extract fractions to induce the genotoxic effect on diploid cells. Both fractions exhibited significant genotoxic effect by increasing the frequency of convertants at the *trp5* locus, while the frequency of revertants at the *ilv1* locus significantly increased only by the ethanolic extract from the root at the doses of 1 and 2 mg/ml. The fractionated water extract from the root was significantly induced the frequency of revertants at the highest concentration (2 mg/ml) whereas the fruit fraction did not induce the frequency of convertants and revertants at the same concentration. The data indicated that the frequency of revertants induced by both extract fraction from the root and the fruit was lesser than the frequency of convertants in the range of concentration. It may be due to the induction of revertant colonies depends on the specificity of compounds to restore the functional ability to synthesize an essential amino acid which would induce both of homologues to formation the revertant colonies.

The induction of total aberrant colonies by the ethanolic extracts from the root and the fruit was detected among Trp^+ convertants on synthetic lacking tryptophan but did not increase regularly with concentrations tested. This result is corresponding to the previous studies of Freeman *et al.* (2006) and Zimmermann *et al.* (1974) who proposed that the frequency of red and/or pink altered colonies involving the *ade2* locus can be scored among surviving yeast cells on complete medium, convertants and revertants on synthetic medium which the frequency of ade2 altered colonies might be higher among Trp^+ convertants than among total colonies [71, 72] Moreover, these finding showed that the incidence of mitotic recombination and other genetic

events by mutagens in different chromosomes was induced as an independent events [72].

The genotoxic effect of the extracts against ethyl methanesulfonate (EMS) as recombinogenic and/or mutagenic agent was performed in this study. Ethyl methanesulfonate (EMS) is one of the most widely used chemical mutagen which has an effect on DNA of organisms. EMS has been used against assessment of genetic effects in a variety of test systems [85, 88, 102, 103]. When testing yeast cells for genotoxic activity in combination with EMS, most of extracts exhibited significant enhancement of EMS-induced the frequency of convertants and revertants. Results from the ethanolic and fractionated water extracts from the root of *A. marmelos* showed significant either inhibitory or enhancement effect of EMS-induced the frequency of convertants and revertants. The induction of total aberrant colonies by treatment of the extracts combined with EMS was detected among total colonies on a complete medium. Both extracts from the root showed inhibitory effect at all concentrations tested whereas the extracts from the fruit showed either inhibitory or enhancement effect of EMS-induced the total aberrant colonies.

It was observed that the treatments in combination with EMS seemed to be genotoxic in D7 greater than the treatments of extracts alone. The data indicated that the combined treatments potentiated the genotoxic effects of both extract fractions and acted synergistically with the extracts in the induction of gene conversion at the *trp5* locus, reverse mutation at the *ilv1* locus as well as total aberrant colonies involving *ade2* locus. This results are in accordance with the study of Hoffmann *et al.*

(2009) which reported that the frequencies of convertants and revenrtants showed strong recombinogenic and mutagenic effect in combined treatments with nitrocrine and BLM that there are a synergistic interaction of two substances to have their effects [104].

The results of the treatments of the extracts alone and the treatments in combination with EMS by yeast-based genotoxic assay demonstrated that the elevated frequencies of genetic events for gene conversion, crossing-over and reverse mutation in yeast depend on some general variability of yeast cells. Fogel and Hurst, (1963) suggested that the induction of coincident recombination in yeast cells depends on general factors such as the process of experiments, pre-conditions, and cell cycle stage [78].

The growth phase of yeast cells is an experimental condition in yeast genotoxic studies. Logarithmic growth phase is more sensitive than stationary growth phase which results from the high enzymatic activities, as compared to stationary growth phase and is able to detoxicate some direct mutagens. Although the response of logarithmic phase cells is greater than the stationary phase cells, the direct mutagen may show cytoxicity to yeast cells [62]. This data is consistent with the study of Monaco *et al*. (1992) which found that MMS-induced genotoxic activity in logarithmic phase cells was higher than stationary phase cells, while the highest CP-induced genotoxic activity was obtained in stationary phase cells [105].

Results obtained with the ethanolic and fractionated water extracts from the root and the fruit indicated that most of the extracts were shown to induce genotoxic effects in *S. typhimurium*, but it was sightly effects in yeast test system. The results were consistent with the study of Bianchi *et al*. (1994) which found that many substances were ineffective in inducing both convertants and revertants in *S. cerevisiae* strain D7, whereas they showed positive result in *S. typhimurium* [106]. The different results in *S. typhimurium* and *S. cerevisiae* yeast cells to respond genotoxic agents might possibly due to limited permeability of yeast cell wall and cell membrane. The passage of several substances play an important role in their chemical structure and the yeast cell wall which acts as an ion exchanger and molecular sieve [107]. According to the increase permeability of yeast cell wall and cell membrane suggested by Staleva *et al*., (1996) which was found that D7ts1 cells enhanced the cell permeability of *S. cerevisiae* cells due to the ts1 mutation. The reponse of D7ts1 cells to genotoxic agents (hydroxyurea benzpyrene, ethyl methanesulfonate and methyl methansulfonate) in the detection of genotoxic action showed higher sensitivity in comparion with D7 strain [108]. Similarly to experiments of environmental pollution, the usage of bacterial cells in the Ames test, yeast D7 test, and D7ts1 test found that D7ts1 test was higher sensitive than the other tests and responded positive results to some samples which were negative in bacterial cells and D7 cells [16]. In addition, the genotoxic effects of substances were studied by other diploid yeast strains which were hypersensitivity to response the effect of substances. The diploid *S. cerevisiae* strain *D7 rad3*, excision-deficient of UV-induced pyrimidine dimers and the *rad52/rad52* diploid strain, deficient in DNA double-strand breaks repair and recombination were

used for studying the cytotoxic and genotoxic effects of cisplatin [79]. Similarly, the study of Schafer *et* al. (2007) used diploid *S. cerevisiae* strain DAN which was more sensitive against DNA-damaging agents for detection of genetic events such as mitotic recombination, forward mutation, and non-disjunction or chromosome loss [68].

In conclusion, the outcome of this study provided the safety and efficacy of the ethanolic and fractionated water extracts from the root and the fruit of *A. marmelos* both in prokaryotic cells and eukaryotic cells. The bacterial reverse mutation test (Ames Test) on *S. typhimurium* and yeast-based genotoxicity assay on diploid yeast *S. cerevisiae* strain D7 could be used for screening the genotoxic and anti-genotoxic potential of extracts. Although both model organisms showed different results to respond all of extracts, it could be used as elementary information to evaluate the genotoxic potential of the root and the fruit extracts in further studies. However, the further studies should be performed using the other model organisms to explicate clearer understanding of the activity of the root and the fruit extracts, for example other fungi or higher sensitivity strains of *S. cerevisiae*, somatic mutation and recombination test (SMART) using *Drosophila melanogaster*, and in mammalian cells which can provide more plausible confirmation on the genotoxic and anti-genotoxic potential of the root and the fruit of *A. marmelos*.

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APPENDICES

APPENDIX A

Manipulation of the tester strains

1. Preparation of Stock Solution and Media

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Use: Minimal agar

Ingredient	per liter
Distilled water	670 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 g
Citric 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 above to warm water in a 2-liter beaker or flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Filter the solutions and transfer 7 ml of VB salt for each tube. Autoclave at 121°C for 20 min.

1.2 40% D-glucose

Use: Minimal agar

Ingredient	140 ml
D-glucose	110 g
Distilled water	140 ml

Add D-glucose to distilled water in a 250 ml beaker and place on a hot plate. Stir it until dissolve completely. Filter the solutions through whatman No.4 and autoclave at 121°C for 20 min.

1.3 Minimal glucose agar plate

Use: Mutagenicity assay

Ingredient	350 ml
Bacto agar	5.25 g
Distilled water	330 ml
VB salts	7 ml
40% D-glucose	17.5 ml

Add Bacto agar to distilled water in a 1-liter flask. Autoclave at 121°C for 20 min. When the solution has cooled slightly, add sterile VB salts and sterile 40% D-glucose. After all the ingredients have been added, the solution should be stirred thoroughly. Appropriately 30 ml of molten agar was poured into each sterile petri dish. It was left until solidify and kept in an incubator at 37°C for 48 h before using.

1.4 Oxoid nutrient broth No.2

Use: Growing culture

Ingredient	100 ml
Nutrient broth no.2	2.5 g
Distilled water	100 ml

Dissolve nutrient broth no.2 in distilled water. Mix it until dissolve completely. Transfer 12 ml of nutrient broth for each 50 ml Erlenmeyer flask (wrapped the flask with sterile gauze or metal foil). Autoclave at 121°C for 20 min.

1.5 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 by microwave oven or hot plate. Mix thoroughly and transfer to 250 ml Erlenmeyer flask. Autoclave at 121°C for 20 min and add 10 ml of a sterile solution of 0.5 mM L-histidine/biotin and mixed thoroughly by swirling.

1.6 0.1 M L-histidine HCl stock

Use: Preparation of 1 mM L-histidine HCl stock

Ingredient	100 ml
L-histidine HCl	2.096 g
Distilled water	100 ml

Dissolve L-histidine HCl (MW 209.63) in distilled water. Autoclave at 121°C for 20 min. Store the solution in a glass bottle at 4°C until use.

1.7 1 mM L-histidine HCl stock

Use: Preparation of 0.5 mM L-histidine/biotin solution

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

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water and autoclave at 121°C for 20 min.

1.8 1 mM biotin stock

Use: Preparation of 0.5 mM L-histidine/biotin solution

Ingredient	100 ml
Biotin	24.43 mg
Distilled water	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 20 min.

1.9 0.5 mM L-histidine HCl-0.5 mM biotin

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 ingredients and autoclave at 121°C for 20 min.

1.10 0.05 M sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)

Use: Preparation of phosphate buffer

Ingredient	1000 ml
Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)	8.9 g
Distilled water to	1000 ml

Dissolve $NaH_2PO_4 \cdot 2H_2O$ (MW=156.01) in distilled water. Stir it until dissolve completely. Adjust the final volume with distilled water to be 1000 ml.

1.11 0.05 M di-sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)

Use: Preparation of phosphate buffer

Ingredient	1000 ml
Di-sodium hydrogen phosphate (Na_2HPO_4)	7.8 g
Distilled water to	1000 ml

Dissolve Na_2HPO_4 (MW=141.96) in distilled water. Stir it until dissolve completely. Adjust the final volume with distilled water to be 1000 ml.

1.12 0.1 M Phosphate buffer (pH 7.0)

Use: Mutagenicity assay

Ingredient	1000 ml
0.05 M $Na_2HPO_4 \cdot 2H_2O$	
0.05 M $NaH_2PO_4 \cdot 2H_2O$	

Mix 0.05 M $Na_2HPO_4 \cdot 2H_2O$ with 0.2 M $NaH_2PO_4 \cdot 2H_2O$ until the mixture to be pH 7.4, and then adjust the final volume to be 1000 ml. Autoclave at 121 °C for 20 min.

1.13 0.02 N sodium hydroxide

Use: Preparation of ampicillin solution

Ingredient	100 ml
Sodium hydroxide (NaOH)	0.08 g
Distilled water	100 ml

Dissolve sodium hydroxide (MW= 39.997) in distilled water and autoclave at 121 °C for 20 min.

1.14 8 mg/ml ampicillin solution (in NaOH)

Use: Tests of ampicillin resistance (to confirm R-factor strains)

Ingredient	100 ml
Ampicillin	0.8 g
0.02 N sodium hydroxide	100 ml

It necessary to sterilize ampicillin solutions but they can be filtered through a 0.22 μm membrane filter. Store it in a glass bottle with a screw cap at 4 ℃

1.15 0.1% crystal violet

Use: Test for crystal violet sensitivity (to confirm *rfa* mutation)

Ingredient	100 ml
Crystal violet	0.1 g
Distilled water	100 ml

Store at 4 ℃ in glass bottle with screw cap. Wrap the bottle with metal foil to protect against light.

2. Recipes for some Reagents

2.1 2 M sodium nitrite

Use: Nitrosation

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

Store in a glass bottle with a screw cap (wrap the bottle with metal foil to protect against light). Autoclave at 121°C for 20 min.

2.2 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 the final volume to be 10 ml. Autoclave at 121 °C for 20 min.

2.3 0.2 N hydrochloric acid

Use: Reaction mixture

Ingredient	100 ml
Conc. Hydrochloric acid	1.66 ml
Sterile distilled water	98.34 ml

Dissolve conc. hydrochloric acid in sterile distilled water. Store in sterile glass bottles with screw caps.

Note: Preparation of 0.2 N hydrochloric acid must be used sterile technique because hydrochloric acid cannot be autoclaved.

2.4 0.0375 mg/ml 1-aminopyrene

Use: Standard solution for mutagenicity assay

Ingredient	2 ml
0.3 mg/ml 1-aminopyrene	250 µl
Acetonitrite	1750 µl

Dissolve 3 mg of 1-aminopyrene in 300 µl of acetonitrite and mix; and subsequently dilute 300 µl of this solution (3 mg/ml 1-aminopyrene) in 2,700 µl of acetonitrite, the solution obtained will be 0.3 mg/ml 1-aminopyrene. Then, dilute 250 µl of 0.3 mg/ml 1-aminopyrene in 1,750 µl of acetonitrite and mix. Store all solution in sterile glass vials with screw caps in a refrigerator. The preparation must be used sterile technique.

3. Precedure for Reisolation and Growing of culture

The tester strain (TA98 and TA100) are grown in Oxoid nutrient broth No. 2 and incubated at 37 °C for 16 hr in a shaking water bath. The rate of rotation should be decreased to about 120 rpm to avoid foaming. These cultures are reisolated by streaking on the minimal glucose agar plates enriched with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1M histidine HCl and 0.1 ml of 1 mM biotin. Incubate at 37 °C for 48 hr. With a sterile wire loop, pick a well-isolated colony for overnight growth in Oxoid nutrient broth no. 2 at 37 °C in a shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of the bacteria for

mutagenicity testing. For each 1 ml of culture, add 0.09 ml of dimethyl sulfoxide (DMSO) into an eppendorf and swirl gently. Store the cultures in a freezer at -80 ℃.

4. Checking Characteristics of Tester Strains

The broth cultures of tester strains (TA98 and TA100) are used to confirm genotypes in the following ways.

4.1 Histidine requirement

The his^- character of the strains is confirmed by demonstrating the histidine requirement for growth on the selective agar plate.

Procedure:

Plate a no histidine and biotin

Plate b 0.1 ml of 1 mM biotin

Plate c 0.3 ml of 0.1 M histidine-HCl

Plate d 0.3 ml of 0.1 M histidine-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strain. Apply 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M histidine-HCl and 0.3 ml of 0.1 M histidine-HCl plus 0.1 ml of 1 mM biotin on the surface of minimal glucose agar of plate b, c and d, respectively; and no application for plate a. Make a single streak of each strain across these plates and incubate at 37 ℃ for 24 hr. The growing of bacteria on histidine plus biotin plate (plate d) is the result of histidine requirement.

4.2 R-factor

The tester strains (TA98 and TA100) should be tested for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria. For this test, minimal glucose agar plates are seeded with the cultures of the strains and a sterile filter paper disc containing ampicillin is placed on the surface of the plates.

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-HCl. Then, add 2 ml of molten top agar containing 0.5 mM histidine-HCl and 0.5 mM biotin. Vortex and poured onto a minimal glucose agar. The plates was rotated in order to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and *rfa* mutation are performed in the same plate by dividing the plate into 2 parts, one for R-factor and the other for *rfa* mutation. For R-factor, filter paper disc containing 8mg/ml solution of ampicillin is applied on the surface of agar by using sterile forceps. Incubate at 37 ℃ for 24 hr. The absence of the clear zone of inhibition around the disc indicates resistance to ampicillin.

4.3 *rfa* mutation

Strains having the deep rough *(rfa)* character should be tested for crystal violet sensitivity. The method is similar to the test of ampicillin resistance.

Procedure:

Pipet 10 µl of a 0.1% solution of crystal violet to the center of sterile filter paper discs and transfer one disc to each of the seeded plates using sterile forceps. Press the disc lightly with the forceps. Incubate the plate at 37℃ for 24 hr. the clear

zone of inhibition around the disc indicates the presence of the *rfa* mutation that crystal violet is transferred into the cell and kill bacteria.

4.4 *uvrB* mutation

The uvrB mutation can be confirmed by demonstrating UV sensitivity in tester strains that contain this mutation.

Procedure:

Add 0.1 ml of 0.1 M histidine-HCl and 0.1 ml of 1 mM biotin onto a minimal glucose agar. The plates was rotated in order to distribute the mixtures and allowed several minutes for agar to become firm. Streak the tester strain cultures across a minimal glucose agar plate. Tester strains (TA98 and TA100) are irradiated for 8 sec at a distance of 33 cm. Incubate the irradiated plates at 37°C for 24 h. Strains with the *uvrB* deletion will grow only on the un-irradiated side of the plate.

5. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic 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 from one experiment to another and from one plate to another, and it is advisable to include at least 3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure:

Add 0.1 ml of DMSO to culture tube. Then, add 0.5 ml of sodium phosphate buffer pH7.4 and 0.1 ml of overnight culture of TA98 and TA100. Incubate the mixture in a shaking water bath at 37°C for 20 min. Then, 2 ml of top agar is added to the mixture. Mix and pour on the minimal glucose agar plate. Incubate at 37°C for 48 h. The his^+ revertants colonies that grow on the minimal glucose agar plate are counted.

APPENDIX B

The mutagenicity and anti-mutagenicity of the extracts using Ames test

Table 14. Mutagenicity of ethanolic extracts and fractionated water extracts from the root and the fruit of *A. marmelos* in acidic condition (pH 3.0-3.5) on *S. typhimurium* strain TA98 (frameshift mutation) without metabolic activation

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]		Mutagenic Index (MI)[b]	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
RE	Positive[c]				
	Spontaneous[d]	17.5 ± 6.36	44.67 ± 16.26		
	0.4	23.5 ± 13.44	83.67 ± 13.65	1.34 ± 1.26	1.87 ± 0.99
	0.8	14.67 ± 5.51	136.33 ± 41.43	0.84 ± 0.62	3.05 ± 2.04
	1.6	14.67 ± 0.58	151.33 ± 7.37	0.84 ± 0.34	3.39 ± 1.40
	3.2	17 ± 4	221 ± 7	0.97 ± 0.58	4.95 ± 1.96
FE	Positive[c]		890 ± 179.88		
	Spontaneous[d]	14.33 ± 2.80	30.33 ± 8.26		
	0.4	18.33 ± 5.16	279.67 ± 89.19	1.28 ± 0.61	9.22 ± 5.45
	0.8	17.5 ± 6.86	743.83 ± 115.30	1.22 ± 0.72	24.52 ± 10.48
	1.6	15.83 ± 7.80	890.17 ± 128.53	1.1 ± 0.76	29.35 ± 12.23
	3.2	12.5 ± 5.43	1046 ± 101.10	0.87 ± 0.55	34.49 ± 12.73
RW	Positive[c]		3800 ± 746.70		
	Spontaneous[d]	34.67 ± 8.33	15.67 ± 5.69		
	0.4	37 ± 4.24	35.67 ± 8.02	1.07 ± 0.38	2.28 ± 1.34
	0.8	47 ± 21	50 ± 7	1.36 ± 0.93	3.19 ± 1.61
	1.6	102.33 ± 39.11	131 ± 42.43	2.95 ± 1.84	8.36 ± 5.74
	3.2	787.6 ± 26.84	183.5 ± 16.26	22.72 ± 6.23	11.71 ± 5.29

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]		Mutagenic Index (MI)[b]	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
FW	Positive[c]		1105.33 ± 116.30		
	Spontaneous[d]	18 ± 3.10	29.5 ± 4.04		
	0.4	21.67 ± 4.13	138.83 ± 31.8	1.2 ± 0.44	4.71 ± 1.72
	0.8	21 ± 4.82	159.33 ± 23.76	1.17 ± 0.47	5.4 ± 1.54
	1.6	19 ± 5.83	260.5 ± 28.18	1.06 ± 0.51	8.83 ± 2.16
	3.2	23.33 ± 4.13	459.67 ± 132.44	1.3 ± 0.45	15.58 ± 6.62

Abbreviations including: ethanolic extract from the root of *A. marmelos*: RE, ethanolic extract from the fruit of *A. marmelos*: FE, fractionated water extract from the root of *A. marmelos*: RW, fractionated water extract from the fruit of *A. marmelos*: FW

[a] Mean ± SD of his^+ revertants per plate of each concentration of the extracts (N=6)

[b] Mutagenic index (MI) is calculated from the average value of a number of revertant colonies of the root/fruit extracts divided by that of spontaneous revertant colonies.

[c] The number of revertant colonies after nitrite-treated 1-aminopyrene

[d] The number of spontaneous revertant colonies

Table 15. Mutagenicity of ethanolic extracts and fractionated water extracts from the root and the fruit of *Aegle marmelos* in acidic condition (pH 3.0-3.5) on *S. typhimurium* strain TA100 (base-pair substitution mutation) without metabolic activation

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]		Mutagenic Index (MI)[b]	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
RE	Positive[c]				
	Spontaneous[d]	127 ± 9.54	134.67 ±43.14		
	0.4	104.67 ± 16.20	195.67 ± 8.74	0.82 ± 0.19	1.45 ± 0.53
	0.8	119.33 ± 0.94	205.33 ± 50.05	0.94 ± 0.08	1.52 ± 0.86
	1.6	146 ± 1.15	296 ± 2.83	1.15 ± 0.10	2.2 ± 0.73
	3.2	134.33 ± 22.23	141.67 ± 38.55	1.06 ± 0.25	1.05 ± 0.62
FE	Positive[c]		528 ± 90.35		
	Spontaneous[d]	94.33 ± 16.97	119.33 ± 11.04		
	0.4	111 ± 19.09	562.33 ± 63.92	1.18 ± 0.41	4.71 ± 0.97
	0.8	134 ± 33.97	634.33 ± 82.43	1.42 ± 0.62	5.32 ± 1.18
	1.6	106.83 ± 23.74	654.33 ± 91.45	1.13 ± 0.46	5.48 ± 1.27
	3.2	101.67 ± 16.00	323.5 ± 92.34	1.08 ± 0.36	2.71 ± 1.02
RW	Positive[c]		458.33 ± 121.96		
	Spontaneous[d]	101.83 ± 21.54	82.83 ± 12.09		
	0.4	102.17 ± 20.30	232.17 ± 35.84	1 ± 0.41	2.8 ± 0.84
	0.8	103.5 ± 18.26	369.33 ± 20.28	1.02 ± 0.39	4.46 ± 0.9
	1.6	117 ± 18.14	465.67 ± 34.44	1.15 ± 0.42	5.62 ± 0.12
	3.2	129.33 ± 27.52	428.5 ± 47.61	1.07 ± 0.54	5.17 ± 1.33

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]		Mutagenic Index (MI)[b]	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
FW	Positive[c]		864.33 ± 151.66		
	Spontaneous[d]	127 ± 9.54	126.67 ± 14.01		
	0.4	112 ± 12.73	285.67 ± 40.53	0.88 ± 0.17	2.26 ± 0.57
	0.8	135.67 ± 12.01	371.67 ± 12.01	1.07 ± 0.17	2.93 ± 0.42
	1.6	169 ± 44.24	169 ± 44.24	1.33 ± 0.45	3.36 ± 0.50
	3.2	819.5 ± 6.36	819.5 ± 6.36	6.45 ± 0.53	4.05 ± 0.77

Abbreviations including: ethanolic extract from the root of *A. marmelos*: RE, ethanolic extract from the fruit of *A. marmelos*: FE, fractionated water extract from the root of *A. marmelos*: RW, fractionated water extract from the fruit of *A. marmelos*: FW

[a] Mean ± SD of his^+ revertants per plate of each concentration of the extracts (N=6)

[b] Mutagenic index (MI) is calculated from the average value of a number of revertant colonies of the root/fruit extracts divided by that of spontaneous revertant colonies.

[c] The number of revertant colonies after nitrite-treated 1-aminopyrene

[d] The number of spontaneous revertant colonies

Table 16. Modification effect of the root and the fruit extracts from *A. marmelos* on mutagenicity induced by sodium nitrite-treated 1-aminopyrene on *S. typhimurium* strains TA98 and TA100 without metabolic activation

Sample	Amount of extract (mg/plate)	TA98		TA100	
		% modification[a]		% modification[a]	
		Inhibition	Enhancement	Inhibition	Enhancement
Ethanolic extract from the root (RE)	Negative control				
	Positive control				
	5	87.82 (s)	-	19.46 (n)	-
	10	93.67 (s)	-	85.26 (s)	-
	15	97.02 (s)	-	101.73 (s)	-
Ethanolic extract from the fruit (FE)	Negative control				
	Positive control				
	5	53.93 (m)	-	74.52 (s)	-
	10	75.02 (s)	-	107.96 (s)	-
	15	86.71 (s)	-	113.86 (s)	-
Fractionated water extract from the root (RW)	Negative control				
	Positive control				
	5	NA	NA	NA	NA
	10	NA	NA	NA	NA
	15	NA	NA	NA	NA
Fractionated water extract from the fruit (FW)	Negative control				
	Positive control				
	5	-	2.11 (n)	5.02 (n)	-
	10	-	3.45 (n)	-	11.57 (n)
	15	21.46 (w)	-	-	15.73 (n)

[a] The percent modification of different extracts expressed either decrease (inhibition) or increase (enhancement) on the mutagenicity of nitrite treated 1-aminopyrene on both strains.
Anti-muatgenic potential: (n) = negligible, (w) = weak, (m) = moderate, (s) = strong
NA = No activity, each experiment was done in sixtuplets.

APPENDIX C

Effect of the extracts on *Saccharomyces cerevisiae* D7

The ethanolic extract from the root (RE)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	20.6667	1.86190	.76012	18.7127	22.6206	18.00	23.00
1	6	23.6667	1.96638	.80277	21.6031	25.7303	21.00	27.00
2	6	24.6667	2.73252	1.11555	21.7991	27.5343	21.00	29.00
3	6	22.5000	1.22474	.50000	21.2147	23.7853	21.00	24.00
4	6	24.5000	2.07364	.84656	22.3238	26.6762	22.00	28.00
Total	30	23.2000	2.41261	.44048	22.2991	24.1009	18.00	29.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.800	4	16.450	3.993	.012
Within Groups	103.000	25	4.120		
Total	168.800	29			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (>control)

(I) conc	(J) conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	3.00000*	1.17189	.028	.3347
0.5	Control	4.00000*	1.17189	.004	1.3347
1	Control	1.83333	1.17189	.178	-.8319
2	Control	3.83333*	1.17189	.005	1.1681

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the root (RE)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	9.6667	2.65832	1.08525	6.8769	12.4564	7.00	13.00
1	6	11.1667	2.56255	1.04616	8.4774	13.8559	8.00	14.00
2	6	11.5000	3.39116	1.38444	7.9412	15.0588	7.00	17.00
3	6	13.8333	2.78687	1.13774	10.9087	16.7580	12.00	19.00
4	6	15.5000	4.08656	1.66833	11.2114	19.7886	11.00	22.00
Total	30	12.3333	3.60396	.65799	10.9876	13.6791	7.00	22.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	128.667	4	32.167	3.243	.028
Within Groups	248.000	25	9.920		
Total	376.667	29			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (>control)

(I) conc	(J) conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	1.50000	1.81842	.456	-2.6357
0.5	Control	1.83333	1.81842	.376	-2.3023
1	Control	4.16667*	1.81842	.048	.0310
2	Control	5.83333*	1.81842	.006	1.6977

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the fruit (FE)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	24.0000	1.41421	.57735	22.5159	25.4841	22.00	26.00
1	6	29.1667	1.94079	.79232	27.1299	31.2034	27.00	32.00
2	6	29.3333	1.36626	.55777	27.8995	30.7671	27.00	31.00
3	6	30.5000	2.25832	.92195	28.1300	32.8700	27.00	33.00
4	6	31.8333	2.31661	.94575	29.4022	34.2645	30.00	36.00
Total	30	28.9667	3.23220	.59012	27.7597	30.1736	22.00	36.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	212.467	4	53.117	14.673	.000
Within Groups	90.500	25	3.620		
Total	302.967	29			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	5.16667*	1.09848	.000	2.6684
0.5	Control	5.33333*	1.09848	.000	2.8350
1	Control	6.50000*	1.09848	.000	4.0017
2	Control	7.83333*	1.09848	.000	5.3350

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the fruit (FE)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	14.1667	2.13698	.87242	11.9240	16.4093	11.00	16.00
1	6	14.5000	2.50998	1.02470	11.8659	17.1341	12.00	18.00
2	6	14.5000	3.39116	1.38444	10.9412	18.0588	10.00	20.00
3	6	16.6667	2.58199	1.05409	13.9570	19.3763	12.00	19.00
4	6	16.6667	2.33809	.95452	14.2130	19.1203	13.00	19.00
Total	30	15.3000	2.69290	.49165	14.2945	16.3055	10.00	20.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37.800	4	9.450	1.370	.273
Within Groups	172.500	25	6.900		
Total	210.300	29			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	.33333	1.51658	.721	-3.1158
0.5	Control	.33333	1.51658	.721	-3.1158
1	Control	2.50000	1.51658	.156	-.9492
2	Control	2.50000	1.51658	.156	-.9492

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the root (RW)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	5.5000	1.04881	.42817	4.3993	6.6007	4.00	7.00
1	6	5.6667	2.58199	1.05409	2.9570	8.3763	2.00	9.00
2	6	6.0000	1.41421	.57735	4.5159	7.4841	4.00	8.00
3	6	6.6667	1.63299	.66667	4.9529	8.3804	5.00	9.00
4	6	6.6667	2.80476	1.14504	3.7233	9.6101	3.00	10.00
Total	30	6.1000	1.93605	.35347	5.3771	6.8229	2.00	10.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.200	4	1.800	.443	.776
Within Groups	101.500	25	4.060		
Total	108.700	29			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	.16667	1.16333	.750	-2.4791
0.5	Control	.50000	1.16333	.634	-2.1458
1	Control	1.16667	1.16333	.378	-1.4791
2	Control	1.16667	1.16333	.378	-1.4791

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the root (RW)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	11.1667	2.04124	.83333	9.0245	13.3088	9.00	14.00
1	6	13.3333	2.58199	1.05409	10.6237	16.0430	10.00	17.00
2	6	13.3333	2.50333	1.02198	10.7062	15.9604	10.00	17.00
3	6	14.5000	2.88097	1.17615	11.4766	17.5234	11.00	18.00
4	6	16.5000	2.94958	1.20416	13.4046	19.5954	12.00	20.00
Total	30	13.7667	3.00211	.54811	12.6457	14.8877	9.00	20.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	90.867	4	22.717	3.331	.026
Within Groups	170.500	25	6.820		
Total	261.367	29			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	2.16667	1.50776	.216	-1.2624
0.5	Control	2.16667	1.50776	.216	-1.2624
1	Control	3.33333	1.50776	.057	-.0958
2	Control	5.33333*	1.50776	.003	1.9042

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the fruit (FW)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	5.1667	1.16905	.47726	3.9398	6.3935	4.00	7.00
1	6	8.1667	2.13698	.87242	5.9240	10.4093	6.00	11.00
2	6	7.6667	2.50333	1.02198	5.0396	10.2938	4.00	11.00
3	6	7.0000	3.09839	1.26491	3.7484	10.2516	3.00	11.00
4	6	6.0000	1.89737	.77460	4.0088	7.9912	4.00	8.00
Total	30	6.8000	2.36934	.43258	5.9153	7.6847	3.00	11.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35.800	4	8.950	1.762	.168
Within Groups	127.000	25	5.080		
Total	162.800	29			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	3.00000*	1.30128	.047	.0405
0.5	Control	2.50000	1.30128	.098	-.4595
1	Control	1.83333	1.30128	.225	-1.1262
2	Control	.83333	1.30128	.540	-2.1262

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the fruit (FW)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	6.1667	1.16905	.47726	4.9398	7.3935	5.00	8.00
1	6	9.3333	1.36626	.55777	7.8995	10.7671	7.00	11.00
2	6	9.1667	1.60208	.65405	7.4854	10.8479	8.00	12.00
3	6	9.8333	1.47196	.60093	8.2886	11.3781	8.00	12.00
4	6	7.8333	1.16905	.47726	6.6065	9.0602	6.00	9.00
Total	30	8.4667	1.85199	.33813	7.7751	9.1582	5.00	12.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	52.800	4	13.200	7.071	.001
Within Groups	46.667	25	1.867		
Total	99.467	29			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (>control)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
0.25	Control	3.16667*	.78881	.001	1.3727
0.5	Control	3.00000*	.78881	.001	1.2060
1	Control	3.66667*	.78881	.000	1.8727
2	Control	1.66667	.78881	.069	-.1273

*. The mean difference is significant at the 0.05 level.

APPENDIX D

Effect of the extracts in combination with EMS in

Saccharomyces cerevisiae D7

The ethanolic extract from the root (RE)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	11.5000	2.66458	1.08781	8.7037	14.2963	8.00	15.00
1	6	18.8333	1.47196	.60093	17.2886	20.3781	17.00	21.00
2	6	22.8333	4.07022	1.66166	18.5619	27.1048	16.00	28.00
3	6	17.0000	2.82843	1.15470	14.0317	19.9683	13.00	20.00
4	6	22.8333	2.92689	1.19490	19.7618	25.9049	19.00	28.00
5	6	7.8333	1.83485	.74907	5.9078	9.7589	6.00	10.00
Total	36	16.8056	6.19133	1.03189	14.7107	18.9004	6.00	28.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1112.806	5	222.561	29.178	.000
Within Groups	228.833	30	7.628		
Total	1341.639	35			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	7.33333*	1.59455	.000	3.0968	11.5698
0.5	Pos.	11.33333*	1.59455	.000	7.0968	15.5698
1	Pos.	5.50000*	1.59455	.007	1.2635	9.7365
2	Pos.	11.33333*	1.59455	.000	7.0968	15.5698
Neg.	Pos.	-3.66667	1.59455	.108	-7.9032	.5698

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the root (RE)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	46.5000	2.58844	1.05672	43.7836	49.2164	43.00	50.00
1	6	36.6667	2.94392	1.20185	33.5772	39.7561	32.00	40.00
2	6	39.5000	2.25832	.92195	37.1300	41.8700	37.00	43.00
3	6	39.5000	1.76068	.71880	37.6523	41.3477	37.00	41.00
4	6	48.8333	2.48328	1.01379	46.2273	51.4394	46.00	53.00
5	6	3.3333	1.63299	.66667	1.6196	5.0471	1.00	5.00
Total	36	35.7222	15.45613	2.57602	30.4926	40.9518	1.00	53.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8199.222	5	1639.844	303.675	.000
Within Groups	162.000	30	5.400		
Total	8361.222	35			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	-9.83333*	1.34164	.000	-13.3979	-6.2688
0.5	Pos.	-7.00000*	1.34164	.000	-10.5646	-3.4354
1	Pos.	-7.00000*	1.34164	.000	-10.5646	-3.4354
2	Pos.	2.33333	1.34164	.303	-1.2312	5.8979
Neg.	Pos.	-43.16667*	1.34164	.000	-46.7312	-39.6021

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the fruit (FE)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	11.6667	2.33809	.95452	9.2130	14.1203	8.00	15.00
1	6	16.1667	2.48328	1.01379	13.5606	18.7727	13.00	19.00
2	6	15.6667	2.42212	.98883	13.1248	18.2085	13.00	19.00
3	6	19.8333	2.22860	.90982	17.4946	22.1721	17.00	23.00
4	6	21.0000	2.82843	1.15470	18.0317	23.9683	16.00	24.00
5	6	4.0000	1.78885	.73030	2.1227	5.8773	1.00	6.00
Total	36	14.7222	6.15333	1.02556	12.6402	16.8042	1.00	24.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1156.889	5	231.378	41.236	.000
Within Groups	168.333	30	5.611		
Total	1325.222	35			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	4.50000*	1.36761	.011	.8664	8.1336
0.5	Pos.	4.00000*	1.36761	.027	.3664	7.6336
1	Pos.	8.16667*	1.36761	.000	4.5331	11.8002
2	Pos.	9.33333*	1.36761	.000	5.6998	12.9669
Neg.	Pos.	-7.66667*	1.36761	.000	-11.3002	-4.0331

*. The mean difference is significant at the 0.05 level.

The ethanolic extract from the fruit (FE)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	23.0000	3.74166	1.52753	19.0734	26.9266	16.00	26.00
1	6	27.3333	1.63299	.66667	25.6196	29.0471	25.00	29.00
2	6	27.3333	2.33809	.95452	24.8797	29.7870	25.00	30.00
3	6	27.6667	2.33809	.95452	25.2130	30.1203	25.00	31.00
4	6	27.8333	1.94079	.79232	25.7966	29.8701	25.00	30.00
5	6	2.8333	.75277	.30732	2.0433	3.6233	2.00	4.00
Total	36	22.6667	9.39909	1.56651	19.4865	25.8469	2.00	31.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2932.333	5	586.467	110.192	.000
Within Groups	159.667	30	5.322		
Total	3092.000	35			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	4.33333*	1.33194	.012	.7945	7.8721
0.5	Pos.	4.33333*	1.33194	.012	.7945	7.8721
1	Pos.	4.66667*	1.33194	.006	1.1279	8.2055
2	Pos.	4.83333*	1.33194	.005	1.2945	8.3721
Neg.	Pos.	-20.16667*	1.33194	.000	-23.7055	-16.6279

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the root (RW)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	13.1667	3.18852	1.30171	9.8205	16.5128	10.00	18.00
1	6	7.1667	.98319	.40139	6.1349	8.1985	6.00	8.00
2	6	11.1667	1.47196	.60093	9.6219	12.7114	9.00	13.00
3	6	15.5000	3.08221	1.25831	12.2654	18.7346	11.00	19.00
4	6	15.8333	1.72240	.70317	14.0258	17.6409	13.00	18.00
5	6	5.6667	1.86190	.76012	3.7127	7.6206	3.00	8.00
Total	36	11.4167	4.43605	.73934	9.9157	12.9176	3.00	19.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	542.583	5	108.517	22.273	.000
Within Groups	146.167	30	4.872		
Total	688.750	35			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	-6.00000*	1.27439	.000	-9.3859	-2.6141
0.5	Pos.	-2.00000	1.27439	.395	-5.3859	1.3859
1	Pos.	2.33333	1.27439	.260	-1.0526	5.7192
2	Pos.	2.66667	1.27439	.162	-.7192	6.0526
Neg.	Pos.	-7.50000*	1.27439	.000	-10.8859	-4.1141

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the root (RW)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	23.1667	3.81663	1.55813	19.1614	27.1720	16.00	26.00
1	6	27.3333	2.94392	1.20185	24.2439	30.4228	22.00	30.00
2	6	27.3333	3.32666	1.35810	23.8422	30.8244	23.00	32.00
3	6	31.6667	3.32666	1.35810	28.1756	35.1578	27.00	37.00
4	6	28.5000	1.37840	.56273	27.0535	29.9465	27.00	31.00
5	6	3.0000	.89443	.36515	2.0614	3.9386	2.00	4.00
Total	36	23.5000	9.98427	1.66405	20.1218	26.8782	2.00	37.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3248.667	5	649.733	81.104	.000
Within Groups	240.333	30	8.011		
Total	3489.000	35			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	4.16667	1.63413	.063	-.1750	8.5083
0.5	Pos.	4.16667	1.63413	.063	-.1750	8.5083
1	Pos.	8.50000*	1.63413	.000	4.1583	12.8417
2	Pos.	5.33333*	1.63413	.012	.9917	9.6750
Neg.	Pos.	-20.16667*	1.63413	.000	-24.5083	-15.8250

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the fruit (FW)

The frequency of convertant colonies

Descriptives

Number_of_convertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	16.6667	4.27395	1.74483	12.1814	21.1519	13.00	25.00
1	6	14.6667	2.06559	.84327	12.4990	16.8344	11.00	17.00
2	6	18.0000	2.82843	1.15470	15.0317	20.9683	14.00	22.00
3	6	22.0000	5.13809	2.09762	16.6079	27.3921	17.00	29.00
4	6	19.6667	2.50333	1.02198	17.0396	22.2938	17.00	24.00
5	6	4.1667	2.31661	.94575	1.7355	6.5978	.00	6.00
Total	36	15.8611	6.58202	1.09700	13.6341	18.0881	.00	29.00

ANOVA

Number_of_convertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1173.472	5	234.694	20.537	.000
Within Groups	342.833	30	11.428		
Total	1516.306	35			

Multiple Comparisons

Number_of_convertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	-2.00000	1.95173	.758	-7.1855	3.1855
0.5	Pos.	1.33333	1.95173	.937	-3.8522	6.5188
1	Pos.	5.33333*	1.95173	.042	.1478	10.5188
2	Pos.	3.00000	1.95173	.414	-2.1855	8.1855
Neg.	Pos.	-12.50000*	1.95173	.000	-17.6855	-7.3145

*. The mean difference is significant at the 0.05 level.

The fractionated water extract from the fruit (FW)

The frequency of revertant colonies

Descriptives

Number_of_revertant_colonies

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	6	26.5000	1.87083	.76376	24.5367	28.4633	24.00	29.00
1	6	25.1667	2.78687	1.13774	22.2420	28.0913	22.00	29.00
2	6	25.5000	1.51658	.61914	23.9085	27.0915	24.00	28.00
3	6	25.1667	1.72240	.70317	23.3591	26.9742	23.00	28.00
4	6	27.5000	1.87083	.76376	25.5367	29.4633	25.00	30.00
5	6	4.5000	1.04881	.42817	3.3993	5.6007	3.00	6.00
Total	36	22.3889	8.34019	1.39003	19.5670	25.2108	3.00	30.00

ANOVA

Number_of_revertant_colonies

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2328.889	5	465.778	132.240	.000
Within Groups	105.667	30	3.522		
Total	2434.556	35			

Multiple Comparisons

Number_of_revertant_colonies

Dunnett t (2-sided)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.25	Pos.	-1.33333	1.08355	.617	-4.2122	1.5455
0.5	Pos.	-1.00000	1.08355	.822	-3.8788	1.8788
1	Pos.	-1.33333	1.08355	.617	-4.2122	1.5455
2	Pos.	1.00000	1.08355	.822	-1.8788	3.8788
Neg.	Pos.	-22.00000*	1.08355	.000	-24.8788	-19.1212

*. The mean difference is significant at the 0.05 level.

Table 17. The induction of mitotic recombination and mutations after treatment of diploid yeast cells of *S. cerevisiae* D7 with the extracts

Sample	Sample conc. (mg/ml)	Color colony frequency					Number of total aberrant colonies	Total aberrant (%)
		Sectored red/pink	Red	Pink	Red/ white	Others		
RE	Control	0	2	0	0	0	2	0.30
	0.25	0	2	0	2	0	4	0.61
	0.5	0	3	1	0	0	4	0.54
	1	0	4	1	0	0	5	0.82
	2	0	1	0	0	0	1	0.16
FE	Control	0	1	0	1	0	2	0.36
	0.25	0	3	0	0	0	3	0.53
	0.5	0	1	1	1	0	3	0.53
	1	0	5	3	0	0	8	1.47
	2	0	1	0	0	0	2	0.36
RW	Control	0	0	0	0	0	0	0
	0.25	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
FW	Control	0	0	0	0	0	0	0
	0.25	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0

Abbreviations including: ethanolic extract from the root of *A. marmelos*: RE, ethanolic extract from the fruit of *A. marmelos*: FE, fractionated water extract from the root of *A. marmelos*: RW, fractionated water extract from the fruit of *A. marmelos*: FW

Table 18. The induction of mitotic recombination and mutations after treatment of diploid yeast cells of *S. cerevisiae* D7 with the extracts and ethyl methane sulfonate (EMS)

Sample	Sample Conc. (mg/ml)	Color colony frequency					Number of total aberrant colonies	Total aberrant (%)
		Sectored red/pink	Red	Pink	Red/ white	Others		
RE	Negative control	0	0	0	0	0	0	0
	Positive control	2	0	0	5	0	7	1.16
	0.25	1	0	0	4	0	5	0.80
	0.5	0	0	0	3	0	3	0.48
	1	0	0	0	3	0	3	0.48
	2	1	0	0	4	0	5	0.85
FE	Negative control	0	0	0	0	0	0	0
	Positive control	0	0	0	8	0	8	1.19
	0.25	0	0	0	10	0	10	1.44
	0.5	3	0	0	11	0	14	2.02
	1	1	0	0	7	0	8	1.16
	2	1	0	0	14	0	15	2.19
RW	Negative control	0	0	0	0	0	0	0
	Positive control	0	0	0	7	0	7	1.09
	0.25	0	0	0	6	0	6	0.90
	0.5	0	0	0	2	0	2	0.30
	1	2	0	0	2	0	4	0.59
	2	0	0	0	3	0	3	0.44
FW	Negative control	0	0	0	0	0	0	0
	Positive control	0	0	0	7	0	7	1.07
	0.25	1	0	0	7	0	8	1.19
	0.5	1	0	0	6	0	7	1.07
	1	0	0	0	7	0	7	1.04
	2	2	0	0	6	0	8	1.18

Abbreviations including: ethanolic extract from the root of *A. marmelos*: RE, ethanolic extract from the fruit of *A. marmelos*: FE, fractionated water extract from the root of *A. marmelos*: RW, fractionated water extract from the fruit of *A. marmelos*: FW

VITA

Name: Miss Arunrat Saeou

Born: October 15, 1988, Songkhla, Thailand

Education: Bachelor of Scicence (Traditional Thai medicine) (First class honor), Faculty of Traditional Thai medicine, Prince of Songkla University, Thailand in 2013.

Proceedings:

Saeou, A., Charoensup, R., Palanuvej, C., and Ruangrungsi, N. Effects of root and fruit extractives from Aegle marmelos on mutagenicity and anti-mutagenicity using Ames test. Proceedings of The 7th Thailand-Japan international academic conference 2014 (TJIA2014), pp. 216-219. University of Tokyo, Kongo Campus, Japan, 2014