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Genotoxic and Cytotoxic Effects of Ben-Cha-Moon-Yai Remedy and It's Ingredients

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ABSTRACT

The mutagenicity and antimutagenicity of Ben-Cha-Moon-Yai remedy and its ingredients including the roots of *Aegle marmelos*, *Oroxylum indicum*, *Dimocarpus longan*, *Dolichandrone serrulata*, and *Walsura trichostemon* were evaluated by the pre-incubation method of Ames test toward *Salmonella typhimurium* strain TA98 and TA100 in an acidic condition (pH 3-3.5) without enzyme activating system. The cytotoxicity of the extracts was performed by brine shrimp lethality testing. The results demonstrated that only the water extracts from the root of *A. marmelos* exhibited direct mutagenicity on both strains whereas most of the extracts exhibited their mutagenicity after treated with sodium nitrite on both strains. It was implied that most of the extracts contained certain precursors that could react with nitrite under acidic condition to produce direct mutagenic products causing frame-shift (TA98) and base-pair substitution (TA100) mutation. In addition, most of the extracts, except the water extract of *A. marmelos* and *D. serrulata* demonstrated mutagenic inhibitory effect against the mutagen induced by nitrite treated 1- aminopyrine on both strains. The potency ranged from negligible to strongly active inhibition. Moreover, most of the root extracts are non-cytotoxic except the ethanol extract of *A. marmelos* exhibited the highest toxicity against brine shrimp nauplii with LC₅₀ of 53.5 µg/ml.

Key words: Ben-Cha-Moon-Yai remedy; Brine Shrimp lethality; Ames test; *Salmonella typhimurium*; Nitrite

Running title: Genotoxic, Cytotoxic, Ben-Cha-Moon-Yai remedy

Abbreviations: Ben-Cha-Moon-Yai remedy: BMY, *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT,

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INTRODUCTION

Ben-Cha-Moon-Yai remedy is one of the Thai traditional medicines notified in Tum ra paad sard song khor. The remedy is composed of the roots of *Aegle marmelos* (L.) Correa ex Roxb. (Rutaceae), *Oroxylum indicum* (L.) Kurz (Bignoniaceae), *Dimocarpus longan* Lour. subsp. *longan* var. *longan* (Sapindaceae), *Dolichandrone serrulata* (DC.) Seem. (Bignoniaceae), and *Walsura trichostemon* Miq. (Meliaceae) each in an equal part by weights [1]. This remedy has been used as an antipyretic, anti-inflammatory and analgesic drug for a long time without scientific approved.

Apart from the roots, the biological activities and phytochemicals of other parts of the plants in Ben-Cha-Moon-Yai remedy have been reported. Previously, the fruits, leaves, stem and roots of *A. marmelos* have been used in traditional medicine as an, anti-inflammatory, antipyretic, analgesic [2] and antiproliferative activities [3]. The leaves extract also showed the hypoglycemic and hypolipidemic effects in rats [4]. The *D. longan* extracts contains significant amounts of polyphenolic compounds, where as *D. longan* fruit, aril, pericarp or seed were reported to have antioxidant property based on their ability to scavenge DPPH, superoxide anion and hydroxyl radicals. The fruit and flower inhibited lipid peroxidation and exhibited free radical scavenging activity [5,6]. *D. longan* seed [7] and fruit pericarp [8] was suggested as potential treatment agent for cancer according to polyphenol rich extracts as well as the anti-proliferation activity against cancer cell line. In addition, polysaccharides from longan fruit pericarp were reported to inhibit tyrosinase [9] and glycosylated activities [10]. It was suggested that these extracts might employed as a potential novel treatment agents for cancer. For instance, it was reported that the extract of *W. trichostemon* stem bark exhibited the antimicrobial against gram positive bacteria [11] and cytotoxicity on cancer cell lines [12]. The extract of *O. indicum* root bark exhibited the protective effect against ethanol-induced gastric mucosa damage and lipid peroxidation reduction determined by malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione levels (GSH) assay [13]. Furthermore, dolichandrosides; a novel phenolic triglycosides was isolated from the branches of *D. serrulata* [14].

The use of herbal medicines for prevention and treatment of various health ailments has been in practice from time immemorial and become more popular over the past decades [15]. Nevertheless, it is still important to determine the toxic property of herbal medicines, especially the safety of the herbs that are being consumed frequently over a long period.

The study was carried out to evaluate the mutagenic and antimutagenic activities of the ethanolic and water extracts from the roots of five plant species and Ben-Cha-Moon-Yai remedy using Ames test. In addition, the cytotoxicity of these extracts was determined in brine shrimp lethality testing. These tests are short-term and simple bench top bioassay used to test toxicity of compounds.

MATERIALS AND METHODS

Chemicals

1-aminopyrene (Aldrich, St. Louis, U.S.A.) was used to interact with nitrite in acid solution to produce a standard direct mutagen of the Ames test. L-histidine monohydrochloride monohydrate and D-Biotin were purchased from Sigma-Aldrich, (St. Louis, U.S.A.). D (+)-Glucose monohydrate, Agar-Agar and Dimethylsulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Oxoid nutrient broth No.2 was obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ammonium sulfamate, sodium ammonium hydrogen phosphate tetrahydrate and crystal violet indicator were bought from Fluka AG (Buch, Switzerland) and Sigma-Aldrich (St. Louis, U.S.A.). Ajax Finechem Pty Ltd. supplied sodium nitrite.

Crude extract preparation

Roots of *A. marmelos*, *O. indicum*, *D. longan*, *D. serrulata* and *W. trichostemon* were collected from Chiang Rai, Tak, Surin and Nakhon ratchasima Provinces of Thailand. They were collected during July – December 2009. All set of crude drugs were authenticated by Ruangrunsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The roots were shade-dried and grinded to coarse powders. The powder of each root was continuously macerated with ethanol and water respectively. The ethanol extracts were filtered through Whatman No.4 and evaporated under vacuum, whereas the water extracts were lyophilized to dryness. The remedy extract was prepared by mixing each extract in the quantity equivalent to the formula. The extract yields were weighed, recorded and stored at -20 °C until use to decrease the possibility of degradation of active compounds.

Preparation of the bacterial suspension

Salmonella typhimurium strain 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*) were kindly provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. All tester strains were grown in an Oxoid nutrient broth No.2 and incubated overnight in a shaking water bath at 37 °C. The culture were reisolated by streaking the bacteria on a minimal glucose agar plates enriched with ampicillin, L-histidine HCl and biotin, then incubated at 37 °C for 48 h. After incubation, picked a well isolated colony with a sterile loop, then overnight cultured in an Oxoid nutrient broth No.2 at 37 °C in a shaking water bath. The mutant strains were confirmed for the genotypes of histidine/biotin dependence, *rfa* marker, *uvrB* deletion gene mutations and presence of plasmid *pMK101*.

Mutagenicity assay

The pre-incubation method of Ames test was employed to determine the mutagenic effect of the root extracts and BMV remedy on both TA98 and TA100 *Salmonella typhimurium* strains without enzyme activating system [16]. Briefly, the ethanol extracts

were dissolved in DMSO, the water extracts and BMY were dissolved in water to the concentration of 25, 50, 100 and 200 mg/ml. Added 200 μ l of each solution to the tube containing 550 μ l of 0.2N HCl to acidify the reaction mixture to pH 3-3.5. Adjusted the final volume to 1 ml with 250 μ l of solvent (DMSO or water). For mutagenic assay with nitrite treatment, adjusted the final volume to 1 ml with 250 μ l of 2M sodium nitrite instead. Each reaction tube was shaken at 37 °C for 4 h then placed in an ice bath for 1 min to stop the reaction. Finally, added 250 μ l of solvent or 2M ammonium sulfamate (for nitrite treatment) and allowed the tube to stand in an ice bath for 10 min. Mixed 100 μ l of this extract mixture with 100 μ l of bacterial suspension and 0.5 ml of 152mM phosphate buffer (pH 7.4), pre-incubated at 37 °C for 20 min then 2 ml of molten top agar containing 5mM L-histidine and 5mM D-biotin was added, mixed well and poured onto a minimal glucose agar plate. The final concentration of the root extracts and BMY remedy were 0.4, 0.8, 1.6 and 3.2 mg/plate. The plates were incubated at 37 C for 48 h and the numbers of *his*⁺ revertant colonies on each plate were counted. DMSO or water was used as a negative control to determine the spontaneous reversion activity. 1-Aminopyrene treated with sodium nitrite was used as a positive control. Ten microliters (tested on TA98) or 20 μ l (tested on TA100) of 1-aminopyrene (0.0375 mg/ml) was mixed with 740 μ l or 730 μ l of 0.2N HCl and 250 μ l of 2M sodium nitrite was added to obtain the final volume of 1 ml [17]. The results were reported as mean revertant colonies per plate \pm the S.D. The mutagenic index (MI) was calculated from the number of revertant colonies of the sample treatment divided by the number of spontaneous revertant colonies. Positive mutagenic effect was considered when the number of induced revertant colonies increase in a dose response relationship manner, at least two doses were higher than spontaneous revertants and at least one dose gave rise to twice over the spontaneous revertant (MI > 2) [18].

Antimutagenicity assay

The antimutagenic 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. Forty microliters (tested on TA98) or 80 μ l (tested on TA100) of 0.075 mg/ml 1-aminopyrene was transferred into the sterile test tube. Then, 710 μ l or 670 μ l of 0.2N hydrochloric acid and 250 μ l of 2M sodium nitrite were added to obtain the total volume at 1 ml. The mixtures were shaken at 37 C for 4 h. Later on, the test tubes were placed in an ice bath for 1 min to stop the reaction mixture. Two-hundred fifty microliter of 2 M ammonium sulfamate was added and allowed the test tube to stand in an ice bath for 10 min. Twenty-five microliter of the mixture above (nitrite-treated 1-aminopyrene) was transferred into the sterile test tube containing various concentrations each extract (5, 10, 15 mg/plate). Distilled water or DMSO was adjusted to the final volume of 100 μ l. Then, the mixture was treated as described in the mutagenicity assay. The percent modification was calculated by the following formula:

$$\% \text{ Inhibition} = [(A-B)/(A-C)] \times 100$$

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. The percentage of inhibition is classified as strong

when it is higher than 60%, moderate ranged from 60-41%, weak ranged from 40-21% and negligible effect when it was 20-0% [19].

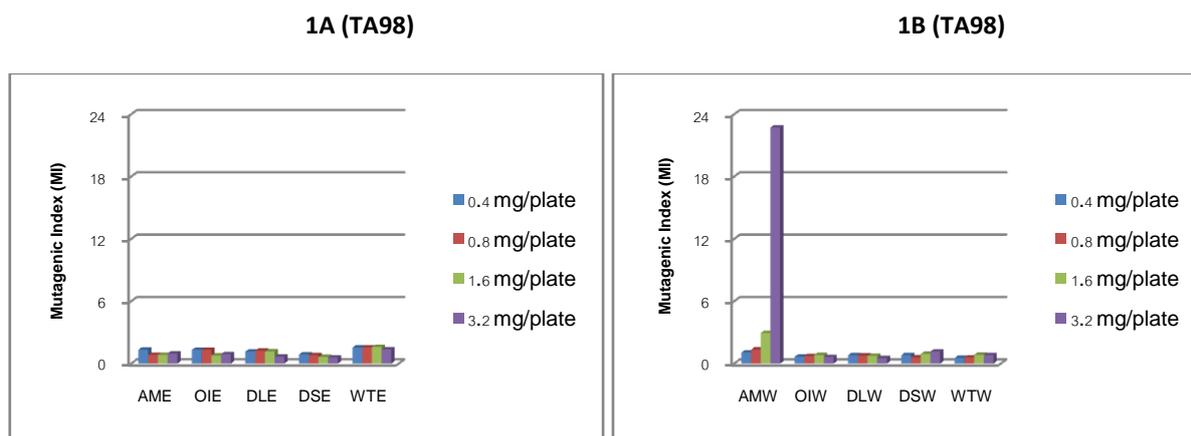
Cytotoxicity in brine shrimp lethality assay

Brine shrimp lethality bioassay was carried out according to the procedure described by Meyer et al., [20]. Brine shrimp eggs were hatched in artificial sea water. After 48 hours of incubation, ten brine shrimps were transferred to each sample vial using a Pasteur pipette and artificial sea water was added to make 5 ml. Filter papers impregnated with extracts at the concentration of 1000, 100 and 10 µg/ml in methanol were air dried before placed in vials containing the brine shrimps. Control was prepared as mentioned above using only methanol instead. Five replicates were prepared for each concentration. The vials were maintained under illumination. Twenty-four hours later, the number of survivors was counted and recorded. As well as the concentration which caused 50% of brine shrimp lethality (LC₅₀ value) was obtained from a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts.

RESULTS

Mutagenicity assay

MI values of BMY remedy and root extracts obtained by the Ames test were shown in **Figure 1-5**. The result demonstrated that only the water extracts from the root of *A. marmelos* exhibited highest direct mutagenicity on both strains. The extract induced 102.33 ± 39.11 (MI=2.95) and 787.67 ± 26.84 (MI=22.72) revertant colonies at 1.6 mg/plate and at 3.2 mg/plate respectively to strain TA98 (**Figure 1B**) and 819.5±6.36 (MI=5.17) revertant colonies at 3.2 mg/plate to strain TA100 (**Figure 2B**).



2A (TA100)

2B (TA100)

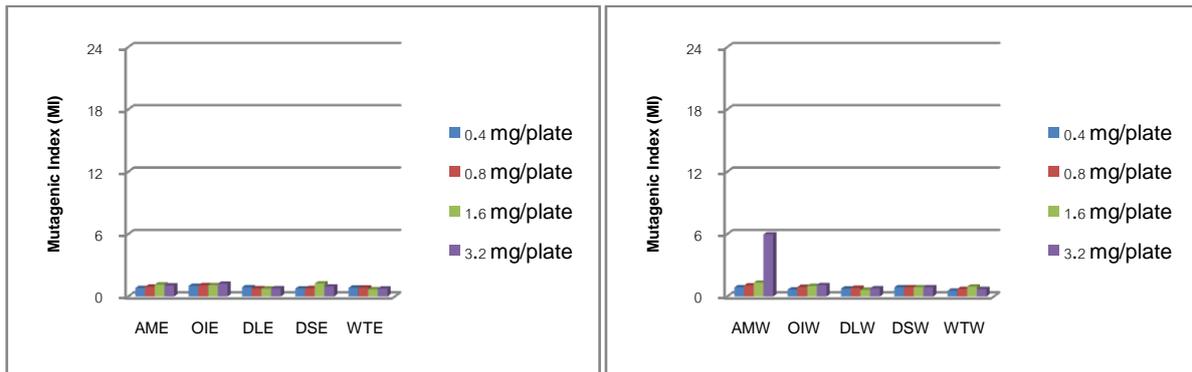
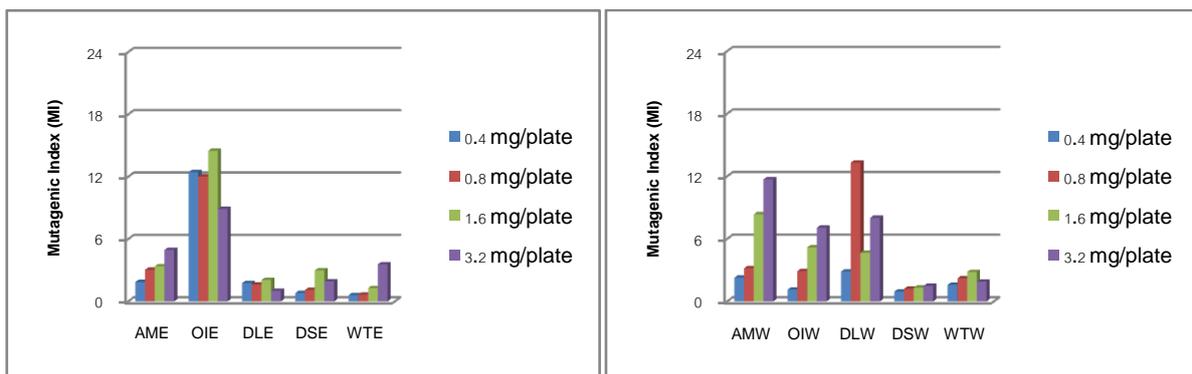


Figure 1-2 The mutagenic index (MI) induced by the ethanol and water extracts from each plant species of Ben-Cha-Moon-Yai remedy without nitrite treated on *Salmonella typhimurium* strains TA98 (1A-1B) and TA100 (2A-2B) using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

It was observed that both ethanol and water extracts of all roots (Figure 3-4) and BMY remedy exhibited their mutagenic effect after they were treated with sodium nitrite (nitrosation) under acidic condition without metabolic activation on both strains. BMY remedy extracts at all tested concentrations exhibited a positive response of mutagenicity after nitrite treated 1-aminopyrene in an acidic condition against *Salmonella typhimurium* TA98 by induced 102 ± 17.09 (MI=5.83), 191 ± 75.43 (MI=10.99), 251.67 ± 63.57 (MI=14.38) and 347 ± 18.36 (MI=19.83) revertant colonies and strain TA100 of 215 ± 35.64 (MI=3.86), 194.67 ± 24.99 (MI=4.4), 276 ± 19.52 (MI=5.56) and 392.33 ± 62.61 (MI=7.9) revertant colonies with dose-response relationship (Figure 5B). However, there were no mutagenic effects exhibited by the ethanolic extract of *D. serrulata* and *D. longan* and the water extracts of *D. serrulata* to strain TA98 and the ethanolic extracts of *D. serrulata* and *W. trichostemon* towards strain TA100.

3A (TA98)

3B (TA98)



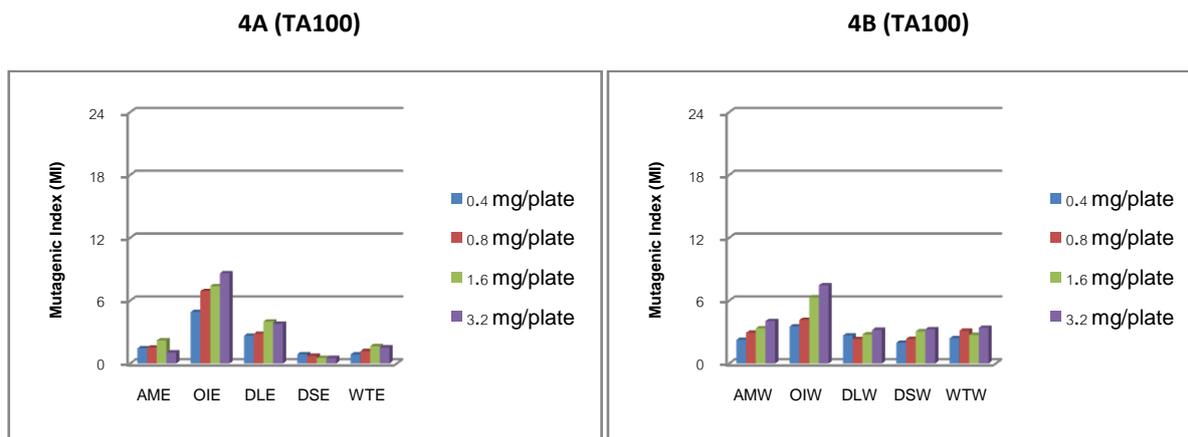


Figure 3-4 The mutagenic index (MI) induced by the ethanol and water extracts from each plant species of Ben-Cha-Moon-Yai remedy with nitrite treated 1-aminopyrene on *Salmonella typhimurium* strain TA98 (3A-3B) and TA100 (4A-4B) using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

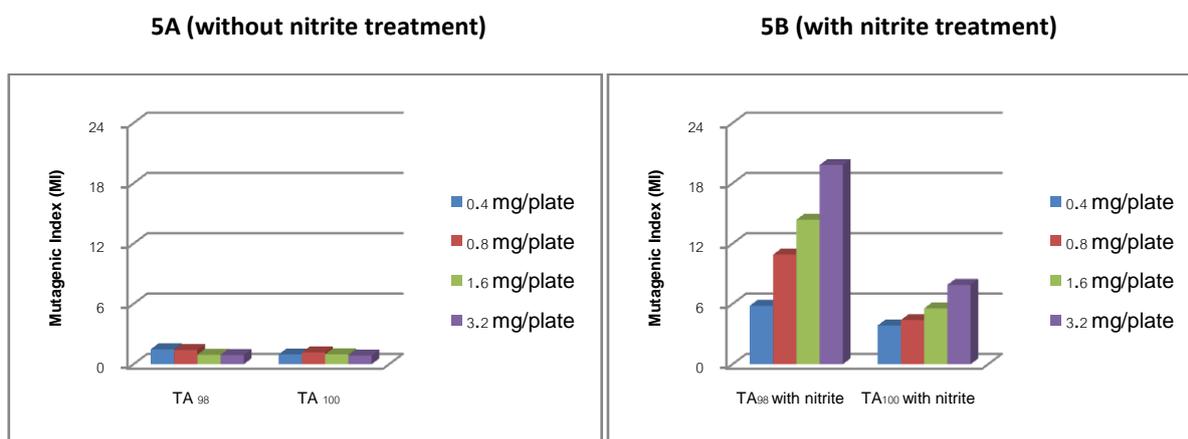


Figure 5 The mutagenic index (MI) induced by the Ben-Cha-Moon-Yai remedy extracts without (5A) and with nitrite (5B) treated 1-aminopyrene on *Salmonella typhimurium* TA98 and TA100 using Ames test. Abbreviations including: Ben-Cha-Moon-Yai remedy: BMY remedy.

Antimutagenicity assay

For antimutagenicity assay, all extracts inhibited mutagenicity effect towards *Salmonella typhimurium* strains TA98 and TA100 (Figure 6A-6B). The effects were ranged from negligible (0–20% inhibition) to strongly active (>60% inhibition). Only the water extracts of *A. marmelos* and *D. serrulata* were not inhibited the mutagenicity on both strains of *Salmonella typhimurium*. However, the BMY remedy exhibited strong antimutagenicity on both strains of *Salmonella typhimurium*. The percentage of inhibition was increased when the doses were increased. Almost all the roots extract and BMY remedy expressed negligible to strong inhibitory effect (> 60%) on both tester strains. On the other hand, the ethanol extracts of *A. marmelos* (5 mg/plate) had negligible effect (19.5%) on *Salmonella typhimurium* TA 100. The moderate antimutagenic activity was observed on 5, 10 and 15 mg/plate of *O. indicum* water extract on *Salmenella typhimurium* TA98 and 5 mg/plate towards TA100. Whereas, the concentration of 5 mg/plate of the water extract

from *D. longan* exhibited the moderate effect on both strains. The moderate (41-60%) to strong antimutagenic (> 60%) effect was observed from the water and ethanol extracts of *O. indicum* toward both strains of *Salmonella typhimurium*, however the water extract at 5 mg/plate exhibited weak inhibitory effect against mutagenicity on strain TA98. All dose of the ethanol extract from *D. serrulata* demonstrated strong antimutagenic effect on both strains, while 5 mg/plate toward strain TA100 exhibited the moderate effect. The strong inhibitory effect was expressed at all concentrations by ethanol extract of *W. trichostemon*, while the weak 20.6% and moderate 51.1% effect were demonstrated when the concentrations of 5 or 10 mg/plate were added to strains TA100, respectively. All of the extracts have dose-related inhibition effect to their mutagenicity of nitrite treated 1-aminopyrene toward *Salmonella typhimurium* strains TA98 and TA100 in the absence of enzyme activating system.

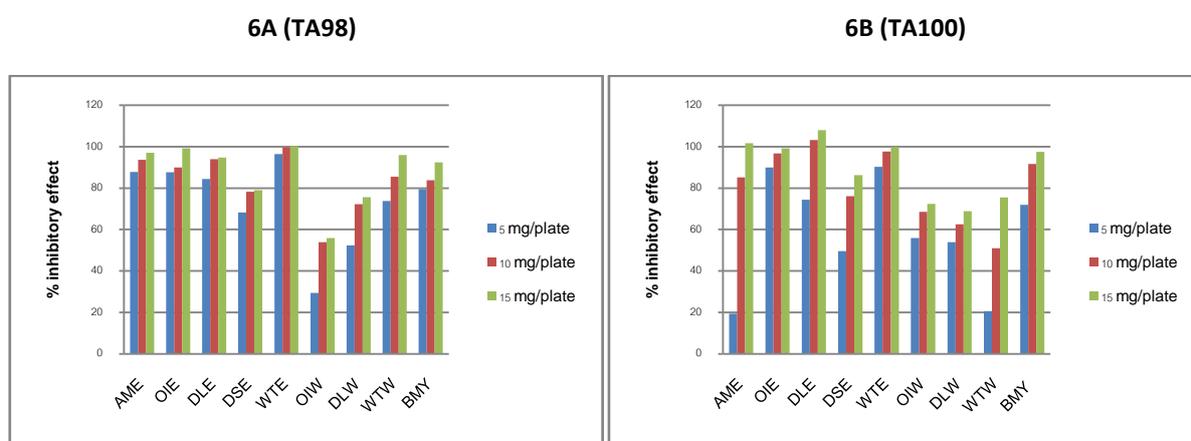


Figure 6 Inhibitory effect of Ben-Cha-Moon-Yai remedy and its components extracts on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *Salmonella typhimurium* strains TA 98 (**6A**) and TA100 (**6B**) using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, Ben-Cha-Moon-Yai remedy: BMY, E: ethanol extract, W: water extract.

Cytotoxicity assay

The results from the brine shrimp lethality testing showed that the ethanol extract of *A. marmelos* exhibited the highest toxicity against brine shrimp nauplii with LC₅₀ of 53.5 µg/ml while BMY remedy showed LC₅₀ of 537.3 µg/ml. The remaining extracts exhibited LC₅₀ of more than 1000 µg/ml.

DISCUSSION

The Ames Salmonella assay is a short-term *in vitro* testing which has gained popularity from the large number of chemical compounds to investigate their genotoxicity and modulation effect on the mutagenic response [21] toward *Salmonella typhimurium* tester strains due to it was a quick and relatively inexpensive assay [22].

The *Salmonella typhimurium* tester strains TA98 and TA100 with histidine-requiring (*his⁻*) auxotrophs were used for detecting and classifying mutagens. Each strain was deficient in excision repair of DNA damage (*uvrB*), ampicillin-resistant R-factors and presence of *pKM101* [22]. They can be reverted back to the wild type by particular

mutagens [16]. In this study, the mutagenic and antimutagenic activity of root extracts and BMY remedy were studied in the absence of enzyme activating system using the pre-incubation method of Maron and Ames [23] to observe the response of the extracts in an acidic condition. Most of the extracts exhibited non-mutagenicity without nitrite treatment in the Ames test toward both strains of *Salmonella typhimurium* under acidic condition without metabolic activation. However, the water extract of *A. marmelos* revealed the mutagenicity on both strains in the present study, whilst the study of Kruawan and Kangsadalampai, [24] demonstrated that the fruit extract from this plant were not mutagenicity toward *Salmonella typhimurium* TA100 in the Ames test.

Most of the extract, except the ethanol extracts of *D. serrulata*, *D. longan* and *W. trichostemon* and the water extracts of *D. serrulata*, were mutagenicity on *Salmonella typhimurium* strains TA98 and TA100 after being treated with sodium nitrite, similar observation was reported by Higashimoto et al. [25]. It was found that three species and Thai medicinal plant extracts were not mutagenic for both strains of *Salmonella typhimurium*, but when the extracts were treated with sodium nitrite, the mutagenicity were observed toward strain TA100. The result was in accordance with the previous study that the nitrosated fraction from the bark of *O. indicum* had been found the mutagenicity on *Salmonella typhimurium* TA98 and TA100 [26]. Besides the roots extract and BMY remedy, many medicinal plants, foods and chemical compounds, show direct-acting mutagenicity after nitrite treatment without metabolic activation. Wakabayashi et al. [27] and Kato et al. [28] also demonstrated that the reaction mixtures showed the mutagenicity to *Salmonella typhimurium* TA98 and TA100 strains after 1-aminopyrene treated with amount of nitrite at pH3 at 37 °C for 4h without metabolic activation.

In the recent years, many research has been employed the *in vitro* assay to determine the genotoxic carcinogen by treatment with the nitrosation reaction mixture or directly with *N*-nitroso compounds; similar to the *in vivo* assays of its biological activity [29]. Previous studied found that people who exposed to the high levels of nitrate have the raise incidences of gastric and liver cancer. Therefore, it has been denoted that the *N*-nitroso compounds is the etiology of human cancer [30].

Nitrite occurs in nitrite-preserved meat of fish, spoiled foods, even nitrate mostly found in foods and vegetables which can be reduced to nitrite by the microbial enzymes. It is the most important precursor to generate the nitrosating agents [31]. It has been denoted that *N*-nitroso compounds are formed by the interaction of nitrogenous compounds with nitrosating agents, the most important of which is acid nitrite [32].

Therefore, when the extracts containing the nitrogen trioxide (N_2O_3) or dihydrogen tetroxide (N_2O_4) with primary, secondary, or tertiary amines, or with secondary amide. [33]³³ reacts with the sodium nitrite (nitrosating agent precursor), it can be generate the carcinogenic *N*-nitroso compounds under the acidic condition with gastric pH [34]. This finding may be common since most natural compounds generally reacted with nitrite and expresses their products that can induce mutations.

In conclusion, this study confirmed that nitrite is a direct-mutagen in certain *Salmonella typhimurium* histidine dependent strains sensitive to frame-shift (TA98) and

base-pair substitutions (TA100) mutations. These results can be suggested that some mutagens and carcinogens may be produced in the human stomach.

The screening for antimutagenicity of plant extract is important in the discovery of new effective anticarcinogenic therapeutic drug [35]. The rationale was due to plant extracts exhibiting antimutagenicity is indication of a possible anticarcinogen [36]. A great number of naturally and synthetic compounds has been known to inhibit the nitrosation reaction. 1-aminopyrene is a derivative of 1-nitropyrene found in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. Previous studied showed that 1-aminopyrene is an important contributor to the direct-acting mutagenicity, as measured by the Ames assay of the diesel particulate extracts [37].

The antimutagenic effect of the roots extract and BMY remedy against the mutagenic reaction product produced from the reaction of 1-aminopyrene treated with nitrite under acidic condition pH 3-3.5 were exhibited in the Ames test. It revealed that most of the extracts exhibited antimutagenic potential ranged from negligible (0-20%) to strong (> 60%) effects toward both strains of *Salmonella typhimurium*. These extracts demonstrated a dose-dependent by inhibitory effect towards *Salmonella typhimurium* TA98 and TA100. This data agree with the previous studied that the methanol extract from the fruit of *O. indicum* also exhibited the strong antimutagenic effect against *Trp-P-1* in an Ames test [38]. The same results were demonstrated in the determination of mutagenic and antimutagenic effects of Ya-rid-si-duang-mahakal which is the one of Thai traditional medicine to treat hemorrhoid and some flower grown in Thailand. It has been denote that there were mutagenic after nitrite treatment and provided the antimutagenic effects against the same condition of this study [39,40]. The antimutagenic potential was also demonstrated in the ethanol extract of *Mucuna collettii* in the Ames test against AF-2 and B(a)P mutagens toward strains TA98 and TA100 of *Salmonella typhimurium* and *rec* assays [41]. Moreover, it has been reported that the fifteen kinds of Thai vegetables exhibited the antimutagenic effects against direct and indirect activating mutagens by using the Ames test with *Salmonella typhimurium* strain TA100 [42].

The present study can be implied that most of the extracts contained certain precursors that could react with nitrite under acidic condition to produce direct mutagenic product causing frame-shift (TA98) and base-pair substitution (TA100) mutation. It provided the evidence to support the safe consumption of Ben-Cha-Moon-Yai remedy and its ingredients at low dose. However, during the use of these remedy or its ingredients, consumer should avoid nitrite containing food items. In addition, the result indicated that the direct acting mutagens formed from interaction between nitrite treated 1-aminopyrene in acid solution could be suppressed by some component, in the extracts.

The preliminary toxicity investigation is brine shrimp lethality testing described by Meyer et al., 1982 and used as a "Benchtop bioassay" for natural medicine discovery [43]. The results indicated the present of potent cytotoxic component of Ben-Cha-Moon-Yai remedy extract and the water extract from *A. marmelos* based on the studied of Meyer et al., 1982 which classified the cytotoxicity of crude extracts into toxic (LC_{50} value < 1000 $\mu\text{g/ml}$) and non-toxic (LC_{50} value > 1000 $\mu\text{g/ml}$). On the contrary, the fruit [44] and leaves

extracts [45] from *A. marmelos* demonstrated non acute-toxicity belong to 6 g/kg in mice and non short-term toxicity for 14 consecutive days in rats, respectively.

However, plant extracts exhibiting a mutagenic or antimutagenic and also cytotoxic effects need to be extensively investigated to determine their possible genotoxicity and cytotoxicity to humans as their safe use in traditional medicine.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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