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Antibacterial activity against four human pathogenic bacteria and anti-inflammatory activity evaluated from five Thai medicinal plants

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Abstract

Microbial infections often produce pain and inflammation. Inflammation is one common and major cause of sufferings now and every time past. Thai medicinal plants which possess biological activities are potential to develop an alternative agent in pharmaceutical products. This study aims to investigate the antibacterial and anti-inflammatory activities of the aqueous, ethanolic and methanolic extracts from five Thai medicinal plants (*Tristaniopsis burmanica, Capparis zeylanica* Linn., *Markhamia stipulata, Caryota maxima* and *Amphineurion marginatum*). Five Thai medicinal plant extracts were subjected to evaluation of their antibacterial activity against four human pathogenic bacteria, including *Vibrio cholerae* (clinical), *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228 and *Escherichia coli* ATCC25922 using the agar well diffusion method. The ethanolic and methanolic extracts of five Thai medicinal plants showed capable of inhibiting the growth of one or more tested bacteria. The analysis of the minimum inhibitory concentrations (MICs) which inhibited the four pathogenic bacteria using the broth microdilution assay showed that MICs of the ethanolic and methanolic extracts were ranging from 0.63 mg mL^{-1} to >10 mg mL^{-1}. Additionally, the anti-inflammatory activity of the extracts was studied using murine macrophage (RAW 264.70) as a model. The results show that the ethanolic and methanolic extracts of *Caryota maxima* and *Amphineurion marginatum* showed anti-inflammatory activity by reduced nitric oxide (NO). Also most of the extracts were not toxic to RAW 264.70 cells. The bioactive compounds analysis of the ethanolic and methanolic extract of *Amphineurion marginatum* was carried out using Gas chromatography-mass spectrometry (GC-MS). The results revealed dominant components were phenol, 3′-Acetyllycopsamine and lupeol. So, the results of this research directly indicate that the *Amphineurion marginatum* extracts provide both antibacterial and anti-inflammatory activities, which could be used as a supplementary agent in pharmaceutical products.

Keywords: Antibacterial activity; Anti-inflammatory; Human pathogenic bacteria; Thai medicinal plant

1. Introduction

The popularity of herbal drugs is increasing all over the world because of lesser side effects as compared to synthetic drugs. Herbal or medicinal plant products in various forms have been available for many hundreds of years for treatment of diseases in both Eastern and Western cultures [1, 2]. Nowadays, the use of phytochemicals for pharmaceutical purpose has gradually increased in many countries. According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants [3]. Plant produces a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens [4].

As tropical regions provide tremendous resource of plants with wide range of biodiversity, various plants have been known to be beneficial; however many species are still not well investigated for their valuable bioactive phytochemicals. The largest class of such beneficial phytochemicals is phenolic compounds which possess physiological or functional properties such as antibacterial and anti-inflammatory [5 – 7]. Among those properties, antibacterial and anti-inflammatory are of our interest.

During inflammatory processes, macrophage plays an important role in response to inflammation via the release of several pro-inflammatory mediators including nitric oxide (NO), prostaglandinE_2 (PGE_2) and pro-inflammatory cytokines (IL-6, TNF-α, IL-1β). These mediators are also produced in many inflamed tissues, enhancing the inflammation-regulatory transcription factors of their mRNAs via the nuclear factor NF-κB pathway [8, 9]. Recent studies have been performed observing antibacterial and anti-inflammatory activities on substances extracted from plants [10].

Nowadays, a rich diversity of plants exists in many regions of Thailand. Some indigenous plants are infrequent found, however their potential bioactivities cannot be neglected [11]. Current social trends in health care show a definite movement towards the use of natural remedies like medicinal plants and away from chemotherapectic regimens. Therefore, five Thai medicinal plants species namely *Tristaniopsis burmanica, Capparis zeylanica* Linn., *Markhamia stipulata, Caryota maxima* and *Amphineurion marginatum* were investigated antibacterial and anti-inflammatory activities. In Thai folklore, these medicinal plants are used for external treatment of skin diseases and wounds healing; however, antibacterial and anti-inflammatory activities have not been reported.

Therefore, the aims of this study were to investigate both antibacterial and anti-inflammatory activities of five Thai medicinal extracts (*Tristaniopsis burmanica, Capparis zeylanica* Linn., *Markhamia stipulata, Caryota maxima* and *Amphineurion marginatum*) which the information obtained, may provide a potential therapeutic approach for inflammation related diseases and application use as ingredients pharmaceutical products. The antibacterial activity of the extracts was teste against four human pathogenic bacteria, including *Vibrio cholerae* (clinical), *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228 and *Escherichia coli* ATCC25922. The anti-inflammatory activity was determined the effect of extracts on NO production using RAW 264.70 murine macrophage cells. Bioactive compounds in crude extracts were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS).

2. Materials and Methods

Bacterial strains

The bacterial strains tested in this study were *V. cholerae* (clinical), *S. aureus* ATCC25923, *S. epidermidis* ATCC12228 and *E. coli* ATCC25922. All bacterial strains were maintained in nutrient agar (NA) slants at 4 °C.

Plant materials

Five plants including *Capparis zeylanica* Linn., *Caryota maxima*, *Markhamia stipulata*, *Amphineurion marginatum* and *Tristaniopsis burmanica* were collected from Dong Sai Thong Learning's Center, Ban Dung, Udon Thani province, Thailand. The plant's species were identified by Applied Taxonomy Research Center, Faculty of Sciences, Khon Kaen University, Khon Kaen, Thailand.

Extraction

Extraction of plant branches was done by using water and organic solvent. The fresh branches of five plants were cut, washed, and air-dried at room temperature for about 10 days. Dried powder weighing about 500 g was then macerated in water or ethanol or methanol for 24 h (Three times). The extracts obtained were then filtered with Whatman filter paper No.1. The aqueous extracts were lyophilized. The ethanolic and methanolic extracts were evaporated at a temperature below 55 °C and concentrated crude of ethanolic and methanolic extract of five Thai medicinal plants was obtained.

Agar well diffusion method

Agar well diffusion method was followed to determine the antibacterial activity. Bacterial cells were cultured at 37 °C with nutrient broth (NB) until their growth stage reached a log-phase (optical density at 600 nm (OD_{600}) ≈ 0.40 – 0.60). Then, Bacteria cell was diluted by NB to final OD_{600} around 0.01 (10^7 CFU mL^{-1}), after that swabbed to 20 mL NA plate. Wells (8 mm) were made in each of these plates using sterile cork borer. One hundred microliter (μL) of extracts (20 μg μL^{-1} in 10% dimethyl sulfoxide (DMSO)) were pipetted into the wells and allowed to diffuse at room temperature for 2 h. The plates were incubated at 37 °C for 18 – 24 h. The diameter of the inhibition zone (mm) was measured. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded. The broad spectrum antibiotic streptomycin disc (10 μg) and 10% DMSO were used as positive and negative control, respectively.

Determination of minimum inhibitory concentration (MIC)

The MIC is defined as the lowest concentration able to inhibit any visible bacterial growth. Briefly, 50 μL of crude extracts (a two-fold serial dilution) were incubated in 96-well plates with 50 μL of a suspension of bacteria at a starting optical density of OD_{600} = 0.001 in NB. Bacterial growth was assayed by measurement of optical density at OD_{600} after 16 – 20 h incubation at 37 °C.

Cell Culture

A murine macrophage cell line (RAW 264.70) was purchased from the American Type Culture Collection (American Type Culture Collection [ATCC], USA). It was seeded in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 μg mL^{-1} of streptomycin, 100 U mL^{-1} of penicillin and 25 μg mL^{-1} amphotericin B, then incubated at 37 °C in a 5% CO_2 humidified atmosphere.

Measurement of Nitric Oxide (NO)

RAW 264.70 cells were incubated with lipopolysaccharide (LPS) (100 ng mL^{-1}), and LPS co-incubated with crude extracts of *M.stipulata, C. maxima* and *A. marginatum* (62.50, 125, 250, 500 and 1000 μg mL^{-1}) at 37 °C for 24 h. Then 100 μL of the culture medium from each sample was lightly mixed with the same volume of Griess reagent and incubated at room temperature for 10 min. Subsequently the absorbance was measured at 540 nm using a microplate reader (BioRad, Model 680, USA). The NO production was calculated as a percentage of control.

Cell Viability

RAW 264.7 cells (1 × 10^5 cells mL^{-1}) were seeded on a 48-well plate overnight. Then, cells were divided into two groups; the first group was incubated with crude extracts of *M.stipulata, C. maxima* and *A. marginatum* at different concentrations (62.50, 125, 250, 500 and 1000 μg mL^{-1}) and another group was co-incubated with 100 ng mL^{-1} of LPS. After incubation for 24 h, RAW 264.7 cell viability was determined by MTT assay [12]. Cell viability was evaluated by comparing their absorbance with those of the control.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS analysis of bioactive compounds of crude extracts was performed on a GC-MS equipment Agilent Technologies 7890A GC system and Agilent Technologies 5975C inert XL EI/CI MSD. Experimental conditions of GC-MS system were as follows: DB 5-MS capillary standard nonpolar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25 µm. Flow rate of mobile phase (carrier gas: He) was set at 1 mL min^{-1}. In the gas chromatography part, temperature program (oven temperature) was 50 °C for 5 min then 10 °C min^{-1} to 280 °C for 12 min and injection volume was 1 µL. A scan interval of 0.5 seconds with scan range of 40 – 600 m z^{-1}. Total GC running time was 40 min and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

3. Results and Discussion

Antibacterial activity of aqueous, ethanolic and methanolic extracts

Amongst the fifteen extracts of five Thai medicinal plant extracts that were investigated for antibacterial activity against four human pathogenic bacteria, including *Vibrio cholerae* (clinical), *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228 and *Escherichia coli* ATCC25922. Ten plant extracts, including the ethanolic and methanolic extracts of *T. burmanica, C. zeylanica* Linn., *M. stipulata, C. maxima* and *A. marginatum* were capable of inhibiting the growth of one or more tested bacteria at 2 mg $well^{-1}$. Whereas all crude extracts of aqueous could not inhibit any tested bacteria. The ethanolic and methanolic extracts from *C. maxima* and *A. marginatum* exhibited inhibitory effect against all tested bacteria. The results indicated that types of plant and extracting solvents significantly impacted on antibacterial activities.

Fig. 1 has showed antibacterial activity of ethanolic extract from five Thai medicinal plants against four human pathogenic bacteria using Agar well diffusion method. The ethanolic extract of *A. marginatum* showed maximum zone of inhibition (25 mm) against *V. cholerae* followed by *S. aureus* (14.67 mm), *S. epidermidis* (14.33 mm) and *E. coli* (13.33 mm). Similarly, the ethanolic extract of *C. maxima* has highest antibacterial activity on *V. cholerae* (22 mm) followed by *S. epidermidis* (11.67 mm), *S. aureus* (11 mm) and *E. coli* (10.33 mm). As well as, the ethanolic extract of *M. stipulata* has showed maximum zone of inhibition (19.33 mm) against *V. cholerae* followed by *S. epidermidis* (15.67 mm) and *S. aureus* (12.83 mm). Whereas the ethanolic extracts of *C. zeylanica* Linn. and *T. burmanica* have showed activity against only *V. cholerae* with inhibition zone 14 mm and 13.67 mm, respectively.

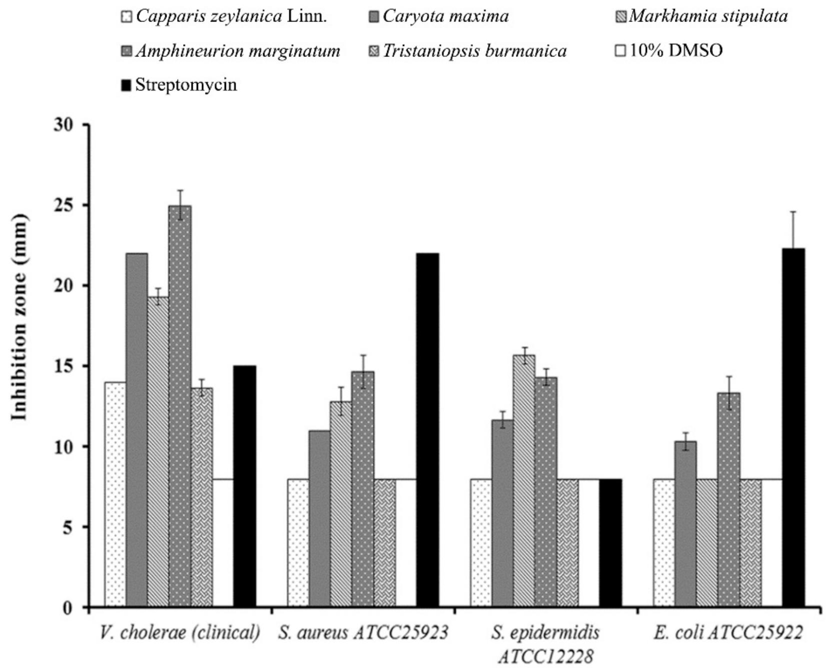

Fig. 1 Antibacterial activity of ethanolic extract of five Thai medicinal plants against human pathogenic bacteria using agar well diffusion method. Diameter of well is 8 mm.

Fig. 2 has showed antibacterial activity of methanolic extract of five Thai medicinal plants against four human pathogenic bacteria using agar well diffusion method. The methanolic extract of *A. marginatum* showed maximum zone of inhibition (20 mm) against *V. cholerae* followed by *S. epidermidis* (12 mm), *E. coli* (12 mm) and *S. aureus* (11.67 mm). Similarly, the methanolic extract of *C. maxima* has highest antibacterial activity on *V. cholerae* (19.67 mm) followed by *E. coli* (17 mm), *S. epidermidis* (14 mm) and *S. aureus* (12 mm). Whereas, the methanolic extract of *M. stipulata* extract has showed maximum zone of inhibition (19 mm) against *S. epidermidis* followed by *S. aureus* (14.83 mm) and *V. cholerae* (14.67 mm). Similarly with ethanolic extract, the methanolic extracts of *C. zeylanica* Linn. And *T. burmanica* have showed activity against only *V. cholerae* with inhibition zone 14.33 mm and 11.33 mm, respectively.

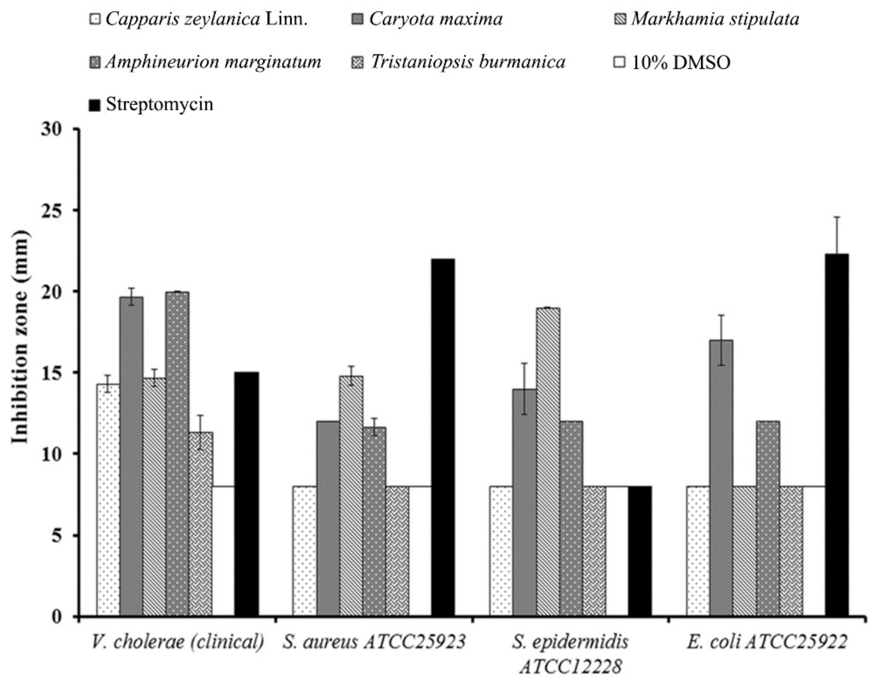

Fig. 2 Antibacterial activity of methanolic extract of five Thai medicinal plants against human pathogenic bacteria using agar well diffusion method. Diameter of well is 8 mm.

Our results similar with previously studied [11, 13, 14] the ethanolic and methanolic extracts of plants had a strong antibacterial activity than other solvents. The mechanisms are yet not clear but thought to be responsible for phenolic toxicity to microorganisms via enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins [15].

When compared antibacterial activity of five Thai medicinal plants extracts against four human pathogenic bacteria with the activity of broad spectrum antibiotic, streptomycin, we found that the ethanolic and methanolic extracts of *A. marginatum* and *C. maxima* exhibited inhibitory effect against all tested bacteria. Whereas streptomycin could inhibit three strains of tested bacteria. The results revealed that these Thai medicinal plants could be used as alternative natural sources for antibacterial substances.

Minimum inhibitory concentration (MIC)

MIC is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The extracts that possessed inhibitory effect were determined for their MIC values by the broth microdilution method. The results showed that MICs of the ethanolic and methanolic extracts were ranging from 0.63 mg mL^{-1} to >10 mg mL^{-1}. The MIC values of the plant extracts are shown in Table 1. The ethanolic and methanolic extracts of *C. zeylanica* Linn., and the methanolic extracts of *C. maxima* exhibited the highest antibacterial activity against *V. cholerae* (clinical) with MICs of 0.63 mg mL^{-1}. The ethanolic extracts of *C. maxima* and *A. marginatum* showed equal antibacterial activity against *S. aureus* ATCC25923 with MICs of 5 mg mL^{-1}. The ethanolic and methanolic extracts of *C. maxima* and *A. marginatum* exhibited the strong antibacterial activity against *S. epidermidis* ATCC12228 and *E. coli ATCC25922* with MICs of 1.25 mg mL^{-1} and >10 mg mL^{-1}, respectively. The methanolic extracts of *M. stipulata* and *A. marginatum* exhibited the highest antibacterial activity against *S. aureus* ATCC25923 with MIC of 5 mg mL^{-1}. The ethanolic and methanolic extracts of *T. burmanica* showed equal antibacterial activity against *V. cholerae* (clinical) with MICs of 2.50 mg mL^{-1}.

Table 1 Minimum inhibitory concentration of the plant extracts.

Medicinal plants	**Minimum inhibitory concentrations (mg mL^{-1})**							
	Ethanolic extracts				**Methanolic extracts**			
	V. cholerae (clinical)	*S. aureus* ATCC25923	*S. epidermidis* ATCC12228	*E. coli ATCC25922*	*V. cholerae* (clinical)	*S. aureus* ATCC25923	*S. epidermidis* ATCC12228	*E. coli ATCC25922*
Capparis zeylanica Linn.	0.63	n.d.	n.d.	n.d.	0.63	n.d.	n.d.	n.d.
Caryota maxima	5	5	1.25	>10	0.63	10	1.25	>10
Markhamia stipulata	5	>10	5	n.d.	>10	5	>10	n.d.
Amphineurion marginatum	1.25	5	1.25	>10	1.25	5	1.25	>10
Tristaniopsis burmanica	2.50	n.d.	n.d.	n.d.	2.50	n.d.	n.d.	n.d.

n.d.; not determined

Effect of Crude extracts on NO Production

NO is synthesized from L-arginine by three nitric oxide synthase (NOS) isomers: endothelial NOS, neuronal NOS, and inducible NOS (iNOS). NO is involved in various physiological functions such as tumor cell killing, host defense, vasodilatation, neurotransmission, and inhibition of platelet aggregation [16]. NO produced by iNOS is involved in immune response; however, when iNOS produces excessive amounts of NO, it can react with superoxide to form peroxynitrite, which results in oxidative damage to cells [17, 18]. NO overproduction in inflammation has become a target for the development of new treatments for inflammatory diseases [19]. The ethanolic and methanolic extracts of *M. stipulata, C. maxima* and *A. marginatum* were selected to determine the anti-inflammatory activity because these extracts had a strong antibacterial activity against tested bacteria. The amount of nitrite, a stable metabolite of NO, was determined using the Griess reaction. The results showed that after inflammation of RAW 264.70 was induced by LPS for 24 h, ethanolic and methanolic extracts of *C. maxima* and *A. marginatum* could reduce the production of NO in a concentration dependent manner, whereas both ethanolic and methanolic extracts of *M. stipulata* did not reduce NO production. The reductions were 7.47 – 25.76% for ethanolic extracts of *C. maxima*, 15.70 – 71.27% for ethanolic extracts of *A. marginatum*, 3.92 – 16.58% for methanolic extracts of *C. maxima* and 16.46 – 54.30% for methanolic extracts of *A. marginatum* compared to that in LPS-stimulated cells. The data showed that the ability to reduce NO production was at highest level in the ethanolic extracts of *A. marginatum* with 71.27% at the highest concentration (1000 µg mL^{-1}), whereas methanolic extracts of *C. maxima* had the lowest ability to reduce NO production. At the highest concentration of methanolic extracts of *C. maxima*, the reduction of NO was 16.58% (Fig. 3). These results similar with An *et al.*, 2016 [20], they found that ethanolic extracts of kiwi berry (*Actinidia arguta*) suppressed the production of NO by LPS-stimulated RAW 264.70 cells in a dose-dependent manner.

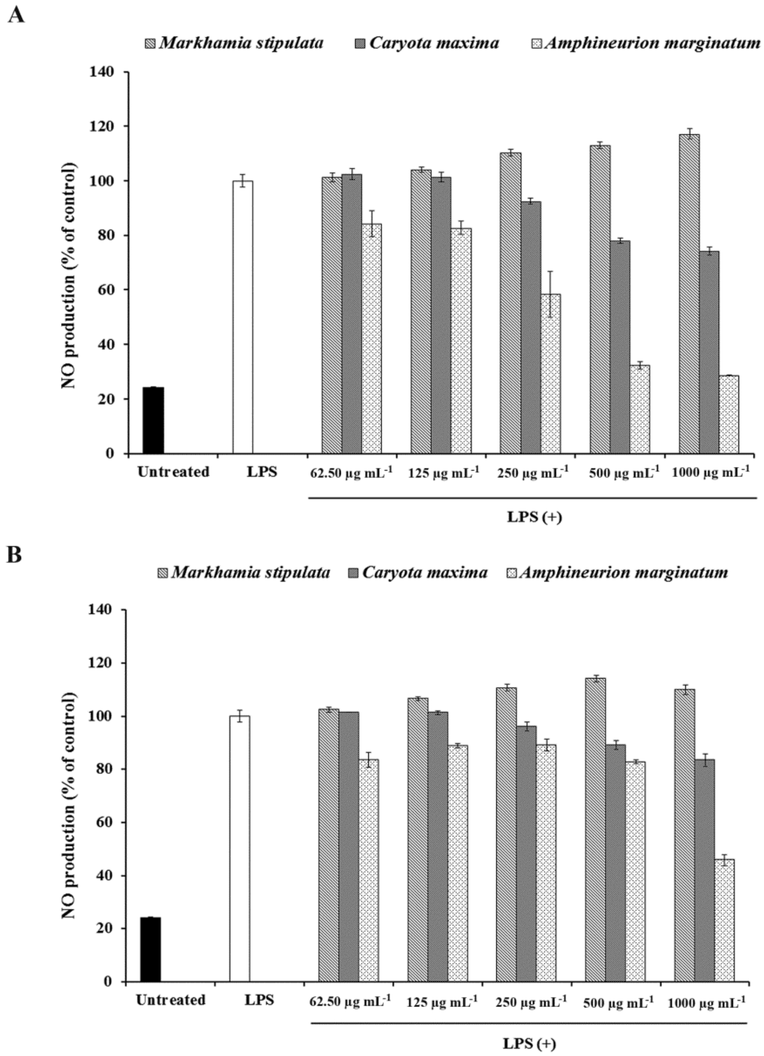

Fig. 3 Effect of three Thai medicinal plants extracts on NO generation in LPS-activated RAW 264.70 Cells were incubated with 100 ng mL^{-1} of LPS and co-incubated with 62.50, 125, 250, 500 and 1000 µg mL^{-1} of Ethanolic extracts (A) and Methanolic extract (B) for 24 h. Each condition media was used to measure nitrite level.

Effect of Crude extracts on Cell Viability

The percentage of cell viability treated with LPS was taken as 100% viability. Investigation of cytotoxic effects of ethanolic and methanolic extracts of *M. stipulata, C. maxima* and *A. marginatum* on the viability of RAW 264.70 cells demonstrated that all extracts showed a proliferative effect on cells and possessed concentration dependence, only the ethanolic eµxtract of *A. marginatum* had cytotoxicity at 1000 µg mL^{-1} (data not shown).

Bioactive compounds

Since the ethanolic and methanolic extract of *A. marginatum* shows significant antibacterial and anti-inflammatory properties. Therefore, crude extracts of *A. marginatum* were selected to determine the bioactive compounds by GC-MS. Based on abundance, the top three major compounds present in both ethanolic and methanolic extracts were phenol, 3′-Acetyllycopsamine and lupeol (data not shown). Phenol or carbolic acid was used as an early antiseptic. 3′-Acetyllycopsamine was used as antibacterial agent. Lupeol exhibited marked anti-inflammatory and anticancer properties [21].

4. Conclusion

This is the first report about antibacterial and anti-inflammatory activities of five Thai medicinal plants extracts including *Tristaniopsis burmanica, Capparis zeylanica* Linn., *Markhamia stipulata, Caryota maxima* and *Amphineurion marginatum*. The results of this research directly indicate that some Thai medicinal plants provide both antibacterial and anti-inflammatory activities, which could be used as a supplementary agent in pharmaceutical products.

5. Acknowledgement

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