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Antibacterial Activity and Stability Evaluation of "Apo-taat" Remedy Extract for Inhibiting Diarrhoea-Causing Bacteria

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ABSTRACT

Apo-taat, a plant-based, Thai traditional medicine that contains *Caesalpinia sappan* and *Phyllanthus emblica*, is used for treating diarrhea. In the present study, the ingredients of the Apo-taat remedy were extracted, analysed and investigated for antibacterial activity and stability testing under forced degradation and accelerated conditions. Apo-taat extract showed good antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serovar Typhi, *Shigella dysenteriae*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Bacillus subtilis* with MIC values of 0.313 to 1.25 mg/mL. The main chemical constituent of Apo-taat extract was brazilin (51.94 mg/g of dried extract) and gallic acid (7.30 mg/g of dried extract). Brazilin and gallic acid were unstable under acid and alkaline conditions but stable under oxidation and thermal degradation and over six months. These results can be concluded that Apo-taat showed good antibacterial activity and stable under oxidation condition, thermal condition and long-term storage at room temperature.

Keywords: Apo-taat remedy, Anti-bacterial activity, *Caesalpinia sappan*, *Phyllanthus emblica*, Stability testing.

Introduction

Infectious diarrhea is caused by pathogen including bacteria, viruses and parasites[1] but the most common cause is bacteria such as *Salmonella*, *Shigella*, *Escherichia coli* and *Staphylococcus aureus*.[2] Symptoms of gastroenteritis are nausea, diarrhea, vomiting, abdominal pain and fever.[3] In 2016, diarrhea was the eighth leading cause of death and there were 1.65 million deaths.[4] Antibiotics including norfloxacin, ciprofloxacin, azithromycin, are the first-line therapies for diarrhea management [5] but overuse or inappropriate use of antibiotics can lead to antibiotic resistant.[6] Thus, many researchers are interested in the development of new antibacterial compounds or natural products to treat bacterial induced diarrhea.[7]

Some plant extracts are used as antidiarrhea agents such as *Acacia nilotica*, *Acanthospermun hispidum*, *Gmelina arborea*, *Parkia biglobosa*, *Vitex doniana*, *Sclerocarya birrea*, *Elephantorrhiza elephantina* and *Schotia brachypetala*.[8,9] In Thailand, herbal medicines are used to treat diarrhea by Thai traditional doctors. Two herbal remedies that are described in national list of essential medicines in Thailand, consist of at least 13 plant ingredients.[10] Therefore, it is difficult to prepare these herbal remedies and replace antibiotics. Apo-taat remedy, a Thai herbal medicine, is used to treat diarrhea but it is not in the list of essential medicines in Thailand. It consists of two species of trees: the bark of *Phyllanthus emblica* and the heart wood of *Caesalpinia sappan* in equal proportions.[11]

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Both components have been shown to possess antibacterial activity. The 50% ethanol extract of the stem of *P. emblica* exhibited antibacterial activity against *S. aureus*, *Staphylococcus epidermidis* and *Salmonella* sp. with minimum inhibitory concentrations (MICs) of 3.2, 1.6 and 3.2 mg/mL, respectively.[12] Eight compounds, including 3-*O*-*α*-Larabinopyranosyloleanolic acid, 3-*O*-*α*-L-arabinopyranosylursolic acid, 16-dehydropregnenolone, periplogenin, betulin, phyllanthol, gallic acid and methyl gallate, have been isolated from the bark of *P. emblica*.[13] While other chemical constituents of *P. emblica* such as gallic acid and methylgallate also showed antibacterial activity against pathogenic bacteria such as *E. coli*, *S. aureus*, *Listeria monocytogenes*.[14, 15]

A crude extract of *C. sappan* displayed good activity against methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus mutans*, *Streptococcus intermedius*.[16,17] Brazilin is the major active constituent of *C. sappan* and has activity against *S. aureus*, *S. epidermidis*, *Bacillus subtilis*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *E. coli*.[18]

However, the development of herbal products needs scientific reports to confirm their efficacy, dosage and stability of product. To date, there are no published data on the antibacterial activity, chemical constituents and stability of Apo-taat. Hence, the present study was conducted to better characterize its constituents, their stability, and antibacterial activity.

Materials and Methods

Plant materials

Bark samples of *P. emblica* was collected in Pathumthani Province, Thailand in December 12, 2019, and those of the heartwood of *C. sappan* were collected at Samutsakorn Province, Thailand in December 20, 2019. All plant materials were identified and authenticated by the herbarium of Southern Centre of Thai Medicinal plants, Faculty of Pharmaceutical Sciences, Prince of Songkla

University, Songkhla Province, Thailand. The specimen reference numbers were SKP049200301 and SKP098031901, respectively.

Preparation of Apo-taat and its plant component extracts

The barks of *P. emblica* and heartwoods of *C. sappan* were cleaned, dried and ground into powder and the aqueous extracts prepared by decoction. For Apo-taat preparation, it was consisted of *P. emblica* (500 g) and *C. sappan* (500 g). Each plant ingredient extract was consisted of 500 g of *P. emblica* or *C. sappan.* The aqueous extracts were obtained by boiling the dried plant powder in water (2 L for Apo-taat extraction and 1 L for plant ingredient extraction) for 30 minutes. The aqueous phase was filtrated through Whatman No.1 filter and dryness using a lyophilizer. The dried extracts were stored at -20°C until used.

Antibacterial Activity

Test organism

A total of seven bacteria reference strains were tested: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *Salmonella enterica* subsp. *enterica* serovar Typhi DMST 22842, *S. dysenteriae* DMST 15111, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* DMST 20651 and *Bacillus subtilis* ATCC 6633. All microorganisms were cultured on nutrient agar (Difco, USA).

Preparation of bacterial inoculum

Bacteria were subcultured on nutrient agar and incubated at 37°C for 18-24 h. Then single colonies were transferred to Muller Hinton broth (Difco, USA) and incubated at 37°C for 2-6 h. The turbidity was adjusted to the 0.5 McFarland standard by a densitometer. The bacteria suspension was diluted with Muller Hinton broth to achieve a final concentration of bacteria suspension of 5×10^5 CFU/mL.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC value of Apo-taat and its plant components extracts was performed using the microtiter plate-based method [19]. The water extracts were dissolved in sterile deionized water and adjusted to 10 mg/mL. All solutions were filtered through a 0.22 µm sterile filter. Stock solutions were diluted with Muller Hinton broth to obtain final concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078, 0.039 mg/mL. All samples were transferred into 96-well plate (50 µL/well) in triplicate to which bacteria suspensions were added (50 µL/well) and incubated at 37°C for 18-20 h. After incubation, 10 µL of resazurin (1 mg/mL; Sigma, Germany) was added to each well and incubated at 37°C for 3 h. The MIC was defined as a minimum concentration of extract with no color change of resazurin. All extracts with no color change of resazurin was transferred onto nutrient agar and incubated at 37°C for 18-24 h. The minimum concentration of extract with no bacterial growth was considered as the MBC. Norfloxacin and vancomycin were used as positive control.

Evaluation of the chemical constituents of the Apo-taat extract

The contents of brazilin and gallic acid were determined by HPLC. The HPLC separation was performed using the C18 reverse phase column (Phenomenax Luna 5 µ C18 (2) 100A analytical column 250 × 4.60mm 5 microns) with a C18 guard column. The mobile phase consisted of 0.1% phosphoric acid in water (A) and acetonitrile (B) with the following gradient: 0-5 min, 100% A, 5-15 min, 85% A, 15-35 min, 75% A, 35-40 min, 0% A, 40-42 min, 0% A, 42-45 min, 100%A, 45-47 min, 100% A at 1 mL/min. Brazilin was monitored at 280 nm while gallic acid was detected at 272 nm. The volume of injection was 10 µL. Brazilin and gallic acid contents were analyzed based on the retention time and spiking with the standard stock solution that was prepared in methanol. The calibration curves of the standard were performed by serial dilutions of methanol to 60-300 µg/mL for brazilin and 10-120 µg/mL for gallic acid.

Stability study of Apo-taat extract under accelerated conditions

The stability study was performed according to the World Health Organization guideline.[20] The water extract of Apo-taat (50 mg) was weighed in each of the 7 vials in triplicate. All vials were placed in the stability chamber at 40°C and 75% relative humidity for 6 months. Sample vials were taken at 0, 30, 60, 90, 120, 150 and 180 d and analyzed for antibacterial activity and chemical constituents in triplicate by MIC and HPLC, respectively.

Forced degradation study of Apo-taat extract

The forced degradation study was separated into four components, including acid hydrolysis, alkaline hydrolysis, thermal degradation and oxidation.[21, 22] For each test, 125 mg of extract was weighed and dissolved in water (1 mL) and then sample solutions were investigated under various conditions include acid hydrolysis, alkaline hydrolysis, oxidative degradation, thermal degradation and analyzed for antibacterial activity, brazilin content and gallic acid content.

Acid hydrolysis study

Stability under acidic conditions was performed by transferring 1 mL of sample solution to a glass tube and then adding 1 mL of 1 N hydrochloric acid and heating the mixture to 80°C for 2 h. After heating, the sample solution was cooled to room temperature and neutralized to pH7 by adding of 1 N sodium hydroxide. The sample solution was adjusted to 12.5 mg/mL in a volumetric flask and filtered through a 0.22 µm syringe filter and analyzed antibacterial activity and chemical content [21, 22].

Alkaline hydrolysis study

The sample solution (1 mL) was transferred into glass tube with screw cap followed by the addition of sodium chloride (1 mL, 1 N) and heated to 80°C for 2 h and left to cool down at room temperature. Neutralization to pH 7 was performed by adding 1 N hydrochloric acid and the volume of sample solution adjusted to 10 mL using a volumetric flask. The solution was filtered and investigated for antibacterial activity and chemical content.[21, 22]

Oxidative degradation study

The stock solution of Apo-taat (1 mL) was added to glass tube to which 1 mL of hydrogen peroxide (30%v/v) was added and heated to 80°C for 2 h and left to cool down at room temperature. The solution was adjusted to 10 mL and filtered through a syringe filter for further study.[21, 22]

Thermal degradation study

The sample solution (1 mL) was transferred into glass tube, heated to 80°C for 2 h and left to cool down at room temperature. Its volume was adjusted to 10 mL and filtered through 0.22 µm syringe filter for further investigation.[21, 22]

Statistical analysis

All analyses were performed in triplicate and expressed as mean ± SEM. Statistical analysis was performed using ANOVA followed by Dunnett's test. Statistical significance was indicated when the p-value was < 0.05.

Results and Discussion

Antibacterial activity of Apo-taat extract and its plant ingredients

The apo-taat, *P. emblica* and *C. sappan* extracts showed broad-spectrum antibacterial activity against all tested bacteria, as shown in Table 1. These results revealed that Apo-taat and *C. sappan* extracts showed highest anti-bacterial activity against MRSA with MIC values of 0.156 and 0.078 mg/mL. Moreover, it had also had the highest inhibition against *S. aureus*, *S.* Typhi, *S. dysenteriae* and *B. subtilis* with MIC values < 1 mg/mL. However, the Apo-taat extract had moderate effect against the Gram-negative bacteria, including *E. coli* and *P. aeruginosa*, with MIC values of 1.25 mg/mL. In addition to plant ingredient extracts, *C. sappan* extract generally showed higher

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antibacterial activity than Apo-taat extract while the antibacterial activity of *P. emblica* extract was lower than the Apo-taat extract.

The aqueous extract of *C. sappan* and *P. emblica* have been reported to inhibit Gram-negative and Gram-positive bacteria such as *P. aeruginosa*, *S.* Typhi, *Enterobacter aerogens*, *E. coli*, MRSA and *S. aureus* as well as *Candida albicans*.[18, 23] The main active constituent of *C. sappan* is brazilin and has potent antibacterial activity by reducing DNA and protein synthesis [18]. The isolated compounds in the bark of *P. emblica* were gallic acid and ellagic acid.[24] Gallic acid showed high potent antibacterial activity against *S. aureus*, *S. epidermidis* and *Klebsiella pneumoniae* (MIC < 10 µg/mL) but ellagic acid showed moderated effect against *S. aureus*, S. epidermidis with MIC values of 1.25 and 5 mg/mL, respectively.[25] The antibacterial activity of the Apo-taat extract is probably best explained by the constituents found in the *C. sappan* and *P. emblica* extracts.

Antibacterial agents or drugs are generally divided into bactericidal or bacteriostatic. A bactericidal drug is defined as one with a MBC/MIC ratio ≤ 4; a ratio > 4 defines a bacteriostatic drug.[26] Our results revealed that all extracts were bactericidal.

Chemical constituent of Apo-taat extract and its plant ingredients

The chemical structures of brazilin and gallic acid are shown in Figure 1 and the HPLC profiles of brazilin, gallic acid, Apo-taat, *P. emblica* and *C. sappan* extracts are shown in Figure 2. The *P. emblica* extract gallic acid whilst the *C. sappan* extract contained brazilin (Figure 2c and 2d). The standard curves for brazilin and gallic acid were plotted (peak area vs. concentration) and showed correlation coefficients of 0.9992 and 0.9994, respectively. The amount of gallic acid in *P. emblica* was 61.08 mg/g of dried extract while the amount of brazilin in *C. sappan* was 148.90 mg/g of dried extract (Table 2) whist the main chemical constituent in Apo-taat extract was brazilin (51.94 mg/g of dried extract) and the gallic acid content was 7.30 mg/g of dried extract (Table 2 and Figure 2b). Apo-taat consisted of *C. sappan* and *P. emblica* in a ratio of 1:1 but, surprisingly, gallic acid in Apo-taat extract decreased more than two-fold when compared to *P. emblica* extract while brazilin decreased about two-fold when compared to *C. sappan*. Apo-taat extract contained *C. sappan* in a ratio of 50% of all plant ingredient, so brazilin content in Apo-taat extract were detected for approximately 50% of the brazilin content in *C. sappan* extract. For gallic acid, it is unstable to the high pH (7-10). The gallic acid in pH 7.0 and 10.0 was degraded with 13.1% and 93.6%, respectively.[27] Apo-taat extract consists of two plant species that may affect to the pH changing of aqueous phase and affect to gallic acid content in the extract.

Antibacterial activity and chemical constituents of Apop-taat extract under forced degradation and accelerated conditions

The Apo-taat extract retained antibacterial activity after forced degradation against all tested bacteria (Table 3). However, Apo-taat had slightly decreased antibacterial activity against *S. dysenteriae* under alkaline hydrolysis, thermal degradation and oxidative degradation (Table 3). Acidic and basic conditions decreased brazilin content in the Apo-taat extract while these conditions significantly increased gallic acid content in Apo-taat extract (Figure 4). However, thermal and oxidative stress had no effect on brazilin and gallic acid contents in Apo-tat extract. On the other hand, Apo-taat extract under forced degradation still showed antibacterial activity against all testing bacteria. *C. sappan* has been reported on forced degradation study. Acidic and alkaline pH reduced antibacterial activity of the aqueous extract of *C. sappan* against *S. aureus*, *S. epidermidis* and *Propionibacterium acne*.[28] Even though, the antibacterial activity of Apo-taat extract was stable under forced degradation, acidic and alkaline condition should be avoided for the product development of Apo-taat extract to prevent the degradation of gallic acid and brazilin.

Brazilin is the main active ingredient of *C. Sappan* and its structure consists of a hydroxyl group that is easily oxidized and converted to brazilein.[29] Under acid and alkaline conditions, the amount of brazilin in Apo-taat extract was reduced (Figures 3 and 4) and is probably due to the oxidation of brazilin to brazilein under acid and alkaline conditions. Brazilein has been reported The Apo-taat extract still showed good antibacterial activity, suggesting that brazilein also has antibacterial properties.[30]

The gallic acid content significantly increased under acid and alkaline conditions (Figures 3 and 4) and may be due to the hydrolysis or oxidation of tannins, including gallotannins, to gallic acid.[31]

In accelerated stability study, the Apo-taat extract had antibacterial activity with similar MIC values against *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, *S.* Typhi, *S. dysenteriae* and *B. subtilis* for the Day 0 to the Day 180 (Table 4), suggesting the Apo-taat extract is stable and can storage. Indeed, HPLC-measured brazilin and gallic acid contents were no different on Day 180 compared to Day 0; the brazilin content in the Apo-taat extract increased by ~20% on Days 60 and 90. Normally, the compounds or extracts that are stable under accelerated stability testing are claimed of at least 12 months shelf life [32]. One may suggest that Apo-taat extract can be preserved for 1 year at room temperature.

Table 1: MIC and MBC values of the aqueous extract of Apo-taat and its plant ingredients

Bacterial strains	Apo-taat extract			*P. emblica* extract			*C. sappan* extract			Norfloxacin		Vancomycin	
	MIC (mg/mL)	MBC (mg/mL)	MBC/ MIC	MIC (mg/mL)	MBC (mg/ mL)	MBC/ MIC	MIC (mg/ mL)	MBC (mg/ mL)	MBC/ MIC	MIC (µg/mL)	MBC (µg /mL)	MIC (µg/mL)	MBC (µg /mL)
S. aureus	0.313	0.313	1.000	0.313	1.250	4.000	0.078	0.313	4.000	0.391	0.781	NT	NT
MRSA	0.156	0.156	1.000	0.313	0.313	1.000	0.078	0.078	1.000	50.000	100.000	0.391	0.391
E. coli	1.250	1.250	1.000	2.500	2.500	1.000	1.250	1.250	1.000	0.023	0.023	NT	NT
P. aeruginosa	1.250	1.250	1.000	1.250	2.500	2.000	1.250	1.250	1.000	0.391	1.563	NT	NT
S. Typhi	0.625	0.625	1.000	1.250	1.250	1.000	0.625	0.625	1.000	0.045	0.781	NT	NT
S. dysenteriae	0.3125	0.625	2.000	0.625	0.625	1.000	0.156	0.313	2.000	0.023	0.023	NT	NT
B. subtilis	0.625	1.250	2.000	1.250	2.500	2.000	0.313	0.313	1.000	0.098	0.098	NT	NT

NT = Not tested

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Table 2: Brazilin and gallic acid content of Apo-taat extract and its plant ingredients

Sample	Brazilin content (mg/g)	Gallic acid content (mg/g)
P. emblica extract	Not detectable	61.080 ± 1.920
C. sappan extract	148.900 ± 10.310	Not detectable
Apo-taat extract	51.940 ± 2.300	7.300 ± 0.300

Data were mean ± SEM (n = 3).

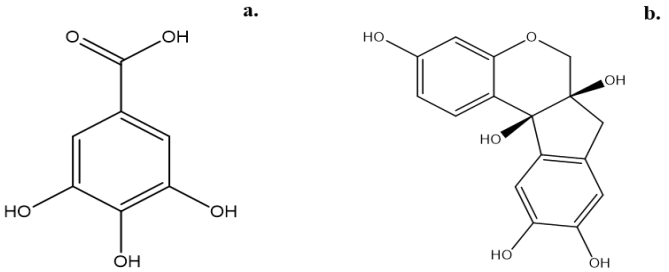

Figure 1: Chemical structures of (a) gallic acid and (b) brazilin

Table 3 Antibacterial activity of Apo-taat extract under various stress conditions

Bacterial strains	Control (Apo-taat extract)		Stress condition							
			Acid hydrolysis		Alkaline hydrolysis		Oxidative degradation		Thermal degradation	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. aureus	0.313	0.313	0.625	1.250	0.313	0.625	0.313	0.625	0.313	0.625
MRSA	0.156	0.156	0.313	0.313	0.156	0.156	0.156	0.156	0.156	0.313
E. coli	1.250	1.250	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500
P. aeruginosa	1.25	1.25	1.25	1.250	1.250	1.250	1.250	1.250	1.250	1.250
S. Typhi	0.625	0.625	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250
S. dysenteriae	0.313	0.625	0.625	1.250	1.250	1.250	1.250	1.250	1.250	1.250
B. subtilis	0.625	1.250	1.250	1.250	0.625	1.250	0.625	1.250	0.625	1.250

Table 4: MIC and MBC values of Apo-taat extract under accelerated condition for 180 days (mg/mL)

Bacterial strains	Day 0		Day 30		Day 60		Day 90		Day 120		Day 150		Day 180	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	0.313	0.313	0.313	0.313	0.313	0.625	0.313	0.625	0.313	0.625	0.313	0.625	0.156	0.625
MRSA	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.313	0.156	0.156	0.156	0.156	0.156	0.156
E. coli	1.250	1.250	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500	2.500
P. aeruginosa	1.250	1.250	1.250	2.500	2.500	2.500	2.500	2.500	1.250	2.500	1.250	2.500	1.250	>5.000
S. Typhi	0.625	0.625	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250	1.250
S. dysenteriae	0.313	0.625	0.3125	0.625	0.313	0.625	0.313	0.625	0.313	0.625	0.313	0.625	0.313	0.625
B. subtilis	0.625	1.250	0.625	1.250	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625

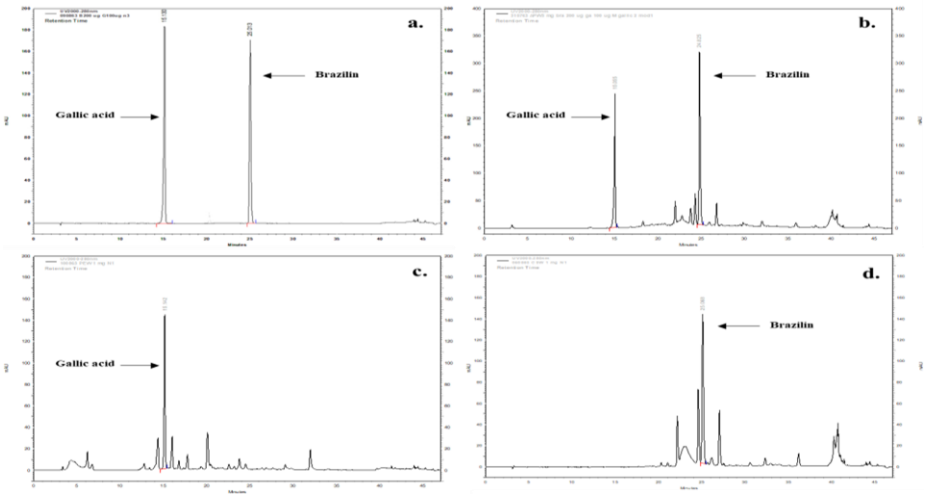

Figure 2: HPLC chromatogram of (a.) gallic acid (100 µg/mL), brazilin (200 µg/mL), (b.) Apo-taat extract (3 mg/mL) , (c.) *P. emblica* extract (1 mg/mL) and (d.) *C. sappan* extract (1mg/mL)

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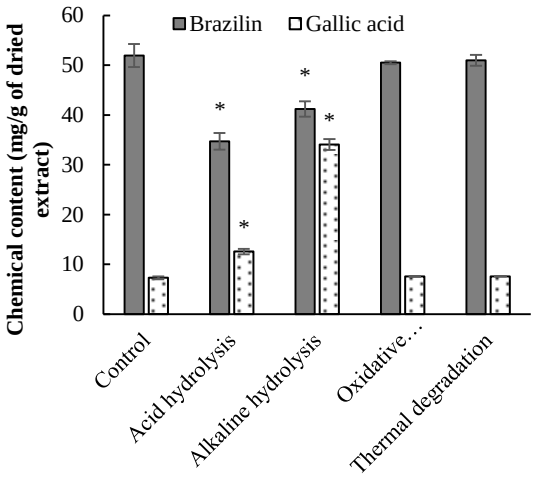

Figure 3: Brazilin and gallic acid content of Apo-taat extract under stress conditions.
Data were mean ± SEM (n=3). *p-value < 0.05 when compared to control

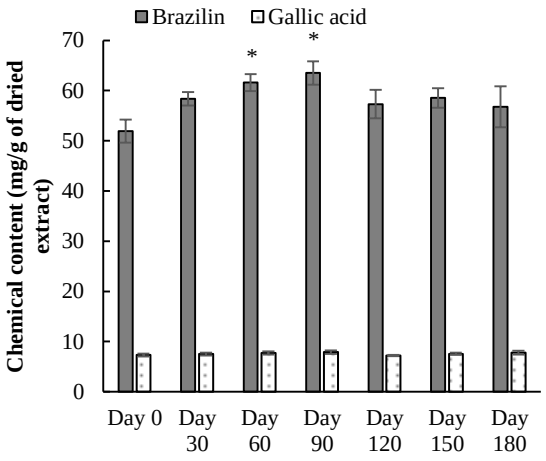

Figure 5: Brazilin and gallic acid content of Apo-taat extracts under accelerated condition for 180 days.
Data were mean ± SEM (n = 3). *p-value < 0.05 when compared to day 0

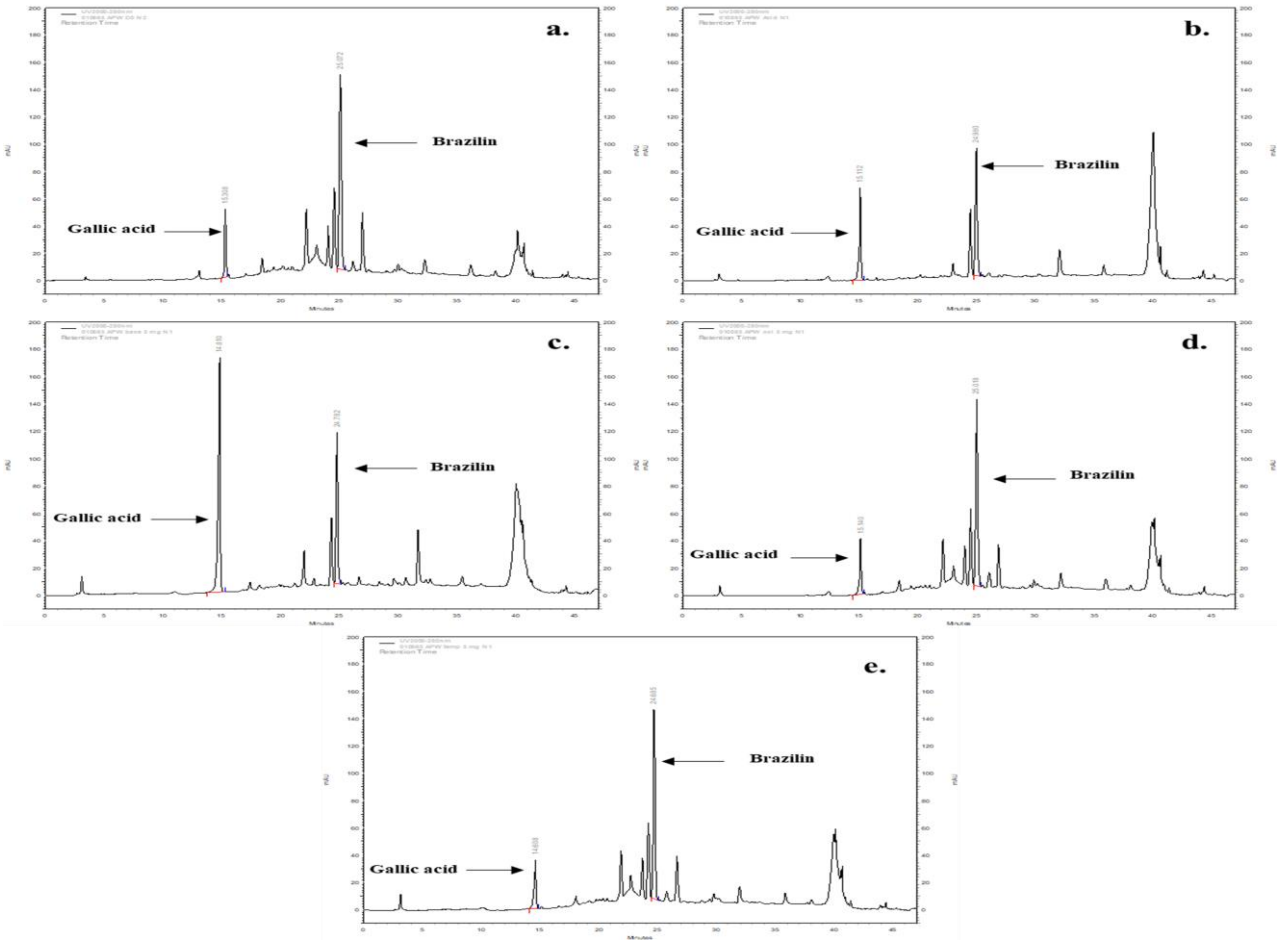

Figure 4: HPLC chromatogram of (a.) Apo-taat extract, (b.) Apo-taat extract under stress conditions including acid hydrolysis, (c.) alkaline hydrolysis, (d.) oxidative degradation and (e.) thermal degradation at concentration of 3 mg/mL

Conclusion

Apop-taat extract contains two active compounds, brazilin as the main active component, and gallic acid; it showed bactericidal activity against both Gram-positive and Gram-negative bacteria. It showed stable chemical content and antibacterial activity under oxidative and thermal degradation. Although it still retained antibacterial activity under acidic and alkaline condition, it showed unstable brazilin and gallic acid content. Thus, the development of product containing Apo-taat extract should be carefully considered to avoid acidic and alkaline pH. Furthermore, there was no change in chemical constituent of the extract on storage under accelerated conditions for 6 months, so the extract can be stored at room temperature for 1 year. Brazilin and gallic acid are stable and could be used to assess the quality control of products containing Apo-taat extract as antibacterial agents. Preclinical studies are needed to see if Apo-taat can be developed as an antibacterial agent. Moreover, the long-term stability testing under other conditions such as 0 and 25°C and toxicity of the extract should be performed in the future.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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