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In vitro Antimicrobial and NO Inhibitory Activities of A Thai Herbal Recipe Against *Cutibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract

Introduction: *Acanthus ebracteatus* (AE), *Curcuma longa* (CL) (rhizome), (leaf and stem), and *Quercus infectoria* (QI) (gall) (ACQ03 recipe) have long been used to treat skin and inflammatory conditions. In addition, it has been used in traditional Thai medicine hospitals to treat acne. This study is aimed to evaluate the antibacterial and anti-inflammatory effects of ACQ03 recipe. Materials and methods: ACQ03 recipe was evaluated antibacterial using broth microdilution, a time-kill assay and nitric oxide inhibitory effects and cytotoxicity activities. The ingredients that make up the ethanol extract in the ACQ03 formulation were identified by LC-QTOF-MS method. Results and discussion: Antibacterial activity of ACQ03 against *C. acnes* DMST14916 had good results, with MIC/MBC values of 64/128 (µg/mL); for *C. acnes* NPRC021, with MIC/MBC values of 128/256 (µg/mL); against 20 strains of *S. aureus*, MICs ranging from 62.5 to > 1,000 and MBC values > 1,000 (µg/mL); of *S. epidermidis* had MIC values ranging from 125 to > 1,000 and MBC values ranging from 500 to > 1,000 µg/mL. NO release in LPS-treated RAW264.7 cells was strongly suppressed by the ACQ03 recipe ethanol extract at concentrations ranging from 1.95 to 16.25 µg/mL, with inhibition values ranging from 43.54 to 93.14 % without cytotoxicity to RAW264.7 cells or L929 cells. Conclusion: ACQ03 recipe could reduce antibacterial and anti-inflammatory effect. It might be suggested that this ACQ03 recipe could be useful for the treatment of acne.

Keywords: Antimicrobial, NO inhibitory, *Cutibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, Traditional Thai medicine

Introduction

An excess of *Cutibacterium acnes* (*C. acnes*), *Staphylococcus aureus* (*S. aureus*), and *Staphylococcus epidermidis (S. epidermidis*) is linked to acne vulgaris, an inflammatory dermatosis [1]. Acne can be treated in a variety of ways, but none of them can fully heal patients' conditions, and many of them have serious side effects [2]. For instance, the overuse of topical and systemic antibiotics has led to the development of resistant strains of *C. acnes* and other skin microbes, making the phenomenon of antibiotic resistance in the context of acne a serious problem [3]. Therefore, patients with acne vulgaris will likely benefit from a medication that may control the inflammatory response and inhibit the growth of *C. acnes*, *S. aureus*, and *S. epidermidis*. Medicinal plants have been used to preserve human health since prehistoric times. Herbs have been used for a variety of reasons, such as medicine, food, drinks, and fragrance. Herbal medicine had a significant impact on early interest in functional foods and nutraceuticals.

Inflammation, resulting in the release of critical inflammatory mediators such as TNF-α, IL-6, and nitric oxide (NO), and prostaglandin E2 (PGE2). Regulating macrophage activation is essential for the management of many inflammatory disorders. Although corticosteroids, immunosuppressants, biologics, and NSAIDs are conventional therapies, extended NSAID usage may lead to considerable adverse consequences, including gastrointestinal, renal, and cardiovascular issues. Due to these constraints, there is a growing interest in investigating medicinal plants that are abundant in anti-inflammatory activity [4].

ACQ03 recipe formed by *Acanthus ebracteatus* (leaf and stem), *Curcuma longa* (rhizome), and *Quercus infectoria* (gall) exhibited remarkable antimicrobial and anti-inflammatory effects corroborating its use in acne vulgaris treatment.

Acanthus ebracteatus Vahl. is a member of the Acanthaceae family and is referred to as Nguag-plaa-moa in Thai. Similar to traditional medicine, the herb Acanthus ebracteatus has been used in Thailand as a wound-healing and anti-inflammatory [5]. The whole leaf is boiled in water to cure skin conditions like rashes. You can apply fresh plant material as a compress to boil or take it orally as a depurative. Menstrual problems are treated by taking fruit orally [6].

Curcuma longa is known in Thai as Kamin-Chun of the Zingiberaceae family [7]. *C. longa* L. (turmeric) is a well-liked herbal remedy in Thailand. is used as a spice and a coloring agent in foods and cosmetics. The volatile oil of the rhizome is used medicinally as a carminative and for its antifungal qualities, whereas yellow curcuminoids are utilized for their ability to reduce inflammation and heal wounds [5]. Most of the yellow bioactive components of *C. longa* rhizomes are curcumin and 2 closely related demethoxy compounds, demethoxycurcumin and bisdemethoxycurcumin. Turmeric has been used to treat respiratory and gastrointestinal issues [8] and has been shown to have anti-inflammatory, hepatoprotective, antitumor, anticancer, and antiviral qualities [9]. Curcuminoids have antioxidant activity and the ability to scavenge free radicals [10,11]. Curcumin's physiological activity and other therapeutic qualities have been the subject of recent publications [12,13].

Quercus infectoria Olivier is known in Thai Ben Ka Nee. In Thai medicine, galls have been used to treat diarrhea, heal wounds and treat inflammatory diseases [5]. Gall is used as a local anesthetic, astringent, antidiabetic, antipyretic, anttremor, and a preventative measure against Parkinson's disease. The tannins found in galls include gallic acid, syringic acid, and ellagic acid. Powdered galls can be used as an ointment to cure hemorrhoids brought on by inflammation of the skin [14].

Dr. Sompond Chanvanichkul and Dr. Teerawat Sudkhaw, traditional doctors at the Traditional Thai Hospital in Prince of Songkla University, Hat Yai, Songkhla Province, used a dried powder of the ACQ03 recipe composed of *Curcuma longa*, *Acanthus ebracteatus*, and *Quercus infectoria* for the treatment of acne [15].

Since an EtOH extract of the mixture of *Curcuma longa, Acanthus ebracteatus* and *Quercus infectoria* showed good inhibitory activity against *Cutibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis* without previous reports of this recipe, this study aimed to determine the antimicrobial and NO inhibitory activities of this recipe to obtain scientific

support for its traditional use. This study aimed to determine the efficacy of an herbal acne gel for the treatment of facial acne vulgaris and determine the antimicrobial and NO inhibitory effects of this recipe to obtain scientific support for its traditional use.

Materials and methods

Reagents

Tryptic soy agar, tryptic soy broth, Mueller-Hinton broth, and Muller-Hinton agar were produced by Difco in France. The ascorbic acid, catechin, gallic acid, and dimethyl sulfoxide (dimethyl sulfoxide) were purchased from the French company Sigma-Aldrich. The supplier of propylene glycol was Vidhyasom Co., Ltd., in Bangkok, Thailand. Tween 80 was employed by Bangkok, Thailand-based P.C. Drug Center Co., Ltd. French Sigma was the origin of erythromycin.

Plant materials

Acanthus ebracteatus (leaf and stem) (**Figure 1**), *Curcuma longa* (rhizome) (**Figure 2**), and *Quercus infectoria* (gall) (**Figure 3**) were collected in 2016 from the Traditional Thai Hospital in Prince of Songkla University. A voucher specimen *Curcuma longa* AH0321121501, *Acanthus ebracteatus*, AH0103050202, *Quercus infectoria* AH1721091403. They are deposited at Applied Thai Traditional Medicine, School of Medicine, Walailak University, Nakhon Sri Thammarat Province, Thailand.

Figure 1 *Curcuma longa*.

Figure 2 *Acanthus ebracteatus*.

Figure 3 *Quercus infectoria*.

Preparation of the crude extracts and isolation

A dried powder of the ACQ03 recipe composed of *A. ebracteatus* (leaves and stem), *C. longa* (rhizome), and *Q. infectoria* (gall) (1.0 kg), 15.365 % yield, was extracted 3 times at room temperature with 9 L of EtOH. Under reduced pressure, the solvent was extracted to produce an EtOH extract (153.65 g) and was kept at 4 °C before the bioassay tests.

Bacterial strains and culture conditions

S. aureus and *S. epidermidis* from acne lesions (n = 20 and n = 16, respectively), *S. aureus* ATCC23235, *S. epidermidis* ATCC 12228, *Cutibacterium acnes* DMST14916 and *Cutibacterium acnes* NPRC021 were kindly provided by the Excellent Research Laboratory on Natural Products, Research Center of Excellence, and Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand. *S. aureus* and *S. epidermidis* were grown on tryptic soy agar (TSA) and incubated at 37 °C for 24 h, after which 3 - 5 colonies were collected, and cultured colonies of *C. acnes* were raised in Mueller-Hinton broth (MHB), which was anaerobically incubated at 37 °C for 3 - 5 h, and brain heart infusion (BHI) broth, which was supplemented with 0.5 % yeast extract and 1 % glucose.

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The Clinical and Laboratory Standards Institute Guidelines [16,17] modified broth microdilution method was used to calculate the test extract's minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). In 96-well microtiter plates, the plant extract (20 µL) was combined with the bacterial suspension (180 µL) in broth medium (supplemented with BHI broth for *C. acne* and MHB for *S. aureus* and *S. epidermidis*). For *S. aureus* and *S. epidermidis*, the plates were incubated at 37 °C for 18 h; for *C. acne*, the plates were incubated at 37 °C under anaerobic conditions for 72 h. The lowest concentration that completely suppressed observable growth was noted as the MIC. An aliquot (10 µL) of the broth without any growth was pipetted onto MHA plates for *S. aureus* and *S. epidermidis* and onto supplemented BHI agar plates for *C. acne* to determine the MBC. The plates were then incubated for each bacterium under the aforementioned.

The MBC was defined as the lowest concentration of the extract that completely prevented bacterial growth. A reference antibiotic, erythromycin (Sigma, France), was used, and 1 % DMSO was used as a negative control. In 3 separate trials, each test was performed 3 times.

Time-kill assay

A time-kill experiment was used to examine the bactericidal activity of the ethanal extract from the ACQ03 recipe [18]. From the 20 isolates, time-kill curves for 2 *S. aureus* isolates were created. TTM007 and TTM017 were the randomly chosen *S. aureus* isolates in this study. Two isolates from 16 total isolates of *S. epidermidis*, TTM010 and TTM015, were used in this study. From the culture on MHB agar, suspensions of *S. aureus* and *S. epidermidis* at the stationary phase of growth were made in 0.85 % NSS. At the MIC, 2 MIC, 4 MIC, and 8 MIC, the bacterial suspensions were

added to MHB broth containing the extract, and the mixture was then incubated at 37 °C. The total number of bacteria in the sample was 5×10^5 CFU mL^{-1}. The samples were collected throughout a 24 h period at 2 h intervals, and the bacteria that survived were grown on MHA. A negative control was generated via the use of DMSO (1 %). There were 2 separate repeats of the test.

Anti-inflammatory activity

Cell viability assay

Cell viability was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 96-well plates and incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere. Following incubation, the media were removed, and the cells were treated with either control media or media supplemented with varying concentrations of ACQ03, *Quercus infectoria* Olivier, *Acanthus ebracteatus* Vahl, or *Curcuma longa* extract (1.95 - 400 µg/mL). After 24 h, MTT solution (10 mg/mL in phosphate-buffered saline) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. The medium was subsequently aspirated, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm via a microplate reader (Metertech, Taipei, Taiwan) [19]. Nontoxic concentrations, defined as those eliciting no significant reduction in cell viability compared with the control group (viability > 80 %), were selected for further nitric oxide production studies.

NO inhibitory assay

RAW 264.7 cells were seeded into 96-well plates at 1×10^5 cells/mL and cultured for a complete day. The media of the experimental group was consequently replaced with phenol red-free DMEM supplemented with 1 µg/mL lipopolysaccharide (LPS), and the incubation period was further extended by 1 day. Nitric oxide (NO) production was evaluated by measuring the nitrites present in the culture supernatant via the Griess reagent test. The liquid phase was mixed with 0.1 % naphthylethylenediamine-HCl and 5 % phosphoric acid with Griess reagent (1 % sulfanilamide). The mixture was incubated for 15 min. Absorbance was read at 540 nm [19] with a microplate reader (Metertech, Taipei, Taiwan). The data are presented as the means ± S.E.M. The % inhibition was calculated as follows:

$$\% \text{ Inhibition} = [(\text{OD control} - \text{OD sample})/\text{OD control}]\times100$$

Cell culture of L929 fibroblasts

The Japanese Collection of Research Resources Cell Bank (JCRB) provided the mouse L929 fibroblasts used in the culture. The cells were maintained at 37 °C in a 5 % CO_2 atmosphere and supplemented with 10 % fetal bovine serum (FBS; Gibco, USA), 1,000 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, USA). The cells were subcultured in trypsin-EDTA solution and were ready for experimentation when they reached approximately 80 % confluence.

***In vitro* cytotoxicity**

Biocompatibility testing complied with ISO 10993-5 [20]. L929 fibroblasts were cultured at 10,000 cells per well, allowing them to develop for a period of 24 h. The cells were subsequently exposed to various concentrations of the test formulation 10, 100, 1,000 and 10,000 mg/mL which were subsequently diluted in the culture medium and left to develop for 24 h before the quantitative MTT assay was conducted. Similar to Chusri and Voravuthikunchai [18] the procedure was conducted according to the protocols described by Sangkaew *et al.* [21]. L929 cells at a density of 10,000 cells per well were cultured in 96-well plates. The next step involved exposure to the ACQ03 formulation at doses ranging from 0 to 10,000 mg/mL for 24 h. The subsequent step was the quantitative MTT assay. To assess cell viability, MTT solutions at a concentration of 0.5 mg/mL were added to the cells briefly and then incubated at 37 °C for 1 h. The formazan crystals were not dissolved. Afterward, the cells were dissolved in 100 µL of DMSO solution, and the absorbance was recorded on a microplate spectrophotometer at 550 nm. The negative control cells were subjected to a 1 % concentration of Triton X-100, whereas the positive control cells were cultivated in growth medium, resulting in 100 % viability. Cell viability was determined via the following mathematical formula:

$$\text{Cell viability \%} = (\text{OD of treatment})/(\text{OD of control})\times100$$

Analysis of chemical compounds by LC-QTOF-MS

The chemical composition of the ethanol extract from the ACQ03 recipe was isolated and clarified via liquid chromatography (LC) and an electrospray ionization quadrupole time of flight mass spectrometer (LC-QTOF-MS). LC separation was performed via an Agilent 1290 Infinity Series HPLC System (Agilent Technologies, USA) and a Zorbax Eclipse Plus C-18 column (5 m, 2.1 and 150 mm; Agilent Technologies, USA). A linear gradient of 90 - 35 % A from 0 - 30 min, a linear gradient of 35 - 10 % A from 30 - 40 min, and a linear gradient of 10 - 90 % A from 40 - 50 min of separation were used. The mobile phase was composed of both 2.0 % v/v acetic acid in water (A) and acetonitrile (B). The sample injection volume was 2 µL, and the solvent flow rate was 0.5 mL/min. The data were collected via a MassHunter WorkStation Software Qualitative Analysis Navigator V8 mass spectrometer.

Results and discussion

Antimicrobial activity results

ACQ03 displayed strong antibacterial activity against strains of *C. acnes*, with MIC/MBC data of 64/128 µg/mL against *C. acnes* DMST14916 and 128/256 µg/mL against *C. acnes* NPRC021. The MIC values are crucial as *C. acnes* is the main pathogen involved in acne pathogenesis, and offers strong inhibitory potential. The ACQ03 recipe exhibited MIC values of 62.5 to > 1,000 µg/mL against 20 strains of *S. aureus*. The MIC_{50} and MIC_{90} values were found to be 500 and 1,000 µg/mL, respectively, MBC_{50} and MBC_{90} values > 1,000 µg/mL (**Table 1**). The reference strain, *S. aureus* ATCC23235, exhibited MIC and MBC values of 125 and 250 µg/mL, respectively, which were lower than the values for most 20 strains of *S. aureus*. While, the ACQ03 recipe demonstrated MIC values of 125 to > 1,000 µg/mL against 16 strains of *S. epidermidis* tested, with MIC_{50} and MIC_{90} values of 125 µg/ml and 500 µg/mL, respectively. The MBC_{50} and MBC_{90} values > 1,000 µg/mL (**Table 2**). Chemograms of the reference *S. epidermidis* ATCC 12228 strain with the MIC and MBC values of 125 and 500 µg/mL, respectively, were found corresponding with those of 16 strains of *S. epidermidis*.

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antibiotic ACQ03 extract of ethanol against 20 strains of *S. aureus*.

***S. aureus* (n = 20)**						
MIC (µg/mL)	**Number of organisms**	**Total number of organisms**	**Cumulative percent (%)**	**MIC_{50} (µg/mL)**	**MIC_{90} (µg/mL)**	**MBC_{50} and MBC_{90} (µg/mL)**
62.5	2	2	10			
125.0	5	7	35			
250.0	2	9	45	500	1,000	> 1,000
500.0	4	13	65			
1,000	5	18	90			
> 1,000	2	20	100			

All strains tested with clindamycin (MIC = 0.488 µg/mL) and *S. aureus* ATCC 23235 presented MIC and MBC values of 125 and 250 µg/mL, respectively.

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antibiotic ACQ03 extracted with ethanol against 16 strains of *S. epidermidis*.

***S. epidermidis* (n = 16)**						
MIC (µg/mL)	**Number of organism**	**Total number of organisms**	**Cumulative percent (%)**	**MIC_{50} (µg/mL)**	**MIC_{90} (µg/mL)**	**MBC_{50} and MBC_{90} (µg/mL)**
125.0	9	9	56.25			
250.0	4	13	81.25	125	500	> 1,000
500.0	2	15	93.75			
1,000	1	16	100			

All strains tested with clindamycin (MIC and MBC ≤ 0.488 µg/mL) and *S. epidermidis* ATCC 12228 presented MIC and MBC values of 125 and 500 µg/mL, respectively.

Time-kill assay results

The time-kill analysis of the ACQ03 recipe against selected of *S. aureus* (TTM007 and TTM017) (**Figures 4** and **5**) and of *S. epidermidis* (TTM010 and TTM015) showed concentration-dependent bactericidal activity (**Figures 6** and **7**). The extract at concentrations of 4× MIC and 8× MIC killed both strains of *S. aureus* rapidly, leading to a > 3 log10 reduction in viable cells within 8 h of exposure. By comparison, the bactericidal effect (> 3 log10 reduction) against *S. epidermidis* strains at 8× MIC was observed at 16 h. These results indicate a bactericidal rather than bacteriostatic action of the ACQ03 recipe against these pathogens, a preferable feature for acne infection treatment. These results indicate a bactericidal rather than bacteriostatic action of the ACQ03 recipe against these pathogens, a preferable feature for acne infection treatment.

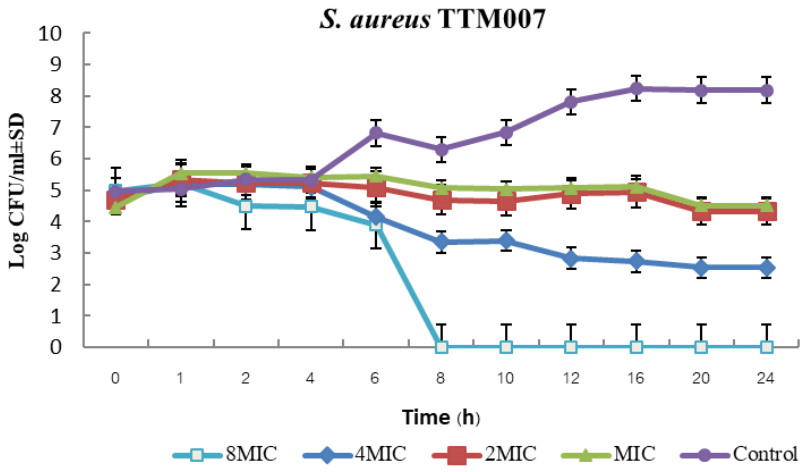

Figure 4 Time-kill curves of *S. aureus* TTM007 after treatment with the ethanol extract of ACQ03. Each symbol indicates the mean ± SD.

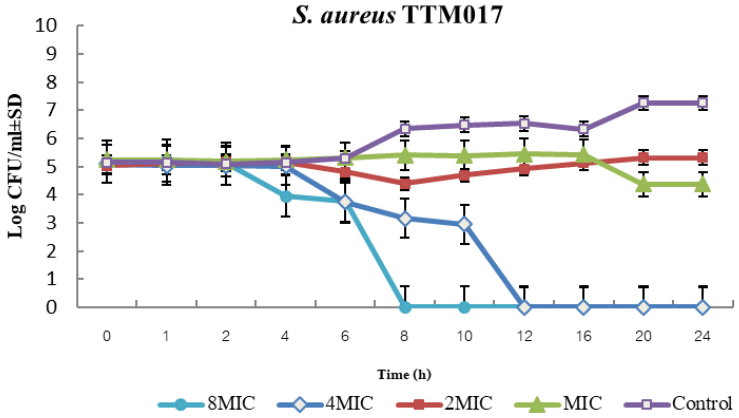

Figure 5 Time-kill curves of *S. aureus* TTM017 after treatment with the ethanol extract of the ACQ03 mixture. Each symbol indicates the mean ± SD.

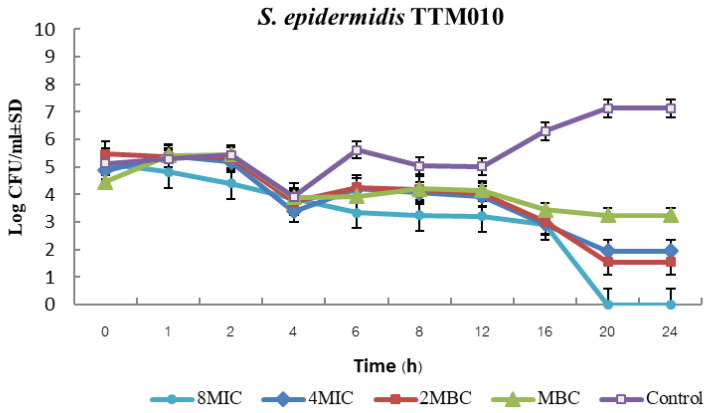

Figure 6 Time-kill curves of *S. epidermidis* TTM010 after treatment with the ethanol extract of the ACQ03 mixture. Each symbol indicates the mean ± SD.

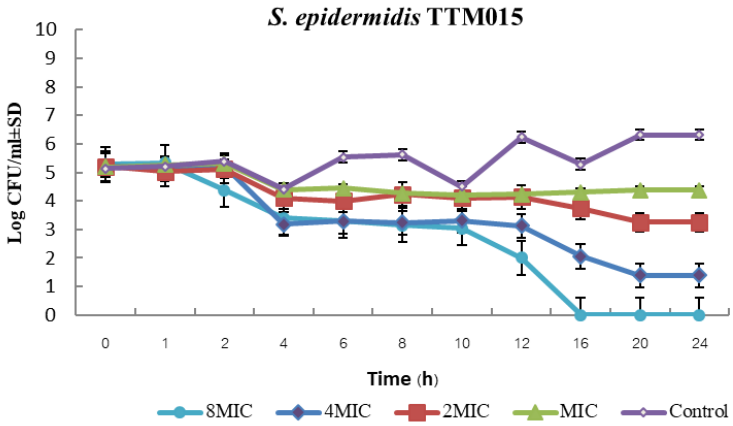

Figure 7 Time-kill curves of *S. epidermidis* TTM015 after treatment with the ethanol extract of the ACQ03 mixture. Each symbol indicates the mean ± SD.

Nitric oxide inhibitory results

NO production was utilized as a model to measure the anti-inflammatory effect of the ACQ03 recipe by scrutinizing its function in the inhibition of nitric oxide (NO) secretion in LPS-stimulated RAW264.7 macrophage cells. The inhibition of NO production by

the extract was dose-dependent and ranged from 43.54 to 93.14 % at concentrations of 1.95 to 15.62 µg/mL (**Figures 8** and **9**). Interestingly, the ACQ03 recipe showed higher NO inhibitory activities than the extracts of single-constituents (QI, AE and CL), indicating combined anti-inflammatory effects of the recipe components. These strong anti-inflammatory activities are especially relevant for the treatment of acne because inflammation is a major part of the acne pathogenesis. The fact that the recipe suppresses NO production shows that the ACQ03 recipe may usefully reduce the inflammatory reaction of both types of acne lesions. Curcuminoids are polar compounds that include mainly curcumin, demethoxycurcumin, and bisdemethoxycurcumin bioactive substances in the rhizome of *C. longa*. Curcumin has been shown to inhibit proinflammatory mediators such as nitric oxide, prostaglandin E2, and cytokines such as TNF-α and IL-6 through inactivation of the NF-κB and MAPK signaling pathways [22]. According to studies by Um *et al.* [23]; Thangapazham *et al.* [24], curcumin treatment can inhibit proinflammatory transcription factors such as NF-κB, activate the peroxisome proliferator activated receptor-gamma (PPAR) cell signaling pathway, and reduce the production of proinflammatory cytokines such as tumor necrosis factor, IL-6, and macrophage inflammatory protein 2 (MIP2), which are dependent on NF-κB. The anti-inflammatory effects of the aqueous extract of *A. ebracteatus* on NO production in J774A.1 cells were investigated. Various concentrations of AE (31.25 - 500 µg/mL) inhibited nitric oxide (NO) production [25]. *A. ebracteatus* showed potent inhibitory activity against the generation of leukotriene B4 [26], which was related to this study.

The effects of the ACQ03, QI, AE, and CL ethanolic extracts on RAW 264.7 cell viability were assessed following a 24-hour incubation with varying concentrations (1.95, 3.91, 7.81, 15.62 and 31.25 µg/mL) of the ethanolic extracts. The MTT assay results indicated that the ACQ03, QI, AE, and CU ethanolic extracts presented the lowest cytotoxicity, with no significant effects on cell viability (more than 80 % viability) observed at concentrations up to 31.25 µg/mL. In contrast, both the AE and CL extracts demonstrated cytotoxicity at 31.25 µg/mL, whereas QI induced toxicity starting at 15.62 µg/mL. On the basis of these findings, concentrations ranging from 1.95 to 15.62 µg/mL were selected for subsequent experiments (**Figure 9**).

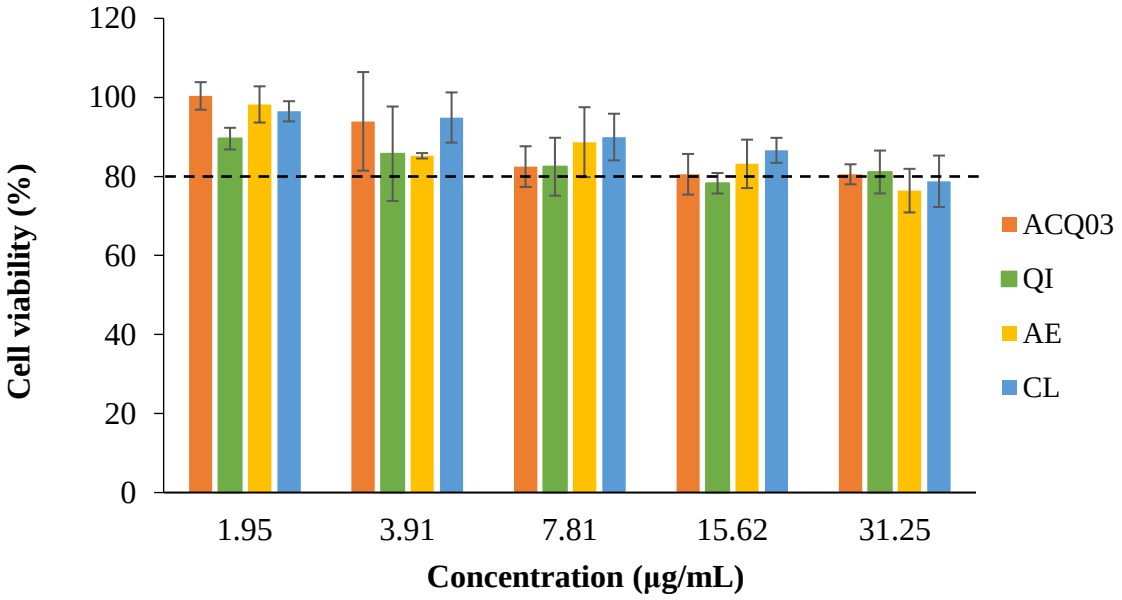

Figure 8 Effects of ACQ03, *Quercus infectoria* Olivier (QI), *Acanthus ebracteatus* Vahl (AE), and *Curcuma longa* extract (CL) ethanolic extracts on cell viability and LPS (lipopolysaccharide)-induced NO (nitric oxide) production in RAW 264.7 cells. The cells were treated with 1.95 - 31.25 µg/mL ACQ03, QI, AE, or CL for 24 h. Cell viability was determined via the MTT assay.

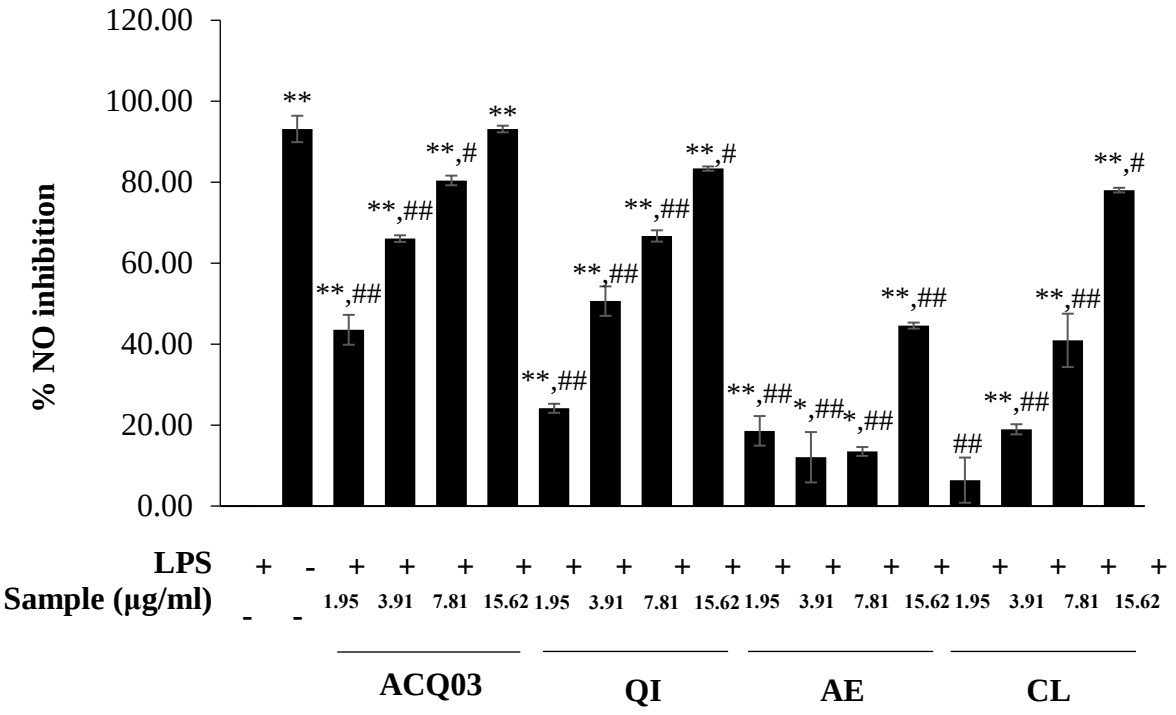

Figure 9 Effects of ACQ03, *Quercus infectoria* Olivier (QI), *Acanthus ebracteatus* Vahl (AE), and *Curcuma longa* extract (CL) on LPS (lipopolysaccharide)-induced NO (nitric oxide) formation in RAW 264.7 cells. The cells were pretreated with 1.95, 3.91, 7.81, or 15.62 μg/mL ACQ03, QI, AE, or CL for 1 h before being treated with 1 μg/mL LPS. After incubation for 24 h, NO production was detected via the Griess test. The values are presented as the means ± SDs of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with the LPS-treated group. # $p < 0.05$, ## $p < 0.01$, compared with the LPS nontreated group.

Biocompatibility testing of medical supplies and equipment is vital for patient safety

With respect to cytotoxicity, gel-containing recipes should be biocompatible (*in vitro* cell culture model). *In vitro* cytotoxicity may reveal changes inside cells, ranging from cell death to minor abnormalities in particular biological functions. Owing to the correlation between mitochondrial dysfunction and cell growth, the MTT assay is often used as a rapid and accurate tool for assessing *in vitro* cytotoxicity. When exposed to mitochondrial enzymes in live cells, the tetrazolium salt MTT may be transformed into insoluble blue formazan [27]. ISO 10993-5 [20] suggested the use of the L929 cell line for testing the cytotoxicity of medical devices. Since the L929 cell line is often used as a model for *in vitro* cytotoxicity experiments, we selected it for our research. **Figures 10** and **11** depict the cytotoxic effects on cell morphology and viability, respectively. Following treatment with 10, 100, 1,000 and 10,000 mg/mL recipe, the percentages of viable L929 cells were 88.95 ± 3.02, 85.42 ± 3.40, 80.61 ± 2.39 and 10.42 ± 1.61, respectively. A percentage of viable cells greater than 80 % was reported at concentrations less than 1,000 mg/mL, indicating the absence of cytotoxic effects. The effect of 10,000 mg/mL on L929 survival was less than 80 %. Therefore, the 1,000 mg/mL formula is preferred since it does not inhibit fibroblast growth. On the other hand, the percent viability of RAW264.7 cells after incubation with various concentrations of the recipe and individual medicinal plants at 1.95 - 400 mg/mL did not significantly differ from that of the control group (1.95 - 31.25 μg/mL) (**Figure 8**).

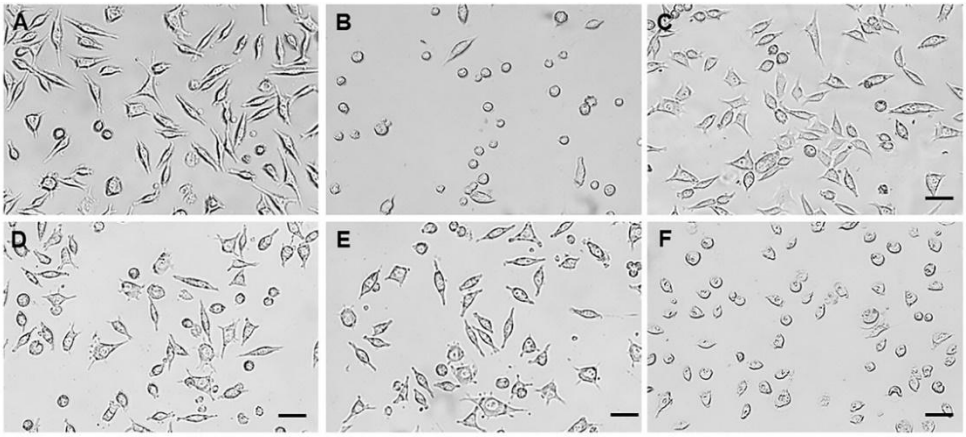

Figure 10 Morphology of L929 cells treated with DMEM as the NC (A), 1 % Triton X-100 as the PC (B), and various concentrations of recipe at 10 mg/mL (C), 100 mg/mL (D), 1,000 mg/mL (E) and 10,000 mg/mL (F). Scale bars = 50 μm.

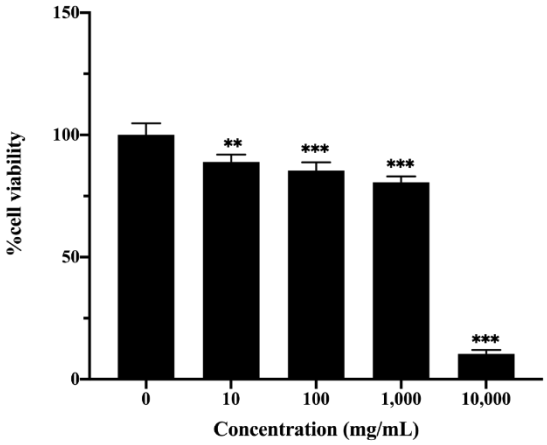

Figure 11 (A) Histograms representing the percentage of viable L929 cells with various concentrations of the recipe ranging from 0 - 10,000 mg/mL. (**, $p < 0.01$ *** $p < 0.001$)

Chemical constituents of the ACQ03 ethanol extract

LC-QTOF-MS analysis

Quadrupole time-of-flight LC-MS was used to analyze the chemical constituents of the ethanol extract from the ACQ03 recipe, and MassHunter Metlin PCD/PCDL was utilized to predict its chemical composition. Most chemicals in the Metlin database and library have matching scores of at least 90 %. All of the identified substances had errors (ppm) that were under 5 %. The ingredients that make up the ethanol extract in the ACQ 03 formulation were identified (**Tables 3 - 4**), which have a probable contribution to the antimicrobial and anti-inflammatory properties of the ACQ03 recipe. In ESI+ 6 compounds including betaine, choline, bisdemethoxycurcumin, demethoxycurcumin, curcumin and quinic acid were detected. In the negative ionization mode (ESI−) 10 compounds were detected: Quinic acid, gallic acid, 4-O-methyl-gallate, quercetin, apigenin, curcumin, pinocembrin, bisdemethoxycurcumin, demethoxycurcumin, and curcumin. The identified compounds are consistent with the known bioactive constituents of the plants in the ACQ03 recipe. Curcuminoids from *C. longa* (curcumin, demethoxycurcumin and bisdemethoxycurcumin) are known for their antimicrobial and anti-inflammatory activity [28]. Anti-inflammatory activities of betaine and quinic acid from *A. ebracteatus* have also been reported [29,30]. It has been reported about the antimicrobial effect of gallic acid, quercetin, and tannins from *Q. infectoria* [31,32].

Table 3 Compounds identified in the ACQ 03 recipe via positive electrospray ionization (ESI+) MS analysis.

No.	RT	Name of the compound	Molecular formulae	m/z	Molecular weight
1.	1.887	Betaine	$C_5H_{12}NO_2$	118.0864	118.087
2.	1.912	Choline	$C_5H_{14}NO$	104.1073	104.1078
3.	17.529	Bisdemethoxycurcumin	$C_{19}H_{16}O_4$	309.1126	308.1052
4.	25.514	Demethoxycurcumin	$C_{20}H_{18}O_5$	339.1229	338.1156
5.	26.192	Curcumin	$C_{21}H_2OO_6$	369.1337	368.1264
6.	1.885	Quinic acid	$C_7H_{12}O_6$	191.0567	192.0639

Table 4 Compounds identified in the ACQ 03 recipe via negative electrospray ionization (ESI-) MS analysis.

No.	RT	Name of the compound	Molecular formulae	m/z	Molecular weight
1.	1.885	Quinic acid	$C_7H_{12}O_6$	191.0567	192.0639
2.	2.588	Gallic acid	$C_7H_6O_5$	169.0164	170.0237
3.	5.613	4-O-Methyl-gallate	$C_8H_8O_5$	183.0305	184.0377
4.	15.118	Quercetin	$C_{15}H_{10}O_7$	301.0355	302.0427
5.	17.402	Apigenin	$C_{15}H_{10}O_5$	269.0455	270.0528
6.	18.558	Curcumin	$C_{21}H_2OO_6$	367.1189	368.1261
7.	23.931	Pinocembrin	$C_{15}H_{12}O_4$	255.0662	256.0735
8.	24.685	Bisdemethoxycurcumin	$C_{19}H_{16}O_4$	307.098	308.1054
9.	25.287	Demethoxycurcumin	$C_{20}H_{18}O_5$	337.1081	338.1155
10.	26.091	Curcumin	$C_{21}H_2OO_6$	367.1197	368.1269

The multi-target mechanism-of-action study for the ACQ03 recipe is fully described and illustrates its anti-microbial and anti-inflammatory properties subject to plaque acne pathology. Its strong activity against *C.acnes* (MIC 64 - 128 μg/mL) is special note, since this organism is critical in acne pathogenesis. Results from the time-kill studies indicated that the recipe has bactericidal activity meaning it can kill acne-causing bacteria rather than just preventing their growth. In addition, the anti-inflammatory activity, indicated by high levels of NO inhibition (43.54 - 93.14 %), could be complementary to its antimicrobial effects by helping to reduce the inflammatory response accompanying acne lesions. This dual action-combining antimicrobial and anti-inflammatory properties-points to the ACQ03 recipe as a promising candidate for use in acne treatment. Its favorable safety profile (low cytotoxicity in therapeutically relevant limited concentrations) also underscores the potential use of this herbal formulation in a clinical setting. The LC-MS/MS analysis successfully identified several bioactive compounds potentially responsible for the observed biological activities, suggesting possible interactions among the constituents.

Conclusions

ACQ03 exhibited potent antimicrobial activity against all acne-associated bacteria as well as potent anti-inflammatory properties with a satisfactory safety profile. These results scientifically corroborate the traditional medicinal use of this herbal combination for treating acne and set the stage for testing its potential as an alternative medicine for acne regarding the standard therapies in clinical settings**.**

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