The antibacterial activity of four Saudi medicinal plants against clinical isolates of Propionibacterium acnes

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ABSTRACT
This study reports the high susceptibility of Propionibacterium acnes to four medicinal plants collected from western regions of Saudi Arabia. Plant extracts were obtained by using methanol and acetone. The susceptibility was tested by agar well diffusion method. Plants of interest, namely, Rhazya stricta, Azadirachta indica, Camellia sinensis and Ocimum basilicum, exhibited good antibacterial activity with minimum inhibitory concentration (MIC) values of 50 mg/mL, 25 mg/mL, 100 mg/mL and 100 mg/mL respectively. The maximum antibacterial effect was observed by A. indica followed by R. stricta. In addition, C. sinensis, and O. basilicum had moderate activities. The results show that these plants could be tested for use in skin care products to prevent or improve acne.

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Introduction
The human skin acts as an external barrier which contains microorganisms that form commensal microbial community or microbiota and are linked with skin health. The most common microbiota found in the skin is Corynebacterium, Propionibacterium and Staphylococcus. The loss in the balance of natural microbial communities has been linked with skin diseases such as acne [1–3]. Acne vulgaris is a chronic disorder, an inflammatory disease of the sebaceous glands and hair follicles of the skin marked by the eruption of pimples and found in areas of increased sebaceous production such as the face, upper arms, chest, and back [4–6]. Disease studies have estimated acne to affect approximately (9.4%) of populations worldwide and consider it one of the most prevalent dermatologic conditions [7]. Some psychological damages are severe and cause depression, low self-esteem, suicidal ideation and anxiety [8, 9]. Propionibacterium acnes is a gram-positive facultative anaerobe, a pleomorphic rod-shaped bacterium, which thrives on the sebum and is believed to cause skin diseases such as acne [10, 11].

Current therapies against acne primarily use 13-cis-retinoic acid and various antibiotics as the first line of defense [12]. Standard oral and topical therapies can have significant side effects, including skin irritation, gastrointestinal upset and the development of drug-resistant bacteria [13–17]. Current treatments are palliative, and there is an urgent need to find additional therapeutic options.

This research aimed to find an appropriate herbal treatment rather than available medicines with side effects to treat acne. Accordingly, the methanol and acetone extracts of four Saudi medicinal plants; Rhazya stricta, Azadirachta indica, Camellia sinensis and Ocimum basilicum were used to control the pathogenesis of P. acnes. Therefore, we proposed this research intending to discover new antimicrobial agents from natural sources as anti-acne agents and limit drug-resistant bacteria.
Materials and methods

Ethical approval

The study was approved by the unit of Biomedical Ethics Research Committee at King Abdul Aziz Hospital, Dermatology Department (Reference No. 230-17). The patients signed informed consent forms before the study.

Propionibacterium acnes clinical isolates

The isolates of *P. acnes* (22 isolates) were taken from patients with mild, moderate and severe acne conditions.

The acne severity was graded as severe, moderate and mild according to the classification of acne severity with some modification [18]. Gender, age, and type of acne lesion were studied. The inflammatory acne lesions were determined based on counting and were (0–5), (6–20) and (21–50) for mild, moderate and severe, respectively. The main exclusion criteria were as follows: antibiotic intake, pregnant women, and patients receiving other therapy.

The bacterial strains were isolated from an inflammatory papule, pustule and cyst to screen for antibacterial activity. Each isolate was taken within a specified surface area of about 16 cm² of the skin divided into three parts: the forehead, cheeks and jaw. An alcohol pad was used above the acne lesion to wipe microorganisms that are found on top of it. Acne lesion was then extracted with a scratch of the tiny thin lancet and the sebum was collected by slight pressure with the hand. In addition, the surface acne lesion was swabbed without an alcohol pad.

The swab was placed in a test-tube containing brain heart infusion (BHI), (Sigma-Aldrich Ltd) or Thioglycolate (THIO; HiMedia) as transport medium. The tubes were incubated for five days at 37°C, followed by ten-fold serial dilution.

Identification of the isolates

Fermentation test

The fermentation test was made according to McDowell *et al.* method with some modification [19]. Reinforced Clostridial Agar (RCA) media (Oxoid Ltd.) supplemented with a bromocresol purple (HiMedia) as an indicator (40 mg/L). About 50 μL of the culture was dispensed on Petri plates and a glass spreader was used to spread the culture. All plates were incubated at 37°C for five days in an anaerobic chamber (Baker Co, UK) in an atmosphere of a mixed gas of (5.5%) hydrogen, (5%) carbon dioxide, and (89.5%) nitrogen. Fermentation was considered to occur when the colonies of *P. acnes* appeared yellow due to propionic acid production.

Molecular identification of strains

DNA extraction. DNA was extracted from pure cultures of *P. acnes* grown in BHI or THIO media after bacteria reached ideal growth. DNA extraction was made based on the binding of magnetic beads to nucleic acids and by combining 200 μL, 20 μL, 200 μL of lysis buffer, proteinase K buffer, and the sample, respectively with occasional shaking following the protocol of DNA/RNA Easy Extraction Kit (Genesig primer design, UK). The tube was incubated at room temperature for 15 min and 500 μL of magnetic beads were added to the solution. The next step was separating of magnetic beads and solution by attracting the beads to the magnetic rack. The beads were washed with 500 μL of wash buffer 1 and 2 separately for seconds then attracting the beads to the magnetic rack and the solution was discarded. Ethanol (80%) was added for seconds then the solution was removed and beads were left to air dry for 10 min. Finally, the elution buffer was added to elute nucleic acids, and the supernatant that contains DNA was stored at −20°C.

The purity of DNA was measured by NanoDrop™ (Thermo Scientific). About 1 μL of elution buffer was used as a blank then reading was obtained by applying 1 μL of DNA sample at 260/280 (the ratio of absorbance).

Polymerase chain reaction (PCR) and amplification of 16S rRNA gene. Universal bacterial primers correspond to regions of the 16S rRNA gene that are highly conserved among divergent groups of prokaryotes and therefore would be expected to amplify partial DNA from the bacteria. The primer locations for *P. acnes* were Forward primer, 5’-AGAGTTTGTACCTGCGCA-3′ (27), and Reverse Primer, 5’-AAGGAGGTGATCAGCGCA-3′ (1525).

The PCR reaction was conducted in a total volume of 25 μL, which contained 1 μL of 10 pmol of each primer, 12.5 μL of the commercial Master mix (GoTaq® Green Master Mix, 2X, Promega). Approximately 1 μL of 200 ng of DNA template was added to the PCRs tube. Nuclease-free water was added to adjust the final volume to 25 μL. PCR was performed with a thermal cycler (Applied Biosystems™ Veriti™ 96-Well Thermal Cycler) and programmed to perform 35 cycles that consist of initial step at 94°C for 5 min, denaturation at 94°C for 30 s, annealing of primers at 60°C for 30 s, extension at 70°C for 1.30 min and a final extra extension of 10 min at 70°C.
Gel electrophoresis. After amplification, PCR products were analyzed by 2% agarose gel electrophoresis in 1X TAE (Tris Acetate EDTA). After the DNA migrated approximately (75%) of the way down the gel, DNA products were compared with molecular size markers (DNA ladder 1 kb, Bio lab Inc, US) and visualized under UV light using a gel documentation system (Biospectrum 410, UVP).

PCR products were sent to Macrogen Co. laboratories (Korea) to determine the sequence and identify the isolated strains. Data were analyzed and optimized by using MEGA X (Molecular Evolutionary Genetics Analysis) program and compared with sequences in the NCBI database.

Plant collection and preparation of powder
To prepare extracts, R. stricta, A. indica, C. sinensis and O. basilicum were collected from western regions (Makkah province) of Saudi Arabia in April 2017. Plant leaves were air-dried, homogenized by chopper blade blender into a fine powder, and stored in air-tight bottles at room temperature until use.

Methanol and acetone extract preparation
About 50 g of powdered plant materials were dissolved in 500 mL of 80% methanol and acetone separately then left in a laboratory shaker at room temperature for 48 h. The extract was filtered using filter paper (Whatman) and concentrated on a rotary evaporator (Eyela ON-1110) at 45 °C. The residue was dissolved in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, US) to obtain a starting concentration of 500 mg/mL. A sterile syringe filter disk (Whatman) with 0.22 μm pore was used to filter the extract and finally stored in dark container glassware at −20 °C until use.

Preparation of different concentrations from a stock solution
Five different concentrations (400 mg, 200 mg, 100 mg, 50 mg) were prepared by diluting the extracts (stock) to the required concentration using sterile distilled water.

Agar well diffusion assay
The agar well diffusion assay was used according to Balouiri et al. [20] with some modification. A loop inoculated with a single colony of P. acnes was dipped inside an Eppendorf tube containing 600 μL of sterile sodium chloride (0.9%) and set visually comparing it to the turbidity of the standard 0.5 McFarland tube. Sterilized cotton swab was used to inoculate freshly made Muller Hinton agar media (Micro-master). Cork borer with 6 mm in diameter sterilized and used to make the pores inside. About 100 μL of each plant extract was dispensed inside the wells and DMSO was used as a negative control. For positive control, Teicoplanin (TEI) 30 μg was used.

The plates were incubated anaerobically upwards at 37 °C for 36 h, and finally, the zones of inhibition were measured and recorded for each extract.

Minimum inhibitory concentration (MIC)
The minimum inhibitory concentration was determined according to Lima and de Aguiar [21] with some modification. About 100 μL of Muller Hinton broth (Micro-master) was dispensed in a 96-well reaction plate. The first row was used as a negative control, the second row was used as a positive control and the third row contained an initial concentration of 100 mg of extract that was serially diluted horizontally up to well-labeled 12.

Approximately 30 μL of bacterial cultures (equal to 0.5 McFarland units) was dispensed in wells except for negative control. All plates were incubated in a sealed tight bag with a gas generating pack (BD, USA) for 36 h at 37 °C. Finally, 20 μL of resazurin (Sigma-Aldrich) was added to each well, then was incubated for two hours. Results read by changes in the color of media and the MIC was the lowermost concentration of plant extract that prevented this change.

Results and discussion
There was a correlation between acne and age. The data showed that high incidence of acne was found in the patients from the age group of 25–34 years, followed by those from the age group of 15–24 years, then the patients from the age group of 35–44 years with 50%, 36% and 9%, respectively. This study also estimated the distribution of acne vulgaris in both genders and the data showed that acne was more prevalent in females than in males: 91% vs. 9%, respectively. Similar results were reported in other studies [22, 23]. In addition, another study reported that females were more likely to develop acne earlier than males [24]. In contrast, other studies showed that acne occurred mostly in the age group of 13–20-year-olds and appeared in males more than in females, and severity was observed in males [25, 26].
In the present study, the severity of acne in patients was classified as a severe, moderate and mild condition. The results showed that 46% had severe acne, followed by 27% who had a moderate condition, and the last group (27%) had a mild acne form. The severity of acne was associated with pustules (59%), cysts (32%) and nodules (9%). The position of acne lesions appeared mostly on the cheeks (50%) followed by the jaw (32%), then the forehead (14%) and near the nose (4%).

The severity of acne in adolescence was associated with pustules and might be due to the production of more sebum, and hormonal changes such as testosterone, progesterone, glucocorticoids, insulin and insulin-like growth factors [27]. On the other hand, Schäfer et al. [28] pointed to the severity of acne associated with cigarette smoking. Other factors include physiological differences between men and women, the sampling site, drug administration, anxiety, study pressure and type of food [29, 30].

**Differentiation of P. acnes among different microbes (fermentation test)**

The fermentation test was considered positive when the color of the media changes from purple to yellow. Different microbes emerged in the early days and *P. acnes* began to appear as visible colonies at five days. *P. acnes* produces propionic acid (pH range 4–5) and colonies appeared as small circular yellowish dots or in an area of a complete change in the color of the medium. Other microbes appeared white or translucent in the purple region of the medium indicating that these microbes did not produce acids. In Gram stain, *P. acnes* cells appeared as rod-shaped bacilli (Figure 1A). After the incubation of pure culture on RCA, *P. acnes* appeared after 48 h, which was described as a small circular, yellowish, opaque, and semi-opaque convex colony. While *P. acnes* grows in a plate, the bacteria produce propionic acid that changes the color of the medium, indicating bacterial growth rate (Figure 1B).

*P. acnes* was isolated anaerobically from the pilosebaceous units; these sites were further occupied by it. These results are in agreement with other studies [31, 32]. *P. acnes* reduces the growth of other microbes such as *Staphylococcus aureus*, *Streptococcus pyogenes* and further maintains an acidic pH in the sebaceous site due to hydrolysis of sebum contents and production of propionic acid [33].

In contrast, the surface acne lesion was dominated by *Staphylococcus epidermidis* and it was described as small round whitish or translucent and flat colonies. This bacterium can gain access to *P. acnes* sites. However, this interaction between *S. epidermidis* and *P. acnes* is important due to the antagonistic properties of *S. epidermidis* against *P. acnes*. These data are also in agreement with previous studies [34, 35].

**Sequencing of 16S rRNA gene**

The 16S rRNA gene is used as a tool for the identification of bacteria because the similarity in this region is found in most microbes with slight differences in the sequence of nucleotides [36]. This has made it possible to identify prokaryotic isolates because the 16S rRNA gene is flanked by a conserved sequence which enables the design of universal primers that can amplify the 16S rRNA from different bacteria [37, 38].

*P. acnes* isolates were identified by molecular test, and the 16S rRNA gene was amplified by PCR using specific primers. All strains (6 out of 22 isolates) of *P. acnes* in the present study were positive for the 16S gene. The PCR products were separated by electrophoresis in a 2% agarose gel (Figure 2).

All samples were subjected to a partial sequencing of the 16S rRNA gene. Sequences were optimized...
LN681574.1 was used as the outgroup (Figure 3).

NR_040847.1, and NR_145912.1. Accession numbers: MH699352.1, KF933807.1, AB777865.1, NR_113028.1, the GenBank database with accession numbers: 99% nucleotide identities with sequences available in

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sequences of known isolates of Propionibacterium and some closely related species. (A) Type of strains. (B) Accession numbers are listed.

using MEGA X software. Nucleotide sequence alignment was done against the corresponding GenBank sequences of known isolates of P. acnes.

The phylogenetic data and BLAST results of 16S rRNA genes showed that P. acnes displayed 99% nucleotide identities with sequences available in the GenBank database with accession numbers: MH699352.1, KF933807.1, AB777865.1, NR_113028.1, NR_040847.1, and NR_145912.1. Accession number LN681574.1 was used as the outgroup (Figure 3).

**Agar well diffusion assay**

In the current study, the mean diameter (±SD) of inhibition zones of each plant extract was calculated after 36 h when the bacteria reached ideal growth. *P. acnes* was inhibited by all plant extracts in all tested isolates with varying degrees between each concentration.

The maximum antimicrobial activity was observed by acetone and methanol extracts of *A. indica* at 500 mg/mL, followed by *R. stricta*, *C. sinensis*, and the lowermost inhibition was achieved by the extracts from *O. basilicum*. In addition, the concentrations (400 mg/mL to 50 mg/mL) of each acetone and methanol extract showed varying degrees of inhibition zones with a correlation between the diameter of the inhibition zones and the concentration of extracts where the inhibition zones decreased as the concentrations decreased.

Each of *A. indica* and *R. stricta* inhibited *P. acnes* at all concentrations tested and showed strong activity against the bacteria, whereas *C. sinensis* and *O. basilicum* had moderate activity. The positive control used was Teicoplanin (TEI, 30 μg) with a mean inhibition zone of 30 ± 1.0 mm.

For *A. indica*, the acetone extract showed strong inhibition against the bacteria and the mean diameter of the inhibition zone at a concentration of 500 mg/mL was 40 ± 1 mm. In addition, even when the concentration was 50 mg/mL, the mean diameter of the inhibition zone was 25.4 ± 1.517 mm. The methanol extract had a similar effect, where the mean diameter at 500 mg/mL was 38.6 ± 1.67 mm, while at 50 mg/mL the mean diameter was also high 28 ± 1.414 mm.

The results showed strong inhibition by extracts of *A. indica* and these were higher than reported before by Charde et al. [39], where *A. indica* at 20 mg/mL had a 20 mm zone of inhibition. In contrast, Nand et al. [40] used methanol extract of leaves and bark of *A. indica* by agar disk diffusion method that showed insignificant effect against *P. acnes*. Another study reported that *A. indica* exhibits moderate inhibitory activity against *P. acnes* [41]. However, Daud et al. [42] showed that *A. indica* has a strong antibacterial activity against *P. acnes*.

Compared to other studies, the effect of *A. indica* against *P. acnes* in the present study may be attributed to differences in the extraction process, where a combination between water and methanol (80%) was used. Some studies agree that solvent combinations have effects mostly on phenolic compounds, antioxidants, and further to release most secondary metabolites [43].

The *R. stricta* methanol extract was more effective with a zone of inhibition of 30.4 ± 2.96 mm (500 mg/mL) when compared to the acetone extract, whose inhibition zone was 25.6 ± 1.94 mm. At 50 mg/mL of methanol and acetone extracts, the mean diameter of the inhibition zone was 11.4 ± 2.074 and 9.2 ± 1.789 mm, respectively. Studies have shown that *R. stricta* has antimicrobial and antifungal activities due to the presence of active compounds found in the leaves such as alkaloids, tannins, triterpenes, glycosides and volatile bases [44–47].

Methanol is the most used solvent in the screening of antimicrobial activities in plant extracts preparation [48]. *R. stricta* showed strong activity against *P. acnes* and that was higher than recorded by the commercial antibiotic itself [49, 50]. Our results are in agreement with Ahmad et al. [51], who showed that the crude extract of *R. stricta* had antibacterial activities against a wide range of gram-positive and gram-negative bacteria.

The *C. sinensis* methanol extract at 500 mg/mL was more effective than the acetone extract and had moderate antimicrobial activity, where the inhibition zone was 18.6 ± 1.67 mm and 16 ± 2.34 mm, respectively. This was higher than previously reported by Nand et al. [52], where the inhibition zone of *C. sinensis* was 13 mm.

Figure 3. Unrooted tree type of *P. acnes*, which was constructed by the neighbor-joining method and show the phylogenetic relationships between members of the genus Propionibacterium and some closely related species. (A) Type of strains. (B) Accession numbers are listed.
On the other hand, *O. basilicum* had moderate to weak antimicrobial activities as compared to *C. sinensis*. The acetone and methanol extracts had similar antimicrobial activities with an inhibition zone of 13 mm and 12 mm, respectively. The methanol extract of *O. basilicum* showed resistance at 100 mg/mL. The inhibitory effect of *O. basilicum* may be due to the presence of a phenolic group, alkaloids, glycoside, flavonoids, anti-inflammatory and anti-oxidant agents, as suggested in other studies [53–55]. The diameters of the inhibition zones obtained using all the plant extracts are shown in Figures 4 and 5 and summarized in Table 1.

**The minimum inhibitory concentration (MIC)**

The change in the color from blue to pink indicated the presence of the bacteria. In the negative control, Resazurin gave blue color indicating the absence of the bacteria. In contrast, positive control turned into pink color. The reaction between Resazurin and extracts in the presence of the bacteria determined the MIC and was noted as the lowermost concentration of plant extract that prevented this color change.

The MIC values of *R. stricta, A. indica, C. sinensis* and *O. basilicum* were determined. The MIC for acetone and methanol extracts of *R. stricta* was 50 mg/mL and this result is reported for the first time. The acetone and methanol extracts of *A. indica* had MIC of 25 mg/mL and it was higher than what has been reported before by Charde et al. [39], where the MIC was 6.0 mg/mL. The MIC values were 100 mg/mL for both *C. sinensis* and *O. basilicum*. By comparison, Nand et al. [52] showed that methanolic and petroleum ether extracts of *C. sinensis* had an antimicrobial effect.

![Figure 4. Antimicrobial activity of acetone plant extracts against *P. acnes* evaluated based on diameter of inhibition zones. Note: Extract concentrations were 500 mg, 200 mg, 50 mg](image4.png)

![Figure 5. The diameter of the inhibition zone by methanol extract of plants against *P. acnes* at 500 mg, 200 mg, 50 mg.](image5.png)
against *P. acnes* with a MIC value of 1.25 mg/mL. In addition, phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides and terpenoids, which indicates that these phytoconstituents may be responsible for the anti-acne activity. Furthermore, a study by Adigüzél *et al.* [56] showed that *O. basilicum* methanol extract was active against a wide range of bacteria in the disk diffusion assay. The result showed that the extract had antibacterial effects and the MIC value was between 62.50 and 500 μl/mL. The results are shown in Figure 6 and summarised in Figure 7.

### Figure 6. Minimum inhibitory concentration (MIC) of plant extracts against *P. acnes*. Representative image. Note: Resazurin in the medium changes its color from blue to pink as an indication of the presence of bacteria. The MIC was the lowermost concentration of plant extract that prevented this change in color. NC, Negative Control, PC, Positive Control, MIC (RA), (RM)= where R for Rhazya, A for acetone, M for methanol, MIC (AA), (AM)= where A for Azadirachta, A for acetone, M for methanol, MIC (CA), (CM)= where C for Camelia, A for acetone, M for methanol, MIC (OA), (OM)= where O for Ocimum, A for acetone and M for methanol.

### Figure 7. Minimum inhibitory concentration of plant extracts against *P. acnes*.

### Table 1. Mean diameters of *P. acnes* inhibition zones obtained using different plant extracts.

| Plants | 50 mg/mL | 100 mg/mL | 200 mg/mL | 300 mg/mL | 400 mg/mL | 500 mg/mL |
|--------|----------|-----------|-----------|-----------|-----------|-----------|
| AM     | 28 ± 1.414 | 29.4 ± 0.894 | 33.8 ± 0.837 | 35.2 ± 1.095 | 37 ± 1.581 | 38.6 ± 1.67 |
| AA     | 25.4 ± 1.517 | 29.2 ± 2.95 | 33.8 ± 1.924 | 35 ± 3.082 | 37.2 ± 1.643 | 40 ± 1.58 |
| RM     | 11.4 ± 2.074 | 15.2 ± 2.387 | 19.2 ± 1.095 | 23.8 ± 1.643 | 26.6 ± 2.074 | 30.4 ± 2.96 |
| RA     | 9.2 ± 1.789 | 13 ± 1.225 | 17.6 ± 2.608 | 22.6 ± 2.302 | 24 ± 2.449 | 25.6 ± 1.94 |
| CM     | 0         | 0         | 12.8 ± 1.789 | 15 ± 2.828 | 16.6 ± 2.074 | 18.6 ± 1.67 |
| CA     | 0         | 0         | 9.1 ± 1.581 | 10.6 ± 1.673 | 13.8 ± 1.924 | 16 ± 1.24 |
| OM     | 0         | 0         | 7.1 ± 1.871 | 8 ± 1.871 | 9.2 ± 1.095 | 12.6 ± 1.51 |
| OA     | 0         | 0         | 8.6 ± 1.342 | 11 ± 2.345 | 13.2 ± 2.38 |

* (AM), (AA)= where A for Azadirachta, M for methanol, A for Acetone, (RM), (RA)= where R for Rhazya, M for methanol, A for Acetone, (CM), (CA)= where C for Camelia, M for methanol, A for Acetone, (OM), (OA)= where O for Ocimum, M for methanol, A for Acetone.
The medicinal plants have potent bioactive compounds, which are proven to have antimicrobial activity against a wide range of bacteria. Therefore, this leads to investigations into those bioactive compounds to decrease the emergence of antibiotic-resistant bacteria [57]. The present study recommends using Saudi medicinal plants against acne-causing microbes. The plant extracts in the present study exhibited good antimicrobial activity against *P. acnes* in vitro. Further studies are needed to isolate the active compounds from *A. indica* and *R. stricta* and to perform toxicity tests against animal and human cell lines.

The emergence of antibiotic-resistant bacteria has been high since the 1980s, and is still a serious problem nowadays with antibiotics that have side effects [58, 59]. Hence, there is a great demand to use alternative medicine and safer treatment. Medicinal plants are generally safer and have low side effects [15].

Until now, the relationship between acne and the causal bacterium is still in debate. It is not clear how *P. acnes* are involved in acne while being a major commensal of the normal skin microbiota [2].

**Conclusions**

In this study, extracts from *R. stricta, A. indica, C. sinensis* and *O. basilicum* exhibited good antibacterial activities against *P. acnes*. The maximum antibacterial effect was observed with *A. indica*, followed by *R. stricta*. In addition, *C. sinensis* and *O. basilicum* had moderate activities.

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**Conflict of interest**

No potential conflict of interest was reported by the authors.

**Data availability**

All data that support the findings from this study are available from the corresponding author upon reasonable request.

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