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Research Article
In vitro Antibacterial Activity of Ethanolic Tanao Si Kan Dang RD1 (*Cannabis sativa* L.) Extracts Against Human Antibiotic-Resistant Bacteria

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Abstract

Background and Objective: A new strain of cannabis, *Cannabis sativa* L. Tanao Si Kan Dang RD1, has been approved and registered by the Rajamangala University of Technology Isan, Thailand. The *C. sativa* is acknowledged for its medicinal properties which demonstrated various therapeutic properties, such as anti-cancer and antibacterial activities. This study aimed to investigate the antibacterial activity of ethanolic extracts from the stems and leaves of the Tanao Si Kan Dang RD1 strain against seven antibiotic-resistant bacteria. **Materials and Methods:** The primary antibacterial activity of ethanolic Tanao Si Kan Dang RD1 extracts were determined using the disc diffusion method, while the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using the broth microdilution method. **Results:** The largest inhibition zone, measuring 12 mm, was observed in leaf extracts against *Pseudomonas aeruginosa* 101. The lowest MIC, at 0.78 mg/mL, was obtained from stem extracts against *Stenotrophomonas maltophilia*. The lowest MBCs, at 12.5 mg/mL, were observed in leaf extracts against *Enterococcus faecalis*, *Acinetobacter baumannii*, multidrug-resistant *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* 101 and stem extracts against *Acinetobacter baumannii*, multidrug-resistant *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* 101. **Conclusion:** This study presents a novel finding regarding the antibacterial activity of ethanolic extracts from the leaves and stems of Tanao Si Kan Dang RD1 against antibiotic-resistant bacteria. The potential application of these cannabis plant extracts in the development of antibiotics capable of combating antibiotic-resistant pathogenic bacteria represents a promising strategy to address a significant global health concern.

Key words: Antibiotic-resistant bacterial activity, Tanao Si Kan Dang RD1, *Cannabis sativa* L., multidrug-resistant *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The cannabis strain "Tanaosri Kan Daeng" is widely recognized, much like its counterparts "Hang Krarok", "Hang Seua" and "Tanaosri Kan Khao". Its distribution is primarily along the Tanaosri and Thongchai mountain ranges, extending to the southern provinces of Thailand, including Nakhon Si Thammarat, Trang, Takhli and Kanchanaburi. The cultivation of the "Tanaosri Kan Daeng" strain is a collaborative effort between the Rajamangala University of Technology Isan and The Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health. The seeds selected for cultivation were obtained from verified sources, as documented in SN 134/2562©, dated May 15, 2562.

The characteristics of the seeds were meticulously examined and found to be consistent with those of the Tanaosri Kan Daeng strain. This assessment was corroborated by data gathered from local communities and traditional healers in the provinces of Tak, Kanchanaburi, Nakhon Si Thammarat and Trang. This information proved crucial in distinguishing this strain from other varieties of cannabis. The Tanaosri Kan Daeng strain is characterized by its unique flowering cluster features and distinctive olfactory profile. The densely arranged flower clusters at the branch tips bear a resemblance to those of the Tanaosri Kan Khao strain. However, a defining characteristic of this strain is the red pigmentation that adorns its branches, stems and leaf stalks. Unlike conventional cannabis varieties, this strain emits a sweet fragrance, akin to ripe fruit and lacks the typical pungency associated with cannabis. This unique combination of characteristics underscores the distinctiveness of the Tanaosri Kan Daeng strain.

Numerous studies have highlighted the therapeutic potential of *Cannabis sativa* L., in addressing a variety of health conditions. These include lowering blood pressure, treating cachexia and schizophrenia, managing anxiety disorders, combating cancer and HIV/AIDS, alleviating gastrointestinal disorders, controlling epilepsy, improving sleep disorders, reducing inflammation and exhibiting antiviral properties against SARS-CoV-1 and SARS-CoV-2[1,2]. Furthermore, it has been used in the treatment of post-traumatic stress disorder, demonstrated antibacterial activity[3], exhibited antifungal activity[4] and has been beneficial in managing glaucoma[5]. The escalating prevalence of antibiotic-resistant infections in humans represents a significant challenge that necessitates immediate attention. The indiscriminate and excessive use of antibiotics has culminated in the emergence of antibiotic-resistant bacterial strains. There have been several reports presenting the antibacterial activity of *Cannabis sativa* extracts, indicating its potential role in addressing this global health concern.

The oil derived from the seeds of *C. sativa* has demonstrated significant antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, as evidenced by an inhibition zone measuring 21-28 mm. It has also shown activity against *Escherichia coli* (15 mm) and *Pseudomonas aeruginosa* (16 mm)[6]. Frassinetti *et al*.[7] reported on the antimicrobial activity of *Cannabis sativa* L. seed extract against *Staphylococcus aureus*, indicating that the extract can inhibit the biofilm producer *S. aureus* ATCC 35556. In a separate study conducted by Isahq *et al*.[8], *Cannabis indica* demonstrated a wide range of antibacterial activity against *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Proteus mirabilis*. Additionally, extracts from the leaves, seeds and stems of the *C. sativa* plant exhibited antifungal properties against *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus oryzae*[8]. While there are limited reports on the antibacterial activity of cannabis extracts against antibiotic-resistant bacteria, this research investigated the antibacterial activity of ethanolic extracts from the stems and leaves of Tanao Si Kan Dang RD1 against seven antibiotic-resistant bacteria. The findings of this study underscore the potential benefits of using cannabis extracts in the development of antibiotics or as a co-treatment for patients infected with antibiotic-resistant bacteria.

MATERIALS AND METHODS

Study area: This research was performed from February, 2022 to December, 2023 Faculty of Agricultural Innovation and Technology, Rajamangala University of Technology Isan, Nakhon Ratchasima and the Microbiology Laboratory, Department of Science and Technology, Faculty of Liberal Arts and Science, Roi Et Rajabhat University, Roi Et, Thailand.

Tanao Si Kan Dang RD1 (*Cannabis sativa* L.) extract preparation: The Tanao Si Kan Dang RD1 stems and leaves were collected from the Rajamangala University of Technology Isan farm (Fig. 1). The plant samples were washed twice using water and cut into small pieces. The plant samples were dried using a hot air oven (POL-EKO-APARATURA company, Wodzisław Śląski, Poland) at 45°C for 72 hrs. The dried Tanao Si Kan Dang RD1 stems and leaves were powdered using an herb blender (WF-20B THAIGRINDER, Thailand). Fifty grams of stems and leaves powder samples were extracted using ethanol by shaking for 3 hrs at room temperature. The extract solution was collected by filtering and dried at 50°C for 2 days. The dried extracts were weighed for percent yield calculation[9]. The stem and leaf extracts were adjusted to the final concentration at 500 mg/mL by adding the dimethyl sulfoxide (DMSO, Sigma).

Fig. 1: Tanao Si Kan Dang RD1 at the Rajamangala University of Technology Isan farm, Nakhon Ratchasima, Thailand

Antibiotic-resistant bacteria inoculum preparation: *Enterococcus faecalis*, *Burkholderia pseudomallei*, *Proteus mirabilis*, *Acinetobacter baumannii*, multidrug-resistant *Klebsiella pneumoniae* (MDR-KP), *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* 101 were obtained from the Department of Clinical Microbiology, Roi Et Hospital, Roi Et, Thailand. The single colony of each bacterium was cultured using 20 mL nutrient broth (NB) at 37°C with shaking overnight before use.

Antibacterial activity screening: The agar disc diffusion was used as antibiotic activity primary screening[10]. Fresh seven antibiotic-resistant bacteria were prepared. A single colony of each bacterium was cultured with NB overnight. The bacterial cells were centrifuged and the cell concentration was adjusted at OD600 to 0.1 before use. One hundred microliters of each antibiotic-resistant bacterium were spread onto a nutrient agar (NA) plate using an aseptic technique. The sterile paper disc (Ø 0.6 mm) was placed onto the NA plate. Ten microliters of ethanolic Tanao Si Kan Dang RD1 stems and leaves extracts were dropped onto a paper disc (triplicated). The NA culture plates were allowed to diffuse for 15 min and then were incubated at 37°C for 24 hrs using a bacterial incubator. The formation of an inhibition zone around the paper disks was measured and recorded. The size of the inhibition zone was measured in millimeters (mm), providing information about the microorganism's susceptibility to the ethanolic Tanao Si Kan Dang RD1 extracts. A larger inhibition zone indicates that the tested bacteria are more susceptible to the extracts.

MICs and MBCs values determination: The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) are two key parameters in antimicrobial susceptibility testing. The MIC and MBC were determined using microbroth dilution as described previously by Rattanasuk *et al.*[11]. The ethanolic Tanao Si Kan Dang RD1 stems and leaves twofold serially diluted in a 96-well culture plate containing NB to obtain various extract concentrations (triplicated). The DMSO was used as a negative control. The antibiotic-resistant bacteria that show susceptibility from antibacterial activity screening were cultured using NB overnight. The bacterial concentration was adjusted at OD600 to 0.1 before being added to a 96-well culture plate. The plates were incubated in a bacterial incubator at 37°C for overnight. Iodonitrotetrazolium chloride (INT) is a monotetrazolium salt used as an indicator dye. Fifty microliters of 2 mg/mL INT were added to each well and the plates were incubated at 37°C for 30 min. The MIC value is the lowest concentration of extract that can inhibit the tested bacterial growth. The MBC is the lowest concentration of extract that can eliminate the tested bacteria that did not produce the color change after INT was added[11].

Data analysis: The data of the inhibition zone was expressed as Mean±Standard Error.

RESULTS AND DISCUSSION

Agar disc diffusion assay: The agar disc diffusion method is a widely utilized technique for assessing the susceptibility of bacteria to antimicrobial agents. This standardized procedure entails the placement of sterile paper discs, impregnated with specific concentrations of antimicrobial substances such as antibiotic drugs and plant extracts, onto an agar medium that has been previously inoculated with the target pathogenic bacteria[12]. The agar disc diffusion assay was used as the primary antibacterial activity careening of *Cannabis sativa* L. (Tanao Si Kan Dang RD1) extracts similar to those previously reported by Rattanasuk and Phiwthong[9]. The Tanao Si Kan Dang RD1 stem and leaves were extracted using ethanol. The ethanolic Tanao Si Kan Dang RD1 stem and leaf extracts were dropped to NA-spreading tested bacteria. The results indicated that the ethanolic leave extract presented the highest diameter of inhibition zone at 12 mm against *Pseudomonas aeruginosa* 101, followed by *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* at 9 mm (Table 1). The result of this research presented a higher diameter of inhibition zone against *Pseudomonas aeruginosa* which Ali *et al*.[6] reported. They reported the antibacterial activity of the petroleum ether extract of the whole *C. sativa* plant inactive against *P. aeruginosa*[13]. The result was similar to the previous report from Isahq *et al*.[8] that the antibacterial activity of the extracts of *C. indica* leaves against *P. aeruginosa* was at 11-16 mm of inhibition zone[8].

For the Tanao Si Kan Dang RD1stem extracts, the result presented that the highest diameter of inhibition zone at 8 mm against *Acinetobacter baumannii*, multidrug-resistant *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* 101 (Table 1). The result from this research was similar to a previous report that presented the antibacterial activity of *Cannabis sativa* extracts that can inhibit the *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*[1,6]. Kaur *et al*.[14] reported that *C. sativa* leaves extracted with methanol, ethanol, acetone and aqueous cannot inhibit the growth of *Pseudomonas aeruginosa*[14]. Rattanasuk *et al*.[11] reported the antibacterial activity of *Cissus quadrangularis* extracts against antibiotic-resistant bacteria. The results indicated that the highest diameter of the inhibition zone at 15 mm against *Enterococcus faecalis* and colistin-resistant *Pseudomonas aeruginosa* (CoR-PA) was obtained from ethanolic *C. quadrangularis* extracts[11]. Novak *et al*.[15] reported the antimicrobial activity of essential oils of *C. sativa*. The result found that all cultivars (cv. Felina 34, cv. Fedrina 74, cv. SwissMix, cv. Kompolti and cv. Secuemi) was inhibited that *Acinetobacter calcoaceticus* growth with the inhibition zone at 5.1-15 mm[15].

MIC and MBC values: The microbroth dilution was used for MIC and MBC determination. The *Cannabis sativa* L. (Tanao Si Kan Dang RD1) stem and leave extracts were two-fold diluted and the tested bacteria were added. The colorimetric assay was used to measure the antibacterial activity. The results indicated that the lowest MIC values of stem and leaf extracts were at 1.56 and 0.78 mg/mL against *Stenotrophomonas maltophilia* (Table 2). The lowest MBC values of stem and leave extracts were at 12.5 mg/mL against *Enterococcus faecalis*, *Acinetobacter baumannii*, multidrug-resistant *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* 101.

The MIC result of *C. sativa* L. (Tanao Si Kan Dang RD1) extracts against *P. aeruginosa* 101 from this research at 6.25 mg/mL is lower than previously reported by Ali *et al*.[6]. They presented the methanolic whole *C. sativa* plant extract had MIC against *P. aeruginosa* at 12.5 mg/mL[6]. Ferrante *et al*.[16] also presented the *C. sativa* extracted with water had MIC at 7.14 mg/mL against *P. aeruginosa*[16]. The CBD had MIC against *E. faecalis* at 1-4 µg/mL and *A. baumannii*, *P. mirabilis*, *K. pneumoniae*, *S. maltophilia* and *P. aeruginosa* at >64 µg/mL[17].

Table 1: Diameter of the inhibition zone of Tanao Si Kan Dang RD1 extracts against human antibiotic-resistant bacteria

	Diameter of inhibition zone (mm+SD)	
Antibiotic-resistant bacterial strain	Leaves	Stem
Enterococcus faecalis	7	7
Burkholderia pseudomallei	7	7
Proteus mirabilis	7	7
Acinetobacter baumannii	9	8
Multidrug resistant *Klebsiella pneumoniae*	8	8
Stenotrophomonas maltophilia	9	8
Pseudomonas aeruginosa 101	12	8

Table 2: MIC and MBC values of Tanao Si Kan Dang RD1 extracts against human antibiotic-resistant bacteria

	Leave extract		Stem extract	
Antibiotic-resistant bacterial strain	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Enterococcus faecalis	6.25	12.5	12.5	25
Burkholderia pseudomallei	12.5	25	12.5	25
Proteus mirabilis	3.12	25	6.25	25
Acinetobacter baumannii	6.25	12.5	6.25	12.5
Multidrug-resistant *Klebsiella pneumoniae*	6.25	12.5	6.25	12.5
Stenotrophomonas maltophilia	1.56	12.5	0.78	12.5
Pseudomonas aeruginosa 101	6.25	12.5	6.25	12.5

MIC: Minimum inhibitory concentration and MBC: Minimum bactericidal concentration

The study about the antibacterial activity using *C. sativa* plant extract, cannabidivarin, essential oils, cannabinol oil extract and Cannabidiol (CBD) against bacteria has been reported. The tested bacterial such as *P. aeruginosa*[18], *Escherichia coli*[19], *Helicobacter pylori*[20], *Legionella pneumophila*, *Moraxella catarrhalis*[17], *Neisseria gonorrhoeae*[21], *Porphyromonas gingivalis*[22], *Brochothrix thermosphacta*[23], *Bacillus* species[24], methicillin-resistant *Staphylococcus aureus* (MRSA)[25], *Cutibacterium acnes*, *Clostridioides difficile*[17], *Enterococcus casseliflavus*[21], *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus faecalis*[24], *Enterococcus gallinarum*[21], *Filifactor alocis*[22], *Listeria monocytogenes*[26], *Streptococcus salivarius*, *Staphylococcus saprophyticus*, *Streptococcus sanguis*, *Streptococcus pneumoniae*, *Staphylococcus mutans*[27], *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*[21], *Salmonella typhimurium*, *Salmonella newington*, *Rhodococcus equi*, *Propionibacterium acnes* and *Micrococcus luteus*, etc.[28]. Only few antibiotic-resistant bacteria were reported.

CONCLUSION

This constitutes the inaugural study demonstrating the efficacy of ethanolic extracts derived from the leaves and stems of Tanao Si Kan Dang RD1 in combating antibiotic-resistant bacteria. The findings indicate that these extracts are capable of eradicating a range of antibiotic-resistant bacteria, including *E. faecalis*, *A. baumannii*, multidrug-resistant *K. pneumoniae*, *S. maltophilia* and *P. aeruginosa* 101. Consequently, the extracts from Tanao Si Kan Dang RD1 hold significant potential for the development of novel therapeutic agents aimed at treating patients afflicted with infections caused by antibiotic-resistant bacteria.

SIGNIFICANCE STATEMENT

This study discovers the antibacterial activity of Tanao Si Kan Dang RD1 extracted ethanol against seven antibiotic-resistant bacteria. This study will help the researcher uncover the critical areas of using ethanolic Tanao Si Kan Dang RD1 extracts as a natural antibiotic that many researchers were not able to explore thus, a new application using the antibacterial activity of ethanolic Tanao Si Kan Dang RD1 extracts against seven antibiotic-resistant bacteria may be arrived at.

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