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Antimicrobial Activity of *Cosmos caudatus* Against *Staphylococcus aureus* and *Escherichia coli.*

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ABSTRACT

Malaysia is known for its richness in ecosystem including medicinal plants and herbs. The use of traditional herbs for medical purposes has been practiced for ages without any proper understanding on how they work. This study aims to produce extract from the leaves of *Cosmos caudatus* (Ulam Raja) and to investigate the antimicrobial activity of the produced extract against *Staphylococcus aureus* and *Escherichia coli.* Ethanolic Soxhlet extraction was carried out to obtain the leaves extract. Product from extraction process was subsequently tested using the Kirby-Bauer disk diffusion test to determine its inhibitory effects against *S. aureus* and *E. coli.* Finally, the bioactive compounds in extracts were profiled using gas chromatography coupled with mass spectrometer (GC-MS). Findings showed ethanol is suitable to be used in obtaining extract from the leaves of *C. caudatus*. The extract produced was dark green and was able to inhibit the growth of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The size of inhibition zones ranging from 22 to 24 mm. The zones of inhibition produced by *C. caudatus* extract in this study are comparable to commercial antiseptic tested simultaneously in the experiment. Analysis of extract using GC-MS revealed several bioactive compounds including palmitic acid, stigmasterol, phytol and neophytadiene These compounds are well known for their medicinal and therapeutic properties such as antimicrobial, antioxidants and antitumor. Additionally, these bioactive compounds are commonly used as ingredients in commercial antiseptics and disinfectants. This study therefore has proven the medicinal properties of *C. caudatus.* Findings from this study can be used to develop plant-based pharmaceutical products which are cheaper, safer, and more environmentally friendly.

1. Introduction

The most common pathogen in human are bacteria. Despite being labelled as the most dangerous microorganism, in actual, only less than 30% of bacteria are pathogen. Most bacteria present on human as normal flora, causing no harm to their host. Staphylococcus aureus inhabit human skin and may cause soft tissue infection including boils, furuncles and cellulites. According to MSD manuals.com, S. aureus is considered as the most dangerous of staphylococcal bacteria. Even though,

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these gram-positive, spherical bacteria are often connected to skin infections, they also can cause pneumonia, heart valve infections, and bone infections. Bloodstream infections of *S. aureus* may eventually cause endocarditis, osteomyelitis, and lung infection. *Escherichia coli* is a group of gram-negative oval-shaped bacteria. Some of them are natural flora in human intestines. However, pathogenic *E. coli* are harmful and may cause infection in intestines leading to diarrhea and bladder infections. Pathogenic *E. coli* infect human via contaminated food and water and sometimes transferred from pets. Many strains from *S. aureus* and *E. coli* are known to have develop resistance to commonly available antibiotics. Therefore, a potent drug to combat this pathogen need to be produced, and natural-based remedies are the best option.

The emergence of antibiotic-resistant bacteria poses a significant threat to public health, and there is an urgent need to discover new antimicrobial agents to combat these pathogens. Natural products have been a valuable source of antimicrobial compounds, and *C. caudatus* has been reported to contain bioactive compounds with potential antibacterial properties. However, the antibacterial activity of *C. caudatus* has not been extensively studied, and the specific compounds responsible for its activity remain unknown. Therefore, this study aims to evaluate the antibacterial potential of *C. caudatus* and identify the bioactive compounds responsible for its activity, which may lead to the discovery of novel antimicrobial agents for the treatment of infectious diseases caused by antibiotic-resistant bacteria.

Utilization of local plants and herbs to combat diseases was and still a common practice in most part of the world. In the past, people tend to reach out for remedies that are easily available to treat illnesses and pains. Without proper knowledge of how it works, our ancestors know that some plants and herbs can help to prevent diseases from occurring and can treat illnesses. Malaysia's flora is exceptionally diverse, with an estimated 12,500 types of seed plants, according to conservative estimates (angiosperms and gymnosperms). Malaya (including Peninsular Malaysia and Singapore) is estimated to contain approximately 7,900 species and 1,500 genera of seed plants whereas Borneo (Sabah, Sarawak, Brunei, and Kalimantan) is estimated to contain approximately 9,000 species and 1,500 genera of seed plants. (Forest Genetic Resources Information No. 20, 2021). Currently, there are more than 2000 local plants species are listed as medicinal plants. The abundance of this natural resource opens possibilities for commercialization, especially in pharmaceutical sector. To enable this, proper process must be established to ensure its competitiveness with current pharmaceutical products, especially in production cost, environmental impacts and effectiveness of the product itself. A study on ten Malaysian plants species showed the therapeutic properties such as antioxidation, antidiabetics, antiinflammation, which explains and validates their uses in traditional and modern medicine [1]. Biosynthesis of silver nanoparticles (Ag-NPs) from walnut tree and bitter melon extracts were found to have high antibacterial activity against both Gram positive and negative bacteria [2,3].

This study aimed to investigate the medicinal properties of *Cosmos caudatus or* locally known as Ulam raja. Figure 1 shows *C. caudatus* as a flowering, invasive plant. They can grow easily and sometimes are being used as an ornamental plant. This plant is grouped in Asteraceae family and can grow up to 75 to 100 cm in height. *C. caudatus* produces flowers and seeds in throughout the year and pollination of its flower is done by insects mostly bees and butterflies. Additionally, the *C. caudatus* flower is androgynous with many discs. It has 5 leaves that are pale in colour with a yellow base and a pink crown that is made up of 8 leaves that are 1 cm in length [4].

Most of Southeast Asia countries, notably Indonesia and Malaysia, is home to *C. caudatus*. This plant has been used traditionally as a medicine for centuries. It is generally known to treat various health problem including in reducing high blood pressure, fever, diabetes, infectious disorders and skin problems [5]. In many Southeast Asian countries, including Malaysia, *C. caudatus* is eaten raw as

an appetizer together with meals. Its exceptional characteristic lead to various studies to investigate its medicinal properties. Studies revealed phytochemicals in *C. caudatus* extract including quercitrin, quercetin glycoside, and rutin [6]. A study by Alvarez *et al.,* in 2021 showed several proanthocyanidins with antioxidants properties, including quercetin glycosides, chlorogenic, neochlorogenic, and cryptochlorogenic acids, as well as (+) catechin, were also present in *C. caudatus*.[7]

Fig. 1. *C. caudatus* (From left to right -Whole plant, Leaves, Flower)

Despite the increasing global demand for natural antimicrobial agents to combat infectious diseases caused by multidrug-resistant bacteria, there is still a lack of sufficient information regarding the potential of *C. caudatus* as a source of antibacterial agents. Although previous studies have reported the presence of various bioactive compounds in *C. caudatus*, including flavonoids, phenolics, and terpenoids, their antibacterial activities have not been fully explored. Therefore, there is a need to investigate the antibacterial potential of *C. caudatus* and identify the specific compounds responsible for its activity.

2. Methodology

2.1 Chemicals and Reagents

All chemicals and reagents used in this study were laboratory-grade, obtained from Merck and Fisher Scientific via local suppliers. All glassware used were washed with soap and tap water and rinsed using distilled water to eliminate the presence of surfactants. Cleaned glassware were dried in an oven at 30°C. All apparatus used in microbiological works were either sterile disposables or washed and autoclaved 30 minutes at 121°C and 15 psi prior using.

C. caudatus was obtained from local wet market. The plant was cleaned and washed using tap water to remove all impurities and finally rinse three times with distilled water. Cleaned *C. caudatus* were oven-dried for 48 hours at 50°C. Dried leaves were ground using Panasonic blender MX-151SP1 to fine powder and kept in a vacuum plastic prior extraction.

S. aureus and *E. coli* were obtained from Culture Collection Centre, Faculty of Applied Sciences, UiTM. Bacteria cultures are maintained on fresh nutrient agar plates and broths. Regular sub-culturing was done to avoid nutrients deprivation of bacteria cultures. Gram staining and microscopic examination of cultures were carried out to ensure the authenticity of bacteria culture. All microbiological works were done using proper aseptic techniques to avoid cross contamination.

Nutrient agar used in this experiment was prepared in a 500 ml bottle by dissolving 14 g of nutrient agar powder in 500 ml of distilled water and autoclaved for 30 minutes at 121°C, 15 psi for sterilization. Sterile media was poured into sterile disposable petri plates and leave to hardened in a laminar flow cabinet. Hardened nutrient agar plates were closed and sealed using parafilm prior use.

Nutrient broth was prepared by dissolving 6.5 g of nutrient broth powder in 500 ml distilled water and sterilized using autoclave. Sterile broths are kept in culture bottles. All growth mediums used in this experiment are prepared a day before the experiment.

Gas chromatography coupled with mass spectrometer used in this experiment was Varian GC/MS 4000.

2.2 Soxhlet Extraction Procedure

Solvent extraction of *C. caudatus* was done using a 1 : 10 (*C. caudatus* : solvent) in a Soxhlet apparatus for 8 hour using ethanol as extraction solvent. Extract produced from the process was filtered using Whatman No. 2 filter paper into a 1000 ml flask, to remove impurities. The remaining solvent was purified using rotary evaporator. Crude *C. caudatus* extract were kept in 250 ml flasks and labeled prior further experiments.

2.3. Disk Diffusion Test

1 ml of liquid cultures of *S. aureus* and *E. coli* were aseptically transferred onto nutrient agar plate using micropipette. A sterile cotton swab was used to spread the inoculum on the entire agar surface. Inoculated plates were allowed to dry for 5 minutes to ensure adsorption of inoculum into the medium. All plates were done in triplicates.

A sterile filter paper disks with 1 cm diameter was used as wafers. Using a sterile forceps, wafers were dipped in the crude *C. caudatus* extract and placed on the inoculated plates with a 30 mm space in between disks. Each disk was press lightly to ensure attachment on the agar surface. Wafers were labeled as (a) pure *C. caudatus* extract, (b) extract diluted in 50% ethanol, (c) extract diluted in 50% methanol, (d) commercial disinfectant, and (e) commercial disinfectant in water (50%).) All plates were incubated overnight at 37°C. After 24 hours of incubation, all plates were taken out from the oven and the clear zone around the disks is measured.

2.4. Characterization of Crude C. caudatus Extract

Crude extract was filtered using a 45 μm filter. GC was performed using a Agilent Technologies 7890A GC System, G3171A, USA equipped DB-5ms column with an inner diameter of 250 mm and 0.25 mm film thickness. A sample volume of 1 mL was injected with a splitless mode into the GC-MS system connected with an MS/MS triple quad detector The initial oven temperature was set to 50°C for 3 minutes, and then increased to a target temperature of 315°C for 10 minutes at a rate 10°C/min. Helium was used as the carrier gas at a rate of 1 mL/min. The injector and ion source temperatures were set at 330°C and 250°C, respectively.

Mass spectra were acquired using a full scan, monitoring mode with a mass scan range of 50 to 550 m/z after a solvent delay of 7 minutes. The spectra for each of the chromatogram peaks were compared with the National Institute of Standards and Technology (NIST) database library and the retention time (RT) index of common primary and secondary metabolites. Chromatogram and list of compounds are printed for further interpretation.

3. Results

3.1 C. caudatus Extract

Figure 2 (a) and (b) show the crude extract in ethanol and extract after ethanol removal respectively. *C. caudatus* extract is dark green with odoriferous smell. It can be seen from both figures that purified extract is darker than the extract in solvent. Soxhlet extractions were done three times to yield approximately 50 ml of crude extract. Extracts were kept in a closed conical flask prior further experiment.

Fig. 2(a). Crude extract in ethanol

Fig. 2(b). Purified extract of *C. caudatus*

Green pigment in extract is caused mainly by the presence of chlorophyll in *C. caudatus* leaves. Additionally, the presence of various flavonoid compounds including anthocyanins and anthocyanidins also contributes to colour of solution. Anthocyanins and anthocyanidins are either orange, red, or blue. The combination of these pigments with chlorophyll, produces dark hue, resulting in the darker shades of green [8].

The presence of flavonoids and other aromatic phytochemicals also caused the odoriferous smell in the liquid extracts [9]. Plants are known to produce aromatic phytochemicals and bioactive compounds which help them to pollinate and as preventive mechanisms against pests. A recent study by Phong et al. demonstrated the presence of tannins, alkaloids, triterpenoid, flavonoids and saponins in extracts of several plants including *C. caudatus* [9]. Even though triterpenes and saponins produce agreeable odour, alkaloids, on the other hand, are characterized by their mousy smell. This contributes to the odoriferous smell in several plant extracts.

3.2 Kirby-Bauer Disk Diffusion Test

Figure 3(a) and (b) showed the inhibition zone caused by *C. caudatus* extracts on *S. aureus* and *E. coli.* Clear zones can be seen surrounding all disks which indicate the bactericidal effects of the tested compounds. Additionally, Table 1 summarizes the diameter of inhibition zones for each tested compound in comparison to a commercially available disinfectant. Disk containing extracts (a), (b)

and (c) exhibited dark zone in their surrounding due to diffusion of extracts' pigment into the agar. Nevertheless, there are no growth of bacteria in those areas.

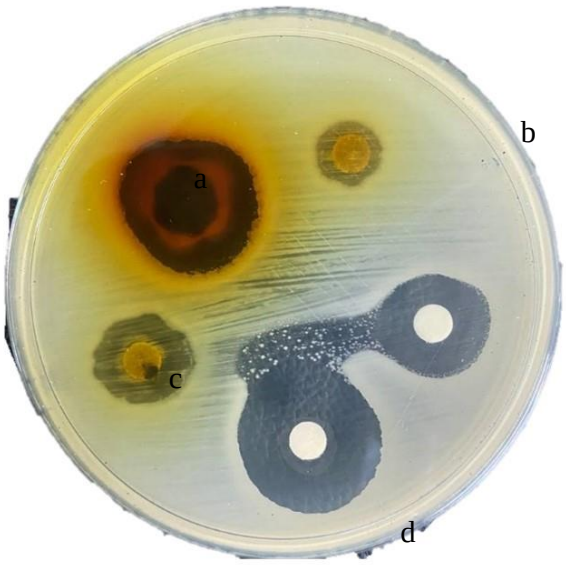

Fig. 3(a). Disk diffusion test for *S. aureus* (a). Pure *C. caudatus* extract, (b). Extract diluted in 50% ethanol, (c). Extract diluted in 50% methanol, (d). commercial disinfectant, (e). commercial disinfectant in water (50%)

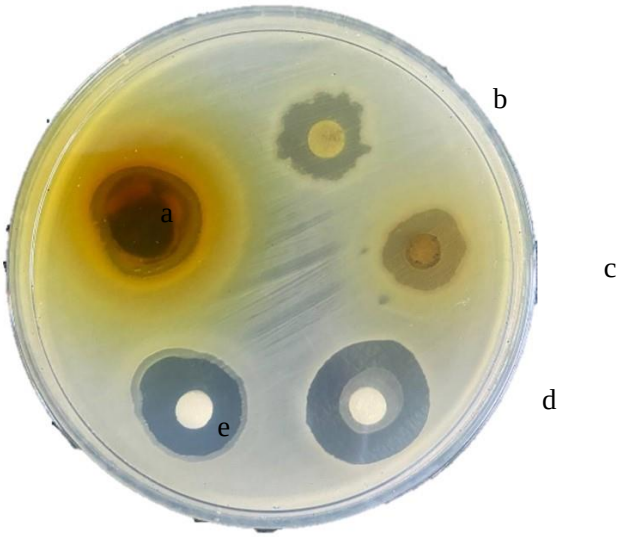

Fig. 3(b). Disk diffusion test for *S. aureus* (a). Pure *C. caudatus* extract, (b). Extract diluted in 50% ethanol, (c). Extract diluted in 50% methanol, (d). commercial disinfectant, (e). commercial disinfectant in water (50%)

Table 1
Diameter of inhibition zones for disk diffusion test

Bacteria strain	(a)	(b)	(c)	(d)	(e)
S. aureus	23 ±0.70	15±1.0	15±0.0	22 ± 0.0	16 ± 0.0
E. coli	22±0.75	15±1.0	10±0.43	22±0.8	16 ± 0.0

The diameter of inhibition zones in *S. aureus* plates ranging from 15 to 23 mm. The smallest zone is produced on *E. coli* plate, around the filter paper disk with extract diluted with methanol and the largest zone is produced on *S. aureus* with wafer dipped in undiluted extracts. Extracts diluted in methanol and ethanol showed reduce in the size of inhibition zones. Alcohols are known for their ability to eliminates microorganisms especially pathogens. This study initially expected the zone of inhibition surrounding disk containing *C. caudatus* extracts with methanol and ethanol will be larger than the extract itself. However, the findings showed the opposite. The reason behind this might be the evaporation of alcohols, leaving the diluted extracts with less antibacterial effects. The other reason is alcohols with less than 50% concentration, which used in this study, are known to have less antibacterial activity [10].

Crude extract of *C. caudatus* was shown to prevent the growth of both *S. aureus* and *E. coli* which represent Gram-positive and Gram-negative bacteria respectively. Sizes for inhibition zone for both crude extracts are comparable to the ones in commercial disinfectant. Gram positive and negative bacteria differ in their cell wall structure where the later has thinner peptidoglycans layer. Compounds such as fatty acids (both unsaturated and saturated) and alcohols break the cells by attacking the cell membrane which eventually eliminate the pathogens [11]. The mode of action of plant extracts seems to be linked to its ability to modulate membrane integrity and to restrain bacterial motility, most likely by its abundant and trace fatty acid contents [12].

The effects of different bactericidal agents vary on different types of microorganisms. Generally, Gram-negative bacteria are more resistant towards antimicrobial agents than Gram-positive due to the presence of an outer membrane surrounding the cell wall. This unique and distinctive feature prevent most antibacterial agents to act against them.

3.3 Active Compounds in C. caudatus Extract

Extracts from tropical plants and herbs are known for their bioactive properties. These compounds are being used extensively for various purposes especially in therapeutic and pharmaceutical products. Findings from this study agreed with this statement as many bioactive compounds were identified in *C. caudatus* extract. Figure 4 demonstrates the GC-MS analysis of crude *C. caudatus* extract and Table 2 summarizes the significant bioactive compounds.

Among the identified compounds includes n-hexadecanoic acid (palmitic acid), stigmasterol, phytol and neophytadiene. These compounds are well known for their inhibitory effects on microorganisms and are commonly used as main ingredients in commercial antiseptics and disinfectants. The medicinal and therapeutic properties of tropical plants are being studied extensively. Various study had proven that these plants not only having bioactive compounds responsible to inhibit the growth of pathogens, but they are also act as antitumor in animals including human [13-15]. The present of the said bioactive compounds are vital for these exceptional properties.

Phytol is a diterpene alcohol, which can be derived from plant chlorophyll and is widely used as a food additive and in medicinal fields. A study by Lee in 2016, demonstrated how phytol induced oxidative cell death in *P. aeruginosa* by increasing the intracellular reactive oxygen species (Lee, 2016). Another study using phytol extracted from *Leptadenia pyrotechnica*, showed this compound is able to suppress the growth of several bacteria including *E. coli, C. albicans A. niger* and *S. aureus* [16].

Neophytadiene, a diterpene, is also known in its role as an anti-inflammatory and an antimicrobial agent. This compound is commonly extracted from plants and algae and can kill or slow the growth of microorganisms, including bacteria, viruses, fungi and protozoans. Extraction using acetone of an African plant, *Markhamia obtusifolia* yielded the presence of neophytadiene (4.38%) and palmitic acid (3.61%) with antifungal and antioxidant potential [17]. Apart from leaf extracts, neophytadiene also present in fruits. Methanolic extract of kiwi fruit revealed the presence various antioxidants including 0.97% of neophytadiene [18].

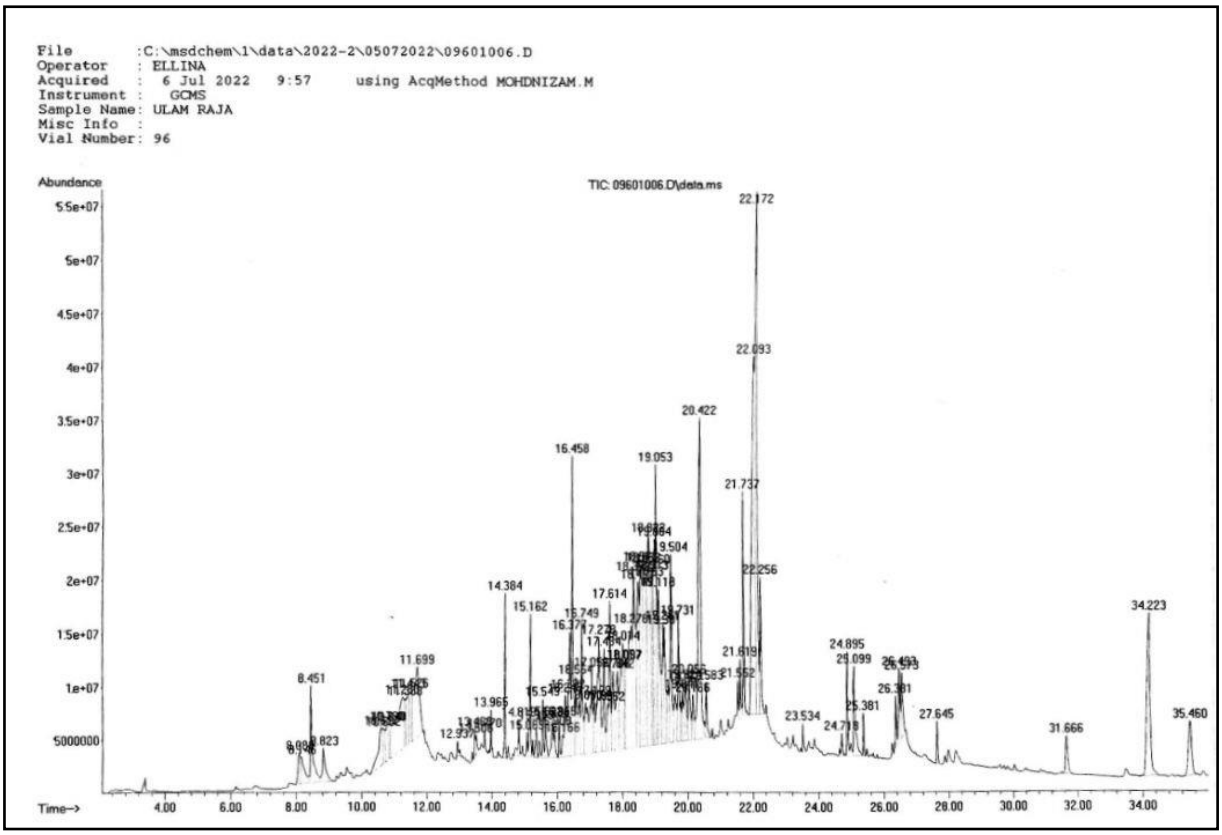

Fig. 4. GC-MS analysis of *C. caudatus* extract

Table 2
Bioactive compounds of *C. caudatus* extract

Number	Proposed compound	RT (min)	Molecular Formula	Peak Area (%)
1	Phytol	19.053	$C_{20}H_{40}O$	1.94
2	n-Hexadecanoic acid	20.422	$C_{16}H_{32}O_2$	5.33
3	Stigmasterol	34.223	$C_{29}H_{48}O$	3.16
4	Neophytadiene	19.053	$C_{20}H_{38}$	1.94

Stigmasterol is an important plant sterol that can inhibits the growth of bacteria and fungi. A study by Yusuf *et al.,* [19] in 2018, demonstrated the effectiveness of stigmasterol isolated from the stem bark of *N. macrophylla* in inhibiting the growth of several human pathogens including methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), *S. aureus*, *Streptococcus faecalis*, *E. coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida krusei* The isolated compound also showed antifungal activity against *Candida* spp., which was comparable to the inhibitory effect of fluconazole. These findings showed that plant extract containing stigmasterol can be used in the development of antimicrobial products for human use. Additionally, stigmasterol has been reported for its anticancer properties against several cancer cell lines including lung, ovary, stomach, and estrogen-dependent human breast cancers.

Another significant compound identified was palmitic acid. Palmitic acid is a saturated fatty acid with a 16-carbon chain mostly found in animals, plants and microorganisms. Many fatty acids are known to have antibacterial and antifungal properties and acid is widely used in a variety of applications, including personal care products and cosmetics. Fatty acids can modulate immune responses by acting directly on T cells. The dietary, conjugated linoleic acid exerts anti-inflammatory effect by decreasing production of the inflammatory mediators such as prostaglandin E2, IL-6, IL-1β,

TNFα, and nitric oxide. The terrestrial plant-derived n-3 fatty acid, α-linolenic acid (ALA) exhibited higher anti-inflammatory effect than the seaweed-derived n-3 fatty acid, docosahexaenoic acid (DHA) [20]. Another study investigated fatty acids including palmitic acids effects on bacteria and fungi showed varying degrees of antibacterial and antifungal activity against *E. coli UPEC*, *P. aeruginosa*, *K. pneumoniae*) and the fungal pathogen *Candida albicans* [13].

4. Conclusions

The present study showed that bioactive compounds from *C. caudatus* can be extracted using simple Soxhlet extraction with ethanol. Extracts are proven potent against the growth of both Gram -positive and Gram-negative bacteria namely *E. coli* and *S. aureus* with zone of inhibition ranging from 22 to 24 mm.

The ability of extracts to inhibit bacteria growth is comparable to a commercial disinfectant. Further analysis of *C. caudatus* leaf extracts revealed several significant bioactive compounds including palmitic acids, stigmasterol, neophytadiene and phytol which are common ingredients used in the production of pharmaceutical products. Further study needs to be carried out to seek feasible technology in incorporating the findings from this study into consumer product.

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