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Valorisation of biomass waste into bioproduct: Potential use of cannabis extracts for bacterial inhibition activity

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Abstract *Cannabis Sativa* is a plant containing two main active components; tetrahydrocannabinol and cannabidiol that can be widely used in pharmacological and medical benefits. Main parts such as seeds, flowers, and leaves are mostly used in several applications; medicines, beverages and foods. While other parts such as stems and incomplete leaves (withered-, dried, and spotted-) were removed to disposal. In this research, cannabis leaves were extracted using 3 solvents namely deionized water, ethanol, and hexane.The extracts were determined to inhibit two pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*). Some parameters; ratios of extracts: solvents (1:5, 1:7 and 1:10) and temperatures (30 °C, 35 °C and 40 °C) were also considered. The results revealed that extracts yield and total phenolic compound (TPC) content slightly increased as all parameters increased. The maximum yield and TPC content were 16.46% and 64.14 mg GAE/g DW when hexane was applied under ratio of 1:10 at 35°C. The extracts obtained from all conditions could inhibit bacterial inoculation. Furthermore, the use of hexane yielded more effective inhibitor than the cases of water and ethanol. Moreover, extracts were also able to inhibit both bacteria at the lowest concentration for tests of minimum inhibition concentration and minimum bactericidal concentration.

Keywords: *Cannabis Sativa*; *Escherichia coli; Staphylococcus aureus;* Minimum inhibition concentration (MIC); minimum bactericidal concentration (MBC).

1. Introduction

Cannabis *(Cannabis Sativa)*, also known as marijuana, a psychoactive plant, is widely found and widespread in central Asia, including Siberia, Persia, India and China. According to Thailand has launched a policy for cannabis liberalization since 2019. It becomes interesting among peoples around the country.

Previous studies have reported that extraction of medicinal plant composted of more active antimicrobial compounds from such as saponins, sterols, flavonoids, phenols, anthraquinones, and etc. In case of cannabis, there are also contained of many active important substances that have pharmacological effect especially two main psychoactive components of tetrahydrocannabinol (THC) and cannabidiol (CBD) that can be widely applied in medical benefits. Furthermore, it also has antimicrobial properties that could be applied to use as antimicrobial material. Since, the problem of antibiotic-resistant micro bial has still serious negative effects on human health until now.

Typically, main parts of cannabis including flowers, leaves and seeds are mostly used in various applications such as medicines, foods, beverages and etc. However, there are some remaining wastes of stems and leaves can be utilised to produce as a value added product under zero waste concept.

Therefore, objectives of the research work were to investigate the extraction of cannabis leaves waste using three different extractants such as deionised water, ethanol, and hexane. Some parameters affecting on extraction yield and the active extracts obtained in term of total phenolic compound (TPC) concentration were also investigated. In addition, minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were tested to evaluate for inhibition activity of two specific pathogenic bacterials (Gram-negative, *Escherichia coli* TISTR073 and Gram-positive, *Staphylococcus aureus* ATCC25923).

2. Methodology

2.1 Preparation of cannabis leaves

Cannabis leaves were weighed at around 1,000 g and ground to a fine powder using spinning blender. Then, the powder was seived by 250 µm metal screen (No.60) and dried in a hot air oven at 105 °C for 3-4 days as shown in Figure 1.

2.2 Extraction of cannabis powder

Cannabis powder was weighed at 10 g using a ratio of the powder and deionized (DI) water at 1:3 (w/v). A mixture was added into Erlenmeyer flasks. Then, it was incubated on orbital shaker under 300 rpm, for 24 h and temperatures were varied at 30, 35 and 40 °C. It should be noted that the experiments were done in triplication for reproducibility. The extracts were then filtered using filter papers (Whatman No.1) and dried in a hot air oven at 50 °C for 24 h. The extract concentration was adjusted into 100 mg/mL as shown in Figure 2. Afterthat, the extracts

mixture was measured in term of total phenolic compounds (TPC) content. The extract containing maximum TPC content was selected to use in further step.

2.3 Optimal condition for cannabis powder extraction

Some parameters affecting on extraction such as ratios of the powder and solvents (1:5, 1:7 and 1:10) and different solvents (DI water, ethanol and hexane) were varied and performed on incubator shaker at 35°C at 300 rpm for 24 h. After that, the extracts was recovered following the precedure mentioned as the previous section.

2.4 Determination of total phenoic compounds (TPC)

Cannabis powder obtained extracted using water were analysed to determine the TPC content. A 0.1 mL extract and 9.9 mL distilled water were pipetted into the test tube. Then, the mixture was diluted to be 100 times. After that, 1 mL of the diluting mixture and 1 mL of 10% v/v Folin-Ciocalteu reagent were mixed in vertex mixer. The mixture was left for 8 min and then a 3 mL of 7% (w/v) sodium carbonate (Na_2CO_3) was added and shaken well. Then, it was left in the dark for 60 min and measured the absorbance at 760 nm using spectrophotometer. It was noted that distilled water was used as a blank solution. Then, the TPC content was calculated comparing to gallic acid standard graph.

2.5 Determination of bacterial inhibition activity

A bacterial inhibition activity was tested using disc diffusion assay. The sterile solution was diluted to get a turbidity value of 0.5 McFarland (equivalent to 1.5 × 108 CFU/mL). The diluent was measured turbidity value using a spectrophoto meter at 600 nm using sterile distilled water as a blank. The 0.1 mL culture was pipetted and spreaded on nutrient agar (NA) as medium plates as shown in Figure 3. The plates were left until dry for 15 min. A volume of 50 µL TPC extracts (100 mg/mL) obtained from water, ethanol and hexane were added in a paper disc. It was placed on a freeze dried NA medium. Sterile distilled water was used as a negative control for testing a Gram-positive bacteria, *S. aureus* ATCC25923 and a Gram-negative, *E. coli* TISTR073. Then, the paper disces were incubated at 37°C for 24 h. The diameter of the area meant a bacterial growth (inhibition zone), was measured in mm.

2.6 Tests of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was considered using broth microdilution methods. In brief, 50 µL liquid NB culture medium was pipetted into all 96 wells plates from rows 2-12 (except row 1). After that, the extract was diluted to get desired concentration and then was added to 100 mL into row no. 1 evey well. The 2-fold dilution was done from row no. 2 until row no. 11. Finally, series of 10 concentrations were obtained folowing row number. After that, 50 µL of bacterial suspension was added into 96 well plates. They were incubated at 37 °C for 18 h and then were read.

For the MBC test, a volume of 10 µL from all wells without turbidity was taken to spread onto N/A medium plates and incubated at 37 °C for 24 hrs. The MBC results were read and impl.

3. Results and Discussion

3.1 Extraction of cannabis leaves

Some parameters, temperature and ratios of cannabis extracts and water, affecting on extraction yields were investigated. The optimal extraction were obtained as shown in Tables 1-2.

Table 1 Cannabis extracts obtained after extracting by water using ratio of 1:3 at various temperatures

Extracting solvent	Temperature (°C)	Extracts (g)	Extracts yields (%)
DI Water	30	0.99 ± 0.06	4.94 ± 0.30
	35	0.95 ± 0.04	4.76 ± 0.18
	40	0.96 ± 0.13	4.76 ± 0.67

Table 2 Cannabis extracts obtained after extracting by water at 35 °C at different ratios of cannabis extract: water

Extracting solvent	Ratio	Extracts (g)	Extracts yields (%)
DI Water	1:3	0.95 ± 0.04	4.76 ± 0.18
	1:5	1.18 ± 0.04	11.76 ± 0.44
	1:7	0.65 ± 0.04	12.72 ± 0.92
	1:10	0.84 ± 0.05	16.46 ± 0.54

Table 1 shows cannabis extracts obtained at 0.99 ± 0.06 g, 0.95 ± 0.04 g and 0.96 ± 0.13 g, respectively, that were equal to percentages of extracts yield at 4.94 ± 0.30 %, 4.76 ± 0.18 % and 4.76 ± 0.67 %, respectively. The highest extraction efficiency reached at 1:3 ratio and 30 °C. In Table 2, when the ratios of cannabis and DI water were varied; 1:3, 1:5, 1:7 and 1:10 at 35 °C, the extracts were obtained approximately 0.95 ± 0.04 g, 1.18 ± 0.04 g, 0.65 ± 0.04 g and 0.84 ± 0.05 g, respectively that were equal to in 4.76 ± 0.18 %, 11.76 ± 0.44 %, 12.72 ± 0.92 % and 16.46 ± 0.54 %, respectively. The condition of 1:10 ratio and 35 °C, giving the highest yield. It was then tested in further. The results obtaine were in agreement with Ahmed et al (2019) and Al Ubeed et al (2022) who found that cannabis leaves were extracted in maxium yield using water compared among several solvents

3.2 Optimal condition for cannabis powder extraction

Table 3 Cannabis extracts obtained after extracting by DI water, ethanol and hexane using ratio of 1:10 at 35°C

Extracting solvents	Extracts (g)	Extracts yields (%)
DI Water	0.84 ± 0.05	16.46 ± 0.54
Ethanol	0.06 ± 0.02	4.76 ± 0.18
Hexane	0.06 ± 0.05	4.76 ± 0.67

It was found that cannabis powder extracted using DI water, ethanol, and hexane under the ratio 1:10 at 35 °C obtained at 0.84 ± 0.05, 0.06 ± 0.02 and 0.06 ± 0.05, respectively. The maximum yield was acheived at 16.46 ± 0.54 when water was used. However, the extract yields were quite low. It might be caused by the evaporation of the solvent used during the extraction period.

3.3 Determination of total phenoic compounds (TPC)

Table 4 shows the TPC contents extracted from water using the ratio of 1:10 under 3 different temperatures

(30, 35 and 40 °C). The TPC were reached at 50.11±0.34, 58.52±1.05 and 57.24± 0.20 mg GAE/g DW, respectively. The hightest TPC content was obtained at 35 °C was 58.52 ± 1.05 mg GAE/g DW). Therefore, cannabis extracts were selected at 35 °C for analysis by ratio variation and further testing for antimicrobial activity. The TPC obtained was agreed with previous work reported by Ahmed et al (2012). However, they could not find the TPC extracted uisng hexane. That was similar result in which the TPC obtained in very small amout.

Table 4 TPC contents extracted by DI water, ethanol and hexane using ratio of 1:10 at 30, 35, 40°C

Extracting solvent	Temperature	TPC (mg GAE/g DW)
DI Water	30	50.11±0.34
	35	58.52±1.05
	40	57.24±0.20

3.4 Determination of bacterial inhibition activity

Table 5 Bacterial inhibition activity test by TPC contents extracted by water, ethanol and hexane

TPC in Extracting solvent	Clear zone (mm)	
	E. coli	*S. aureus*
DI Water	12.00±0.81	21.66±0.47
Ethanol	23.00±0.81	13.66±1.24
Hexane	9.83±1.43	7.3±0.47

In Table 5, In case of *S. aureus* a clear or inhibition zone showed in bigger than *E. coli* after the TPC extracted by water was dropped in the plate as shown in Figure 4. On the other hand, it was less inhibited than that of *E. coli* in both cases of ethanol and haxane (13.66±1.24 and 7.3±0.47 mm) as shown in Figure 5. It would be caused by Gram-positive bacteria having an encapsulated cell membrane which was mainly contained of hydrophobic peptidoglycan. Therefore, it was clearly that Gram-negative bacteria mostly consited of hydro phobic lipopolysaccharide. TPC extracted by low polar or non polar substances of ethanol and hexane performed better than water.

3.5 Tests of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

Table 6 MIC Test for TPC extracted by DI water, ethanol and hexane

TPC in Extracting solvent	Minimum Inhibition Concentration (MIC) (mg/mL)	
	E. coli	*S. aureus*
DI Water	25	12.5
Ethanol	12.5	25
Hexane	0.05	0.05

The minimum concentration of TPC extracted by water, ethanol, and hexane can inhibit bacterial growth in terms of minimum inhibition concentration (MIC). As summarised in Table 6, the lowest concentrations of TPC extracted by water, ethanol and hexane for *E. coli* were 25, 12.5 and 0.05 mg/ml, respectively. Meanwhile, in case of *S. aureus* were 12.5, 25 and 0.05 mg/ml, respectively. It was clearly found that hexane-derived cannabis extract was able to inhibit the bacterial growth even using in minimum concentration.

In Table 7, the minimum bactericidal concentration (MBC) was found at the lowest concentrations of water, ethanol and hexane for E. coli 50, 25 and 0.05 mg/ml, respectively while the case of S. aureus, the MBC were 25, 50 and 0.05 mg/ml. In addition, the TPC extracted by hexane showed the best MBC results among them. extracted cannabis extracts Able to disinfect even with a small concentration of extract. It would be suggested that the antimicrobial activity would be determined for other yeast, mold or other pathogetic bacteria. These were agreed with previous works who reported that the TPC extraced from cannabis leave could control in the antimicrobial activity. In addition, they also have mentioned that the inhibitory effect could be enhanced when TPC extracts were used in combinations of other leat extract such as quercetin, gallic acid, catehic and etc. (Ali, et al (2012), Chakraborty, et al (2018), Hoda, et al (2019), Anumudu, et al (2020), Shah et al (2020).

Table 7 MBC Test for TPC extracted by water, ethanol and hexane

TPC in Extracting solvent	Minimum Bactericidal Concentration (MBC) (mg/mL)	
	E. coli	*S. aureus*
DI Water	50	25
Ethanol	25	50
Hexane	0.05	0.05

4. Conclusion

The optimum condition for cannabis extracts in terms of % yield and TPC content was acheived under specific ratio of an extracting solvent: extracts and temperature. The extracts could obviously inhibit both in *S. aureus* and *E. coli*. However, the case of *S. aureus* gave better result than the case of *E. coli*. The MIC and MBC results were confirmed for all extracting solvents used, especially in case of hexane, which showed the highest inhibition and sterilization efficiency. Interestingly, This current study can be used as a guideline for further applications.

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Figure 1 Cannabis powder derived from cannabis leaves

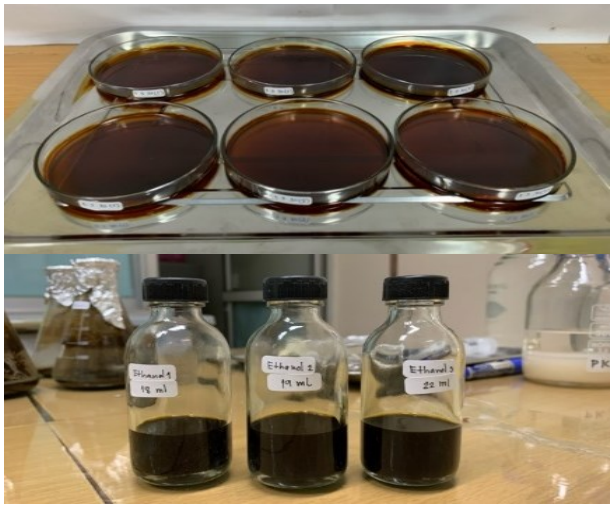

Figure 2 Cannabis extracts (upper) after drying and (lower) after adjusting into 100 mg/mL

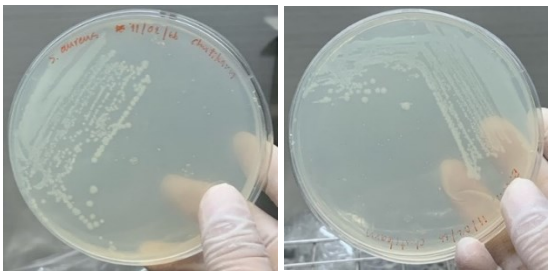

Figure 3 (left*) S. aureus* ATCC25923 and (right) *E. coli* TISTR073 grown after streak plates

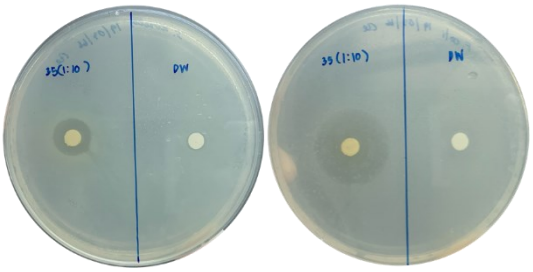

Figure 4 Clear zone by cannabis extracts using deionised water and deionised water control on (left) *S. aureus* ATCC25923 and (right) *E. coli* TISTR073

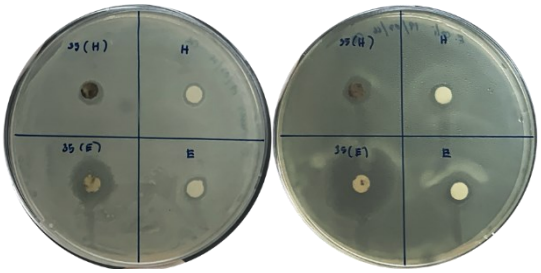

Figure 5 Clear zone by cannabis extracts using ethanol (E) and hexane (H) and their control on (left) *S. aureus* ATCC25923 and (right) *E. coli* TISTR073