

Antimicrobial Properties of Optimized Microwave-assisted Pyrolygneous Acid from Oil Palm Fiber

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ABSTRACT

The demands for an antimicrobial agent of natural origin has increasingly grown in recent years due to the adverse health effects from the long-term application of conventional chemical-based agent. To date, there are very few reports available on the use of pyrolygneous acid produced in optimized condition, as antimicrobial agent. Thus, this study reports on the evaluation of the antimicrobial properties of oil palm fiber-derived pyrolygneous acid produced using microwave heating at optimum condition (MWPA). The optimized MWPA was concentrated and extracted using ethyl acetate and was further determined for its antimicrobial properties against Gram positive and Gram negative bacterial strains through Minimum Inhibitory Concentration (MIC) and Minimum Biocidal Concentration (MBC) analysis. Results obtained shows that MWPA exhibited high inhibition zone that varied between 13 ± 0.58 to 28.67 ± 0.88 mm toward all strains screened in the present study with low range of MIC value from 0.651 ± 0.13 to 1.563 ± 0.00 mg/ml. This indicated that CPAEA MWPA derived from OPF exhibited a strong antimicrobial activity and might be projected as alternative natural source as antimicrobial agent.

INTRODUCTION

The antimicrobial agent is a substance that has the capability to kill or inhibit the growth of microorganisms. Many researchers have focused on elucidating the potential of natural products as a new source of bioactive molecules due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas such as metabolic diseases and anti-infective (Nasir *et al.*, 2015). To date, natural products are still one of the major sources to provide new drug molecules mainly due to its rich phenolic contents that is a proven antimicrobial compound. Various researchers have investigated the potential of a wide range of plant sources and microbial extracts, essential oils, pure secondary metabolites and newly synthesized molecules as an antimicrobial agent using many well-published methods (Balouiri *et al.*, 2016).

Pyrolygneous acid (PA) is a crude condensate obtained by condensing the smoke produced during pyrolysis process

of plant biomass under the absence of oxygen. This liquid is a complex mixture of highly oxygenated liquid consisting of 80-90% of water and 10-20% organic compounds with a distinctive smoky odor as well as reddish-brown and acidic pH in nature. Moreover, PA normally comprised of a complex mixture namely guaiacols, catechols, syringols, phenols, vanillins, acetic acid, ester, derivatives of furan and pyran (Wanderley *et al.*, 2012). In fact, the presence of phenolic compounds, organic acid as well as carbonyls has generally contributed to the antimicrobial activity of PA. According to Karou *et al.* (2005), polyphenols are well documented to have microbicide activities against a huge number of pathogenic bacteria. In addition, oxidized polyphenols also have inhibitory activity against bacterial growth. Few factors have been related to the mechanism of polyphenols toxicity against microbes such as cell envelope transport proteins, inhibition of hydrolytic enzymes (proteases and carbohydrases) and non-specific interactions with the carbohydrates (Mirkarimi *et al.*, 2013). Amongst examples for PA derived from plant biomass that have been shown containing antimicrobial properties include PA from *Rhizophora apiculata* (Ibrahim *et al.*, 2014), oil palm shell (Achmadi *et al.*, 2013) and *Eucalyptus urograndis* (Araújo

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et al., 2018). However, very few reports are available on the antimicrobial properties of PA derived from oil palm fiber (OPF) that was produced using microwave-assisted heating in optimum condition, which is ultimate to be elaborated in this study.

MATERIALS AND METHODS

The extraction process of microwave-assisted pyrolygneous acid (MWPA)

MWPA was produced from the pyrolysis of oil palm fiber at optimized condition (540°C within 23 minutes of holding time with 86.74 g of AC load; data for optimization work not shown as not the focus for this study). It was then filtered through Whatman filter paper No.4 followed by extraction using 99.5% of ethyl acetate (EA) using method proposed by [Rungruang and Junyapoon \(2010\)](#) with slight modification as follows; the MWPA:EA mixtures (1:1.5) was homogenously shaken for 3 minutes under ambient condition in a 250 ml separatory funnel. It was allowed to stand around 30 minutes where the top layer (EA extract) was collected in a 250 ml flat bottom flask while the remaining residues in the sample were re-extracted twice using fresh EA. The combined supernatants were then concentrated using rotary evaporator (Laborota-4003, Heidolph) for 10-15 minutes at 80°C where the pooled and concentrated extracts were termed as a concentrated pyrolygneous acid extract of ethyl acetate (CPAEA) of MWPA.

Characterization of MWPA

The CPAEA was analyzed using GC-MS (QP500, Shimadzu) according to [Zhai *et al.* \(2015\)](#) with a modification as follows; 100 µl of CPAEA was dissolved in 2 ml of 95% methanol (HPLC grade) and was filtered using 0.2 µm membrane syringe filter before it was injected into the GC-MS column. About 1 µl of the sample was injected into the capillary column (BXP-5) with a diameter of 29.4 m × 0.25 mm, the film thickness of 0.25 µm, the flow rate of 2 ml/min and he as the carrier gas. The sample was injected using the splitless injection conducted at a split ratio of 25:1 with 300°C of injection temperature according to the following scheme; 50°C for 2 min with a heating rate of 10°C/min up to 300°C. The final temperature around 325°C was held for 10 minutes and the total runtime for each sample was around 37 minutes. The electron ionization used was 70 eV in mass spectrometry (MS) detection and the mass fragment was detected between 400 and 500 m/z. The ion source temperature and transfer line were set at 200°C and 300°C respectively. The GS-MS profile obtained was identified using database available in the National Institute of Standards and Technology (NIST), USA.

Antimicrobial activity assay

Bacterial strain screening

The antimicrobial properties of optimized CPAEA were evaluated against four different bacterial strains namely; *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Lactobacillus plantarum* WICC B18 and *Escherichia coli* ATCC 25922. All cultures were subcultured on selective agar slants namely, Simmon Citrate Agar (SCA, Merck), Hicrome™ *Bacillus* Agar (HBA, Fluka) McConkey Agar (MA, Merck), Baird-Parker

Agar (BPA, Merck) and de Man, Ragosa, Sharpe Agar (M.R.S.A, Oxoid) prior to incubation at 37°C for 24 hrs. All cultures were kept at 4°C until further used. All strains were sub-cultured in nutrient agar (20 g/L, Merck, Germany) every two weeks to maintain viability.

Disk diffusion method of antimicrobial assay

The disk diffusion method was carried out according to [Ibrahim *et al.* \(2014\)](#) with a slight modification as follows; sufficient amount of a 24-hours bacterial culture (grown on respective agar plates) was transferred (using cotton swab) into a series of sterilized bottle samples (27 mm × 61 mm) until the turbidity reaches the equivalent of 0.5 McFarland standard (1.5 × 10⁸ cells/mL) or can be accurately measured using a spectrophotometer with a 1-cm light path at 625 nm which corresponding to an absorbance reading of 0.08 to 0.1 ([Bandet *et al.*, 2014](#)). Then, 100 µl of the cell suspension was pipetted onto a nutrient agar plate and was distributed uniformly using the spread plate technique. Following this, sterilized antibiotic disc (Whatman, United Kingdom) with 6 mm diameter was impregnated with 30 µL of 100.0 mg/mL of CPAE extract, prior to air dried (in a sterilized condition). It was then placed onto the nutrient agar and incubated for 24 hours at 37°C. The experiments were carried out in triplicates and the antimicrobial activities were evaluated by measuring the diameter of the halo-zone formation around the disc. Chloramphenicol (30 µg/mL, Oxoid) and methanol were used as positive and negative control respectively.

Relative percentage inhibition

The relative percentage inhibition growth of CPAEA towards all strains was determined using the following equation [Naz and Bano \(2012\)](#);

$$\text{Relative percentage inhibition of test extract} = (100 \times (X - Y)) / (Z - Y), \quad (1)$$

where X denotes total area of inhibition of the test extract, Y is the total area of inhibition of the solvent, and Z is the total area of inhibition of the standard drug. The total area of the inhibition was calculated using area = πr^2 , where r is the radius of the zone of inhibition.

Minimal inhibitory concentration (MIC)

The MIC evaluation was analyzed based on the microdilution method using 96 plates well according to [Elshikh *et al.* \(2016\)](#) and [Wiegand *et al.* \(2008\)](#) with some modifications. Initially, 100 µL of nutrient broth (NB) was transferred into each of the 96 wells microplate (Kartell®). Then, 100 µL of CPAEA was pipetted and transferred into the 1st well and thoroughly mixed. After well mixing, 100 µL of the mixture was transferred into the 2nd well to make it a two-fold dilution. This procedure was repeated until the 10th well. Then, 20 µL inoculum of each bacterial suspension (in sterile distilled water equivalent to 0.5 McFarland solution) was pipetted into each well from the 1st to the 11th well. The plate was then incubated at 37°C for 24 hours and the MIC value was taken as the first well showing no visible growth by using the naked eyes ([Balouiri *et al.*, 2016](#)). The experiments were carried out in triplicates for CPAEA and the results were expressed

as means of three experiments. The 11th well contained nutrient broth and inoculum while the 12th well contained nutrient broth only. Both acted as control and blank respectively.

Minimum biocidal concentration (MBC)

The analysis of MBC was immediately carried out by streaking the mixture content directly from wells with concentrations higher than the MIC value as recommended by Elshikh *et al.* (2016) using a sterilized cotton swab. The mixture content of each strain that has a concentration higher than the MIC well was inoculated on a nutrient agar plate followed by incubation at 37°C for 20-24 hrs. Each plate was divided into three compartments to make it as triplicate and the MBC value was determined when no microbial growth was detected.

RESULT AND DISCUSSION

Chemical constituent of CPAEA from OPF

The chemical composition of CPAEA was determined using GCMS analysis. The ion chromatogram as well as the relative contents of the compounds identified as depicted in Figure 1 and Table 1, respectively. From the 15 major peaks observed, phenol was identified as the dominant compound with a peak area of approximately 68.25% of the total fractions. Higher fractions of phenol compound observed was high due to the high final temperature applied (540°C) during pyrolysis process as agreed by Wei *et al.* (2010) and Huang *et al.* (2013). This was followed by the 2-Butenedinitrile, (E)- which accounted to 4.75% of the total fractions. Other compounds detected include benzene, alkene, pyran, toluene, hexane, pyridine, amide and nitrogen compounds.

Table 1: Major chemical constituents in optimized CPAEA of MWPA via GCMS analysis.

Peak	Retention Time (min)	Compound Identification	Area (%)	MW (g/mol)
1	1.1897	Benzene	1.4133	78.11
2	1.2241	2-Butenedinitrile, (E)-	2.2886	78.074
3	1.2527	2-Butenedinitrile, (E)-	2.4593	78.074
4	1.4758	Acetamide, 2-fluoro-(amide)	3.11	77.058
5	1.9222	2-Cyclopenten-1-one (ketone)	1.1671	82.102
6	2.4314	2H-Pyran, 3,4-dihydro-(pyran)	0.9209	84.118
7	2.8091	Phenol	68.2507	94.111
8	2.9292	1,2-Difluoroethane (alkane)	0.2637	66.051
9	3.324	Cyclohexane, 1,4-dimethyl-, cis-	0.6028	112.216
10	3.5128	2-Chloro-5,5-dimethyl-1-phenyl-3-hexen-1-ol	1.7171	236.737
11	3.7246	o-Toluidine(toluene)	4.1198	107.16
12	3.9535	2(1H)-Pyridinone, 1-methyl-(pyridine)	3.5875	109.1259
13	5.1551	2-(2,2-Dimethylvinyl)thiophene (thiophene)	1.3834	138.230
14	6.2308	2-(2,2-Dimethylcyclopropyl)thiophene (thiophene)	0.7413	152.257
15	19.5058	1H-1,3-Benzimidazole-1-acetonitrile, 2-(difluoromethyl)-	0.7705	207.180

*MW = molecular weight.

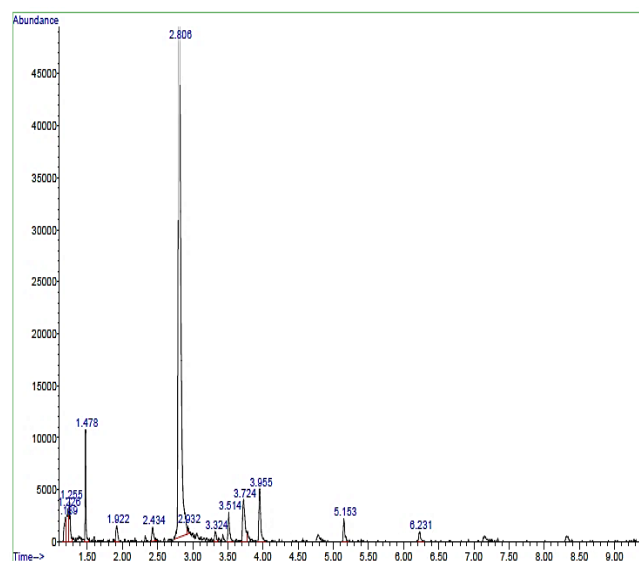


Fig. 1: GCMS ion chromatogram of optimized CPAEA of MWPA from oil palm fiber.

A similar observation was reported by Mathew *et al.* (2015) with 69.5% of phenol present in the dichloromethane extract of pineapple waste-based pyrolygneous acid. Phenol compounds were derived mostly from the thermal degradation of lignin with the cross-linked structures of the phenylpropane units cracking such as hydroxyl- and methoxy-substituted from macromolecular lattice in lignin during the pyrolysis process. In addition, the presence of phenols, ketone, and organic acid compounds also gives an indication of the potentially high antimicrobial properties of CPAEA (Ibrahim *et al.*, 2013). Another constituent such as pyran and its derivatives which includes 2H-pyran, 3,4-dihydro- and 2(1H)-pyridinone, 1-methyl- accounted for 4.51% of the total fractions, of which these compounds contributed to the brown color and smoky odor of the PA (Montazeri *et al.*, 2013). Interestingly, the polycyclic aromatic hydrocarbon (PAH) such as pyrene and phenanthrene, that is highly carcinogenic, were not present in the CPAEA which indicated that highly aromatized pyrolygneous acid can be produced from microwave-assisted pyrolysis heating.

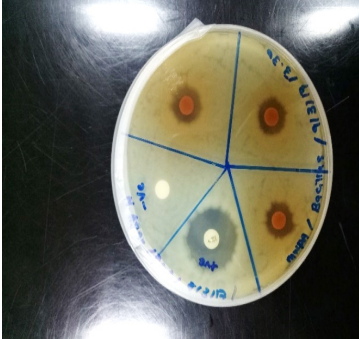
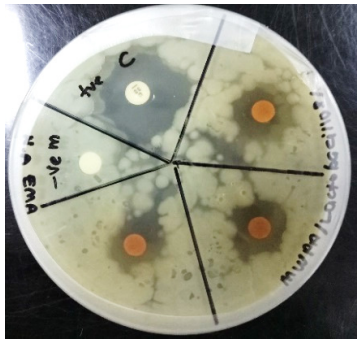


Antimicrobial properties analysis

Inhibition zone activity of CPAEA

All screening bacteria strains observed on a selective agar plate after 24 hr incubation was depicted as in Figure 2. The antimicrobial activity of the optimally-produced CPAEA was evaluated based on the diameter of inhibition zone towards all strains tested. The inhibition zone activity of CPAEA towards all strains tested obtained after 24 hr incubation at 37°C was depicted as in Table 2. It was observed that the inhibition zones on all agar plates were clearly formed around the disc that has been impregnated with CPAEA. The largest diameter for inhibition zone, DIZ (22-32 mm) was obtained for disc impregnated with chloramphenicol (30 ug/ml) compared to the CPAEA (13-28 mm). However, no inhibition zone was observed for disc added with methanol. The antimicrobial activity of CPAEA as observed in this study can be attributed to the presence of phenolic compound and its derivatives as suggested by Ibrahim *et al.* (2013) and Montazeri *et al.* (2013). According to Mohapatra *et al.* (2011), the bacterial strain was sensitive to the sample extract if the DIZ was greater than 11 mm and resistance to the sample if the diameter was less than 7.

Thus, it explained that all the bacterial strains tested in the present study were sensitive and susceptible to CPAEA, which is also comparable to that reported by Araújo *et al.* (2018) where a DIZ in the range of 15 to 25 mm was recorded when the pyroligneous acid from wood of *Eucalyptus urograndis* and *Mimosa tenuiflora* were tested against *Escherichia coli*, *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans*. In this study, the growth of *E. coli* ATCC 25922 was strongly inhibited by CPAEA (DIZ value of 28.67 ± 0.88 mm) followed by *L. plantarum* WICC B18 (16.67 ± 0.33 mm), *S. aureus* ATCC 25923 (16.33 ± 0.33 mm) and *B. cereus* ATCC 10876 (13 ± 0.58 mm). This relatively substantial antimicrobial capability of CPAEA can be due to the presence of hydroxylated phenolic compounds such as catechol (two OH-groups) and pyrogallol (three OH-groups) which is known to be highly toxic to the microorganism (Cowan, 1999). Fullerton *et al.* (2011) reported that the antimicrobial activities were due to the oxidized phenol and hydroxyl group present on the phenolic ring. The hydroxylation process increased with increasing number of hydroxyl group, which in turn increased the antimicrobial activity.

Table 2: Inhibition zone activity by disc diffusion method of CPAEA MWPA towards different strains after 24 hrs at 37°C.

Antimicrobial activity of CPAEA MWPA towards different strains	
<p><i>Bacillus cereus</i> ATCC 10876</p>  <p>+C = 22 mm DIZ: 13 ± 0.58 mm</p>	<p><i>Lactobacillus plantarum</i> WICC B18</p>  <p>+C = 25 mm DIZ: 16.67 ± 0.33 mm</p>
<p><i>Escherichia coli</i> ATCC 25922</p>  <p>+C = 32 mm DIZ: 28.67 ± 0.88 mm</p>	<p><i>Staphylococcus Aureus</i> ATCC 25923</p>  <p>+C = 23 mm DIZ: 16.33 ± 0.33 mm</p>

*+C = chloramphenicol (30 ug/ml) as positive control, n = 3.

The result from the present study is comparable with that reported by other researchers such as *Wei et al.* (2010) where the application of PA produced from walnut shell resulted in DIZ values of 21.2 mm and 18.6 mm against *E. coli* and *S. aureus* respectively. Another report by *Mohapatra et al.* (2011) showed the higher susceptibility of Gram-negative bacteria towards raw honey extracted with DIZ value of 26.49 ± 6 mm for *E. coli* MTCC 1687 compared to Gram-positive bacteria such as *S. aureus* MTCC 737 (8.58 ± 3 mm) and *B. cereus* MTCC 430 (11.11 ± 6 mm). One plausible reason for this condition is the effect of solvent extract used. *Mehmood et al.* (2012) reported that ethyl acetate extract of PA produced from *Cichorium intybus* seeds has

strong antimicrobial activity against *E. coli* with DIZ about 22.5 mm compared to 21 mm against *S. aureus*. This situation could be due to Gram-negative bacteria having a much thinner cell wall compared to Gram-positive bacteria, hence allowing more contacts between phenolic compounds, as that present in CPAEA, to rupture the cell wall which would ultimately lead to the cell lysis. Comparatively, *Yang et al.* (2016) found that *S. aureus* 985 was more susceptible to PA from *Litchi chinensis* compared to *E. coli* 25257 with DIZ of 19 mm and 15.2 mm respectively. However, no further reasoning was conclusively included in this matter.

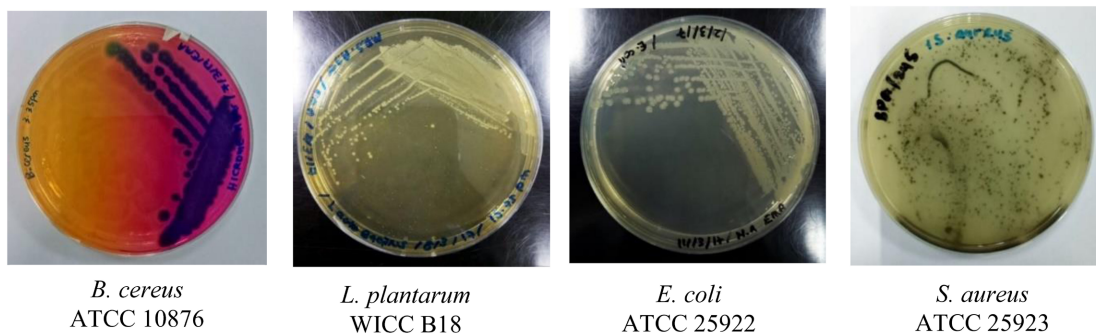


Fig. 2: Bacteria cultured on selective agar plate after 24 hr incubation at 37°C.

Relative inhibition of CPAEA

The relative growth of inhibition of CPAEA towards all strains was evaluated against chloramphenicol (30 ug/ml) and the results obtained are as shown in Figure 3. CPAEA managed to significantly inhibit the growth of *E. coli* ATCC 25922 with percentage inhibition of 79.55%, followed by *S. aureus* ATCC 25923 (46.79%), *L. plantarum* WICC B18 (41.07%) and *B. cereus* ATCC 10876 (29.69%). A previous study by *Naz and Bano* (2012) on the methanolic extracts of *Ricinus communis* leaf showed relatively high inhibition percentage of around 69.2 to 75% against *S. aureus*, *B. subtilis*, *P. aeruginosa* and *K. pneumoniae*. Meanwhile, the crude extract of *Calotropis gigantea* leaves demonstrated relative percentage inhibition around 46.04 to 188.52% against *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *E. coli* (*Kumar et al.*, 2010). This might be due to the different plant extract used, of which the plant extract from leaves has a higher phenolic compound that leads to high inhibition activity towards strains tested and *C. gigantea* leaves were well published as a medicinal purpose. Thus present finding shows that the ability of CPAEA MWPA to penetrate the cell wall led to the successful antimicrobial activity due to the fact that there were specific interactions between cell wall compartments with the bioactive compounds in plant extract itself as agreed by *Hyldgaard et al.* (2012). In addition, the antimicrobial activity varies considerably and might be mediated by a variety of antimicrobial compounds in plant extract used as suggested by *Ibrahim and Lim* (2015).

Evaluation of minimum inhibitory concentration

The result of MIC observation after 24 hr incubation at 37°C for all CPAEA towards bacteria strains noticed that the 11th

well for all strains was highly turbid which indicated there was a visible bacterial growth compared to the absence of bacteria growth in 12th well that display a clear broth solution. In addition, the turbidity for all CPAEA against bacterial strains in each plate was observed from 1st to 10th well and the well of lowest concentration at which no turbidity formed has been marked as the MIC. The MIC values for CPAEA against all bacterial strains after 24 hrs of incubation are as summarized in Figure 4. CPAEA showed good antibacterial activity against all strains with MIC values ranging from 0.651 to 1.563 mg/ml. It was found that *E. coli* ATCC 25922 was the most susceptible to CPAEA with MIC value of 0.651 ± 0.13 mg/ml, followed by *S. aureus* ATCC 25923 (0.78 ± 0.00 mg/ml), and 1.56 ± 0.00 mg/ml for both *B. cereus* ATCC 10876 and *L. plantarum* WICC B18. This finding correlated well with the results obtained for DIZ of which *E. coli* shows the highest DIZ among other strains tested. Similar trends of observation were also reported by *Yang et al.* (2016) where generally, the highest DIZ exhibited by sample extract would demonstrate the lowest MIC value and vice versa. In addition, *Mohapatra et al.* (2011) also reported the MIC value exhibited by raw honey extracted against *E. coli* and *B. cereus* were approximately at 0.625 mg/ml.

Comparatively, the MIC value obtained in the present study was lower than MIC value for PA extract towards antifungal activity (3.13-6.25 mg/ml) reported by *Ibrahim et al.* (2013b). This directly indicates that CPAEA from OPF exhibited stronger antimicrobial effect against bacteria than fungi. A similar situation was also supported by *Cunha et al.* (2017) where lowest MIC value indicates that the plant extract has strong bioefficacy and antimicrobial potential towards bacterial strains. It is noteworthy that value of MIC generally does not give an indication of the mode of action such as cidal or static. This was due to the viable

cells might be still in the MIC well even without visible growth if the antimicrobial agent had a bacteriostatic effect against the bacterial strains tested as suggested by Wiegand *et al.* (2008). This makes it imperative to carry out the microbial biocidal concentration (MBC) assay in order to ascertain the bactericidal effect of CPAEA.

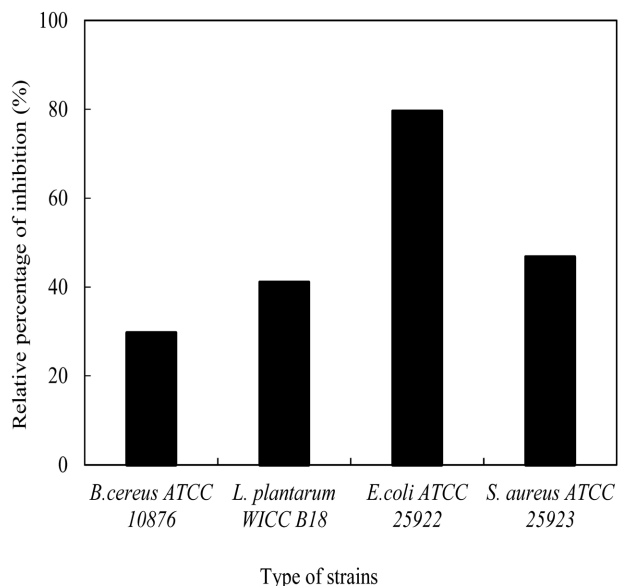


Fig. 3: Relative percentage inhibition of CPAEA MWPA towards all bacteria strains.

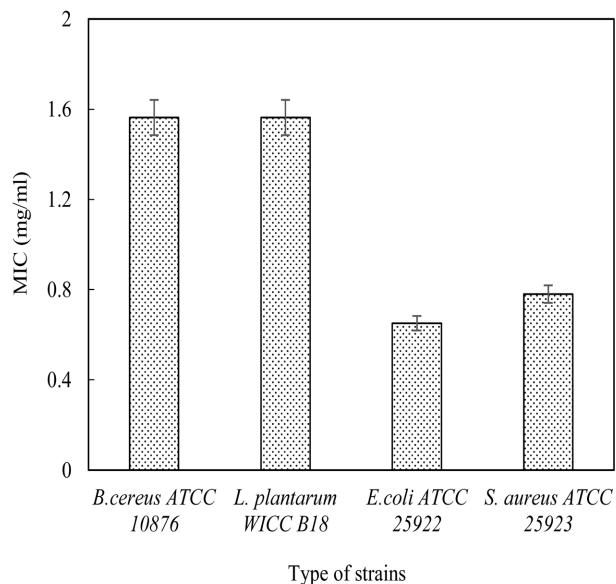


Fig. 4: The MIC value of CPAEA MWPA toward all strains analyzed after 24 hr.

Minimum biocidal concentration analysis

The lowest concentration of antimicrobial agent needed to kill 99.9% of the particular bacterium normally could be determined using the Minimum Biocidal Concentration (MBC) assay. Results for the determination of MBC for CPAEA against each bacterial strains were as tabulated in Table 3. It was noticed

that the MBC values obtained was higher than MIC values in the range of 1.563 to 6.25 mg/ml. The present finding was comparable with previous studies by Elsheikh *et al.* (2016) and Kang *et al.* (2011) that reported the MBC values obtained were higher than MIC values against all strains tested. From the table, the value of MBC against *B. cereus* was determined at 3.125 mg/ml (6th well) and 1.563 mg/ml (7th well) towards both *S. aureus* and *E. coli*. However, the MBC value for *L. plantarum* was determined at the 5th well which corresponds to the 6.25 mg/ml of CPAEA. Low MBC values indicate that the plant extract of CPAEA has a strong antimicrobial activity. Kang *et al.* (2011) suggested that the accumulation of bioactive compounds present in plant extract, such as that in CPAEA, in the bacterial cell membrane fractions resulted in energy depletion and membrane disruption leading to cell damage and lysis. Therefore, findings from MIC and MBC assays help to suggest that the CPAEA has a good potential to be applied as bacteriostatic and bactericidal agents.

Table 3: The biocidal activity and the MBC of CPAEA MWPA against all strains tested after 24 hr incubation at 37°C.

Types of bacteria strains	MBC (mg/ml)
<i>B. cereus</i> ATCC 10876	3.125 ± 0.00
<i>S. aureus</i> ATCC 25923	1.563 ± 0.00
<i>L. plantarum</i> WICC B18	6.25 ± 0.00
<i>E. coli</i> ATCC 25922	1.563 ± 0.00

CONCLUSION

The ethyl acetate extract of concentrated pyroligneous acid (CPAEA) produced from oil palm fiber (OPF) in optimized condition, showed strong antimicrobial activity when evaluated against both Gram Positive and Gram Negative bacteria. The relative growth inhibition zone was varied between 29 to 79% with low MIC values of less than 1.6 mg/ml. This further underlines the characteristic of CPAEA as a highly potential natural source for antimicrobial agent.

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