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WELL DIFFUSION METHOD FOR EVALUATION OF ANTIBACTERIAL ACTIVITY OF COPPER PHENYL FATTY HYDROXAMATE SYNTHESIZED FROM CANOLA AND PALM KERNEL OILS

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Hydroxamic acids and their derivatives have low toxicities and show wide range of biological activities. Copper complexes of phenyl fatty hydroxamic acids (Cu-PFHs) were prepared in a biphasic organic / aqueous medium from phenyl fatty hydroxamic acids (PFHAs) and copper nitrate. The products were separated by decantation of organic phase from aqueous phase followed by evaporation of the solvent. Elemental analysis, UV-Vis spectra and FTIR spectra showed that Cu-PFHs were formed in the solution from the complexation of PFHAs and copper ion. The antibacterial activity of PFHAs and Cu-PFHs from canola and palm kernel oils were investigated against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* using well diffusion method. The results showed that Cu-PFHs have higher antibacterial activity compared to PFHAs. Antibacterial activity of Cu-PHAs from canola oil against *E.coli* was significantly higher than chloramphenicol and cefotaxime.

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1. Introduction

Hydroxamic acids have low toxicities and show wide range of biological activities due to their chelating properties with metal ions [1]. Hydroxamic acids and their derivatives also are weak organic acids with different applications [2] such as: anti-malarial drugs [3], tumour inhibitor drugs [4], enzyme inhibitors [5], growth factors [6], cell-division factors [7] and also as metal chelators [8-10]**.** One of the earliest reports on the biological properties of hydroxamic acids goes back to 1950, when the anti-tubercular effects of some salicylhydroxamic acid derivatives were

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investigated [11]. A series of quinolines containing a cyclic hydroxamic acid group were prepared and tested for their antibacterial activity [12]. The results showed that the anti bacterial activity of 3-alkylquinoline hydroxamic acids was influenced by the size of the alkyl group and the nature of other substituents in the molecule. Other cyclic hydroxamic acids were also reported to inhibit bacterial growth [13]. A number of quinoline N-oxides were prepared and oxidized to quinoline hydroxamic acids using lead tetra acetate and their antibacterial properties against the *S. aureus* and *E. coli* was investigated. According to the results of Coutts, et al., 1970, the antibacterial activity of the hydroxamic acids depend on the type of the substituent on the quinoline nucleus as well as its benzene ring. Wang and Lee studied the antibacterial properties of some of aromatic hydroxamic acids and showed that 2-naphthoylhydroxamic acid was "effective" against *E. coli* and *Streptococcus faecalis* and "slightly effective" against *Bacillus cereus* and *Proteus morganii*. Benzoylhydroxamic acid, salicylhydroxamic acid and indole-2-carbohydroxamic acid were "slightly effective" against some bacterial strains [14]. They concluded that the hydroxamic acid function was essential for the antibacterial activity of the hydroxamic acid compounds.

Hydroxamic acids were extensively studied as bioligands as their metal complexes perform effective roles in biological systems [15]. Copper and ferric complexes of G1549, a new cyclic hydroxamic acid antibiotic isolated from culture broth of *Pseudomonas alcaligenes,* showed moderate activity against *Trichomonas vaginalis* and some Gram-positive bacteria [16]. Topical application of G1549 and its copper and ferric complexes protect Guinea pigs against cutaneous problems. Other researchers synthesized several ring hydroxamic acids and their copper and iron complexes and showed that Cu(II) and Fe(III) complexes of the ring hydroxamic acids had antibacterial activity [17]. Recently Sharma et al. prepared vanadium (IV) complexes of phenoxyacetohydroxamic and cinnamylhydroxamic acids in predetermined molar ratios in THF and methanol. The antibacterial activity of the newly synthesized complexes (vanadium precursor and ligands) was measured by the minimum inhibitory concentration (MIC) method and the results showed that these complexes had better antibacterial activity comparable to the free ligands [18].

Several methods had been reported for the synthesis of fatty hydroxamic acids [19-21]. Recently our group has reported enzymatic synthesis of fatty hydroxamic acids based on canola oil [22], fatty hydroxamic acids derivatives based on canola and palm kernel oils [23] and methyl fatty hydroxamic acids based on Jatropha seed oil [24]. In another of our group recent studies, Jahangirian, et al. reported the synthesis of phenyl fatty hydroxamic acids (PFHAs) from phenyl hydroxylaminolysis of canola and palm oil using lipase as catalyst [25]. The main advantage of their method is the use of cheap and available oils such as canola and palm oils. The canola and palm oils used in the synthesis contained different composition of saturated and unsaturated natural fatty acids with 8 to 22 carbon atoms in their aliphatic chain meaning that the procedure is applicable to other vegetable oils, too. Moreover, the reaction was catalyzed by the immobilized lipase catalysts and had the following advantages of enzymatic reactions: high selectivity, environmental friendly, energy saving and mild reaction conditions. We reported then the antibacterial activity of PFHAs based on canola oil by dick diffusion methods [26].

The present study's aim to use the PFHAs synthesized from canola and palm kernel oils to preparation copper complex of PFHAs (copper phenyl fatty hydroxamate (Cu-PFHs)) by reaction of PFHAs and copper nitrate solution. The PFHAs and Cu-PFHs based on canola and palm kernel oil will then be tested for their antibacterial activity against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) using well diffusion method. This is the first report on the antibacterial activity of PFHAs based on palm kernel oil and Cu-PFHs based on canola and palm kernel oils.

2. Materials and methods

2.1. Chemicals and bacteria

Phenyl hydroxylamine (PHA) was prepared based on the method described by Vogel, et al. [27]. PFHAs were obtained through the reaction of PHA with canola or palm kernel oils using Lipozyme TL 1M catalyst according to our method the method proposed by Jahangirian, et al., [25]. Hexane and absolute methanol were supplied by Systerm Co. (Selangor, Malaysia). Sodium

acetate and copper (II) nitrate were purchased from Sigma Aldrich (Missoure, USA). Krystal brand of canola oil was supplied by FFM Berhad (Selangor, Malaysia) and palm kernel oils were supplied by Malaysian palm Oil Board (MPOB), Malaysia. Mueller-Hinton agar (MHA) Difco (Detroit, Michigan, USA), chloramphenicol and cefotaxime Sigma Alderich (Missoure, USA) were used to test the antibacterial activity of the synthesized compounds against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923).

2.2. Preparation of Cu-PFHs

Copper complexation of PFHAs was performed by stirring the mixture of PFHAs and copper nitrate solutions in a biphasic medium. Briefly, 1 g of PFHAs were dissolved in 150 ml hexane and mixed with 500 ml of copper nitrate solution (buffered by sodium acetate at pH = 6.3) ($[Cu^{2+}] \sim 10$ mM). The mixture was stirred at 300 rpm at ambient temperature for 20 minutes and the organic phase was separated from aqueous phase using separator funnel. To achieve the maximum complex formation, the PFHAs in organic phase was reacted with another 500 ml of copper nitrate solution and the organic phase was separated from aqueous phase. Cu-PFHs were obtained after removing hexane in a rotary evaporator. Cu-PFHs based on canola oil were deep green color liquids while Cu-PHAs based on palm kernel oil were deep green color semi solid (pasty).

2.3. Evaluation of Antibacterial Activity

Antibacterial activities of PFHAs and Cu-PFHs were evaluated using well diffusion method on Mueller-Hinton agar (MHA). The inhibition zones were reported in millimeter (mm). *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were used as references for the antibacterial assay of Cu-PFHs. Briefly, MHA agar plates were inoculated with bacterial strain under aseptic conditions and wells (diameter=6mm) were filled with 50 µl of the test samples and incubated at 37°C for 24 hours. After the incubation period, the diameter of the growth inhibition zones was measured. Eighteen to 24 hrs single colonies on agar plates were used to prepare the bacterial suspension with the turbidity of 0.5 McFarland (equal to 1.5×10^8 colony-forming units (CFU)/ml).Turbidity of the bacterial suspension were measured at 600 nm.

The canola oil, palm kernel oil and hexane were used as negative standards while chloramphenicol and cefotaxime were used as positive standards. All tests were performed in triplicate.

2.4. Characterization

Elemental analyzer (model 932 LECO, USA) was used to measure the amount of PFHAs based on the nitrogen content. Perkin-Elmer 1650 Infrared Fourier Transform Spectrometer was used for FTIR spectra recording and Shimadzu UV-Vis Spectrophotometer (Model UV-1650 PC) was used to view UV-Vis spectra.

3. Results and discussion

3.1 Antibacterial activity

Results of the inhibition zone values for PFHAs and Cu-PFHs against *E. coli* and *S. aureus* are presented in Figure 1 and Table 1. According to Figure 1PFHAs and Cu-PFHs showed high antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*E. Coli*) bacteria. Both PFHAs and Cu-PFHs had better antibacterial activity against *E. Coli* than *S. aureus* ($p<0.001$, except PFHAs from canola oil $p<0.05$) (Table 1). Higher concentration of PFHAs and Cu-PFHs resulted in an increase in the antibacterial activity. The results also indicated that metal complexation increased the antibacterial activity of PFHAs (Table 1). Both PFHAs and Cu-PFHs from canola oil had better antibacterial activity against *S. aureus* comparing to the respective

PFHAs ($p<0.001$) and Cu-PFHs ($p<0.05$) from palm kernel oil. This phenomenon can be explained by the higher mobility of the PFHAs and Cu-PFHs obtained from canola oil than those obtained from palm kernel oil as the alkyl branches of palm kernel oil are high in saturated hydrocarbon while canola oil is high in unsaturated hydrocarbon. Antibacterial activity of Cu-PFHs from canola and palm kernel oils against *E. coli* was stronger than antibacterial activity of chloramphenicol ($p<0.01$) (Table 1). Antibacterial activity of Cu-PHAs from canola oil on *E. Coli* was also more than cefotaxime ($p<0.05$) while the antibacterial activity of Cu-PFHs from palm kernel oil on *E. coli* was almost similar to cefotaxime ($p<0.1$) (Table 1). On the other hand antibacterial activity of Cu-PFHs from canola oil on *S. aureus* was slightly stronger than chloramphenicol ($p<0.1$).

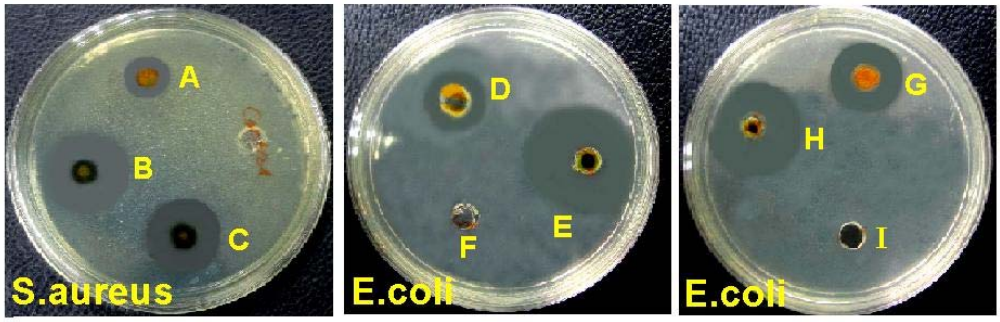

Fig. 1. Inhibition zone of solution of PFHAs and Cu-PFHs in hexane on E. coli and S. aureus. PFHAs from palm kernel oil 50% (A,G). Cu-PFHs from canola oil 50% (B). Cu-PFHs from palm kernel oil 50% (C,H). PFHAs from canola oil 20% (D). Cu-PFHs from canola oil 20% (E). hexane 99 % (F). Canola oil 99% (I).

Table 1. Growth inhibition of E. coli and S. aureus by PFHAs and Cu-PFHs in hexane using well diffusion method.

Compound	Concentration in hexane (%)	Growth Inhibition on *E. Coli* (Mean ± SD)	Growth Inhibition on *S. aureus* (Mean ± SD)
PFHAs from canola oil	20	14.7 ± 0.3	13.2 ± 0.7
	30	15.6 ± 0.6	14.1 ± 0.5
	40	16.7 ± 0.3	15.0 ± 0.5
	50	17.4 ± 0.4	15.8 ± 0.5
Cu-PFHs from canola oil	20	26.3 ± 0.7	16.4 ± 0.7
	30	26.9 ± 0.7	17.5 ± 0.5
	40	27.8 ± 0.4	18.8 ± 0.8
	50	28.4 ± 0.4	19.5 ± 0.3
PFHAs from palm kernel oil	20	14.1 ± 0.7	7.8 ± 0.5
	30	14.9 ± 0.6	8.4 ± 0.5
	40	15.8 ± 0.7	9.5 ± 0.4
	50	16.4 ± 0.7	10.3 ± 0.3
Cu-PFHs from palm kernel oil	20	20.8 ± 0.8	16.3 ± 0.4
	30	21.3 ± 0.8	16.9 ± 0.6
	40	21.7 ± 0.8	17.5 ± 0.5
	50	22.1 ± 0.6	18.0 ± 0.5
Control positive	Chloramphenicol 50	19.4 ± 0.3	18.7 ± 0.5
	Cefotaxime 50	23.1 ± 0.4	25.4 ± 0.3
Control negative	Hexane 99	NO	NO
	Canola oil 99	NO	NO
	Palm kernel oil 99	NO	NO
	PHA 10	NO	NO

NO: not observed

3.2. Characterization

3.2.1. Elemental Analysis

Elemental analysis showed that the nitrogen content in the Cu-PFHs from palm kernel oil was 4.128%, indicating that there was 2.949 mmol of phenyl fatty hydroxamic acid groups in 1 gram of the product. However the Cu-PHA from canola oil contained only 3.479% nitrogen which indicates that there was 2.485 mmol of phenyl fatty hydroxamic acid groups in 1 gram of the product.

3.2.2. Fourier Transform Infrared Spectroscopy (FTIR)

Table 2 shows the absorption peaks in the FTIR spectra of PFHAs and Cu-PFHs from canola oil. In PFHAs spectra the peaks at 2800 to 3100 cm^{-1} correspond to O—H stretching that was wider and lower in intensity compared to free O—H probably due to the formation of intermolecular hydrogen bonding. The peak at 3068 and 3008 cm^{-1} correspond to =C—H stretching and the peaks at 2924 and 2854 cm^{-1} correspond to —C—H stretching of the long chain of alkyl. The peaks at 1739 and 1708 cm^{-1} correspond to C=O stretching which splits to two branches and shifted to upper frequencies due to Fermi resonance (Pavia, et al., 2001). In addition the peaks at 1477 and 1437 cm^{-1} correspond to C=C stretching while the peaks at 1299 and 1071 cm^{-1} correspond to =C—N and —C—N stretchings. Finally the peaks at 761 and 682 cm^{-1} correspond to mono substitution of aromatic ring. In Cu-PFHs spectra the peaks at 3067and 3006 cm^{-1} correspond to =C—H stretching and the peaks at 2923 and 2854 cm^{-1} correspond to —C—H stretching for the long chain of alkyl while the peak at 1742 cm^{-1} correspond to C=O stretching. In addition the peaks at 1475 and 1440 cm^{-1} correspond to C=C stretching, the peak at 1299 cm^{-1} corresponds to =C—N stretchings, the peaks at 1164 cm^{-1} correspond to —C—O stretching that appeared due to connection of copper to C—OH. Finally the peaks at 761 and 682 cm^{-1} correspond to mono substitution of aromatic ring. The FTIR spectra of PFHAs and Cu-PFHs from palm kernel oil were almost similar to the spectra of PFHAs and Cu-PFHs from canola oil.

Table 2. Numerical Presentation of FTIR Spectra of PFHAs and Cu-PFHs from canola oil

Compound	Wavelength (cm^{-1})	Chemical bond assignment
PFHAs	3068, 3008	=C—H stretching
	2924, 2854	—C—H stretching for long chain alkyl
	1739, 1708	C=O stretching for hydroxamic acid
	1477, 1437	C=C stretching
	1299	=C—N stretching
	1071	—C—N stretching
	761, 682	Correspond to mono substitution aromatic ring
Cu-PFHs	3067, 3006	=C—H stretching
	2923, 2854	—C—H stretching for long chain alkyl
	1742	C=O stretching for hydroxamic acid
	1475, 1440	C=C stretching
	1297	=C—N stretching
	1164	—C—O stretching
	764, 685	Correspond to mono substitution aromatic ring

3.2.3. UV-Vis Spectroscopy

UV-Vis spectra of canola oil, PHA, PFHAs and Cu-PFHs showed that Cu-PFHs from canola oil were successfully formed in this study (Figure 2).

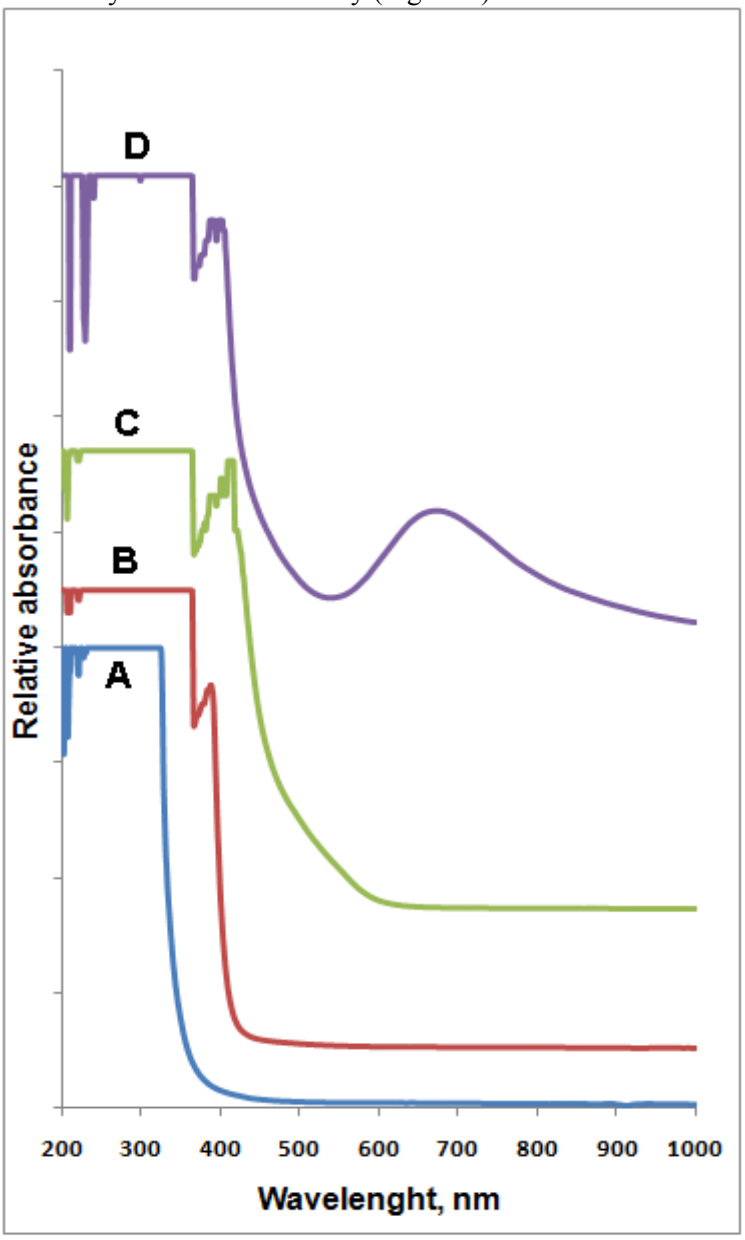

Fig. 2. Comparison of UV-Vis spectra of canola oil (A), PHA (B), PFHAs(C) and Cu-PFHs (D). Concentration of all compounds: 50 mM.

No distinct absorbance peak was observed in the spectrum of canola oil (A). In the spectrum of PHA (B), the peak appeared at 390 nm might have been resulted from the hydroxyl amine functional group on benzene ring due to n → p^* electronic transition. In the spectrum of PFHAs (C), the mentioned peak was shifted to upper region (415 nm) due to coupling of carbonyl group and forming of hydroxamic acid group. Finally in the spectrum of Cu-PFHs (D), the mentioned peak was shifted to lower region (410 nm) due to coupling of copper for formation of copper hydroxamate. Also in Cu-PFHs spectrum (D), another peak appeared in the visible region of 673 nm due to the coordinate covalent bonds between copper and oxygen atoms of PFHAs. Similarly, consideration of UV-Vis spectra of palm kernel oil, PHA, PFHAs and Cu-PFHs from palm kernel oil (figure was not shown) indicated that the Cu-PFHs complex was also successfully

formed. In addition, the obtained Cu-PFHs were green and this is in agreement with green colour of copper fatty hydroxamic acids based on canola oil [22], copper fatty hydroxamic acid derivatives based on palm kernel and canola oils [9] and of copper complex of methyl fatty hydroxamate based on Jatropha seed oil [24].

4. Conclusions

This study was the first report on antibacterial activity of Cu-PFHs from palm kernel oil and canola oil. Advantages of the method are as follows: the preparation method of PFHAs ligand and its copper complex is simple, the technique uses vegetable oils which are cheap and easily available and uses of enzymatic reaction in the preparation of PFHAs is an energy saving method towards achieving green chemistry. Elemental analysis, UV-Vis. and FTIR spectra showed that Cu-PFHs were produced from the complexation of PFHAs and the copper nitrate solution and PFHAs and Cu-PFHs showed high antibacterial activity. Antibacterial activity of Cu-PFHs was stronger than PFHAs and the antibacterial activity of Cu-PFHs and PFHAs from canola oil were stronger than the respective compounds from palm kernel oil. The antibacterial activity of Cu-PFHs and PFHAs on *E. coli* was stronger than *S.aureus* and antibacterial property was increase with higher concentration of PFHAs and Cu-PFHs. The main point of the current study was that the antibacterial activity of Cu-PFHs from canola oil on *E. coli* was significantly stronger than antibacterial activity of the known antibiotic drugs such as chloramphenicol and cefotaxime while antibacterial activity of Cu-PHAs prepared from palm kernel oil on *E. Coli* was only slightly higher than chloramphenicol.

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