Journal of Medicinal Plants Research Vol. 5(19), pp. 4826-4831, 23 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Antibacterial effect of phenyl fatty hydroxamic acids synthesized from canola oil

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Accepted 3 May, 2011

Phenyl fatty hydroxamic acids (PFHAs) were synthesized in a biphasic organic/aqueous medium from canola oil and phenyl hydroxylamine (PHA). The reaction was carried out in a sealed flask, incubated in water batch shaker at 39°C for 72 h and catalyzed by immobilised lipase (Lipozyme TL IM). The products were then separated by filtration, solvent extraction followed by evaporation of the solvent. Elemental analysis, ^{1}H NMR (hydrogen nuclear magnetic resonance) and FTIR (Fourier transform infrared) spectra showed that PFHAs were produced from the reaction of PHA and canola oil. The antibacterial activity of PFHAs dissolved in hexane were investigated against gram-positive (that is, *Staphylococcus aureus*) and gram-negative bacteria (that is, *Escherichia coli*) by the disk and well diffusion methods using Mueller-Hinton Agar (MHA). The results showed that PFHAs have high antibacterial activity and their antibacterial property on *E. coli* is stronger than on *S. aureus*. The antibacterial property of PFHAs increased with the increase in PFHAs concentration.

Key words: Antibacterial activity, phenyl fatty hydroxamic acids, canola oil, enzymatic reaction.

INTRODUCTION

Hydroxamic acids and their derivatives are weak organic acids and have low toxicity. They also are attractive and important compounds for researchers due to their wide biomedical and analytical applications. These compounds have been widely applied as food additives, growth factors, antibacterial agents, fungicidal agents, tumour inhibitor drugs, metal chelators, rare earth mineral collectors, enzyme inhibitors, cell-division factors and antimalarial drugs. (Kurzak et al., 1992; Gao et al., 1995; Tsafack et al., 1995; Dankwardt et al., 2001; Agrawal et al., 1999; Lockhart et al., 1995; Gijbels et al., 1994; Nakagawa et al., 1995). Hydroxamic acids were generally synthesized by the chemical reaction of alkyl or aryl ester with hydroxylamine in high alkaline medium. Fatty hydroxamic acids were synthesized from hydroxylamine and free fatty acids or their methyl esters by Servat et al. (1990) using either lipase or Mucor Miehei as the catalyst. Vaysse et al. (1997) carried out biosynthesis of fatty hydroxamic acid in a biphasic lipid/aqueous medium from fatty acid or fatty acid methyl ester and hydroxylamine by using lipase-acyltransferase from *Candida parapsilopsis* as the catalyst.

Coutts et al. (1970) prepared a number of quinoline N-oxides and quinoline hydroxamic acids and evaluated their antibacterial properties against *S. aureus* and *E. coli*. They showed that most of the compounds show antimicrobial activity which depends on the position of the nitro-group on the quinoline nucleus. Bravo and Lazo (1993) synthesized hydroxamic acids from 2,4-dihydroxy-1,4-benzoxazin-3-one or 4-hydroxy-1,4-benzoxazin-3-one and then used them as antibacterial agents against the *S. aureus, E. coli* and *Candida albicans*. The results showed that the synthesised products have moderate antibacterial effect. Pepeljnjak et al. (2005) prepared

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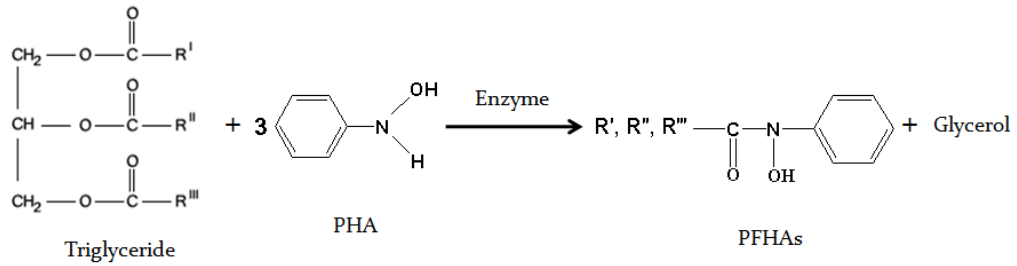

Scheme 1. The reaction equation of phenylhydroxylaminolysis of triglycerides. Enzyme = lipozyme. R', R'', and R''' = hydrocarbon chains of fatty acids.

several hydroxamic acids such as N-benzyl-N'-hydroxysuccinamide (1), poly [α,β-(N-hydroxy)-DL-aspartamide] (2) and poly [α,β –(N-hydroxy-N-methyl-DL-aspartamide] (3) and applied them as antimicrobial agents on ten Gram-positive and seven Gram-negative species of bacteria, five *Candida* species, four dermatophyte species and three mould species separately. They indicated that Compound (1) has antimicrobial activity only on *S. aureus* ATCC 29213 and *Pseudomonas aeuruginosa* 91 but other compounds (2,3) have antibacterial activity on majority of test microorganisms except mould species (Pepeljnjak et al., 2005). Recently Agarwal et al. (2010) synthesized several hydroxamic acids such as: N-phenylbenzohydroxamic acids, N-p-tolylbenzohydroxamic acid, N-o-tolylbenzohydroxamic acid, N-m-chlorophenylbenzohydroxamic acid, N-p-carboxyphenylbenzohydroxamic acid, acetohydroxamic acid, benzohydroxamic acid and salicylhydroxamic acid. The compounds were tested in vitro for growth inhibitory activity against bacteria *Enterobacter cloacae*, *E. coli, P. aeuruginosa, Proteus vulgaris* and *S. aureus* (coagulase positive and coagulase negative) by using the disc-diffusion method. Their results showed that acetohydroxamic acid and benzohydroxamic acid exhibited wide spectrum of activity, as they were highly active against all cultures followed by salicylhydroxamic acid. All of the other tested compounds exhibit moderate antimicrobial activity against all species of bacteria tested. In this investigation, we carried out the synthesis of phenyl fatty hydroxamic acids (PFHAs) by the reaction of phenyl hydroxylamine (PHA) and canola oil catalyzed by immobilised lipase. The synthesised PFHAs were tested antibacterial activity against *E. coli* (Gram-negative) bacteria and *S. aureus* (Gram-positive) bacteria by the disk and well diffusion methods. This paper is the first report about antibacterial activity of PFHAs synthesized from canola oil.

MATERIALS AND METHODS

Hexane, diethylether, and absolute methanol were supplied by Systerm Co., Malaysia. Sodium hydroxide, zinc powder, sodium chloride, ammonium chloride, nitrobenzene were purchased from Aldrich Co., USA. Lipozyme TL IM and was obtained from Novo Nordisk, Denmark. Krystal brand of canola oil was supplied by FFM Berhad, Malaysia, Mueller-Hinton agar (MHA) Difco brand was obtained from Voigt Global Distribution, USA. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were from clinical isolates which conformed to the recommended standards of the Clinical and Laboratory Standards Institute, CLSI, 2000. The commercial antibiotic of chloramphenicol and cefotaxime were obtained from Sigma-Aldrich Co., USA.

Synthesis of PFHAs

Phenyl hydroxylamine (PHA) was prepared using the method described by Vogel (1978). Phenyl fatty hydroxamic acids were synthesized by Phenyl hydroxylaminolysis of triglycerides. Phenyl hydroxylaminolysis was carried out by shaking the mixture of the reactants, which contained 10.0 g phenyl hydroxylamine dissolved in 80 ml of distilled water, 11.0 g canola oil dissolved in 120 ml hexane and 960 mg of the lipozyme TL IM in a 500 ml flask sealed using Teflon film.

The mixture was shaken at 120 rpm and 39°C in a water bath shaker for 72 h. The product was separated from the reaction mixture as follows. First the enzyme was filtered and the filtrate was transferred into a separation funnel for separation of aqueous phase from organic phase. The organic phase in the funnel was shaken with 100 ml distilled water for removal of glycerol residue, and then with two times 100 ml HCl solution (2 M) for removal of the unreacted PHA. Hexane was then removed by rotary evaporation to obtain mixture of PFHAs and unreacted oil. Finally PFHAs were extracted by using 150 ml absolute methanol from the unreacted oil and then recovered by rotary evaporation. Scheme 1 shows the phenyl hydroxylaminolysis.

Evaluation of antibacterial activity

The *in vitro* antibacterial activity of the PFHAs samples was evaluated against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) by the disc and well diffusion methods. This was done by determining the inhibition zones (mm) in Mueller-Hinton agar. In the disc diffusion method, the sterile paper discs (6 mm in diameter) were impregnated 4 times with 5 µl of PFHAs solution in hexane and left to dry at 37°C for 24 h in a sterile condition. The bacterial suspension was prepared by making a saline suspension of isolated colonies grown for 18 to 24 h on agar plate. The suspension was adjusted to match the tube of 0.5 McFarland turbidity standard using the spectrophotometer at 600 nm, which equals to 1.5×10^8 colony-forming units (CFU)/ml. The surface of MHA was completely inoculated using a sterile swab, which steeped in the prepared

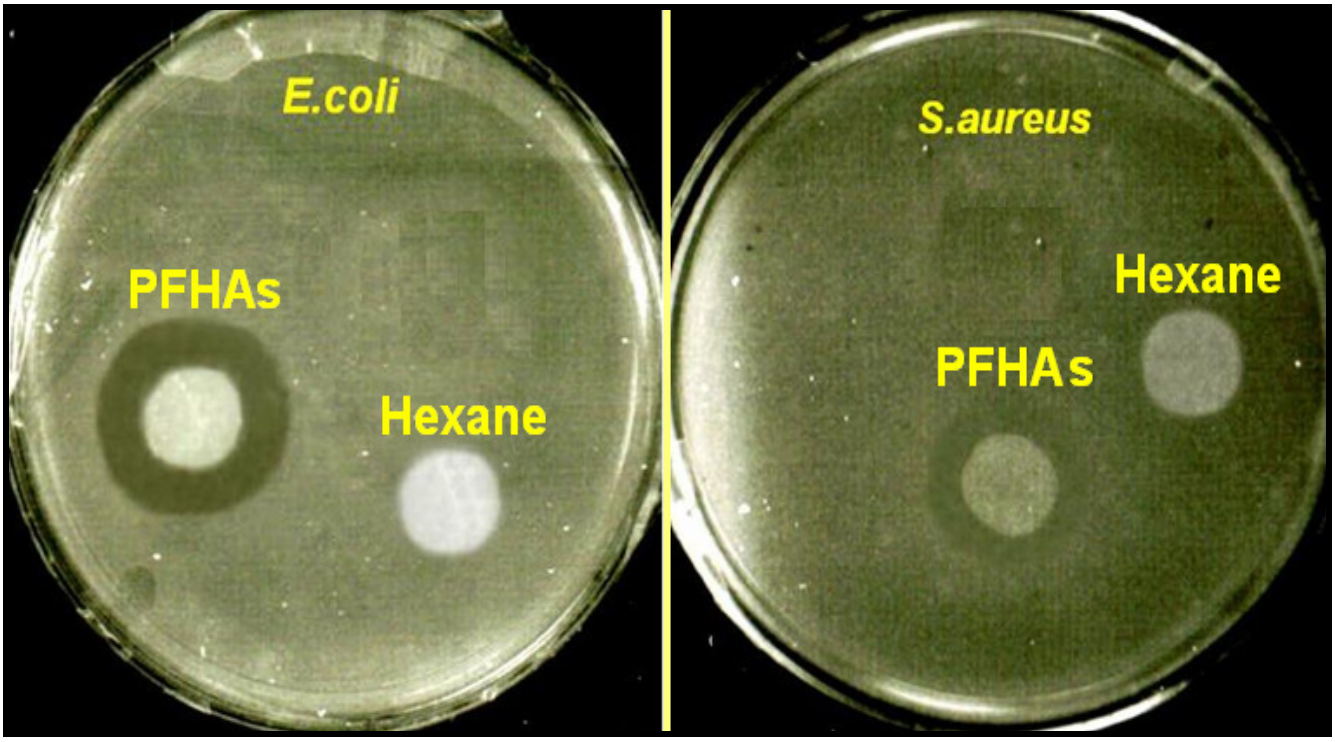

Figure 1. Comparison of the inhibition zone of solution of PFHAs in hexane tested on *E. coli* and *S. aureus* by disc diffusion method.

Table 1. Inhibition of growth of *E. coli* and *S. aureus* by PFHAs in hexane at different concentrations (% w/w) (C1 = 20, C2 = 30, C3 = 40, C4 = 50) in disc diffusion method.

Bacteria	Inhibition zone (mm)							
	PFHAs				Control negative			Control positive
	C1	C2	C3	C4	Hexane 99%	Canola oil 99%	PHA* 10%	CTX C 50% 50%
E. coli	9.5±0.4	9.8±0.2	10.6±0.3	11.7±0.3	NA**	NA	NA	21.8±0.5 16.7±0.4
S. aureus	8.5±0.2	9.4±0.2	10.1±0.1	10.5±0.1	NA	NA	NA	23.6±0.4 16.4±0.4

* Percentage of PHA in hexane = 10% w/w, ** NA = Not appearing, CTX = Cefotaxime, C = Chloramphenicol.

suspension of bacterium. Finally, the impregnated discs were placed on the inoculated agar and incubated at 37°C for 24 h. After incubation, the diameter of the growth inhibition zones was measured. Canola oil, hexane and solution of PHA in hexane (10%) were used as the negative controls while chloramphenicol and cefotaxime were used as the positive controls for the antibacterial essay.

All tests were done in three replicates. In the well diffusion method, all conditions were the same as disc diffusion method except that instead of using paper discs, wells of 6 mm in diameter were constructed on the surface of the MHA and then 50 µl of PFHAs solutions or control samples were applied.

Quantification and characterization of PFHAs

The amount of PFHAs in the synthesised product was estimated based on nitrogen content, determined by elemental analyzer (model 932 LECO, USA). PFHAs were characterized by spectral data. A Perkin-Elmer 1650 Infrared Fourier transform spectrometer was used for recording FTIR spectra and a NMR spectrophotometer (Model 400 Joel Ltd., Tokyo, Japan) for ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra.

RESULTS AND DISCUSSION

Antibacterial activity

Inhibition zone values were obtained for the synthesized PFHAs against *E. coli* and *S. aureus* by disc and well diffusion methods. The images and the sizes of inhibition zones using disc diffusion method are presented in Figure 1 and Table 1. Figure 1 shows that the PFHAs gave high antibacterial activity against Gram-negative and Gram-positive bacteria. The antibacterial activity on

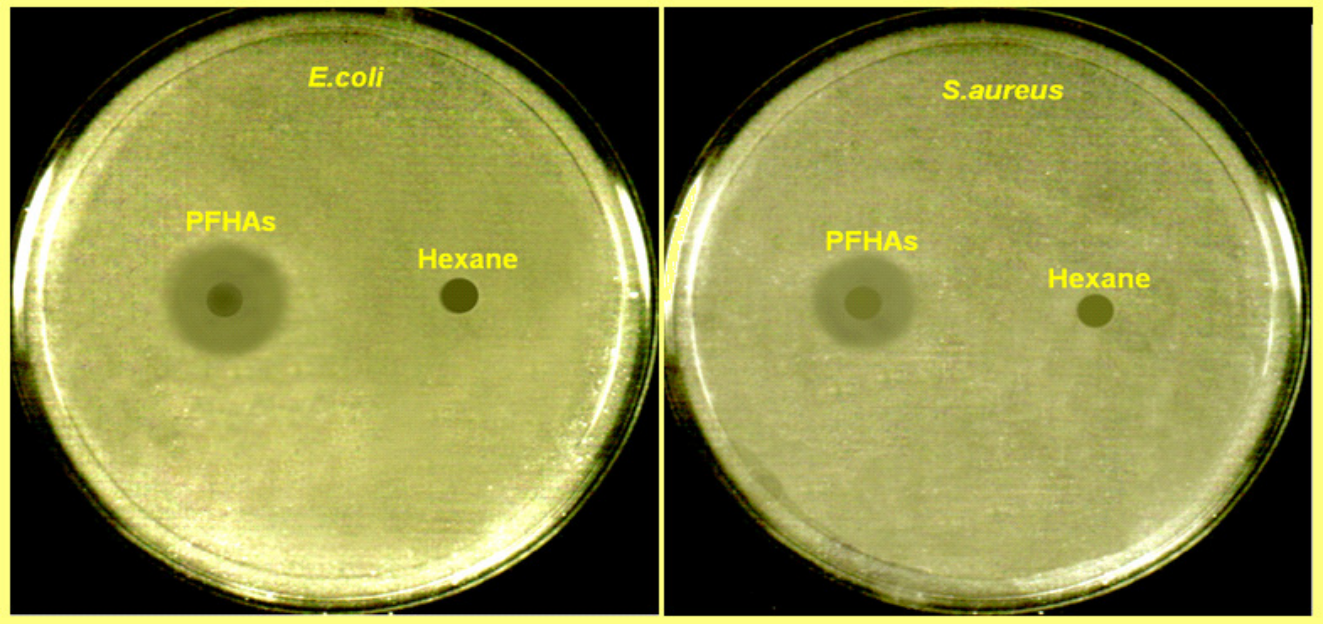

Figure 2. Comparison of the inhibition zone of solution of PFHAs in hexane tested on *E. coli* and *S. aureus* by well diffusion method.

Table 2. Inhibition of growth of *E. coli* and *S. aureus* by PFHAs in hexane at different concentrations (% w/w) (C1 = 20, C2 = 30, C3 = 40, C4 = 50) in well diffusion method.

Bacteria	Inhibition zone (mm)								
	PFHAs				Control negative			Control positive	
	C1	C2	C3	C4	Hexane 99%	Canola oil 99%	PHA* 10%	CTX 50%	C 50%
E. coli	15.5±0.4	15.9±0.5	17.1±0.4	17.7±0.3	NA**	NA	NA	23.1±0.4	19.4±0.3
S. aureus	14.1±0.4	15.4±0.5	16.4±0.6	16.9±0.2	NA	NA	NA	25.4±0.3	18.7±0.5

* Percentage of PHA in hexane = 10% w/w, ** NA = Not appearing, CTX = Cefotaxime, C = Chloramphenicol.

the Gram negative bacteria was higher than on the Gram positive bacteria. In addition, the data in Table 1 shows that antibacterial activity of PFHAs increased with the increase in concentration of PFHAs. The results of the images and the sizes of inhibition zones using well diffusion method are presented Figure 2 and Table 2. Similar to disc diffusion method, the results show that the PFHAs antibacterial activity on the Gram negative bacteria is higher than on the Gram positive bacteria (Figure 2) and also the antibacterial activity of PFHAs increases while concentration of PFHAs increases (Table 2).

The results also show that the antibacterial activity of PFHAs using the well diffusion method is higher ($p < 0.01$) compared to disc diffusion method. This was expected since in the well diffusion method the PFHAs samples were applied directly in to the well constructed on agar where the bacteria were cultured. However there are no significant different ($p > 0.01$) in the activity of both positive controls (antibiotic drugs) using the well diffusion method compared to disc diffusion method. This implies that the PFHAs is more mobile compared to controls. Although the antibacterial activity of PFHAs is significantly lower ($p < 0.001$) compared to antibacterial activity of the controls, antibacterial activity of PFHAs could be better *in vitro* due to higher mobility and hydrophobicity properties of the compound. These results also indicate that well diffusion method is better than disc diffusion method for evaluating antibacterial effect of phenyl fatty hydroxamic acids.

Quantification and characterization of PFHAs

Elemental analysis

Elemental analysis showed that the nitrogen content in the synthesized PFHAs from the canola oil was 3.83%. This indicates that there was 2.736 mmol of phenyl fatty hydroxamic acid groups in one gram of the product.

Table 3. Numerical presentation of FTIR spectrum conducted on PHA, canola oil and PFHAs.

Compound	Wavelength (cm^{-1})	Chemical bond assignment
PHA	3242	N—H stretching for amine
	2959	=C—H stretching
	3139	O—H stretching for amine
	1472,1419	C=C stretching
	1301	=C—N stretching
	747, 686	Correspond to mono substitution aromatic ring
Canola oil	2923, 2857	—C—H stretching for long chain alkyl
	1743	C=O stretching
	1455	C=C stretching
	1159	C-O stretching for ester
PFHAs	3068, 3008	=C—H stretching
	2924, 2854	—C—H stretching for long chain alkyl
	1739, 1708	C=O stretching for hydroxamic
	1477, 1437	C=C stretching
	1299	=C—N stretching
	1071	—C—N stretching
	761, 682	Correspond to mono substitution aromatic ring

Fourier transform infrared spectroscopy (FTIR)

The absorption peals of the FTIR spectra of PHA, canola oil and PFHAs are shown in Table 3. In the PHA spectra the peaks at 3242 and 3139 cm^{-1} correspond to N—H stretching and O—H stretching respectively, while the peaks at 1592, 1472 and 1419 cm^{-1} correspond to C=C stretching, the peak at 1301 cm^{-1} corresponds to =C—N stretching and the peaks at 747 and 686 cm^{-1} correspond to mono substitution of aromatic ring (Pavia et al., 2001). In the canola oil spectra the peaks at 2923 and 2857 cm^{-1} correspond to —C—H stretching for the long chain of alkyl, while the peaks at 1743, 1455 and 1155 cm^{-1} correspond to C=O stretching, C=C stretching and C—O stretching, respectively (Pavia et al., 2001). In PFHAs spectra the peaks at 2800 to 3100 cm^{-1} correspond to O—H stretching that was sharper compared to PHA probably due to formation of intermolecular hydrogen bonding.

The peak at 3008 cm^{-1} corresponds to =C—H stretching and the peaks at 2924 and 2854 cm^{-1} correspond to —C—H stretching for 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, the peaks at 1299 and 1071 cm^{-1} correspond to =C—N and —C—N stretchings, respectively and finally the peaks at 761 and 682 cm^{-1} correspond to mono substitution of aromatic ring.

^{1}H Nuclear magnetic resonance (^{1}H NMR)

The formulation of PHA and the major PFHAs that based on the canola oil are presented in Figure 3. ^{1}H NMR spectra of product showed the signals at a(0.92, 0.93 and 0.93 ppm), b(1.30 and 1.33 ppm), c(1.63, 1.65 and 1.66 ppm), d(2.04 and 2.06 ppm), e(2.33 and 2.35 ppm), f(2.81, 2.83 and 2.84 ppm) and g(5.39, 5.40, 5.41 ppm) corresponding to the alkyl branch hydrogens of the PFHAs (Figure 3). These signals were confirmed for the alkyl branch hydrogens of oleic, linoleic and linolenic acids (Lie Ken Jie et al., 1997; Lie Ken Jie, 2001). In addition Takenaka et al. (2009) reported the ^{1}H NMR signals of aromatic ring hydrogen's of PHA appeared at 6.96 to 7.01 ppm (labelled by i, j, k) and 7.26 to 7.28 ppm (labelled by h, L). However ^{1}H NMR spectra of the product in this study showed these signals shifted to the upper regions at 7.37 to 7.53 ppm (labelled by i, j, k) and 8.20 to 8.33 ppm (labelled by h, L) due to the formation of PFHAs.

Conclusion

This paper is the first report which describes the antibacterial activity of PFHAs synthesized from PHA and canola oil. Among the advantages of this synthesis is the use of easily available oil as well as the utilisation of enzymatic reaction for the purpose of good energy saving in order to achieve green chemistry. Elemental analysis,

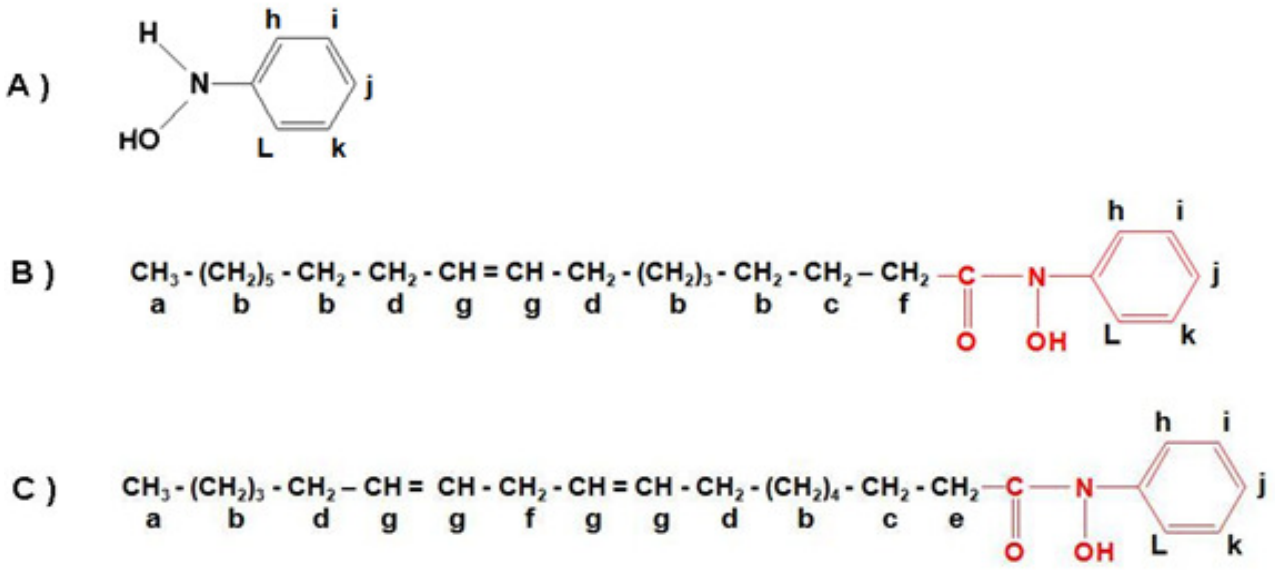

Figure 3. Formulation of phenylhydroxylamine (A), phenyl oleo hydroxamic acid (B), phenyl linoleo hydroxamic acid (C).

^{1}H NMR and FTIR spectra showed that PFHAs were produced from the reaction of PHA and the oil. Also in this paper we showed that PFHAs have high antibacterial activity and comparable with the antibiotic drugs such as chloramphenicol and cefotaxime. The results showed that the antibacterial activity of PFHAs on *E. coli* is stronger than on *S. aureus* and also antibacterial property increase when the concentration of PFHAS increases.

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