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Dual COX/LOX Inhibition: Screening and Evaluation of the Effect of Pyroligneous acid Fractions of Palm kernel Shell as an Anti-inflammatory agents

(Penghambatan ganda COX/LOX: Pemeriksaan dan Penilaian Kesan Asid Pyroligneous Fraksi Shell Kernel Sawit sebagai Ejen Anti-radang)

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ABSTRACT

Inflammation is treated using Steroidal (SAIDs) and Non-steroidal (NSAIDs) anti-inflammatory drugs. These synthetic drugs act by inhibiting the activity of inflammatory mediators (Prostaglandins and Leukotrienes) and are known to have various side effects. The use of natural products as alternatives is gaining prominence due to effective therapeutic function with reduced side effects. Palm kernel shell biomass can be thermo-chemically converted into Pyroligneous Acid (PA), which have been found to contain phenols that have antioxidant activities and potential anti-inflammatory effects. In this study, the PA is derived from slow pyrolysis of palm kernel shell by fractionation using column chromatography. The fractions are then screened for Total Phenolic content using Folin ciocalteau assay and free radical scavenging activity using FRAP and DPPH procedures. Anti-inflammatory activity of COX and LOX pathways with the screened fractions, is determined using the LOX-5 and COX-2 assay by ELISA method. The fractions were found to have phenolic and free radical scavenging activity with high percentage yield. The fractions were also found to have dual inhibition for COX and LOX enzymes with IC_{50} values ranging for COX enzymes IC_{50} (17.04% - 48.42%) and LOX with IC_{50} (5.23% -53.34%). The findings of the present study indicate the potential for use of various fractions of pyroligneous palm kernel shell as a viable source of anti-inflammatory agents.

Keywords: Palm kernel shell, Pyroligneous acids; Anti-inflammation; COX-2 and LOX-5.

ABSTRAK

Keradangan dirawat menggunakan ubat-ubatan anti-radang Steroidal (SAID) dan bukan steroid (NSAID). Ubat-ubatan sintetik ini bertindak dengan menghalang aktiviti mediator peradangan (Prostaglandin dan Leukotrienes) dan diketahui mempunyai pelbagai kesan sampingan. Penggunaan produk semulajadi sebagai alternatif semakin terkenal kerana fungsi terapeutik yang berkesan dengan kesan sampingan yang berkurang. Asid Pyroigneous dihasilkan dari penukaran termokimia biomass shell kernel kelapa sawit, yang didapati mengandungi fenol yang mempunyai aktiviti antioksidan dan kesan anti-radang. Dalam kajian ini, asid Pyroligneous yang dihasilkan daripada pirolisis lambat shell kernel kelapa sawit dengan fraksinasi menggunakan kromatografi lajur.;Fraksi tersebut kemudian ditayangkan diperiksa untuk kandungan Total Phenolic menggunakan Folin ciocalteau prosedur dan aktiviti pembasmian radikal bebas menggunakan prosedur FRAP dan DPPH. Fraksi didapati mempunyai aktiviti pemangkasan radikal fenolik dan bebas dengan hasil peratusan yang tinggi. Fraksi tersebut juga didapati mempunyai penghambatan ganda untuk enzim COX dan LOX dengan nilai $IC_{lima\ puluh}$ yang meliputi enzim COX $IC_{lima\ puluh}$ (17.04% - 48.42%) dan LOX dengan $IC_{lima\ puluh}$ (5.23% -53.34%). Hasil kajian ini menunjukkan potensi untuk menggunakan pelbagai pecahan shell kernel sawit pyroligneus sebagai sumber ejen anti-radang.

Kata kunci: Shell kernel sawit; anti-keradangan; asid Pyroligneous; COX-2 dan LOX-5

INTRODUCTION

Inflammation is an essential response to external stimuli such as injury and infection (Medzhitov 2010). This complex process results in the release of chemical mediators such as prostaglandins and leukotrienes. These mediators are responsible for certain inflammatory responses (Sala, Zarini, and Bolla 1998; Martel-Pelletier et al. 2003). Inflammation is commonly treated using Corticosteroids (gluco-corticosteroids) and NSAIDs (Non-steroidal anti-inflammatory drugs), which also provide relief from pain (analgesic) and fever (anti-pyretic) (Cuzick et al. 2007). The

mechanism of action of these drugs is through the inhibition of the enzymes of the cyclo-oxygenase (COX) and lipoxygenase pathways (LOX-5). These enzymes are responsible for the production of prostaglandins and leukotrienes mediators that are released during inflammation (Ciriaco et al. 2013). Corticosteroids act by inhibiting Phospholipase A_2 enzyme thus mitigating the release of arachidonic acids and its mediators (Ciriaco et al. 2013), while the NSAIDs act by inhibiting the COX-2 enzyme. The different COX isoforms (COX-1 and COX-2) identified with different gene codes, are involved in different functions (Smith, Garavito, and DeWitt 1996). COX-1 is associated with haemostatic functions and COX-2 associated with pathological functions of inflammation. This became the basis of selective COX-2 inhibitors (Martel-Pelletier et al. 2003). The inhibition of the COX-2 enzyme pathway by the NSAIDs may lead to substrate diversion of arachidonic acid metabolism to the second main pathway i.e. the lipoxygenase (LOX) pathway. Thus this can lead to increased production of LTs resulting in inflammatory responses (Bertolini, Ottani, and Sandrini 2002). The continuous use of these drugs, produce unwanted side effects such as; gastric toxicities (Green 2001; Higuchi et al. 2009), renal toxicities (Wang et al. 2007; Weir 2002), joint disorders (Jacob and Kumar 2015; Vane and Botting 1998; Botting 2000), cardiovascular diseases and related disorders (Huang, Ou, and Prior 2005; Solomon et al. 2005). Despite these associated side effects, the estimated use of inflammatory related drugs is expected to rise especially in the elderly (McCarberg 2013). The associated side effects of NSAIDs makes it necessary to substitute them with green alternatives (Lucetti et al. 2010).

Palm kernel shell biomass has been found to contain phenols. Polyphenolic activity present in these sources are found to have antioxidant and anti-inflammatory (Jiang and Dusting 2003; Zhang et al. 2011) activity. Pyroligneous acids (PA) are a complex blend of highly oxygenated liquid produced by condensation through thermochemical pyrolysis of biomass(Mathew and Zakaria 2015). This dark brown liquids have been found to exhibit antioxidant and free radical scavenging activity (Loo, Jain, and Darah 2007; Rungruang and Junyapoon 2010; Loo, Jain, and Darah 2008; Loo, Jain, and Darah 2007)). The antioxidant activity has been correlated to the presence of phenolic compounds which are widely distributed in natural products (Hagerman et al. 1998) with pronounced potential as anti-cancer (Reddy et al. 2009) and anti-inflammatory agents (Ho et al. 2013).

The PA contains the pyrolytic lignin comprising of phenolic compounds and their derivatives (Czernik and Bridgwater 2004). These organic compounds are high value compounds with potential for use in various chemical and pharmaceutical industries. The quality of these bio-oils is largely determined by the biomass material used (Kim 2015). The structure and yield of PAs is dependent on the biomass feedstock and conditions used in production (Demirbas 2007).

Traditional treatment of inflammation using PAs has been well documented (Ma et al. 2013). However, there is little scientific research on its efficacy and quantification. For example, previous studies by (Lee et al. 2011) and (Ho et al. 2013) respectively have shown that anti-inflammatory activity (in-vivo and in-vitro) of oak and bamboo PA act by decreasing the expression of inflammatory mediators. Oil palm TRF has also been shown to affect cellular mediators (Wu, Liu and Ng 2008) such as nitric oxide, prostaglandin E_2 etc. Hence, the possibility of utilizing palm kernel shell (PKS) from oil palm biomass, as an anti-inflammatory agent is highly possible.

This study attempts to extract phenols from palm kernel shell for use as an inhibitor to the COX and LOX pathway, thereby serving as a natural substitute for synthetic inflammatory drugs.

METHODOLOGY

The palm kernel shell sample (PKS) were collected from Felda palm oil mill processing plant in Johor, Malaysia. The sample cleaned and sun dried in the sun for a week at ambient temperature. Pyrolysis was carried out on the sample to convert to liquid pyroligneous acid. The condensate product of pyrolysis was obtained using a designed laboratory reactor. Optimized conditions were achieved at temperature 429^{o}C-519^{o}C, with heating rate 1.34^{o}C/min, nitrogen flow rate of 0.4L/min , residence time (39 mins and 35 mins), and condensing temperature 7.2^{o}C. The temperature was set at (400-519^{o}C) to produce the PA using the (PicoLog Recorder software, version 5.23.0) which was refined using ethyl-acetate (Rabiu and Zakaria 2017; Ma et al. 2014).

The concentrated ethyl-acetate pyroligneous acid (EPA-PKS) was separated into different fractions using a column chromatography (1.5 cm internal diameter. × 80 cm), silica gel (0.063-0.200 mm, Merck, Germany) as stationary phase, with different solvent combinations of solvents (n-Methanol-Ethyl acetate and n-Hexane-Ethyl acetate) and ratio combination.

The EPA-PKS was analysed for antioxidant activity using (TPC, DPPH, FRAP). The total phenolic content was determined using the Folin-Ciocalteau reagent with gallic acid used as a standard expressed as (mg/g) (Liu et al. 2007 and Loo 2012). The DPPH was determined using the method of Ma et.al. (2014) and the FRAP was determined using the reducing antioxidant activity of the sample expressed as (TE/g) Trolox equivalent (Guilllen and Manzons 2002; Loo 2007). The different fractions from column chromatography were screened for phenolic and antioxidant activities. The fractions with the highest phenolic and antioxidant activities where then characterised and identified using the Gas Column- Mass Spectrometry system (Agilent 190915 GC. The sample was injected in three washes using injector pressure and split flow rate were set at 9.78 psi and 28.2 mL/min respectively with helium gas at a flow rate of 7.0 mL/min used as the carrier gas, with run time set at 60 mins. The NIST database was used in characterizing the different compounds with manually assisted elucidation used for identification of compounds. The results of the GC-MS analysis of the different fractions

are shown in Figure 2-4 the results identify the presence of 16 different compounds including phenols, aldehydes, ketones, esters and sugar.

LIPOXYGENASE (LOX) INHIBITION ASSAY

The lipoxygenase inhibitor screening assay kit measures the formation of hydro-peroxides (PGG_2), following the lipoxygenation of fatty acids. A purified LOX (purified soya beans) was used as standard to screen for various inhibitors of LOX enzymes. The assay protocol requires the addition 10 ul of the PA fractions to the enzyme prior to starting the reaction in a 96 micro well plate pre-coated with a target specific antibody. The reaction was initiated with the addition of arachidonic acid /to serve as a substrate. Chromogen was also added to stop catalysis and then the plate was placed on an orbital shaker for 10mins. The blank, positive control and 100% initial activity reactions were also performed in specific order of reaction. The plate was then covered and placed on a shaker for five minutes. The cover was removed and absorbance measured at 490-500 nm using a plate reader using ELISA reader (FL 600 Microplate Reader, Bio-Tek). The percent inhibition for each inhibitor was calculated using the following equation:

$$I(\%) = \frac{CA - TA}{CA} \quad (1)$$

Where I is the percentage inhibition, CA is the control activity and TA is the test activity.

CYCLOOXYGENASE (COX) INHIBITION ASSAY

The COX-2 inhibitor screening assay directly measures the prostanoids (PGF2α) produced by reduction of COX derived from PGH_2 by the action of $SnCl_2$. The reaction system consists of a reaction buffer, haem, enzyme, substrate and inhibitors acting at 37°C for twenty minutes with background and enzyme controls. The reaction starts with the addition of arachidonic acid and incubated for two minutes at 37°C. The reaction was stopped with addition of saturated stannous chloride solution after five minutes at room temperature.

AChE tracer and antiserum was added and the place incubated for 18 hours at room temperature on an orbital shaker. The plate was finally developed with Ellman's reagent for 60 minutes and also placed on an orbital shaker at room temperature in a dark room. The absorbance was read at 405-420 nm. The percent inhibition for each inhibitor was calculated using Equation (1).

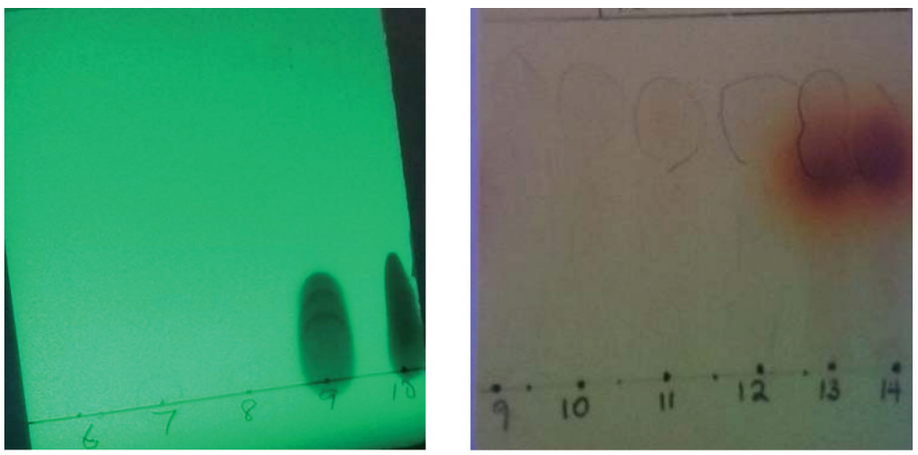
STATISTICAL ANALYSIS

The values are expressed as mean ± standard deviation (S.D) of triplicate measurements calculated using the Microsoft excel spreadsheet. The relationship between the total phenolic (TPC) and radical scavenging activities (FRAP) was established by linear regression using Pearson's coefficient. The significance differences from the respective controls was tested using Pearson's t-test SPSS Statistics package for each set of experiment.

RESULTS AND DISCUSSION

Pyroligneous acid yield of 40.44 wt. % was achieved and the results obtained are similar to previous results recorded by Demibras, (2007) with yield of 21-44 %. In addition to PA produced, solid char and condensable gases were also produced. Analysis of the phenolic content in the PA sample showed a concentration of 123.82 µg/ml GAE. The high phenolic concentration is an indication of potential antioxidant and free radical scavenging activity. The free radical activity of the sample was confirmed using the FRAP assay which showed the sample to have a high antioxidant activity of 361.02 ± 0.29 TE/g.

Fractionation of the EPA-PKS sample also resulted in elution of approximately 112 samples of 250 mL each from different solvent combinations. Thin layer chromatography (TLC) was used to identify fractions with similar compounds. This resulted in twenty three fractions being identified on the basis of similarities in their retention factor ($R_{F)}$ as shown in Figure 1.



(a) (b)

FIGURE 1. TLC plates used in identifying samples of EPA-PKS fractions with similar compounds

Figure 1 (a) shows profile of compounds in F_9 and F_{10} separated on the basis of difference in their retention time. Whilst Figure b shows similarities in different Fractions F_{11-12} and F_{13-14}.

The values of different separated fractions were found to have high phenolic content, while fractions F_9 (181.75 ± 17.0 (µg/g), F_{11-12} (168.36 ± 10.29 (µg/g)), F_{21-25} (174.95 ± 0.39 (µg/g)) and F_{26-30} (181.76 ± 15.54 (µg/g), were identified to have the maximum phenolic content. Further screening of the fractions using DPPH assay revealed strong scavenging activity of IC_{50} within range of 23.97 to 52.5%. High FRAP concentrations of F_9 (286.63 ± 2.56), F_{21-25} (331.80 ± 4.60) and F_{25-30} (318.16 ± 6.34) were also detected.

With application of GC/MS analysis, the presence of 16 different compounds of phenols, aldehydes, ketones, esters and sugars were detected in fractions 9, 21-25 and 26-30. Phenol and Phenol 2-methoxy were found to have high percentage concentrations of 79.34% and 20.65% respectively in fraction F_9. Phenols are essential starting materials in the synthesis of aspirin which has high inhibition for the cyclooxygenase enzyme. Furthermore, in $F_{(21-25)}$, 11 different compounds from compounds were identified with varying concentrations. They include; Phenol (4.89%), Maltol (4.91%), 2-Furancarboxaldehyde -5-hydroxymethyl (1.69%), Hydroquinone (1.67%), Ethyl- cyclopropane carboxylate (1.52%), Benzoic Acid, 4 - Hydroxy- (43.31%), Benzoic Acid, 4-Hydroxy- (21.99%), Benzaldehyde, 4-hydroxy-3,5-dimethoxy- (2.60%), Ethanone, 1-(4-Hydroxy-3, 5-Dimethoxyphenyl) - (4.44%), D-Nor leucine, N-(2-Hloroethoxy) Carbonyl-, Propyl Ester (2.37%) and 2-Pentanone, 1-(2,4,6-Trihydroxyphenyl) (10.62%). Finally in fraction $F_{(26-30)}$, three compounds were identified as Phenol (43.14%), 1, 4:3, 6-Dianhydro –alpha – D - Glucopyranose (22.53%) and Phenol, 3 - ethyl-, acetate (34.33%).

The GC-MS profile of Figure 2 include constituent compounds (a)- Phenol (b) Phenol-2-methoxy.

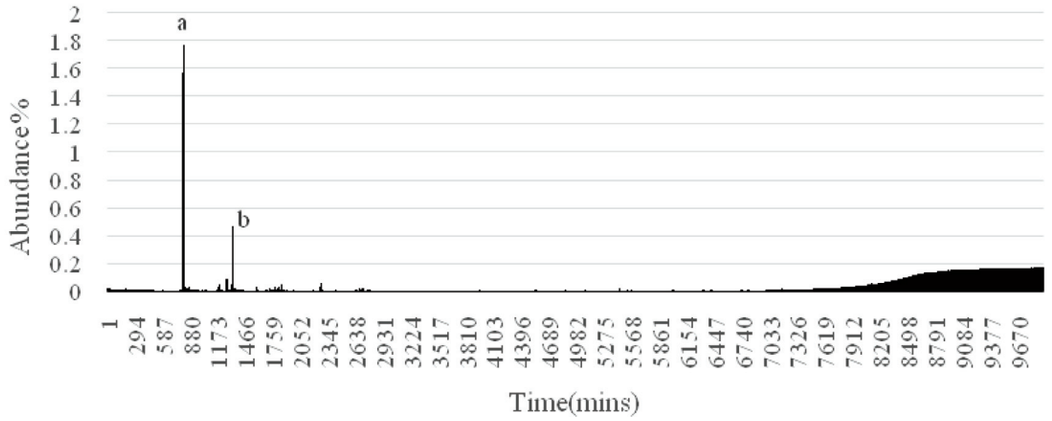


FIGURE 2. GC-MS profile of the pyroligneous fraction F_9 of palm kernel shell

The GC-MS profile of Fraction F_{21-25} is shown in Figure 3 and found to include constituent compounds (a)- D-norleucine N 3-chloro ethoxy carbonyl (b)- Maltol (c)- Benzoic acid4-hydroxy (d) Phenol (e) Aceto-syringone.

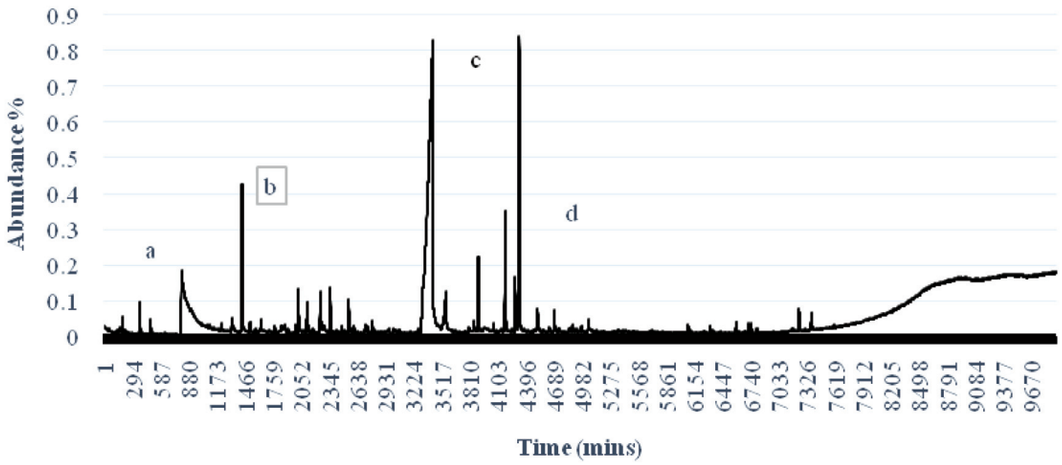


FIGURE 3. GC-MS profile of the pyroligneous fraction F_{21-25} of palm kernel shell

While Figure 4 shows the GC-MS profile of Fraction $F_{25\text{-}30\ which}$ include constituent compounds (a)-Phenol (b)- 1, 4, 3, 6 Di-anhydo-alpha-D- Glucopyranose and (c) Phenol-3-ethylacetate in different concentrations.

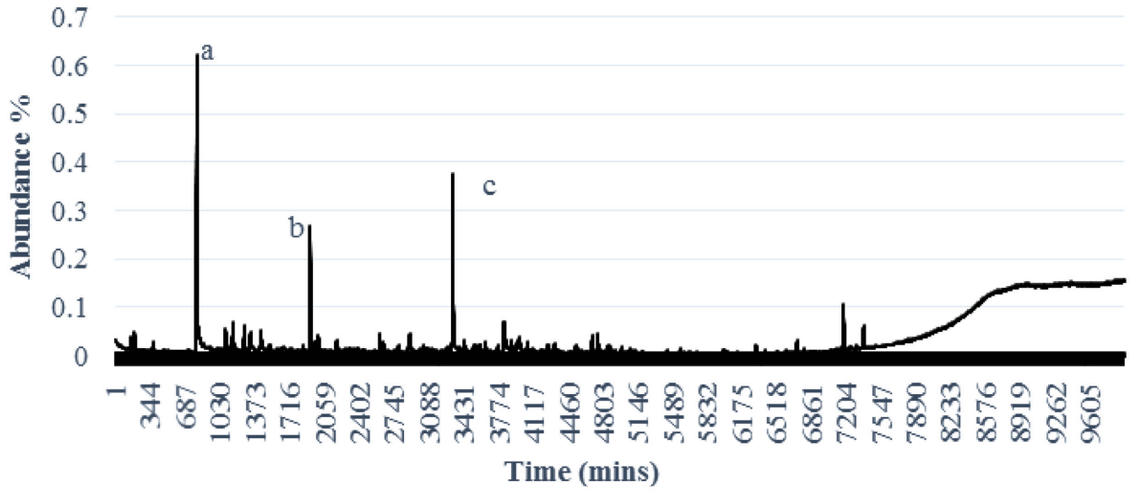


FIGURE 4. GC-MS profile of the pyroligneous fraction $F_{26\text{-}30}$ of palm kernel shell

SCREENING OF PYROLIGNEOUS ACID FRACTIONS FOR EFFECT ON COX -2 ACTIVITY

The results obtained in all fractions in this study were found to contain bioactive compounds. Table 1 represents COX-2 inhibition activity expressed as IC_{50} percent. The values are expressed as Mean ± SD of the three independent experiments. It can also be observed from Figure 1 that all three fractions (F_9, $F_{21\text{-}25}$ and $F_{26\text{-}30}$). The results of the Pearson's T-test showed a value of $P > 0.05$ indicating no significant difference between the fractions and the control standard used thus indicating a potency for the COX-2 enzyme.

From Table 1, it can also be noticed that fraction F_9 had high inhibition of COX enzyme at 17.04 % which can be ascribed to the phenol concentration. Fraction $F_{21\text{-}25}$ was also shown to have high inhibition for the COX enzyme with IC_{50} at (18.56%). This can be attributed to the high concentration of Benzoic acid, Hydroxyl (43.31%). In addition, fraction $F_{26\text{-}30}$ with IC_{50} of 48.42% inhibition had a high phenol concentration of 43.14%. Having p values of above 0.05, all the fractions examined have thus shown a potent inhibition of COX activity.

TABLE 1. The effect the different fractions of EPA-PKS on COX activity

Sample	IC50 (%)
Fraction (F9)	17.04 ± 0.02
Fraction (F21-25)	18.56 ± 0.02
Fraction (F26-30)	48.42 ± 0.03
Capsirin (+ve Ctrl)	19.70 ± 0.01

SCREENING OF PLANT EXTRACTS FOR EFFECT ON LOX ACTIVITY

The results of IC_{50} values of fractions (F_9, $F_{21\text{-}25}$ and $F_{26\text{-}30}$) are as shown in Table 2. The results are expressed as percent inhibition of LOX activity and values are expressed as Mean ± SD of three independent experiments. It can be observed that the examined fractions tested using Pearson's T-tests showed a $P > 0.05$ thus indicating no significant difference in effectiveness of fractions for the LOX enzyme. Thereby showing the potency of the fractions and control standards for inhibition.

TABLE 2. The effect of different fractions of EPA-PKS on LOX activity

Sample	IC_{50} (%)
Fraction (F_9)	53.34 ± 0.04
Fraction ($F_{21\text{-}25}$)	5.25 ± 0.03
Fraction ($F_{26\text{-}30}$)	10.07 ± 0.02
NDGA(+ve Ctrl)	40.35 ± 0.04

NDGA (Nordihydroguaiaretic Acid)

The values show specific phenolic compounds and their derivatives present in each fraction that could provide anti-inflammatory activity. Having p values of above 0.05, all the fractions examined have thus shown a potent inhibition of COX/LOX activity.

CONCLUSION

The results of the total phenolic and antioxidant activities have conclusively established the presence of bioactive compounds in different pyroligneous acid fractions extracted

from palm kernel shell. The study has also confirmed presence of bioactive molecules with potential antioxidant and anti-inflammatory activity. The results make strong pharmacological justifications on the use of oil palm biomass as a viable source value added compounds while simultaneously reducing waste.

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