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Extraction and Identification of Water-Soluble Compounds in Palm-Pressed Fiber by SC-CO_2 and GC-MS

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Abstract: The residual oil recovered from palm-pressed fiber is known to contain high level of carotenes (4,000 mg $kg^{-}1$ to 6,000 mg kg^{-1}) and vitamin E such as tocopherols and tocotrienols (2,000 mg kg^{-1} to 3,000 mg kg^{-1}). In this study, the water-soluble compounds in fresh palm-pressed fiber were recovered by supercritical carbon dioxide (SC-CO_2) extraction. The concentration of these compounds in palm-pressed fiber oil recovered was determined in the range of 1,000 mg $kg^{-}1$ to 2,000 mg kg^{-1}. A total of 12 compounds have been identified from this water-soluble extract with molecular weights ranging from 126 amu. to 208 amu.. Preliminary results showed that these water-soluble compounds possess superior antioxidant properties screening using FRAP (Ferric Reducing Ability of Plasma) and TEAC (Trolox Equivalent Antioxidant Capacity) assays.

Key words: Palm-pressed fiber, phenolic, water-soluble compound, supercritical carbon dioxide

INTRODUCTION

Plant phenolics form an extensive group of compounds, which prevent oxidation of lipids and may have beneficial effects on human health. These phenolic compounds occur naturally in plant sources such as olive oil[1,2], tea and fruits[3], rice bran[4], oat groats and hulls[5] and herbs[6]. They appear to have desirable medicinal properties. Some have been reported to be anti-tumor agents and to exhibit anti-viral and anti-microbial activities[7,8], hypotensive effects[9] and antioxidant properties[10]. Recent evidence suggested that phenolics may play an important role in the regulation of plant metabolism[11]. For example, flavonoids have been shown to be naturally occurring auxin transport regulators[12].

In short, the plant phenolics play a major role in both plant and animal health. Although much basic research still remains to be done, it is possible that many of these compounds, either as isolates or in conjunction with other compounds, may be used in both agricultural and pharmaceutical fields. Developing an understanding of the distribution and existence of phenolics in vegetable oils will give an assessment of the antioxidant behavior of this important group, which possibly contributes to the oil stability and its nutritional properties.

Over the years, the most abundantly available natural water-soluble antioxidant is vitamin C (L-ascorbic acid). It is a powerful water-soluble antioxidant, which is vital for the growth and maintenance of tissues in human. It is produced in higher plants, in the liver and kidney of higher animals, but not in human, bats and a variety of fishes. Therefore, human need to have access to sufficient ascorbic acid from adequate dietary sources or supplements in order to maintain a good health. Commonly available food sources of ascorbic acid are citrus fruits, potatoes, peppers, green leafy vegetables, tomatoes and berries. However, recent development has found that vegetable oil such as olive oil and its by-products contain considerably high amount of water-soluble phenolic compounds with excellent antioxidant activities[13].

Over the last two decades, fat-soluble vitamins (tocopherols and tocotrienols) and pro-vitamin A (α- and β-carotenes) from palm oil have received much attention in the field of research for their nutritional attributes. The sterilization process in the mill uses steam to terminate the activity of the enzyme in the palm fruits has possibly removed most of the water-soluble components from the fruitlets. In view of this, investigation was carried out to study the existence of water-soluble compounds especially the phenolics content in the by-products from the palm oil mill, which have direct contact with the process water.

MATERIALS AND METHODS

Fresh palm-pressed fiber was collected from MPOB Palm Oil Mill Technology Center, Negeri Sembilan, Malaysia. CP grade carbon dioxide (CO_2) with 99.995% purity was purchased from Malaysia Oxygen Berhad. The 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium persulfate, ferric chloride, ferrous chloride and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used were of analytical or chromatographic grade and were purchased from Merck, Germany.

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The SC-CO_2 extraction system was designed by Jasco, Japan (Model CO-960) completed with a column oven (Jasco CO-960), intelligent HPLC pump (Jasco PU-980), back pressure regulator (Jasco 880-81) and extraction vessel (Thar Designs, Inc. USA). The CO_2 chiller was purchased from Polyscience, USA.

The identification of compounds was carried out by GC (Agilent 6890 Series, USA) coupled with MS Detector (Agilent 5973N, USA). The capillary column used was HP5MS (30m × 0.25mm × 0.25μm) from USA.

Extraction: The fresh palm-pressed fiber was immediately kept under nitrogen blanketing at 0°C prior to extraction. It was ground into fine particles (2 - 5 mm length) and 600 gram of the fiber was loaded into one liter high-pressure extractor. The SC-CO_2 extraction system used is shown in Fig. 1. Approximately 5.0 mL min^{-1} of CO_2 at -5°C was pumped into the extraction vessel at the operating pressure of 300 bar and temperature of 60°C. The fiber was soaked in CO_2 for 20 min for equilibration before the dynamic extraction was carried out for three hrs.

The sample collected was heated at 60°C and transferred into a 50 mL separating funnel. 20 mL of *n*-hexane and 5 mL of distilled water was added and the water layer (bottom) was separated from the organic phase. Subsequently 5 mL of *n*-hexane was added to the water phase for complete separation of residual oil substances. The water layer was then dried with nitrogen and kept at -5°C prior to analyses.

Analysis of Sample by GC-MS: Approximately 0.02 gram of sample was weighed into a 1.5 mL GC vial. The sample was diluted with dichloromethane. 1.0 μl of sample was injected (splitless injection) into GC-MS. The injector temperature was set at 240°C, ionization voltage was 70eV, MSD transfer line temperature was 280°C and carrier gas, helium at a flow rate of 1.0 mL min^{-1}. The oven temperature programming was as follows: initial temperature at 100°C hold for 2 min, temperature ramping at 2°C min^{-1} to 290°C and hold at final temperature for 10 min.

Antioxidant Capacity Measurement: The antioxidant capacity was carried out using FRAP (Ferric Reducing Ability of Plasma) and TEAC (Trolox Equivalent Antioxidant Capacity) assays. *TEAC assay*: A stable stock solution of $ABTS^{\bullet+}$ was prepared by reacting 7 mmol L^{-1} aqueous solution of $ABTS^{\bullet+}$ with 2.45 mmol L^{-1} potassium persulfate and allowed to stand in dark for 12 to 16 hrs after which the dark blue solution was produced. For daily analysis, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 AU at 734 nm. 10 μL of diluted sample was spike into one

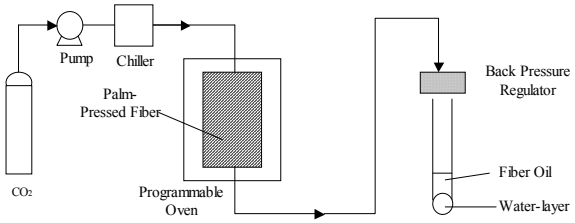

Fig. 1: Schematic diagram of SC-CO_2 extraction system

milliliter of the resulting solution and absorbance was recorded at 30°C at 1, 4 and 6 min after mixing. Results were expressed as TEAC in mmol Trolox equivalent at 1 mg mL^{-1} of sample. *FRAP assay*: The FRAP reagent was prepared by mixing TPTZ (2.5 mL, 10 mM in 40 mM HCl), 25 mL of acetate buffer and 2.5 mL of $FeCl_3 \bullet H_2O$ (20 mM). 3 mL of freshly prepared working FRAP solution was mixed with 100 μL of diluted sample. The absorbance at 593 nm was read after 30 min incubation at 37°C. The change of absorbance at 0min and 30 min was calculated and related to Fe (II) standard solution which is linearly proportional to the concentration of antioxidant. The final results were converted to mmol Trolox equivalent at 1 mg mL^{-1} of sample.

RESULTS AND DISCUSSION

Fresh palm-pressed fiber contained 30 wt.% to 50 wt.% of moisture contributed by steam condensation during sterilization and water injected in the screw-press of palm fruits. A substantial amount of water condensate is removed during sterilization process, which is subsequently drained off into the sludge pond as palm oil mill effluent. A process for the production of water-soluble antioxidant with potential nutraceutical applications from palm oil mill effluent using combination of centrifugation and membrane filtration technologies has been reported earlier[14]. The product was riched in flavonoids and phenolic compounds with potent antioxidant capacity and free radicals scavenging activities. However, there are still numerous unidentified compounds in the pool of the mixture and yet to be discovered. In addition, the feed material (sludge) is possibly contaminated with all types of dirt and the purification processes are difficult.

The current study aimed to recover the natural phenolic from the primary source, palm-pressed fiber, which is a cleaner feedstock and readily available from palm oil mills. The advantages of using CO_2 as the extraction medium are (a) the extracted water layer is clear without any solid contaminants and sediments, (b) able to concurrently recover the residual oil remaining in the fiber and (c) it is a solvent-free process.

Identification of water-soluble compounds by GC-MS fragmentation pattern: The percentage of water-soluble phenolics in fiber oil recovered from fresh palm-pressed fiber ranges from 0.1 wt.% to 0.2 wt.%. This group of compounds was very volatile and contributed to good aroma when exposed to the atmosphere. The identification of compounds was carried out using GC-MS. The total ion chromatogram of the water layer and structures of some phenolic compounds in palm-pressed fiber are shown in Fig. 2 and 3, respectively.

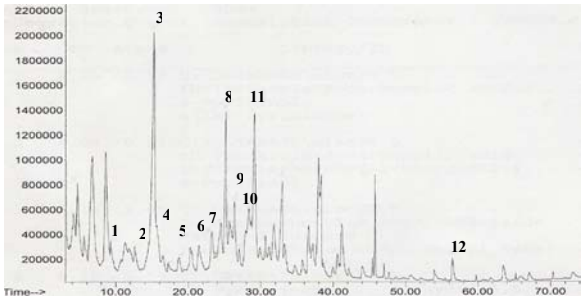

Fig. 2: Total ion chromatogram of water-soluble components from palm-pressed fiber

Compounds Identified by MS
1. 2-methoxy-4-vinylphenol
2. Vanillin or 3-methoxy-4-hydroxy-benzaldehyde
3. Vanillyl alcohol or 4-hydroxy-3-methoxy benzyl alcohol
4. 3,4-dimethoxy-benzenemethanol
5. 3-methoxy-4-hydroxy-phenylethanol
6. 3,4,5-trimethoxyphenol
7. Methyl-(2-hydroxy-3-ethoxy-benzyl)-ether
8. 4-hydroxy-3,5-dimethoxy-benzaldehyde
9. Benzoic acid, 4-hydroxy-3-methoxy-ethyl ester
10. 3,5-dimethoxy-4-hydroxy-benzylalcohol
11. 3-methoxy-4-hydrophenyl-methyl methanoate
12. 3,5-dimethoxy-4-hydrocinnamalde

Most of the phenolic compounds identified in palm-pressed fiber oil were found to have similar molecular structures with those in olive oil. These phenolics were at least double substituted at benzene ring at *ortho* and *para* positions except for 3-methoxy-4-hydroxy-phenylethanol (compound 5) and methyl-(2-hydroxy-3-ethoxy-benzyl)-ether (compound 7) containing *meta* substitutions. Due to the relatively small molecular weights of these compounds (126 amu. to 208 amu.), identification based on mass spectra was satisfactorily determined.

The side chains of phenol ring were substituted with alkyl ether, alkyl alcohol, alkyl ester, aldehyde, short chain alkyl or ethylene. The formation of benzylium ion by cleavage of all the side chains resulted in the formation of *m/z* 77 ion indicating the existence of aromatic ring in all the phenolics molecules. The cleavage in ß of aromatic ring (benzyl cleavage) forms a resonance stabilized tropylium ion $[C_7H_7]^+$ of *m/z* 91, which confirmed the benzyl entity of these molecules. The m/z of tropylium ion will deviate depending on the cleavage of side chains at benzene ring. The formation of these electrons stabilized species is shown by the mass spectral fragmentation of 2-methoxy-4-vinylphenol (compound 1) as in Fig. 4.

The *m/z* 91 and *m/z* 89 are observed in the mass spectral confirmed the tri-substitutions of compound 1.

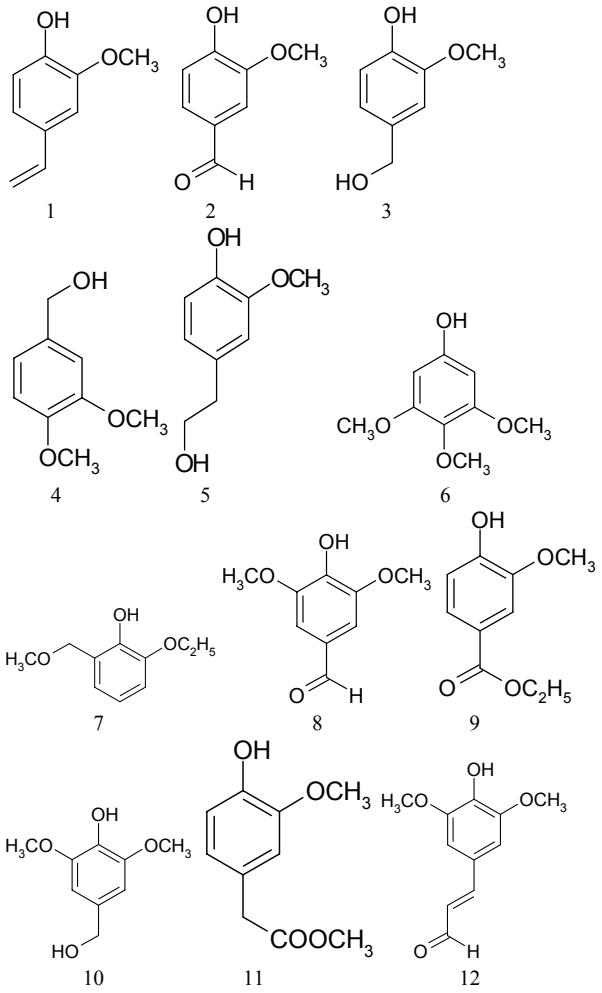

Fig. 3: Water-soluble phenolic compounds in palm-pressed fiber

Elimination of an unstable carbon led to the formation of the benzylium ion that stabilized the delocalized electrons in the ring. Other characteristic fragmented ions are *m/z* 135 $[M-15]^+$, *m/z* 107 $[M-28]^+$ and *m/z* 121 $[M-15-24]^+$. The $[M-28]^+$ fragment ion indicates that an ethylene group is present.

The mass spectral fragmentation of peak 2 confirmed the presence of an aldehyde group in vanillin or 3-methoxy-4-hydroxy-benzaldehyde (compound 2). The simple cleavage of hydrogen ion attached to carbonyl group in aldehyde gives a based peak *m/z* 151 $[M-1]^+$ with one mass unit less than the molecular ion as also clearly shown in compound 8. The observation of strong peak at *m/z* 109 is probably due to the cleavage of CO from fragmented species after β

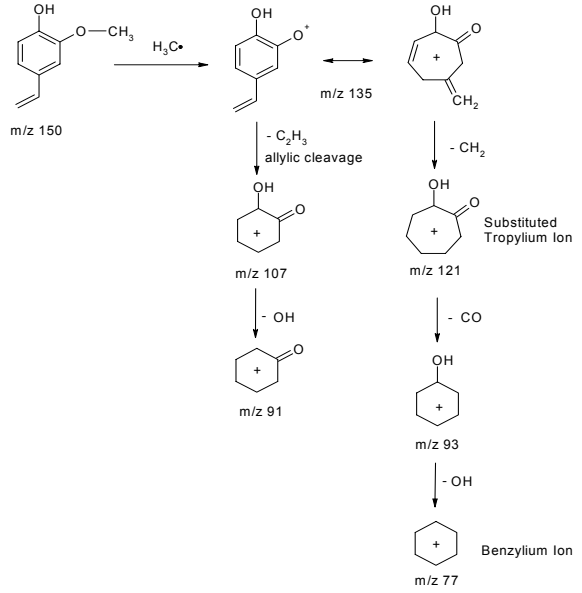

Fig. 4: Mass Spectral Fragmentation of 2-methoxy-4-vinylphenol

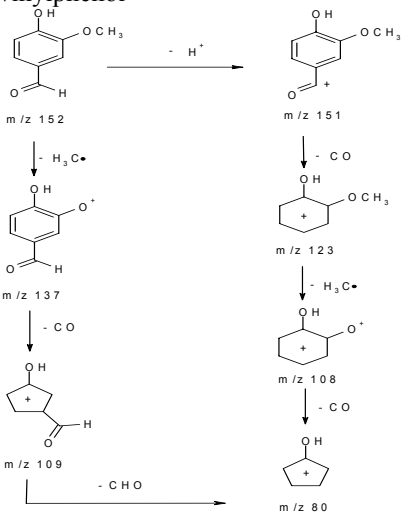

Fig. 5: Mass fragmentation of vanillin or 3-methoxy-4-hydroxy-benzaldehyde

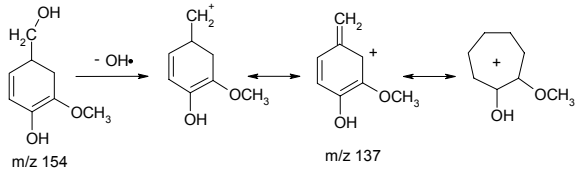

Fig. 6: Resonance structure of tropylium ion

cleavage of aromatic ring to yield [hydroxyl-aldehyde-$C_5H_5]^+$ and *m/z* 80 is fragmented by both *m/z* 152 and *m/z* 151 routes as shown in Fig. 5.

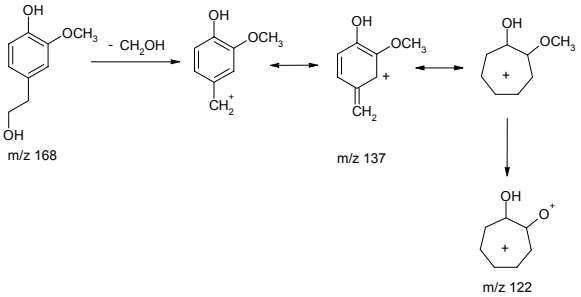

Fig. 7: Mass spectral fragmentation of 3-methoxy-4-hydroxy-phenylethanol

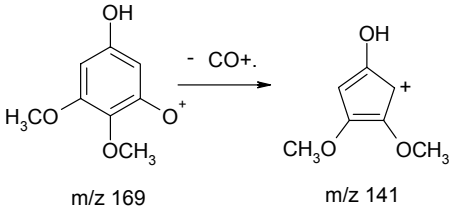

Fig. 8: Mass spectral fragmentation of 3,4,5-trimethoxyphenol

The fragmentation species of vanillyl alcohol or 4-hydroxy-3-methoxy benzyl alcohol (compound 3) characterised by *m/z* 137 $[M-OH]^+$, *m/z* 123 $[M-CH_2OH]^+$, m/z 107 $[M-CH_2OH-CH_3]^+$, m/z 93 $[M-CH_2OH-OCH_3]^+$, *m/z* 77 $[M-CH_2OH-OCH_3-OH]^+$ and *m/z* 65 $[C_5H_5]^+$. The peak at *m/z* 137 is stabilized by the resonance structure of tropylium ion resulted from cleavage in β of aromatic ring (Fig. 6). The β-bond is cleaved preferentially because of the charge can be stabilized by the aromatic ring.

The aromatic ring in 3,4-dimethoxy-benzenemethanol (compound 4) is di-methoxy and mono-methyl alcohol substituted with molecular ion

(*m/z* 168) as based ion in mass spectral. Another two intense peaks are observed in the mass spectral fragmentation at *m/z* 151 and *m/z* 139 as contributed by the formation of substituted tropylium ion $[M\text{-}OH]^+$ and cleavage of one methoxy group $[M\text{-}OCH_3]^+$. The other characteristic peaks are *m/z* 125, *m/z* 109, *m/z* 97, *m/z* 79 and *m/z* 67.

Compound 5 (3-methoxy-4-hydroxy-phenylethanol) is methoxy and ethyl alcohol substituted at *ortho* and *para* positions. The based peak at *m/z* 137 in mass spectral fragmentation is shown by β cleavage of methyl alcohol in aromatic ring at *para* position $[M\text{-}31]^+$, which formed the tropylium ion. The subsequent bond breaking at *ortho* positioned methoxy group gives another characteristic peak at *m/z* 122. The fragmentation of compound 5 is shown in Fig. 7.

Compound 6 (3,4,5-trimethoxyphenol) is tri-methoxy substituted at *meta* and *para* positions. The high probability of single cleavage of any methyl groups resulted in *m/z* 169 $[M\text{-}15]^+$ as based peak. Peak *m/z* 141 is observed by cleavage of carbonyl group $[M\text{-}CH_3\text{-}CO]^+$ as illustrated in Fig. 8. Other characteristic peaks at *m/z* 126 $[m/z\ 141\text{-}CH_3]^+$ and *m/z* 111 $[m/z\ 126\text{-}CH_3]^+$ are fragmented species after succeeding cleavage of methyl groups.

Apart from single ether substituted phenolics, di-ether substituted methyl-(2-hydroxy-3-ethoxy-benzyl)-ether (compound 7) was also found in the phenolics mixture. Two fragmented benzylium ions are produced by cleavage of ethyl ether (*m/z* 137 as based peak) and di-methyl ether (*m/z* 123). Species *m/z* 164 is observed

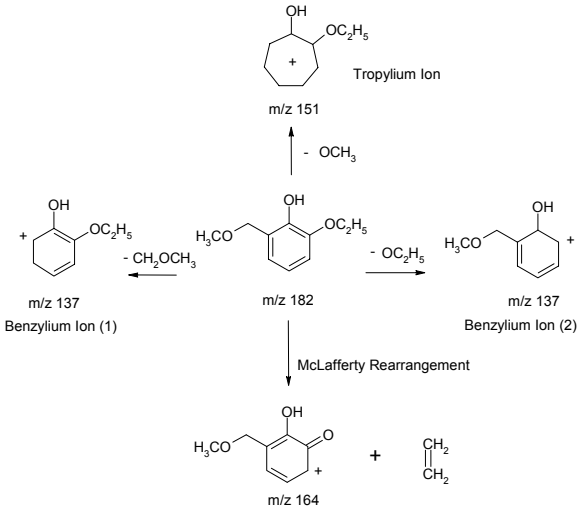

Fig. 9: Mass spectral fragmentation of 4-hydroxy-3,5-dimethoxy-benzaldehyde

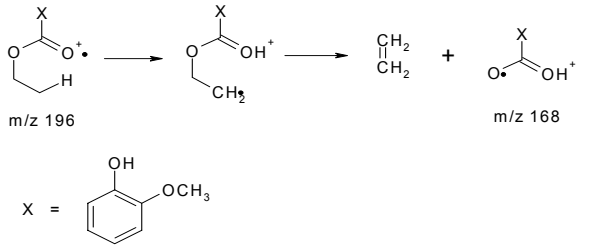

Fig. 10: McLafferty Rearrangement Mechanisme of Benzoic acid, 4-hydroxy-3-methoxy-ethyl ester

by elimination of ethylene group as ethoxy benzene readily undergone McLafferty rearrangement. The α cleavage of benzyl entity also formed tropylium ion characterised by *m/z* 151. The mass spectral fragmentation of compound 7 is given in Fig. 9.

Compound 9 (benzoic acid, 4-hydroxy-3-methoxy-ethyl ester) is attractively proposed to contain both ester and ether substitutions in phenol ring. The based peak of *m/z* 151 is fragmented species of ethoxy cleavage at carbonyl group to form stabilised tropylium ion. An interesting species at *m/z* 168 $[M\text{-}28]^+$ is proposed to have analogous McLafferty rearrangement mechanism in which an ethylene group is eliminated from carbonyl group as described in Fig. 10.

A fairly stable tropylium ion formed by cleaving the methyl ester substitute from benzyl entity contributed to the based peak (*m/z* 137) of 3-methoxy-4-hydrophenyl-methyl methanoate (compound 11). A simple mass spectral fragmentation was observed with *m/z* 122 and *m/z* 107 confirming the presence of methoxy group in the compound.

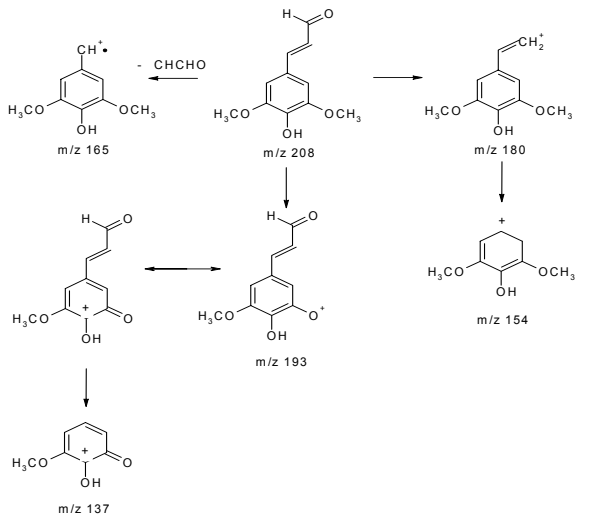

Fig. 11: Mass spectral fragmentation of 3,5-dimethoxy-4-hydrocinnamalde

Compound 12 (3,5-dimethoxy-4-hydrocinnamalde) contains three substitutions with two methoxy groups and one ethylene aldehyde group. The species of *m/z* 193, *m/z* 180, *m/z* 165, *m/z* 154 and *m/z* 137 are generated as shown in Fig. 11.

***In vitro* antioxidant capacity measurement:** Four types of samples namely α-tocopherol standard; water layer, oil layer and mixture of oil and water layer were studied for their antioxidant capacity. The mixture of water-soluble compounds recovered from the water layer of palm-pressed fiber extract have shown good antioxidant capacity based on comparative study against pure α-tocopherol.

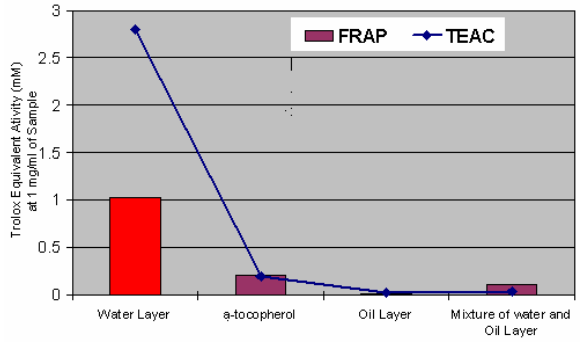

Fig. 12: Antioxidant activity of palm-pressed fiber phenolics

The antioxidant test results are shown in Fig. 12. The TEAC and FRAP assays are based on the electron-transfer reaction which can be represented by simple redox reaction. Water-soluble compound in water extract of palm-pressed fiber was found to possess superior anti-oxidant capacity with 10 times more effective than pure α-tocopherol based on TEAC value obtained. Removing of water layer from the oil layer has significantly reduced the antioxidant capacity of the oil to 8 times lower as indicated by FRAP values obtained. No correlations have been found for TEAC and FRAP values for the sample tested in this study. Regardless of the assay applied, the antioxidant capacity of this phenolic-riched water layer has shown to be an effective reducing agent. However, the discovery of palm phenolic-riched fraction requires more in depth investigation on clinical research as these assays do not measure in vivo stability, bioavailability, retention of compounds in tissue and reaction rate.

CONCLUSION

The water layer extract of fresh palm-pressed fiber has been found to contain mixture of phenolic compounds. Thorough study of the complete identification of the unknowns presence in the palm-pressed fiber water layer extract is required for screening the potential powerful antioxidant in the extract and possible synergistic relationship.

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