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Phytotoxicity of Phenolic Acids Extracted from Palm Oil Dry Solids

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ABSTRAK

Pengekstrakan ke atas pepejal kering kelapa sawit (PODS) telah dilakukan bagi mengenalpasti kompaun terlarutkan air yang terlibat dalam kefitotoksikan PODS. Ekstrak akueus PODS telah dilakukan sekatan berturutan dengan beberapa pelarut organik. Setiap ekstrak kering telah dibiocerakinkan untuk aktiviti perencatan terhadap pertumbuhan radikel tomato. Perencatan maksimum ke atas pertumbuhan radikel diperolehi pada ekstrak dietil eter, yang menghasilkan 53.3% pertumbuhan berbanding kawalan. Pemisahan pecahan eter menggunakan kromatografi turus menghasilkan satu pecahan toksik, RM10, yang hanya dapat menampung 30% pertumbuhan radikel. Perbandingan pecahan tersebut dengan 14 kompaun fenolik sintetik ke atas kromatograf lapisan nipis menunjukkan persamaan antara empat kompaun tersebut. Analisis lanjut menggunakan kromatografi cecair berkeupayaan tinggi menunjukkan pecahan RM10 terdiri dari asid vanilik. Namun demikian, pecahan RM10 lebih merencatkan pertumbuhan radikel tomato berbanding asid vanilik sintetik.

ABSTRACT

A study on the extraction of palm oil dry solids (PODS) was conducted to identify the water-soluble compounds involved in the phytotoxicity of PODS. The aqueous extract of PODS was sequentially partitioned using various organic solvents. Each of the dried extracts was bioassayed for inhibitory activity on the growth of tomato radicles. Maximum inhibition of radicle growth was observed in the diethyl ether extract, resulting in 53.3% growth compared with control. Further separation of the ether fraction using column chromatography resulted in a single toxic fraction, RM10, which caused only 30% radicle growth. The fraction was compared with 14 synthetic phenolic compounds using thin-layer chromatography and was observed to be similar to four of the compounds. Further analysis by high performance liquid chromatography revealed that the RM10 fraction comprised vanillic acid. However, the RM10 fraction was more inhibitory to the growth of tomato radicles than synthetic vanillic acid.

INTRODUCTION

The increase in palm oil production has generated abundant waste effluent from the palm oil mills. Due to the pollution problem from the effluent, several treatment methods have been developed, producing different types of by-products. Palm oil dry solids (PODS) is one of the by-products generated from mills equipped with a decanter drier system. It contains substantial amounts of plant nutrients and is currently being applied to soil as an organic fertilizer. However, PODS must be properly decomposed before it can be applied to soil. Growth of plants, especially vegetables, has been shown to be adversely affected by undecomposed PODS or other forms of palm of mill effluent (POME). Application of raw or partially decomposed PODS to sandy tailing soil has been shown to inhibit growth of tomato and spinach seedlings (Radziah *et al.* 1997). Zulkifli and Rosmin (1990) observed low yields of cowpea and mustard greens grown on sandy tailing soil with undecomposed POME added. The inhibitory effect of raw effluent has been associated with the presence of lipid and volatile substances (Lim 1986). However, such effects are reduced when the material is completely decomposed. Growth of vegetable seedlings increased when

grown on soil containing decomposed PODS (Radziah *et al.* 1997).

It has been shown that decomposed PODS contains higher quantities of plant nutrients, especially nitrogen, and low lipid contents. Interactions of nutrients and improved physical and biological properties finally lead to improved plant growth. However, there is little information which explains the inhibitory effects of undecomposed PODS or other forms of POME.

The phytotoxicity of plant residue which reduces growth of plants has been associated with the presence of various organic compounds including phenolic compounds (Rice 1984) which are widely distributed in various plant species. These compounds are either leached from plant residues or are formed as by-products during residue decomposition in soil. Several phenolic acids such as ferulic, *p*-coumaric, *p*-hydroxybenzoic, syringic, vanillic acids and *p*-hydroxybenzaldehyde have been identified in extracts of wheat mulch (Lodhi *et al.* 1987), rice residues (Chou and Lin 1976) and sorghum-sudan grass hybrid (Weston *et al.* 1989). The activities of various compounds in soil are often attributed to the water-soluble fractions. The allelopathic effects of phenolic compounds could also be due to both the water-soluble and free form compounds (Whitehead *et al.* 1983). Growth is adversely affected as these water-soluble compounds are taken up by plant roots which inhibit nutrient uptake and affect various physiological processes (Rice 1984). Currently, the potential phytotoxicity of soluble compounds in raw PODS has not been properly characterized. The soluble compounds in PODS could be similar to those in other crop residues since PODS is a by-product originating from plant material i.e. oil palm fruit. Identification of these water-soluble compounds is essential in order to better understand the nature of phytotoxicity and mechanisms involved in its formation, thus enabling proper management of the effluent. Therefore, the following studies were conducted to extract and identify the water-soluble compounds present in raw PODS which are inhibitory to plant growth.

MATERIALS AND METHODS

Extraction of Water-soluble Phytotoxic Compounds

Raw PODS was collected from the Rantau palm oil mill, Negeri Sembilan. The dark granular material, previously described by Radziah *et al.* (1997), contained 19.4% C, 1.44% N, 0.32% P, 1.32% K, 1.46% Ca, 0.32 Mg, 12.0% lipid and the pH was 5.0 (1:5 in H_2O). It was kept dry in the cold room at 8 ± 1°C before use to prevent microbial decomposition. The method of extracting the water-soluble compounds was adapted from that of Weston *et al.* (1989). The flow diagram of the extraction and partitioning procedures used is shown in *Fig. 1*. One hundred gram batches of dried PODS were extracted for 6 hours with 500 ml distilled water in a 1-l conical flask on an orbital shaker, and the suspension was left to stand in the refrigerator for one hour at 4 ± 1°C. The supernatant was decanted and centrifuged at 5,000 rpm for 15 min and the clear brown solution obtained transferred into a clean flask, and the residue returned to the original flask and re-extracted using 250 ml water. A total of 750 ml of water was used to extract every 100 g PODS. The fine

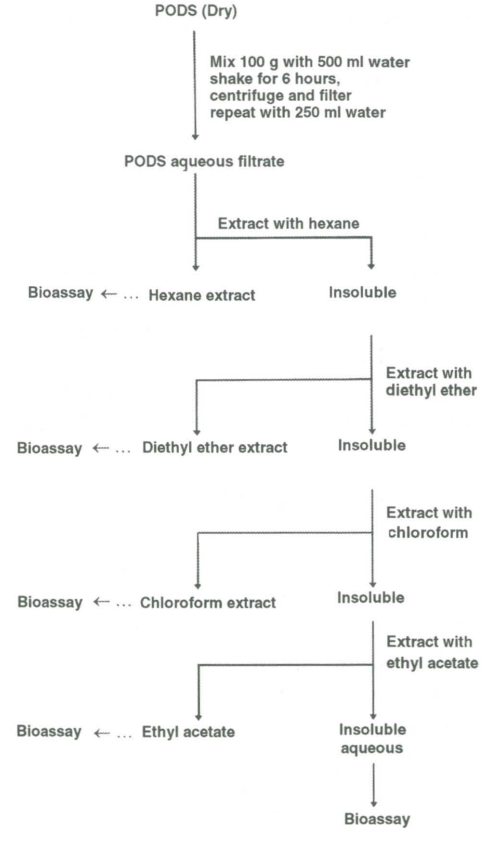

Fig. 1. Flow diagram of the extraction and partitioning procedure of PODS

particulates in the aqueous extract were removed by vacuum filtration through Whatman No. 4, 1 and 42 filter papers sequentially. The extraction procedure was repeated until sufficient aqueous extract was collected for further use. A total of 2.2 kg PODS was used during the extraction process, giving 16.5 l of aqueous extract.

Partitioning of Aqueous PODS Extract

The aqueous PODS extract was sequentially partitioned with hexane, diethyl ether, chloroform and ethyl acetate. The solvents were used to separate the polar and non-polar compounds present in the aqueous PODS. The separation procedure was conducted in batches using 1 l of aqueous extract each time. Each litre of the aqueous extract in a 2-l separatory flask was partitioned six times with 200 ml of the respective four solvents. The extracts were transferred to clean flasks, dried with anhydrous $MgSO_4$ and filtered. The solvent was subsequently removed from each extract by rotary evaporation at 35-40°C. The degree of inhibitory properties of each extract was then evaluated on growth of tomato radicles as described below.

Bioassay of the Solvent Soluble Fractions

Each extract (yellowish brown residue) was weighed and redissolved in chloroform to form a solution with a concentration of 1.0 mg residue ml^{-1} chloroform. Ten millilitres of the remaining aqueous solution were then freeze-dried and redissolved in water to form a similar concentration of 1 mg ml^{-1}. One millilitre of each extract was then placed in separate sterile glass petri dishes (90 x 15 mm), lined with a double layer of Whatman No. 1 filter paper. The chloroform was allowed to evaporate overnight before adding 3 ml sterilized distilled water, to a final concentration of 334 µg residue ml^{-1}. The control dish was given 1 ml chloroform which was allowed to evaporate overnight before the addition of 3 ml distilled water. Ten uniform pregerminated tomato seeds were then placed equidistantly in each dish and incubated in the dark at 30°C. Radicle length of each seedling was measured 72 h after placement in the dish. The activity of the extracts was expressed as percentage radicle growth of control. The bioassay for each extract was replicated three times.

Separation of the Active Fractions by Column Chromatography

The bioassay conducted on the different extracts showed that the diethyl ether extract was the most toxic to growth of tomato radicles (Table 1). This extract was further separated using column chromatography. The ether extract (4.4 g) was precoated with silica gel (60, Baker, 200-250 mesh) and loaded into a silica gel column (30 cm x 6 cm). The column was eluted with petroleum ether, gradually increas-

TABLE 1
Quantity of solvent extracts partitioned from 2.2 kg PODS and their effects on growth of tomato radicles

Solvent extract	Quantity extracted (g)	Recovery (%)	Radicle length ± standard error	Radicle growth (% of control)
Hexane	7.26	0.33	35.8 ± 5.0	115.0
Diethyl ether	4.40	0.20	16.6 ± 1.3	53.3
Chloroform	1.54	0.07	24.0 ± 1.4	77.1
Ethyl acetate	1.98	0.08	30.7 ± 1.9	98.7
Remaining aqueous	ND	ND	41.4 ± 3.1	132.1
Control (H_2O)	-	-	31.1 ± 2.8	100.0
LSD (0.05)			8.4	20.9

ND - not determined

ing the amount of chloroform and methanol. Two hundred fractions of 100 ml each collected from the column were monitored using thin-layer chromatography (TLC) (Kieselgel 60 F_{254} by Merck, 0.2 mm layer) and the profiles were visualized under UV light at 254 nm. Fractions with similar profiles under the UV light were combined to form 11 fractions. During the combination process, precipitation was observed in fractions 9, 10 and 11. These fractions were then separated further into chloroform and methanol soluble fractions. The respective fractions at the concentration of 334 µg ml^{-1} was bioassayed for their inhibitory activity using two controls. Chloroform was used as control for fractions soluble in chloroform and methanol for fractions soluble in methanol. There was strong inhibitory activity in one of the fractions (Table 2), which gave a single spot on the TLC, was active under UV light and was hence selected for further characterization

Comparison of the Unknown Fraction with Standard Phenolic Compounds

The active unknown fraction obtained (designated as RM10) was tentatively identified by comparing its migration profile with the other synthetic (standard) phenolic compounds. The RM10 fraction and the standards were subsequently dissolved in methanol, spotted on TLC plates using a mobile phase of 6:4 acetone-chloroform, and their profiles visualized under UV (254 nm) light. The 14 standard compounds used were: 4-hydroxybenzaldehyde, vanillin, acids of *p*-hydroxybenzoic, ferulic, *p*-coumaric, vanillic, caffeic, gentisic, *p*-hydroxyphenyl acetic, sinapic, gallic, salicylic, *trans*-cinnamic and syringic (Fluka Chemical Company).

HPLC Profile of the Unknown Fraction

The identity of the unknown fraction RM10 was further confirmed by using high performance liquid chromatography (HPLC). The RM10 fraction and the four standard samples (ferulic, *p*-hydroxybenzoic, vanillic and *p*-coumaric acid) were dissolved singly in methanol to form a concentration of 1 mg ml^{-1}, and filtered through 0.45 µm Millipore membrane. Ten microlitres of the sample was injected into the Waters HPLC system fitted with an absorbance detector model 460 set at 254 nm, a pump model 510 and a reverse-phase µ Bondapak C_{18} column (300 mm x 3.9 mm). The isocratic elution solvent used consisted of a mixture of methanol, ethyl acetate and acetic acid in the

TABLE 2
Recovery of different fractions from column chromatography of diethyl ether extract and their effects on growth of tomato radicles

Fractions	Quantity recovered (mg/4.4 g)	Recovery (%)	Radicle length (mm) ± standard error	Radicle growth (% of control)
R1-R3	126	2.9	ND	ND
R4	488	11.1	37.0 ± 3.8	92.5
R5	752	17.1	31.5 ± 4.4	78.7
R6	1271	28.9	38.2 ± 2.5	95.7
R7	869	19.8	34.0 ± 1.3	85.1
R8	158	3.6	35.6 ± 2.2	88.9
R9 $CHCl_3$	194	4.4	15.7 ± 0.4	39.2
R9 MeOH	45	1.0	30.0 ± 2.6	68.7
R10 $CHCl_3$	27	0.6	13.9 ± 1.7	34.8
R10 MeOH	184	4.2	13.2 ± 4.3	30.2
R11 $CHCl_3$	3	0.1	39.0 ± 1.0	97.5
R11 MeOH	31	0.7	24.9 ± 4.8	56.9
Control ($CHCl_3$)[a]			36.6 ± 3.3	100.00
Control (MeOH)[b]			41.7 ± 4.6	100.00
LSD (0.05)				18.8

ND - not determined
a for fractions dissolved in $CHCl_3$
b for fractions dissolved in MeOH

ratio of 35:1:2, at a flow rate of 2 ml min^{-1} (Blum *et al.* 1984).

Effect of Various Concentrations of RM10 Fraction and Standard Phenolic Acids on Growth of Tomato Radicles

The bioassay technique described previously was used to compare the toxicity of RM10 fraction with standard phenolic acids. The activity of RM10 was assessed with four standard phenolic acids of similar TLC profiles. The standards used were: ferulic, *p*-hydroxybenzoic, vanillic and *p*-coumaric acids. The concentrations used were; 0, 250, 500, 750 and 1000 µg ml^{-1}. Growth response curves were then drawn to determine the concentration of each compound responsible for causing 50% inhibition (I_{50}) on elongation of tomato radicles.

RESULTS

Extraction of Water-soluble Phytotoxic Compounds

Various amounts of water-soluble organic fractions were extracted from PODS. The highest amount of dry fraction recovered from the partitioning procedure was the hexane extract (7.26 g), followed by diethyl ether extract (4.4 g) (Table 1). The hexane extract probably consisted of lipids and non-polar compounds which were present in PODS in substantial amounts. The hexane extract made up 0.33% and diethyl ether extract 0.20% of the total dried PODS used. Smaller amounts of residues were obtained from the chloroform and ethyl acetate extracts.

Results from the bioassay studies showed that different solvent extracts significantly ($P \leq 0.01$) influenced elongation of tomato radicles. The maximum inhibitory activity of radicle growth was observed in the diethyl ether extract where radicle elongation was significantly reduced to 53.3% of the distilled water control. This indicated that the ether had extracted out most of the inhibitory compounds present in the aqueous PODS. The chloroform and ethyl acetate extracts exhibited lesser inhibition on radicle growth. The hexane extract as well as the remaining crude aqueous extract, however, stimulated growth of tomato radicles. Improvement in radicle elongation, especially in the remaining aqueous fractions, indicated absence of the toxic compounds.

Isolation and Characterization of Phytotoxins

Since the greatest inhibitory effect was obtained from the diethyl extract, this extract was further separated by column chromatography and each new fraction recovered was bioassayed for inhibitory activity. Two hundred fractions were collected from the separation process and were monitored by using TLC. Fractions with similar TLC profiles were then combined to form 11 distinct fractions. Fractions R9, R10 and R11 which formed precipitations were further extracted by dissolving the individual fraction in chloroform and methanol. Three additional fractions were obtained from this extraction (Table 2). The flow chart of the separation procedure is shown in *Fig. 2.*

Results of the bioassay of all fractions (R4-R11) showed significant ($P \leq 0.01$) differences in their inhibitory activities (Table 2). High inhibition on radicle growth was concentrated in three fractions, R9 which is soluble in $CHCl_3$, R10 which is soluble in $CHCl_3$ and R10 which is soluble in MeOH. Among these three fractions, the R10 in MeOH (designated as RM10) had the lowest radicle growth of 30.2%. This brown, viscous fraction was found to produce a single

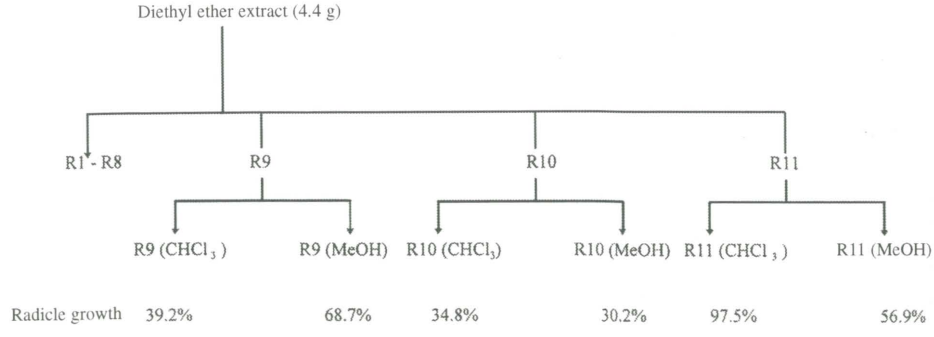

Fig. 2. Separation of the diethyl ether extract and the effect on growth of tomato radicles

spot on the TLC and was strongly visible under UV light (254 nm). Although the other two fractions (R9-$CHCl_3$ and R10-$CHCl_3$) showed almost similar activity to RM10, they did not give a distinct spot on the TLC, and thus required further separation for identification. No attempt was made to identify them in this study.

The amount of RM10 fraction recovered from the crude diethyl ether extract using the column chromatographic procedure was very small (only 184 mg/4.4 g or 4.2%) (Table 2). This indicates that the active RM10 fraction which could be toxic to plant growth was only 0.0084% (dry weight) of the original raw PODS.

The unknown RM10 fraction was tentatively identified by comparing its TLC profile with the standard phenolic acids. The results showed that this fraction was similar to 4 of the standards: ferulic, *p*-hydroxybenzoic, *p*-coumaric and vanillic acids (*Fig. 3*). The identity of RM10 was further confirmed by injecting the sample into HPLC using the reverse-phase µ Bondapack C_{18} column. A single peak at the retention time of 2.89 min was obtained (*Fig. 4*). Further coelution with known standards indicated that RM10 was similar to vanillic acid. The retention times of the respective standards are shown in Table 3.

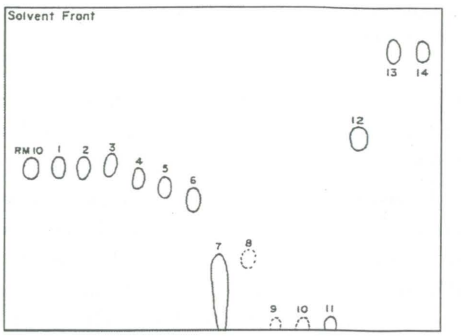

Fig 3. Thin-layer chromatography profiles of fraction RM10 and standard phenolic acids with mobile phase of acetone : chloroform (6:4)

**Key: 1) p-hydroxybenzoic acid; 2) ferulic acid; 3) p-coumaric Acid; 4) vanillic acid; 5) sinapic acid; 6) syringic acid; 7) caffeic acid; 8) p-hydroxyphenyl acetic acid; 9) gentisic acid; 10) salicylic acid; (11) trans-cinnamic acid; 13) 4-hydroxybenzaldehyde; 14) vanillin*

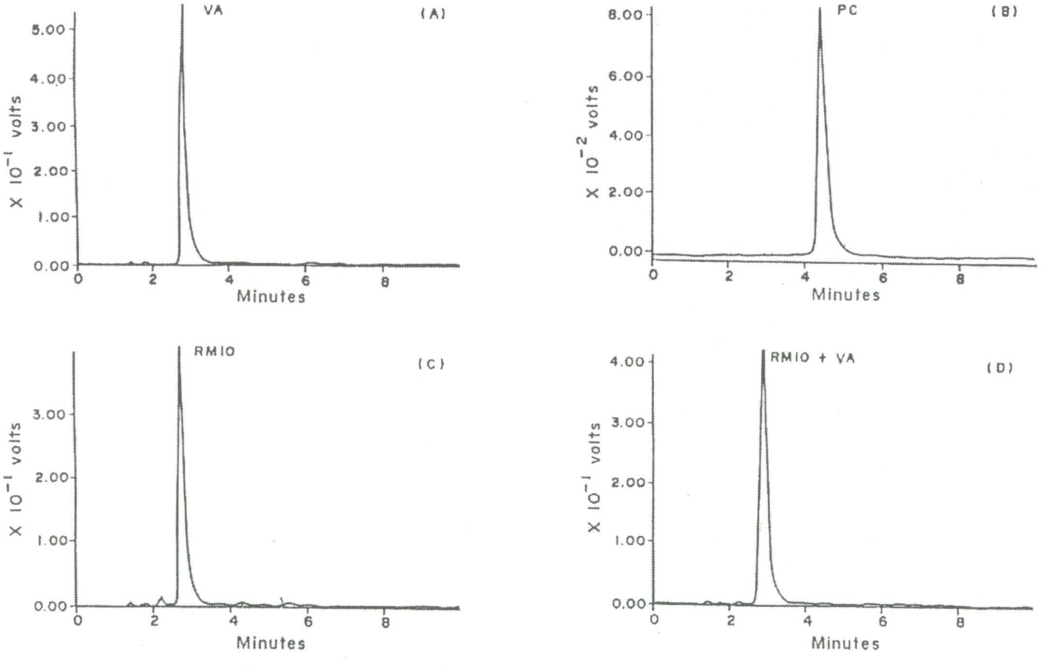

Fig 4. HPLC chromatograms of (A) vanillic [VA], (B) p-coumaric [PC], (C) RM10 and (D) RM10 + VA, using C_{18} column with flow rate of 2 ml min^{-1} and solvent of 35% methanol, 1% ethyl acetate and 2% acetic acid

TABLE 3
Retention times of the unknown and standard phenolic compounds

Phenolic acid	Retention time (min)
Ferulic	5.40
p-coumaric	4.64
p-hydroxybenzoic	3.02
Vanillic	2.89
Extract RM10	2.89

Growth Response of Tomato Radicles to Various Concentrations of RM10 and Standard Phenolic Acids

The length of tomato radicles was found to be significantly ($P \leq 0.01$) affected by the concentrations of RM10 and the standard phenolic acids (ferulic, vanillic, *p*-coumaric and *p*-hydroxybenzoic acids) (*Fig. 5*). Radicle elongation was reduced with increase in concentration of RM10 and phenolic acids. This indicated that the toxicity of these compounds increased with increase in concentration. The percentage of radicle growth showed negative liner responses to increasing concentration of the compounds.

p-coumaric acid was found to be the most toxic to growth of tomato radicles of all the compounds. Total inhibition (no growth) on radicle elongation was observed at 500 μg ml^{-1} *p*-coumaric acid concentration. Ferulic acid was least toxic to the growth of tomato radicles. At low concentrations, the activity of RM10 was close to that of *p*-coumaric acid. However, the activities differed as the concentration of compounds increased to 500 μg ml^{-1}. Total lack of growth of tomato radicles was observed as the concentration of these compounds increased to 1000 μg ml^{-1}, i.e. the highest concentration studied.

The 50% radicle growth inhibition (I_{50}), i.e. the compound concentration at which 50% of the radicle elongation was inhibited, was also calculated from the growth response curves in *Fig. 5*. The RM10 fraction was more toxic then *p*-hydroxybenzoic, vanillic and ferulic acids. The descending order of toxicity of the compounds with increasing I_{50} values recorded was *p*-coumaric acid (194 μg ml^{-1}) > RM10 (292 μg ml^{-1}) > *p*-hydroxybenzoic acid (434 μg ml^{-1}) > vanillic acid (466 μg ml^{-1}) > ferulic acid (524 μg ml^{-1}).

DISCUSSION

Results from the extraction procedures used showed that most soluble toxic compounds present in raw PODS were concentrated in the diethyl ether extract. This extract was found to reduce the growth of tomato radicles by 46.7% of control (Table 1). Most of the earlier findings have also shown that the toxic compounds found in other crop residues are soluble in polar solvents such as ether (Weston *et al.* 1984). It was also observed that the toxicity of the remaining aqueous PODS was completely eliminated after removal of the toxic extracts, where growth of tomato radicles was found to be stimulated. Further separation of the ether extract using column chromatography recovered a more toxic RM10 fraction. The amount of this phytotoxic compound present in raw PODS was small (0.0084%). Hence, under normal conditions the application of a low amount of raw PODS to soil would probably cause minimum inhibition on

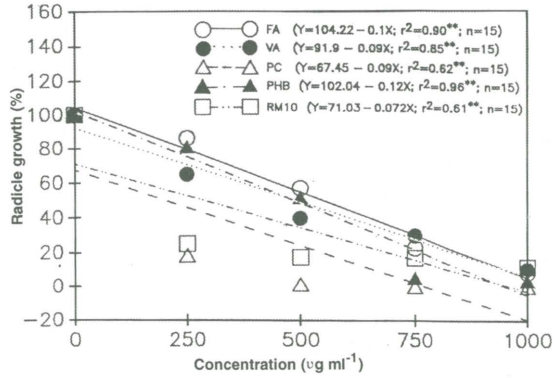

Fig 5. Growth of tomato radicles in relation to concentration of phenolic acids: ferulic (FA), vanilic (VA), p-coumaric (PC), p-hydroxybenzonic (PHB) and fraction RM10

growth of plants. Degradation of these compounds is rapid in well-drained soils. However, continuous application of raw PODS to clayey soil with long periods of water saturation could probably lead to accumulation of these phytotoxic compounds.

Although RM10 was confirmed to be vanillic acid on the HPLC, its inhibitory effect on growth of tomato radicles was higher than that caused by the synthetic vanillic acid. The higher toxic effect of RM10 was probably a result of being bound to other compounds which have higher toxic effect. No attempt was made to purify RM10 in the present study.

The activity of RM10 was also compared to the activity of the other toxic phenolic compounds (besides vanillic acid) commonly found in soil: ferulic, *p*-coumaric and *p*-hydroxybenzoic acids (Lodhi *et al.* 1975). The inhibitory effect exhibited by RM10 was found to be intermediate between that of *p*-coumaric and vanillic acid (*Fig. 5*). This indicates that the phytotoxic compound in PODS is present in mixed form. This is to be expected since under natural conditions, phenolic acids usually occur in mixed rather than in single form. The phytotoxic effects of organic residues as observed is probably the result of the strengthening effect of complex organic mixtures (Rasmussen and Einhellig 1977; Blum 1996).

Results of the present study showed that the RM10 fraction was more toxic then standard ferulic, vanillic, and *p*-hydroxybenzoic acids, but less toxic than *p*-coumaric acid on the growth of tomato seedlings. However, the inhibitory activity of RM10 extracted from PODS was low compared to the activity of some other standard phenolic compounds. The concentration of RM10 which caused 50% reduction in radicle growth (I_{50}) of tomato was 292 μg ml^{-1}. This value was higher than other compounds reported to be phytotoxic. Vanillic and *p*-coumaric acids at as low a level as 100 μg ml^{-1} were reported to inhibit growth of rice seedlings, seed germination and growth of ryegrass, lucerne and wheat (Hartley and Whitehead 1985). Weston *et al.* (1989) observed that *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde at concentrations of 70-140 μg ml^{-1} reduced radicle growth of curly cress, lettuce and radish by 50%. This indicates that the degree of toxicity of compounds differs with plant species. The tomato seeds used in the study were more tolerant to the presence of phenolic acids than other seeds. The choice of plant species in the bioassay technique is crucial in determining the inhibitory activity of phenolic compounds.

The amount of the inhibitory compound extracted from PODS in the present study was only an estimate and could not therefore reflect the actual amount present in the entire PODS. This study could only focus on compounds that are soluble in water and not on the total compounds present. The amount of toxic compounds may also vary with the type of POME. There are variations in the physical, chemical and biological properties of POME generated from different palm oil mills which use different methods of effluent treatment (Lim 1986). Raw POME needs to be decomposed in order to eliminate the inhibitory effect on plant growth. The proper management of POME applied to soils is important in order to reduce its phytotoxic effect, while obtaining optimum benefits for plant growth. Decomposition is important to reduce the phytotoxicity of various types of POME in order to convert them to valuable organic fertilizer.

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