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Research Article

Analysis and identification of phenolic compounds in *Dioscorea hispida* Dennst

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Abstract

Hydroxybenzoic acid, hydroxycinnamic acid and their derivatives were found in extracts of *Dioscorea hispida* Dennst tuber (Kloi tuber). Extraction was done with 60% aqueous MeOH, hydrolyzed and fractionated into fractions by SPE C_{18} cartridge by using aqueous MeOH (10-60% MeOH). The identification was achieved by Rp HPLC and ESI-LC-MS in negative ion mode. The compounds found present in the peel were caffeic acid, chlorogenic acid, p-hydroxybenzaldehyde and methylester of protocatechuic acid (methoxyprotocatechuate). Only methyester of protocatechuic acid was found in the flesh of Kloi tuber. Most of the identified compounds were in the 20% MeOH fraction. Antioxidant potential of Kloi tuber was also investigated, the DPPH value for 20% MeOH peel and flesh fraction were EC_{50} 1.05 x 10^2 µg/ml for the peel fraction and EC_{50} 2.04 x 10^2 µg/ml for the flesh fraction. FRAP values were 3.26 x 10^2 µmol Fe^{2+}/mg for peel and 2.93 x 10^2 µmol Fe^{2+}/mg for flesh. The total phenol content of peel was 0.068 ± 0.04 mg GAE/mg and 0.085 ± 0.05 mg GAE/mg for flesh.

Keywords: Kloi tuber, FRAP, DPPH, chemical analysis, Rp HPLC, ESI-LC-MS, antioxidant, yam, Thailand.

Introduction

Phenolic compounds are ubiquitously present in fruit and vegetables commonly consumed by humans. The compounds are useful as dietary antioxidants to serve as a protective factor against oxidative cellular damage. Oxidative damage is associated with the generation of reactive oxygen species (ROS). ROS have the ability to induce alterations of genetic material by oxidized specific DNA-bases or indirectly by oxidizing proteins or lipids important in maintaining the function of individual cells. The produced functional damage can trigger events such as mutagenesis, carcinogenesis and aging. Under normal physiological conditions, cells counterbalance the production of ROS with antioxidants [1, 2]. Therefore, an adequate dietary supplement of antioxidants is relevant for maintaining good health.

Tubers of *Dioscorea* spp., which are commonly known as yams, are an important foodstuff in the tropical and subtropical regions of the world, with world production in 2003 being approximately 40 million tonnes [3]. Some species of yam tubers have been shown to contain substantial amounts of phenolic compounds [4, 5, 6], hence these tubers could be a good source of dietary antioxidants.

In Thailand, a wild *Dioscorea* species found in the forest all over the country is *Dioscorea hispida* Dennst, usually referred to as *Kloi* in Thai. Its tubers are used to make a dessert called *Kao Nuew Kloi*. Although there has been several studies on the toxins and lactonic alkaloids, particularly dioscorine, [7, 8, 9] to date no previous work has been carried out on the phenolic compounds in the tubers of *Dioscorea hispida* Dennst. Thus, this research is aimed to study phenolic compounds in Kloi tubers and the antioxidant activity in both peel and flesh of the tuber. The analysis will be useful for potential uses of the tuber as a food supplement, supplying dietary antioxidants and as well as for commercial utilization as an active ingredient in the cosmetic or pharmaceutical industry. Additionally, increased study on *Kloi* could add to the likelihood of exploitation of the species as an economic plant and bring about further work on its cultivation. Figure 1 shows the plant and tubers of *Dioscorea hispida* Dennst.

Figure 1. Plant and fibrous tubers of *Dioscorea hispida* Dennst.
(courtesy of Medplant/Mahidol)

Materials and Methods

Phenolic standards

The commercial phenolic standards used in this study were caffeic acid, chlorogenic acid, catechin, ferulic acid, gallic acid, sinapic acid, syringic acid, vanillic acid and quercetin, provided by Sigma Aldrich (Australia). Standards were dissolved in ethanol, working solutions were prepared daily by appropriate dilution with ethanol to make 100 – 200 mg/L.

Plant material

Three *Kloi* tubers (*Dioscorea hispida*) were collected from the forest in the Chaibadan district of Lopburi province. The tubers weighed between 10–16 kilograms and each tuber consisted of 4-5 lobes. The tubers were washed to remove dirt, sliced and blanched for 5 min. to inactivate enzyme polyphenol oxidase, then sun-dried. The peel and flesh were separately ground by electrical mill. The powdered peel samples from the three tubers were pooled together, likewise for the powdered flesh.

Extraction of phenolic compounds

One hundred grams of dried peel powder of *Kloi* tuber was macerated in 60% methanol in a shaker for 48 hr (1g *Kloi* : 7 ml solvent). The experiment was performed in duplicate. After filtration, the filtrate was concentrated to dryness by rotary evaporator at 48^{o}C then weighed and diluted to 20 ml with 60% MeOH. The solution was washed with 30 ml petroleum ether (2 x 15 ml) to remove chlorophyll, carotenoid and lipids. Twenty ml of absolute ethanol (2 x 10 ml) was used to precipitate protein from the solution. After centrifugation at 32,000 rpm for 20 min, the supernatant was concentrated by rotary evaporator at 48 ^{o}C, weighed and subjected to hydrolysis. The dried flesh powder of *Kloi* tuber was treated in the same manner.

Hydrolysis of the extract

The extract was made up to 10 ml with 60% MeOH and hydrolyzed under reflux with 8 ml of 1.5 M HCl for 90 min. The hydrolysate was adjusted to pH 7 with 1M NaOH, centrifuged and the supernatant was concentrated to approximately 5 ml by rotary evaporator at 48^{o}C, then made up to 10 ml with 60% MeOH at pH 7. A two-fold dilution of the solution was performed prior to fractionation with C_{18} SPE. The dilute sample was labeled as crude extract, determination of total polyphenols content, DPPH assay and FRAP assay were subsequently performed.

Fractionation of the extract

Mega Bond Elut C_{18} cartridges (Varian; 10 gm adsorbent) were used to fractionate the phenolic compounds in *Kloi* tuber. The fractionation method for the neutral phenolic and acidic phenolics was patterned after Jarworski and Lee [10]. Preconditioning of C_{18} Mega Bond Elut was done firstly with 20 ml MeOH and subsequently with 20 ml deionized water at pH 7. Ten ml of the crude extract from hydrolysis was applied on top of a cartridge. Fractionation was done by using a stepwise elution of aqueous MeOH in the following manner; 10 % MeOH, 20% MeOH, 40% MeOH, 60% MeOH and 80% MeOH, the amount of aqueous MeOH at each concentration for stepwise elution was 71 ml. Each fraction was evaporated under vacuum to dryness by rotary evaporator at 48^{o}C, weighed and made up to 10 ml with the corresponding aqueous MeOH solvent. The neutral fraction was labeled as SPE1 and the acidic fraction as SPE2. Total phenolic content, DPPH assay and FRAP assay for each fraction were subsequently determined.

HPTLC analysis

The crude extract, each SPE1 fraction of both peel and flesh and standard compounds were concurrently chromatographed on an HPTLC plate (Silica gel Merck 60, $F_{254.}$) The solvent

system for developing the chromatogram was chloroform: methanol: water (6.5: 3.0: 0.5; v/v). The chromatogram was observed under visible light, UV at 254 nm and 366 nm, after spraying with 1:1 mixture of 1% aqueous $FeCl_3$ and 1% $K_3Fe(CN)_6$ and 20% H_2SO_4 in 50% MeOH. R_f values of individual compounds were recorded.

The crude extract, each SPE2 fraction of both peel and flesh were concurrently chromatographed with standards on HPTLC plate in the same manner as the SPE1 fractions.

Antioxidant assays

The antioxidant assays performed were DPPH assay after Yildirim *et al.* [11] with modification, FRAP assay after Benzie and Strain [12] and total polyphenol assay after Kahkonen *et al* [13]. The assays were carried out in triplicate. Ascorbic acid (vitamin C), vitamin E and quercetin were employed as standards.

Rp HPLC analysis

The experiment was done on a Varian HPLC, column and condition employed were as follows: C_{18} column (Agilent, Zorbax ODS, 5 µm, 4.5 mm x 250 mm); mobile phase 5 mM ammonium formate buffer (pH 3.0) with 10% methanol (solvent A) and 5 mM ammonium formate buffer (pH 3.0) with 70% methanol (solvent B); injection volume 20 µl., gradient elution from 0% B to 100% B; flow rate 1 ml/min; run time 120 min. The standards used were caffeic acid, chlorogenic acid, catechin, ferulic acid, gallic acid, sinapic acid, syringic acid and vanillic acid.

The SPE1 samples analyzed were 20% MeOH, for both the peel and flesh sample. The SPE2 fractions analyzed were the 20% MeOH flesh sample. The chromatogram was monitored at 254 nm with a PDA detector, retention time and the UV absorption spectrum of each component separated was recorded.

The parameter developed for Rp HPLC was then further used in the LC-MS experiment for the identification of components of interest.

LC-MS analysis

LC-ESI-MS analysis was conducted on a PE SCIEX API 365. The LC part was performed on a Perkin Elmer Series 200 micro LC pump equipped with a Series 200 autosampler. The conditions used for the LC separation were those obtained from Rp HPLC experiment by using the same column at a flow rate of 1 ml/min. An Applied Biosystem 785A UV detector set at 254 nm was used to monitor the chromatographic process. The analyses were done in a negative ion mode since initial experiments in which conditions for the standard compounds were optimized showed that more information was obtained in the negative ion mode. The conditions for mass spectrometry were as follows: drying gas (nitrogen) flow 5 L/min; nebulizer flow 10; drying gas temperature 400^{o}C; capillary voltage -4000 V; orifice -34 V; ring -153; Q_o 8.3 V; flow at split end before entering mass spectrometer ie. 0.2 ml (to mass spectrometer): 0.8 ml (to UV detector at 254 nm). The program Masschrom version 1.1 was operated for system control of the MS and Multiview version 1.4 was used for data acquisition.

Standards, i.e. caffeic acid, chlorogenic acid, catechin, ferulic acid, gallic acid, sinapic acid, syringic acid and vanillic acid were subjected to LC-MS analysis and the chromatograms and fragmentation patterns served as in-house library.

Results and Discussion

HPTLC was used for the assessment of the effectiveness of the fractionation step carried out by the SPE technique. The presence of analytes was detected by visualization under UV light at 254 and 366 nm., and also with a 1:1 mixture of 1% $K_3Fe(CN)_6$ and 1% $FeCl_3$ (v/v) spraying reagent. Phenolic compounds appeared as blue colour. Table 1 shows Rf values and colour of all fractionated peel and flesh of SPE1 samples. It is obvious that the active compounds extracted are phenolic compounds, however none of the available phenolic acids and flavan-3-ol standards were found in both peel and flesh sample of SPE1.

Table 1. R_f values and colour with 1:1 mixture of $K_3(Fe(CN)_6)$ and $FeCl_3$.

Fraction	Peel		Flesh	
	R_f value	Colour	R_f value	Colour
Crude	0.48 0.79	Blue Blue	0.27	Blue
10% MeOH	0.48 0.71	Blue Blue	0.27 0.30	Blue Blue
20% MeOH	0.25	Blue	0.19	Blue
40% MeOH	0.20 0.65	Blue Blue	0.19	Blue
60% MeOH	0.16	Blue	0.19	Blue

Antioxidant assays

The antioxidant potential of each SPE1 fraction for both peel and flesh SPE1 was determined by total phenol assay, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay) and ferric ion reducing antioxidant power assay (FRAP assay). The results are presented in Tables 2 and 3.

Table 2. Data on total polyphenol, DPPH and FRAP assay of Peel fractions.

Sample	Total phenol assay mg GAE/mg	DPPH assay EC_{50} µg/ml	FRAP assay µmol Fe $^{2+}$/mg
10% MeOH	0.019 ± 0.00	7.95×10^2	0.25×10^2
20% MeOH	0.068 ± 0.04	1.05×10^2	3.26×10^2
40% MeOH	0.056 ± 0.03	1.40×10^2	3.25×10^2
60% MeOH	0.018 ± 0.00	4.27×10^2	1.06×10^2
dl-tocopherol		2.61	5.88×10^3
ascorbic acid		2.50	11.27×10^3
quercetin		0.98	22.67×10^3

Table 3. Data on total polyphenol, DPPH and FRAP assay of Flesh fractions.

Sample	Total phenol assay mg GAE/mg	DPPH assay EC_{50} µg/ml	FRAP assay µmol Fe^{2+}/mg
10% MeOH	0.040 ± 0.02	5.10×10^2	0.93×10^2
20% MeOH	0.085 ± 0.05	2.04×10^2	2.93×10^2
40% MeOH	0.092 ± 0.11	4.76×10^2	2.79×10^2
60% MeOH	0.026 ± 0.00	6.67×10^2	0.97×10^2
dl-tocopherol		2.88	5.71×10^3
ascorbic acid		2.62	11.29×10^3
quercetin		1.05	23.50×10^3

There is consistency in the total polyphenol, DPPH and FRAP assays in that high values were obtained for the total polyphenols and FRAP assay and low values were obtained for the DPPH assay. The antioxidant activity of the standards was found to be much superior to all fractions from the peel and flesh samples. This is probably due to the low concentration of antioxidant compounds present in the fractions.

In both the peel and flesh samples a correlation between the total phenol assay and antioxidant activity (FRAP assay) was observed for the peel fractions with an r^2 value of 0.8775 and for flesh fractions, the r^2 value was 0.9758 which revealed the robustness of the assay methods employed.

The particular fraction from SPE1 chosen for further analysis by HPLC and LC-MS was the 20% MeOH fraction for both the peel and flesh samples. In the peel sample the 20% MeOH fraction gave the highest value for all the assays employed, therefore the 20% MeOH fraction of the flesh sample was analyzed for comparison.

Rp HPLC and LC-MS analysis of standards

A summary of HPLC analyses on the mixture of standards and on the individual standards are shown in Table 4.

Table 4. Summary of HPLC analyses on the mixture of standards.

Peak	Standards	Mixture of standards		Single standard	
		t_R (min)	λ_{max} (nm)	t_R (min)	λ_{max} (nm)
1	Gallic acid	7.061	214, 269	6.928	214, 269
2	Catechin	19.387	250	25.147	202, 277
3	Chlorogenic acid	28.792	323	28.229	323
4	Vanillic acid	31.872	259, 290	31.448	259, 290
5	Caffeic acid	32.907	320	33.896	320
6	Syringic acid	36.888	216, 273	36.624	216, 273
7	Ferulic acid	52.944	320	51.947	320
8	Sinapic acid	55.365	234, 321	54.981	234, 321

As can be seen from the results in Table 4, retention times and absorption spectra of the phenolic acids were in good agreement between the runs with pure standards and the run with the mixture of standards. The λ_{max} values for the spectra agreed well with the literature. The elution order

was the same as reported in the literature, where hydroxybenzoic acids eluted first and then hydroxycinnamic acids [14].

The conditions developed for the separation of standards was then transferred to the LC-MS experiment. The LC-MS experiment on each standard compound also served to establish an in-house library of the fragmentation pattern for each compound. The summary of LC-MS data are shown in Table 5.

Table 5. Summary of LC-MS results.

Standards	MW	m/z (abundance)
Caffeic acid	180	135(100), 179(86)
Catechin	290	289(100), 245(20)
Chlorogenic acid	354	353(15), 191(100)
Ferulic acid	194	193(64), 178(40), 150(20)
Gallic acid	170	169(100), 125(60)
Sinapic acid	224	223(100), 208(40), 164(64)
Syringic acid	198	197(100), 182(74), 153(38)
Vanillic acid	168	167(100), 152(95), 122(50), 108(60)

These results above demonstrate that the method developed can be applied to identify phenolic compounds**.**

Rp HPLC and LC-MS analysis of Peel and Flesh

The particular fraction from SPE 1 chosen for further analysis by HPLC and LC-MS was 20% MeOH fraction for both peel and flesh sample. Table 6 summarizes the characteristics of components in the 20%MeOH SPE1 peel fraction.

Table 6. HPLC ESI-MS characterization of components in the 20% MeOH SPE1 peel fraction.

Retention time (min)		λ_{max} (nm)	$[M-H]^-$ (m/z)	Other ions (m/z)
LC	TIC			
10.18		229, 282		
26.65	26.62	230, 280	121.2 (100)	362.2 (20)
38.60	38.42	218, 260	167 (100)	152 (23), 107 (41)
74.32	74.15	202, 322	353 (100)	341 (68)
	77.19		361.2 (100)	243 (24)
83.15	83.26	237, 282	177 (100)	357.2 (80), 133 (80)
	89.33		199.2 (100)	283 (18)

The UV absorption spectrum of the 20% MeOH SPE1 peel fraction (Table 6) did not show any absorption maxima in the ranges 330–360 and 250–270 nm. Absorptions in these two wavelength ranges are characteristic of flavonoids, with the two absorptions originating from electronic transitions on the B and A ring, respectively. Therefore, the compounds of the 20% MeOH SPE1 peel fraction probably do not belong to flavonoid class.

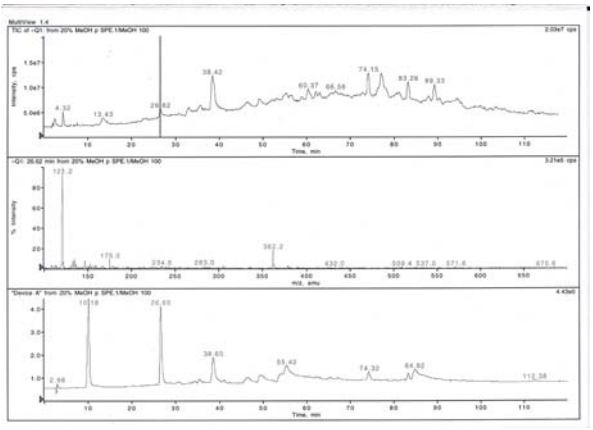
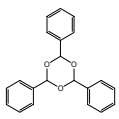


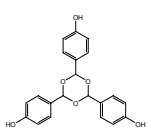
Figure 2. ESI total ion chromatogram and LC with mass spectrum of the component at t_R 26.62 min.

The compounds eluting at t_R 10.18 min and t_R 26.65 min (Table 6) showed the same UV absorption maxima, ie at 230 and 280 nm, implying that their structures might be similar. However, the peak at t_R 10.18 min did not give any response on the ESI total ion chromatogram (Fig 2) suggesting that the compound is not effectively ionized to form a negative ion. This tends to suggest that it is not a phenolic compound although the UV absorption would support such an assignment. Nevertheless, the compound eluting at t_R 26.65 min gave a mass spectrum (negative ion mode). The mass spectrum, abundances in parentheses, had an abundant ion at m/z 121.2 (100) and another ion at m/z 362 (20). The m/z 362 ion was three times higher mass than the m/z 121 ion, suggesting that it could be a condensed structure of the m/z 121 ion, probably a trimer. Given that many of the relevant compounds will be phenolic acids of relatively low molecular mass, it is possible that the compound which gives rise to the m/z 121 ion is benzoic acid (C_6H_5COOH, MW 122). It is known that carboxylic acids can easily dimerise but are less likely to trimerise, so benzoic acid was ruled out. *p*-Hydroxybenzaldehyde ($C_7H_6O_2$, MW 122) was reported to be an oxidation product of the phenolic acid, *p*-coumaric acid [15]. Thus, the appearance of the m/z 121 ion could possibly be due to the presence of *p*-hydroxybenzaldehyde. Aliphatic aldehydes, such as acetaldehyde, can undergo trimerization to become paraldehyde and analogues. Benzaldehyde, the fundamental aromatic aldehyde, can be trimerized to the benzaldehyde trimer (Scheme 1) giving the compound $C_{21}H_{18}O_3$ with molecular mass of 318 as reported by Stampa [16]. Thus the m/z 362 ion is thought to be due to the cyclic trimer of *p*-hydroxybenzaldehyde (Scheme 1), analogous to paraldehyde, and the m/z 121 ion is due to *p*-hydroxybenzaldehyde (Scheme 2), either in its own right or as a fragment ion of the trimer.

The absorption maximum (λ_{max}) in the spectrum of *p*-hydroxybenzaldehyde occurs at 280 nm as reported by Antolovich [15], the observed absorption maxima for the compound eluting at t_R 26.25 min were at 230 and 280 nm (Table 6) and it was found that the 230 nm peak is of much lower intensity than the 280 nm peak.



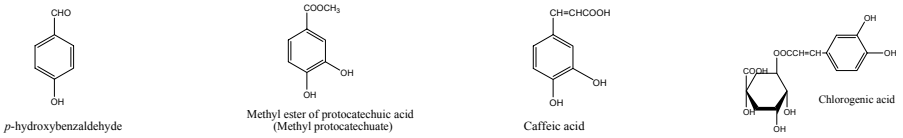
Benzaldehyde trimer



***p*-Hydroxybenzaldehyde trimer**

Scheme 1. Structure of trimers.

On the basis of the evidence discussed above, the compound eluting at t_R 26.25 min is strongly thought to be *p*-hydroxybenzaldehyde.



Scheme 2. Phenolic compounds found in *Kloi* (*Dioscorea hispida* Dennst).

The compound eluting at t_R 38.60 (Fig 3) had UV absorption maxima at 218 and 259 nm. These absorptions are different from those associated with the former two chromatographic peaks (t_R at 10.18 and 26.25 min). The ESI-MS fragmentation pattern revealed ions at m/z 167(100), 152(23) and 107(41). Assuming that the m/z 167 ion is $[M - H]^-$, the m/z 152 ion could be assigned to $[M\text{-}H\text{-}CH_3]^-$ and the m/z 107 ion to $[M\text{-}H\text{-}CH_3\text{-}CO_2]^-$. This suggests that the molecule, of probable molecular mass 168, has both a methyl group and a carboxylic acid group present. It can be deduced from the fragments and the likely molecular mass that the molecular formula is probably $C_8H_8O_4$. It is further known that the compound is phenolic in nature. The compound is likely to be a phenolic acid derivative; two possible compounds are: (i) the methyl ester of protocatechuic acid; or (ii) a methoxyhydroxybenzoic acid.

Vanillic acid is a methoxyhydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, having the formula $C_8H_8O_4$. The mass spectrum of vanillic acid from the in-house library (Table 5) showed ions at m/z 167(100), 152(95), 122(50) and 108(60). The retention time was 24.87 min. The m/z 122 ion was not observed in the mass spectrum of the compound eluting at t_R 38.60 min thus it is unlikely to be vanillic acid. The presence of m/z 107 ion possibly arises from a 59 amu loss ($-COOCH_3$) from the m/z 167 ion suggesting that the parent compound could be an ester. It is proposed that the compound eluting at t_R 38.60 min is the methyl ester of protocatechuic acid (Scheme 2).

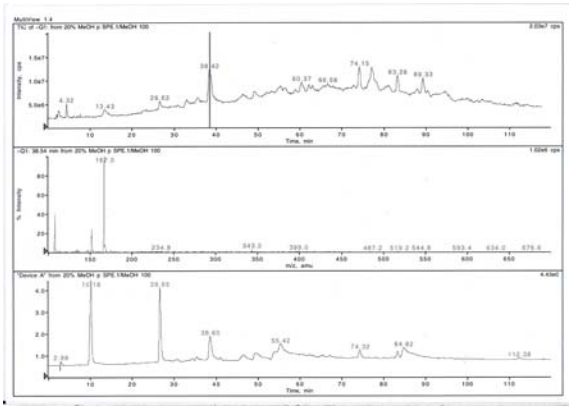
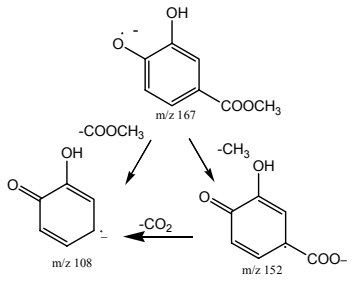


Figure 3. ESI total ion chromatogram and LC with mass spectrum of the component at t_R 38.42 min. of 20% MeOH SPE1 Peel fraction.

The presence of both m/z 167 and 108 ions were in accordance with a reference mass spectrum of 3,4-dihydroxybenzoic acid methylester ie methyl protocatechuate [17]. Moreover, λ_{max} calculated for methyl protocatechuate in ethanol is 262 nm [18], where 259 nm was the absorption maxima in aqueous methanol of this compound. The shorter wavelength by 3 nm was justified because methanol is a more polar solvent than ethanol. Apparently, it is thought to be

good evidence that the compound is the methyl ester of protocatechuic acid. A proposed pathway of fragmentation in the mass spectrometer [19] is shown below (Scheme 3):



Scheme 3. Fragmentation pathway of the parent (m/z 167) ion.

The mass spectrum derived from the chromatographic peak at t_R 74.32 min exhibited a complicated fragmentation pattern. The pattern showed a strongly abundant ion at m/z 353 (100), with less pronounced peaks at m/z 342 (60), 371 (30) and 153 (40). The fragments of abundance 20% and lower were at m/z 488, 444, 309, 263, 219, 191 and 176. It is probable that the chromatographic peak contained co-eluting compounds, with their parent ions having m/z values of 353 and 153, respectively.

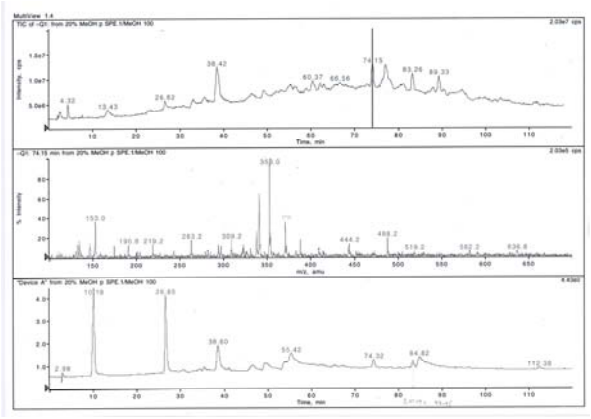


Figure 4. ESI total ion chromatogram and LC with mass spectrum of the component at t_R 74.15 min of 20% MeOH SPE1 peel fraction.

For the compound of molecular mass 354, the fragment ions occur at m/z 309, 263, 219 and 191. The presence of these ions can be rationalized in the following way: (i) loss of 44 amu from m/z 353 give rise to m/z 309; (ii) loss of 46 amu from m/z 309 gives m/z 263; (iii) loss of 44 amu gives m/z 219; and finally (iv) loss of 28 amu gives m/z 191. The loss of 44 amu from m/z 353 indicated that this compound contains the COOH group, implying that the parent compound is an acid. Its spectrum revealed λ_{max} at 323 nm (Table 6) which is the λ_{max} of chlorogenic acid. However, the appearance of fragmentation pattern in the mass spectrum was not similar to that of standard chlorogenic acid which showed the base peak at m/z 191(100) ion and an m/z 353 (30) ion. The anomaly would be justified by the complicated nature of this peak since it was a coelution peak. One of the compounds eluting at this time might be chlorogenic acid.

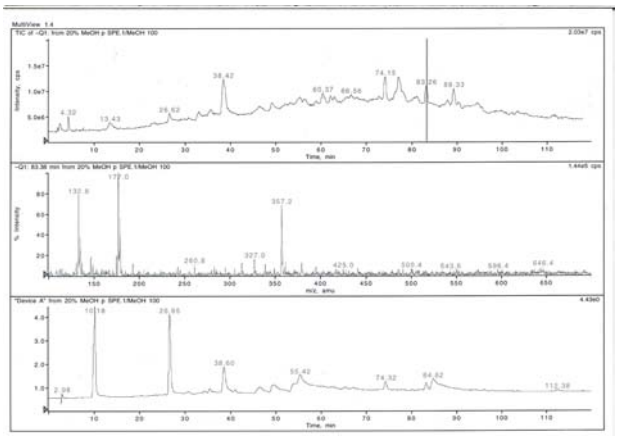


Figure 5. ESI total ion chromatogram and LC with mass spectrum of the component at t_R 83.26 min of 20% MeOH SPE1 peel fraction.

The mass spectrum of the compound eluting at t_R 83.26 min exhibited a base peak at m/z 177 and abundant ions at m/z 357.2 (60) and m/z 133 (80). It should be noted that the peak at m/z 133 was not a single clear peak but appeared as a doublet with a peak at m/z 135 (60) ion. Likewise the peak at m/z 179 (40) appeared adjacent to the peak at m/z 177. It is likely that loss of 44 amu (CO_2) from the m/z 177 ions yielded the m/z 133 ion. This relationship could link the m/z 179 and 135 ions. It is thought that the parent compound has a carboxyl group (-COOH) in the structure and is probably a phenolic acid. The presence of less abundant ions at m/z values 2 units higher could suggest the presence of an element with abundant isotopes 2 amu apart eg. Cl.

From the in-house mass spectra library (Table 5), caffeic acid showed abundant ions at m/z 179 (100) and 135 (90) which accords with the spectrum observed here. The compound present in the sample is probably caffeic acid. The ion at m/z 357 may occur due to some dimerisation of caffeic acid. Dimeric compounds of MW 358 formed from the coupling of two caffeoyl radicals have been reported in the literature. It is noted that many isomers are possible [20, 21]. The high abundance of the ions at m/z 177 may occur because of fragmentation of the dimer or through the formation of doubly-charged ions of the dimer. The data suggest that the ion at m/z 357 could be due to a dimer of caffeic acid which may either be doubly-charged or fragment in the mass spectrometer to yield ions with m/z ca. 177.

The ESI total ion chromatogram for 20% SPE 2 flesh fraction (Fig 6) of the compound eluting at t_R 39.15 min revealed the same fragmentation pattern the same as spectrumof the compound eluting at 38.60 min for the 20% MeOH SPE1 peel fraction. In addition the retention times were similar ie. 39.15 and 38.60 min. On the basis of the previous assignment, the compound eluting at t_R 39.15 min for the 20% MeOH SPE2 flesh fraction is also methyl ester of protocatechuic acid or methyl protocatechuate.

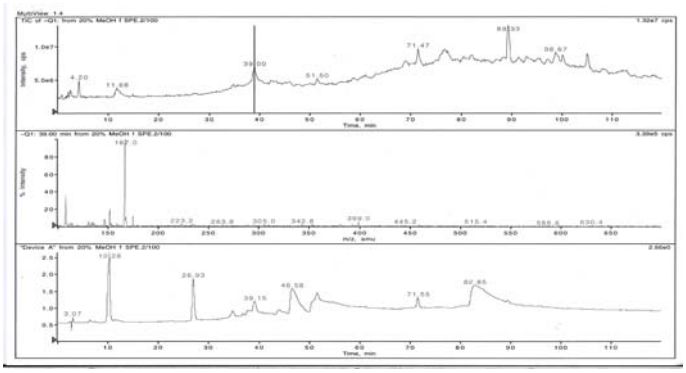


Figure 6. ESI total ion chromatogram and LC with mass spectra of the component at t_R 39.15 min (LC chromatogram) of 20% MeOH SPE2 flesh fraction.

Conclusion

The findings indicated the presence of hydroxybenzoic acids and hydroxycinnamic acids and their derivatives in *Kloi* tuber. The compounds were present in aldehyde and ester forms. Caffeic acid, chlorogenic acid, *p*-hydroxybenzaldehyde and methyl protocatechuate were found in the peel of *Kloi* tuber. In the flesh of *Kloi* tuber only methyl protocatechuate was found**.** However, there were still some unidentified compounds present in both peel and flesh samples.

It was shown in Tables 2 and 3 that the antioxidant activities of the 20% MeOH SPE1 fractions of both peel (EC_{50} 1.05 x 10^2 µl/ml) and flesh (EC_{50} 2.04 x 10^2µl/ml) were very low when compared to standards (Vitamin E, Vitamin C and quercetin), even though the total antioxidant activity reported arose from a mixture of phenolic acid in the 20% MeOH SPE 1 fractions. It appears that phenolic acids were present in only small amounts in *Kloi* tuber. However, the total phenolic content reported in the literature for other yam *Dioscorea* spp [5] were relatively high.

This anomaly might arise from the sample preparation procedure. It is probable that 90 min hydrolysis time was not long enough to liberate all phenolic acids present in *Kloi* tuber. Another explanation might be attributed to the pH used in the fractionation process, a neutral pH may not be suitable for separation of phenolics. With the -OH groups present in the structure, phenolic compounds are weakly acidic. It would be more appropriate to work at acidic pH to inhibit the ionization of the phenolic compounds. In addition, for the antioxidant assay, it would be more appropriate to have a phenolic acid like caffeic acid as the reference standard instead of quercetin since all compounds identified were phenolic acids and phenolic acid derivatives.

Unlike fruit juice or wine, the sample matrix of *Kloi* tuber consists of various components, including carbohydrate, lipids and protein. The development of a single step sample preparation to achieve effective separation may be impossible. Therefore, SPE could serve as a preparative step prior to a purification step in qualitative analysis of *Kloi* tuber. In this study, there were quite a number of constituents in each fraction separated, as seen from HPLC and HPTLC chromatograms. However, even though the sample still had some impurities, LC-MS was able to successfully separate the compounds and some compounds were identified through interpretation of the mass spectra.

From the preliminary findings herein and the documented nutritive value, *Kloi* tuber is a promising commodity to be tapped as a source of phytochemicals for the pharmaceutical and

cosmetic industries as well as to be used as a dietary antioxidant . Further study of *Kloi*, from the perspectives of chemistry, both qualitative and quantitative, nutrition and organized cultivation are necessary to further promote the use of *Kloi* tuber.

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References

1. Klauing, J.E. and Kamendulis, L.M. (2004). The role of oxidative stress in carcinogenesis. **Annual Reviews in Pharmacology and Toxicology,** 44, 239-267.

2. Willcox, J.K, Ash, S.L. and Catignani, G.L. (2004). Antioxidants and prevention of chronic diseases. **Critical Reviews in Food Science and Nutrition,** 44, 275-295.

3. Bhandari, M.R. and Kawabata, J. (2005). Bitterness and toxicity in wild Yam (*Dioscorea* spp.). **Plant Foods for Human Nutrition**, 60, 129-135.

4. Martin, F.W. and Ruperte, R. (1976). The polyphenols of *Dioscorea alata* (Yam) tubers associated with oxidative browning. **Journal of Agricultural and Food Chemistry,** 24, 67-70.

5. Muzac-Tucker, I., Asemota, H.N. and Ahmad, M.H. (1993). Biochemical composition and storage of Jamaica Yams (*Dioscorea* spp.). **Journal of the Science of Food and Agriculture,** 62, 219-224.

6. McAnuff, M.A., Omoyuri, F.O., Sotelo-Lopez, A. and Aswmota, H.N. (2005). Proximate analysis and some antinutritional factor constituents in selected varieties of Jamaica yams (*Dioscorea* and *Rajana* spp.). **Plant Foods for Human Nutrition**, 60, 93-98.

7. Ayer, D.E., Büchi, G., Reynolds Warnhoff, P. and White, D.M. (1958). The Structure of Dioscorine. **Journal of the American Chemical Society,** 80 (22), p. 6146.

8. Leete, E. and Michelson, R.H. (1987). The biosynthetic incorporation of [methyl-14C,6-2H,3H] trigonelline into dioscorine in *Dioscorea hispida*. **Journal of the American Chemical Society,** 109 (20), pp 6179-6181.

9. Webster, J., Beck, W. and Ternai, B. (1984). Toxicity and bitterness in Australian *Dioscorea bulbifera* L. and *Dioscorea hispida* Dennst. from Thailand. **Journal of Agricultural and Food Chemistry,** 32 (5), pp 1087-1090.

10. Jarworski, A.W. and Lee, C.Y. (1987). Fractionation and HPLC determination of grape phenolics. **Journal of Agricultural and Food Chem**istry, 35, 257-259.

11. Yildirim, A., Mavi, A. and Kara, A.A. (2001). Determination of antioxidant and antimicrobial activity of *Rumex crispus* L. extracts. **Journal of Agricultural and Food Chemistry,** 49, 4083-4089.

12. Benzie, I.F.F. and Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a measure of "antioxidant power". The FRAP assay. **Analytical Biochemistry**, 239, 70-76.

13. Kahkonen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.-P., Pihlaja. K., Kujala, T.S. and Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. **Journal of Agricultural and Food Chemistry,** 47, 3954-3962.

14. Swasitang, P., Tucker, G., Robards, K. and Jardine, D. (2000). Isolation and identification of phenolic compounds in *Citrus sinensis*. **Analytica Chimica Acta**, 417, 231-240.

15. Antolovich, M., Bedgood, D.R., Bishop, A.G., Jardine, D., Prenzler, P.D. and Robards, K. (2004). LC-MS investigation of oxidation products of phenolic antioxidants. **Journal of Agricultural and Food Chemistry,** 52, 962-971.

16. Stampa, G.B. (1969). Polymers from benzaldehyde: A copolymer with dihydropyran and the cyclic trimer. **Macromolecules**, 2, 203-206.

17. McLafferty, F. W. and Stauffer, D.B. (1989). The Wiley/NBS Registry of Mass Spectra Data, John Wiley & Sons, New York.

18. Williams, D.H. and Flemming, I. (1989). Spectroscopic Methods in Organic Chemistry, 4th ed., Mc Graw-Hill, New York.

19. Justesen, U. and Arrigoni, E. (2001). Electrospray ionization mass spectrometric study of degradation products of quercetin, quercetin-3- glucoside and quercetin-3-rhamnoglucoside, produced by in vitro fermentation with human faecal flora. **Rapid Communications in Mass Spectrometry,** 15, 477- 483.

20. Cillers, J.J.L. and Singleton, V.L. (1991). Characterization of the products of nonenzymatic autooxidative phenolic reaction in a caffeic acid model system. **Journal of Agricultural and Food Chemistry,** 39, 1298-1303.

21. Tazaki, H., Taguchi, D., Hayashida, T. and Nabeta, K. (2001). Stable isotope-labelling studies on oxidative coupling of caffeic acid via o-quinone. **Bioscience, Biotechnology and Biochemistry,** 65, 2613-2621.