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Identification, Isolation and Antioxidant Activity of Pheophytin from Green Tea (*Camellia sinensis* (L.) Kuntze)

Lia Kusmita[a], Ika Puspitaningrum[a], Leenawaty Limantara[b*]

[a]*STIFAR "Yayasan Pharmasi", Letjend Sarwo Edie Wibowo KM 1, Semarang 50193*
[b]*Ma Chung Research Center for Photosyntehtic Pigments, Ma Chung University, Villa Puncak Tidar N 1,Malang 65151*

Abstract

Tea is a plant that can grow in different countries in the world. Green tea is one of the types of tea which is most beneficial for health such as anticarcinogenic, antibacterial, antitumor, antivirus, and antioxidant. In this research, identification of green tea pigments (*Camellia sinensis* (L.) Kuntze) were conducted by using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) equipped with photodiode array detector. By TLC analysis, five spots were detected and they were β-carotene, pheophytin *a,* chlorophyll *b*, xanthophyll, and pheophorbide *a.* An analysis using HPLC showed the existence of 14 peaks consisting of 10 chlorophyll peaks and their derivatives as well as four carotenoid peaks. Pheophytin isolation was conducted using column chromatography and antioxidant activity was assayed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Antioxidant activity of pheophytin *a* IC_{50} = (573 ± 0.23) mg · L^{-1} corresponded to that of β-carotene marker IC_{50} = (550 ± 0.26) mg · L^{-1}. Antioxidant activity of the green tea crude extract had IC_{50} = (250 ± 0.21) mg · L^{-1} which was twice higher than β-carotene marker**.**

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Keywords: Antioxidant activity, green tea, hplc-pda; pheophytin

Nomenclature

IC_{50}	inhibition concentration 50
Rf	retardation factor

* Corresponding author. Tel.: +62 813 2636 0303; fax:+62 341 550 175.
E-mail address: leenawaty.limantara@machung.ac.id

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1. Introduction

Green tea is a very popular health drink in the world. Green tea is now preferred as it is believed to be able to contribute much more benefit for health compared with black tea or oolong tea. Research into the pharmacological effects of green tea for human health has been conducted, such as anticarcinogenic, antibacterial, antitumor, antivirus, and antioxidant[1]. Green tea consists of essential oil, tannin, caffeine, vitamin, and pigment[2].

The pigments of green tea consist of chlorophylls and carotenoids. Chlorophylls are dominant pigments of fresh green tea leaves[3], but easily degrade, turning into pheophytin and pheophorbide after heating and storing processes[3-6]. In green tea, pheophorbides content is lower than pheophytins. This is due to the fact that the chlorophylase enzyme is made non-active during the post-harvesting and steaming process whereas the formation of pheophytin is attributable to the storing process[3]. During the increase in storage time, the acidity is also increasing. The result is a reaction between chlorophylls and acids, in which acid removes the magnesium ion and replaces it with two hydrogen atoms giving an olive-brown solid, pheophytin. The reaction between chlorophyll *a* and acid is depicted in Fig.1.

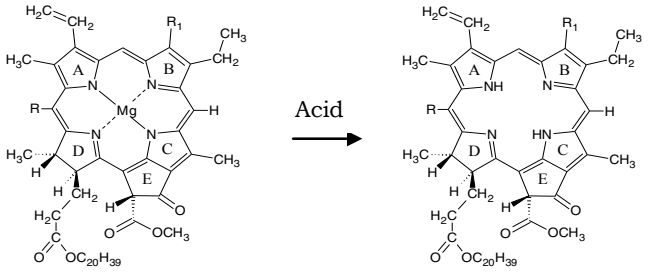


Fig. 1 The chemical structure of (a) Chlorophyll *a* and (b) Pheophytin *a* . Pheophytin forming reaction from chlorophyll due to acid effect[14]

The formation of pheophytin occurs *in vivo* as well as *in vitro*. Pheophytin occurs naturally in the plant leaves and is important as the first electron carrier intermediate in the electron transfer pathway of photosystem II in plants. Furthermore, pheophytin has been reported to have antioxidant activity[7]. Here, we report our preliminary study in isolation and identification of pheophytin from green tea leaves as well as to assay the antioxidant activity of it.

2. Materials and methods

The main material is green tea (*Camellia sinensis* (L.) Kuntze) obtained from tea plantation Rumpun Sari Medini, Ltd. Boja, Kendal Regency, Central Java, Indonesia, and β-carotene marker (E-Merck, no. 1,02236). The chemicals used were acetone, hexane, diethyl ether, methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), silica GF_{254}, and silica gel 60

2.1 Pigment extraction

Ten grams of sample were grounded and extracted by using a mixture solution of acetone : methanol (3 : 7, v/v). During the extraction, calcium carbonate was added as a neutralizing agent and sodium ascorbate as an antioxidant to prevent further oxidations. The extraction was done as quick as possible to avoid further oxidations or enzymatic degradations. Then, the extract was filtered and the residue was re-extracted until the color of residue became pale as an indicator of complete pigment extraction. The resulting extract was partitioned with diethyl ether. The diethyl ether layer was dried with nitrogen gas[8].

2.2 Thin-layer chromatography (TLC)

The pigment composition of green tea crude extract was analyzed with silica gel GF_{254} as stationary phase and hexane : diethyl ether : acetone (6 : 3 : 2, v/v/v) as mobile phase[9]. The colour of each spot on TLC plate was observed and the R_f value was calculated.

2.3 High-performance liquid chromatography (HPLC)

HPLC analysis was performed with Shimadzu HPLC equipped with photodiode array (PDA) detector and column temperature controller (Shimadzu, Kyoto, Japan) using a Shim-pack vp-ods C_{18} column (250 × 4.6 mm i.d.) (Shimadzu, Kyoto). Pigment was eluted with a gradient system using the flow speed of 1.0 mL per min at 25 ºC. The solution used was A: methanol : acetonitrile : 0.25 M pyridine solution (50 : 25 : 25, v/v), and B: methanol : acetonitrile : acetone (20 : 60 : 20, v/v). The pigments were detected with a PDA detector and evaluated on 430 nm wavelength[10].

2.4 Pheophytin isolation and identification.

The pheophytin was isolated from green tea crude extract by column chromatography with silica gel 60 as stationary phase and hexane : diethyl ether : acetone (6 : 3 : 2, v/v/v) as mobile phase. Purity of pheophytin was analyzed with TLC and UV-Vis spectrophotometer.

2.5 Antioxidant activity.

Purified pheophytin, green tea crude extract, and β-carotene marker was dissolved with acetone and was made with several concentrations. Antioxidant activity was measured using spectrophotometer at 517 nm wavelength. Assays were done according to the method reported by Panovska et.al[11]. The percentage of antioxidant activity was calculated using the formula:

$$x = \frac{[DPPH]_0 - [DPPH]_5}{[DPPH]_0} \times 100\% \quad (1)$$

$[DPPH]_0$ = initial concentration of DPPH
$[DPPH]_s$ = remain concentration of DPPH

3. Results and discussion

3.1. Identification of pheophytin using TLC method.

The chromatogram of TLC is shown in Fig. 2. The pigments were separated as can be seen from their spot color. The R_f value of each spot was calculated and then they were compared with literature.

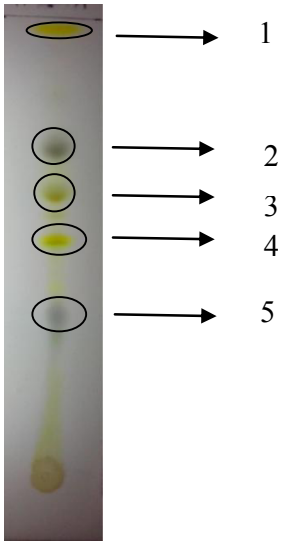


Fig. 2. The result of separation of green tea crude extract by TLC using silica gel GF_{254} and mobile phase hexane : diethyl ether : acetone (6 : 3 : 2, v/v/v)

The identification of pigments from green tea crude extract by above chromatography is shown in Table 1. Spot 1 showed yellow color and had the R_f value of 0.96. It was identified as β–carotene. Similarly, spot 2 (grey color) had R_f value of 0.60 and identified as pheophytin. The results were in accordance with literature, which states that pheophytin has *Rf* 0.59 to 0.60, and the spot is grey with the same method[9]. Spot 3 was identified as chlorophyll *b* because it was yellowish green with R_f 0.42. Spot 4 was orange with R_f 0.36 and identified as xanthophyll. Spot 5 showed black with R_f 0.20 and was determined to be pheophorbide *a*.

Table 1. Identification of green tea pigment extract by Thin Layer Chromatography method

Research result				**Literature[9]**	
No	Spot colour	R_f	Identification	Spot colour	R_f
1	Yellow	0.96	β-carotene	Yellow	0.9 – 1.00[9]
2	Grey	0.60	Pheophytin *a*	Grey	0.59 – 0.65[9]
3	Yellowish green	0.42	Chlorophyll *b*	Yellowish green	0.39 – 0.42[9]
4	Orange	0.36	Xanthophyll	Orange	0.25 – 0.28[9]
5	Black	0.20	Pheophorbide *a*	Black	0.17 – 0.20[9]

3.2. Pheophytin identification by HPLC equipped with PDA detector

Subsequently, HPLC was performed as it is more sensitive, selective, and speedy[12]. The use of PDA detector is convenient method to identify[13] and obtain spectral properties of the pigments by post-analysis. The chromatogram of the result of separation by HPLC is shown in Fig. 3.

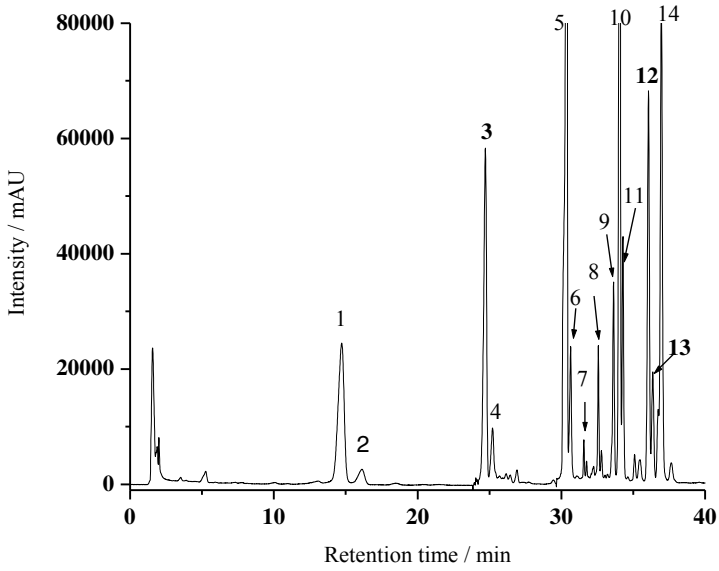


Fig. 3. HPLC profile of green tea crude extract. The detection was carried out at 430 nm with flow rate of 1 mL per min^{-1}. Chromatographic conditions are described in the experimental part. For peak numbers, see Table 2.

Table 2. Identification of green tea pigments by HPLC.

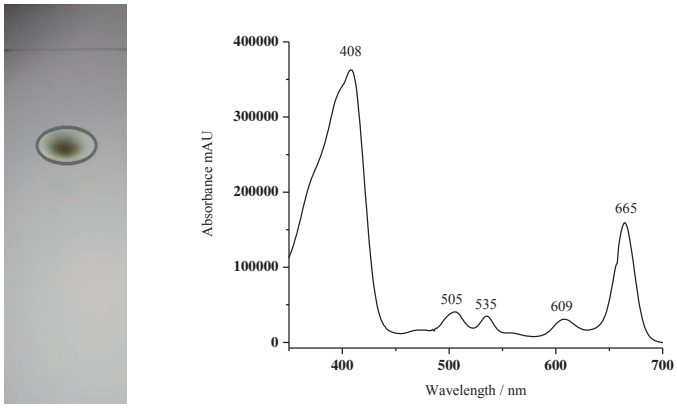
No	t_R	Component	λ_{max}(nm)					
1	14.72	Pheophorbide *b*		435		526	599	654
2	15.60	Pheophorbide *b* epimer		434		525	598	653
3	24.71	Pheophorbide *a*	409		505	539	607	665
4	25.21	Pheophorbide *a* epimer	409		505	539	607	665
5	30.36	Antheraxanthin		446	474			
6	30.64	Violaxanthin	417	438	469			
7	32.57	Chlorophyll *b*		456			596	645
8	33.63	Pheophytin *b*		434		527	600	653
9	34.06	Pheophytin *b* epimer		434		527	599	653
10	35.78	Pheophytin *a* allomer	408		505	535	609	665
11	36.06	Pheophytin *a*	408		505	535	609	665
12	36.35	Pheophytin *a* epimer	408		505	535	609	666
13	36.73	α-carotene	(423)	447	473			
14	36.95	β-carotene		452	475			

The identification was conducted by observing the retention time and the spectral pattern produced, and they were then compared with literature[5,10]. The chromatogram shows that there are 14 peaks, consisting of 10 types of chlorophyll and their derivatives and four peaks of carotenoid. The result of pigment identification is shown in Table 2. A peak with retention time at 35.78 min^{-1}; 36.06 min^{-1}; and 36.73 min^{-1} was identified as pheophytin *a* and their derivatives because it has the same spectral pattern and wavelengths as in literature[5,10]. The separation condition shows that the polar compounds will elute first and non-polar compound will come later. Taken into account that the peak at 36.06 min^{-1} was the pheophytin *a*, we could assign that the peak at 35.78 min should be an allomer of pheophytin *a*, while the peak at 36.73 min^{-1} was assigned as the epimer. Chlorophyll *a* was not found in the identification of pigments in green tea crude extract both by TLC and HPLC. It is known that chlorophyll *a* to chlorophyll *b* ratio of higher plant such as tea leaves is 3 : 1[4]. However, chlorophyll *a* can easily degraded into pheophytin due to heating process compared with chlorophyll *b*[3]. Usually, tea is made by treating tea leaves through

the process of withering, scrolling, and drying, successively, that can also lead into degradation of chlorophyll *a*. In addition, it has been reported that the long storage time will lead to increase the acidity and thus lead to degradation of chlorophyll too[4].

3. 3. Isolation of pheophytin a by column chromatography

The grey band corresponds to pheophytin a was fractionated. The band was then identified with TLC and UV-Vis spectrophotometer. The pheophytin *a* purity test was carried out by TLC to see how many spots are produced, as the number of spots in chromatogram will inform the number of compound. One grey spot was found on TLC of pheophytin, but no other pigment bands were seen. The chromatogram of purified pheophytin *a* is shown in Fig. 4a.



(a) (b)

Fig. 4 (a) Chromatogram of isolated pheophytin *a* and (b) Pheophytin *a* spectrum pattern in acetone

The spot had R_f value of 0.60 and its color was grey and it was identified as pheophytin *a* in accord with literature using the same method[9]. Further, the spectrum pattern and its maximum wavelength of pheophytin *a* isolated were determined by UV-Vis spectrophotometer. The pattern of the spectrum are shown in Figure 4b. The result showed that pattern and the maximum wavelength at 408 nm; 505 nm; 535 nm; 609 nm and 665 nm is the same as the literature of pheophytin *a*[14].

3. 4. Antioxidant activity test by DPPH method

The DPPH method is frequently used because it works not only more quickly, selectively, sensitively and stable, but also it is easy to use. The IC_{50} value that was measured suggests the concentration that works effectively to inhibit the free radical activity at 50 %. If the IC_{50} is small than the compound has high antioxidant activity. The histogram of IC_{50} concentration of isolated pheophytin, β-carotene, and green tea crude extract is shown in Fig. 5**.**

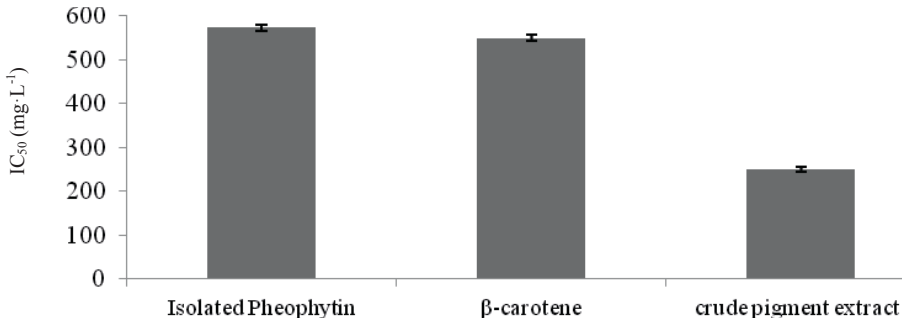


Fig. 5. The histogram of IC_{50} concentration of isolated pheophytin *a*, β-carotene, and green tea crude pigment extract using DPPH method.

IC_{50} concentration of isolated phephytin was (573 ± 0.23) mg · L^{-1}, while, β-carotene marker and green tea crude extract were (550 ± 0.26) mg · L^{-1} and (250 ± 0.21) mg · L^{-1}, respectively. The value of IC_{50} obtained from isolated pheophytin did not differ much from β-carotene marker. The presence of π-conjugated bonds in pheophytin *a* and β-carotene enable them to act as antioxidants through electron transfer mechanism. In pheophytin, especially, the conjugated double bonds in the porphyrin ring can act as electron transfer that will stabilize a radical compound[7]. Pheophytin acts as quencher of reactive species which causes DNA and cell oxidative damage[15].

Fig. 5 shows that the crude pigment extract had lower value of IC_{50} concentration in comparison to the isolated pheophytin and β-carotene. This value means that the antioxidant activity of the crude pigment extract is twice higher than the activity of β-carotene. This result had been expected on the reason that the crude pigment extract contained mixtures of pheophytin and β-carotene, as well as other compounds that could work in synergy as antioxidant.

Conclusion

TLC analysis of the pigments from green tea crude extract showed five spots; they are β-carotene, pheophytin *a*, chlorophyll *b*, xanthophyll, and pheophorbide *a*. Analysis by HPLC showed that there are 14 peaks, comprising 10 peaks of chlorophyll and their derivatives, and four carotenoid peaks. The antioxidant activity of pheophytin *a* had (573 ± 0.23) mg · L^{-1} and was the same as β-carotene marker of (550 ± 0.26) mg · L^{-1}. Green tea crude extract had antioxidant activity of IC_{50} = (259 ± 0.21) mg · L^{-1} that was twice higher than the activity of β-carotene.

Acknowledgement

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