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Full Length Research Paper

Estimation of antioxidant activity in natural rubber (*H.brasiliensis*) using TPC, FRAP, CUPRAC, ABTS, DPPH, ORAC assays, individual phenolic and individual flavonoid quantity

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Natural antioxidant in natural rubber is very useful to react with oxygen or ozone to protect natural rubber from undesirable chemical oxidations. There are various methods used to determine the quantity of antioxidant in food that may be applied to estimate natural antioxidant in natural rubber. The main objective of the present work is to study the effects of different extracted solvents (mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol) on the detected quantity of antioxidant in three grades of natural rubber; Air dry sheet (ADS), Ribbed smoked sheet No.3 (RSS3) and Standard thai rubber 20 (STR20). Various methods of the estimation of antioxidant in natural rubber were also investigated by using total phenolic content (TPC) assay, ferric reducing antioxidant power (FRAP) assay, cupric ion reducing antioxidant capacity (CUPRAC) assay, 2,2′-Azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, oxygen radical absorbance capacity (ORAC), individual phenolic and individual flavonoid. It was found that the mixture of cyclohexane:methanol showed the highest antioxidant activity verified by TPC, FRAP, ABTS, DPPH and ORAC. Individual phenolic and flavonoid exhibited quite different among solvent types, phenolic types and flavonoid types. Moreover, almost all of the results showed that ADS exhibited lower antoxidant activity than that of STR20 and RSS3.

Keywords: antioxidant, flavonoid, natural rubber, phenolic

INTRODUCTION

Antioxidant is a chemical that react preferentially with traces of oxygen or ozone to protect materials from undesirable chemical oxidations (Hiller and Herber 1960). Natural antioxidant in natural rubber composes of phenolic

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acids, flavonoids, tocopherols, tocotrienols, vitamin E, vitamin C, β-carotene, proteins, enzymes, lipids and small molecules of other antioxidants. A small amount of phenolic compound and polyphenoloxidase enzyme in natural rubber (Wititsuwannakul et al., 2002; Madsa-I and Cheewasedtham 2011), can be able to act as the antioxidant in natural rubber. In plants, the enzyme is more commonly called polyphenoloxidase, suggesting that its primary substrates are polyphenolic compounds. Carotenoids in rubber (Wititsuwannakul et al., 2002; Sakdapipanich et al., 2007), are also one of antioxidant types in nature. Moreover, a small amount of lipid in natural rubber (Blackley 1997), can be also caused antioxidant by the oxidation of lipid. Proteins in natural rubber (Madsa-I and Cheewasedtham 2011; Blackley 1997), can be able to act as an antioxidant because proteins can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties (Elias et al., 2008).

In food and agricultural science, many methods such as TPC, FRAP, CUPRAC, ABTS, DPPH, ORAC assay, individual phenolic and individual flavonoid are used to verify amount of antioxidant in natural plants such as legumes[7], Granny Smith apple *(Malus sylvestris*) (Zulkifli et al., 2012), Pink-Flesh Guava (*Psidium guajava L.*) (Musa et al., 2011; Musa et al., 2015). Kesum (*Polygonum minus*), ginger (*Zingiberofficinale*), turmeric (*Curcuma longa*)[11], guava *(Psidium guajava*), Chakonan mango *(Mangifera indica* var. Chakonan), Navel orange *(Citrus sinensis*var. Navel), crataegus specie (Özyürek et al., 2011), wild mushroom (*Pleurotus Porrigens)* (Yim et al., 2012), and several edible mushrooms (Hung and Nhi 2012). Therefore, it could be applied to estimate the quantity of antioxidant in natural rubber.

In different plants, types of solvents affect antioxidant values. For example, in the extraction of legumes, 50% of acetone exhibited the highest TPC for yellow pea, green pea, chickpea, and yellow soybean. Moreover, acidic 70% acetone (+0.5% acetic acid) showed the highest TPC and FRAP values for black bean, lentil, black soybean and red kidney bean. In addition, 80% acetone gained the highest DPPH scavenging activity for yellow pea, green pea, chick pea types and yellow soybean[7]. Furthermore, in the leaves of *Adhatoda Vasicanees*, water extraction gave the highest TPC value than that of the mixture of ethanol:water in the ratio 50:50, and petroleum ether respectively[15]. These results proved that different solvents affect the value of antioxidant activities. Therefore, the methods to extract antioxidant in natural rubber were also investigated to obtain the maximum quantity of detected antioxidant.

In overall, this study aims to investigate the use of TPC, FRAP, CUPRAC, ABTS, DPPH, and ORAC assay to characterize antioxidant activity in the three grades (ADS, RSS3 and STR20) of natural rubber. Furthermore, many types of individual phenolic acids and flavonoids detected by HPLC method were also determined. Different solvents were also investigated to gain the best way of extraction by using the mixtures of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol. The benefit of the results might be point out the way or the method to gain and measure natural antioxidant quantities in natural rubber in the future.

MATERIALS AND METHODS

ADS and RSS3 were collected from Office of the Rubber Replanting Aid Fund of Khokpantan, Pattani province, South of Thailand. STR20 was collected from Pattani local factory. Acetone and methanol were obtained from Sigma Aldrich companies. Cyclohexane was supplied from Fisher Scientific, UK. Chloroform was supplied from R&M Chemicals, UK. Folin Ciocalteu phenol reagent, ferric chloride ($FeCl_3 \bullet 6H_2O$), and HCl were obtained from Merck (Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 2,4,6-tris(2pyridyl)-s-triazine (TPTZ), gallic acid, 4-hydroxy benzoic acid, caffeic acid, p-coumaric acid, ferulic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), myricetin, quercetin, luteolin, kaempferol, apigenin and sodium acetate trihydrate were purchased from Sigma (USA) while glacial acetic acid was from Mallinckrodt Baker (USA). Sodium carbonate and others chemicals were purchased from RDH (Germany). All chemicals and reagents used in the study were analytical grade.

Extracted sample preparation

Natural rubber samples; ADS, RSS3 and STR20 were weighted for 3 g and dissolved in 100 mL in the volume ratio of 4:1 of the solvent mixtures i.e. chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol. The mixtures were stirred overnight with magnetic stirrer. The rubber was coagulated out by pouring the rubber solution gradually in 300 mL of methanol. The remaining solvent in 600 mL of beaker was evaporated in fume hood at room temperature. The 2nd and 3rd extraction processes were repeated by dissolving the previous coagulated rubber from the 1st step in the same solvent type and spent overnight. The rubber solution was coagulated again by methanol with the same method and kept to dissolve again. The remained methanol solutions from the last step of 2nd and 3rd extraction were collected into the same beaker. It was then evaporated in fume hood. A small amount of methanol was then added to dissolve the remaining in the beaker and collected into vial. The methanol solution in the vials was evaporated to be more concentrated. Methanol was used to top up the solution to exactly 10.00 mL. All samples were filtrated by using syringe filters (0.45µm) to remove the impurity and

transferred to new vial. The samples in the vials were used to test various methods of antioxidant activity.

Determination of total phenolic content (TPC)

The total phenolic content of a natural rubber extract solution was determined by a modified Folin-Ciocalteu method[9]. A total phenolic content standard curve was prepared by using gallic acid as the reference. A 100 µL of the extract solution was added by 0.50 mL of ten folds water diluted Folin-Ciocalteu reagent, mixed well and left for 5 min. Then, 1 mL of 7.5% Na_2CO_3 was added, after which the mixture was kept in the dark for 2 hrs, at room temperature. The absorbance was measured at 765 nm wavelength by a UV-visible Spectrophotometer (BMG Labtech, Germany). The results are expressed as milligrams gallic acid equivalent (GAE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the gallic acid standard curve, additional dilution would be done.

Determination of ferric reducing antioxidant power (FRAP)

FRAP assay was also used to estimate the total antioxidant capacity (Gulcin et al., 2011). FRAP assay standard curve was prepared by using Trolox as the reference. FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the volume ratio of 10:1:1[9]. The FRAP reagent (1 mL) was mixed with extracted samples (100 µL) and then the mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at wavelength of 595 nm by UV-visible Spectrophotometer (BMG Labtech, German). The result was expressed as milligrams of Trolox equivalents (TE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the trolox standard curve, additional dilution would be done.

Determination of cupric reducing antioxidant capacity (CUPRAC)

10 mM Cu(II), 7.5 mM neocuprine alcoholic solution and 1 M ammonium acetate buffer (pH 7) solutions were mixed in the volume ratio of 1:1:1 to become CUPRAC reagent. 100 µL of the rubber extracted samples and Trolox standard solution were added by 1 mL of the CUPRAC reagent. The absorbance was measured at 450 nm after 30 minutes (Özyürek et al., 2011; Apak et al., 2004), by using UV-visible Spectrophotometer (BMG Labtech, German). The result was expressed as milligrams of Trolox equivalents (TE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the trolox standard curve, additional dilution would be done.

Determination of 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) assay

7.4 mM ABTS solution and 2.6 mM potassium persulfate solution were mixed in equal quantities and allowed to react for 12 hrs at room temperature in the dark (Arnao et al., 2001). The solution was then diluted by mixing ABTS reagent with methanol to obtain an absorbance of 1.0 unit at 734 nm using a UV-visible Spectrophotometer (BMG Labtech, Germany). Fresh ABTS reagent was prepared for each assay. The rubber extracted samples (100 µL) were dropped into test tube, and the 1.0 unit absorbance ABTS solution was added and kept in dark for 2 hrs. The values were obtained by measuring the wavelength at 734 nm by UV-visible Spectrophotometer (BMG Labtech, German). The result was expressed as milligrams of Trolox equivalents (TE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the trolox standard curve, additional dilution would be done.

Determination of free radical by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of extracted sample was determined by using stable free radical of DPPH. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm (Zulkifli et al., 2012; Maizura et al., 2011; Prakash 2001; Babu et al., 2013), and shows purple in color. The decrease in absorbance occurs when the odd electron of DPPH radical pairs with hydrogen from a free radical of antioxidant to form the reduced DPPH-H (Prakash, 2001). In this experiment, DPPH stock solution was prepared by dissolving 24 mg DPPH in 1 L of methanol. Before test, the DPPH solution was obtained by mixing the DPPH stock solution with methanol to obtain an absorbance of 2.0 units at 517 nm. The rubber extracted samples (100 µL) were dropped into test tube, and 1 mL of 2.0 units absorbance DPPH solution was added, and kept in dark for 1 hr. The values were obtained by measuring the wavelength at 517 nm by UV-visible Spectrophotometer (BMG Labtech, German). The result was expressed as milligrams of Trolox equivalents (TE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the trolox standard curve, additional dilution would be done.

Determination of oxygen radical absorbance capacity (ORAC) assay

The ORAC procedure used an automated plate reader FLUO Star Omega (BMG Labtech, German) with 96-well

plates. The condition of analysis was conducted in phosphate buffer pH 7.4 at 37ºC. 2, 2'- azobis (2-amidino-propane) dihydrochloride was used to generate peroxyl radical which was to be prepared freshly at every run. Fluorescein was used to be the substrate[21]. The standard curve of Trolox was between 0 and 50 mM. The result was expressed as mM of Trolox equivalents (TE) per 100 g of initial natural rubber sample weight (IW). If the absorbance value of sample was over the linear range of the trolox standard curve, additional dilution would be done.

Determination of individual phenolics and flavonoid

For individual phenolics, the chromatographic system consisted of a Shimadazu LC-2010A liquid chromatography (Japan) coupled with diode array detector (SPD-M20A) (Shimadzu, Japan). C18 column (Symmetry 5µm, 4.6 mm x 150mm, Waters, USA) was used for separation. Solvent A consisted of 0.1% formic acid, and solvent B consisted of 100% methanol and the flow rate was 1 mL/min.[22] The standard curve of gallic acid, 4-hydroxy benzoic acid, caffeic acid, p-coumaric acid and ferulic acid were used to determine quantity of individual phenolic acid in ADS.

For the determination of flavonoid, 1% formic acid (55%) in methanol was used to be the mobile phase at 0.9 ml/min flow rate for 20 min. The standard curve of myricetin, quercetin, luteolin, kaempferol and apigenin were used to determine quantity of individual flavonoid in the samples of ADS, RSS3 and STR20.

Statistical analysis

Data is expressed as means ± SD. Correlation analysis was performed by MINITAB® 16 software or Excel. Statistically significant comparisons of the mean values for each experiment were performed by one-way ANOVA, followed by the Fischer test ($P < 0.05$ confidence levels).

RESULTS AND DISCUSSION

For TPC, FRAP, CUPRAC, ABTS and DPPH results (table 1-5) which were based on electron transfer mechanism (Dasgupta and Klein 2014) most of the data of ADS, RSS3 and STR20 natural rubbers extracted by the mixture of cyclohexane: methanol showed probably the highest value of antioxidant detected. The possible reason may be due to the forming of intermolecular hydrogen bonds of the mixture of cyclohexane: methanol with the antioxidant (AH) (Nenadis and Tsimidou 2002). In case of methanol and acetone, the oxygen atoms in methanol have stronger capacities than that of acetone to accept H bonds from antioxidant. Since oxygen atoms are in excess, all labile hydrogen atoms will form H bonds (Max and Chapados 2005). Therefore, methanol can form intermolecular hydrogen bonds with AH more than that of acetone leading to higher antioxidant activity. Moreover, cyclohexane base solvent may extract some more antioxidant than that of chloroform because of the solubility parameter of the solvent. The natural rubbers (□=16.8) can be dissolved completely in the present of cyclohexane in the extraction medium since these compounds are more dissolvable in cyclohexane than that of chloroform solvents because the solubility parameter of cyclohexane (□=16.8) is closer to natural rubber (□=16.8) than that of chloroform (□=18.7). After the natural rubber is dissolved, the antioxidant could be migrated to stay in acetone or methanol phase because of its solubility parameter. Therefore, the more the rubber was dissolved, the more extracted antioxidant was obtained.

For the different types of natural rubber, RSS3 and ADS which come from the same latex and almost the same process, RSS3 showed more detected antioxidant activity than that of ADS. The possible reason may be due to the smoke process in RSS3 production. The smoke that comes from wood burning may bring some antioxidant from wood plant (Kjällstrand and Petersson 2001). In RSS3 process, the rubber wood plant composing of some antioxidants is used in fabrication process. These antioxidants may be contaminated by RSS3. Therefore, more antioxidant in RSS3 than that of ADS were obtained. For STR20, the main raw rubber materials used to produce STR20 were cup lumps which came from different shelf life and the contamination. Moreover, normally all of the cup lumps were accumulated for a certain period of time in the humid conditions before entering the production process. During this accumulation stage, the fermentation by bacteria and algae occurred, which leads to higher detected phenolic compounds. Bacteria, for example, *Bifidobacterium lactis*, *Lactobacillus gasseri*, and *Escherichia coli* in colonic microbiota species can release hydroxycinnamates from chlorogenic acid which is a derivative of phenolic compound (Duda-Chodak et al., 2008). Moreover, the hydrolysis or carbon-ring cleavage of bacteria can be transformed to flavonoid type which can be detected by antioxidant activity. In addition, some antioxidant and flavonoid might be changed with time and environment before the process of STR20 production. Therefore, antioxidant results of STR20 is quite high and varies, although it was passed through many processes of washing. Most of the results of antioxidant activity showed in STR20 were not significantly different when cpmpared with RSS3 for TPC, CUPRAC, ABTS and DPPH methods. Only in the FRAP method there was a significant difference in antioxidant activity in RSS3 (higher results) when compared to that of STR20 and ADS, respectively. In the FRAP value, the results shown significantly different (higher when compared to that of TPC and other techniques) because the rubber contained carotenoids (Sakdapipanich et al., 2007; Max and Chapados 2005), which may play a role in antioxidant capacity. Moreover,

Table 1. Total phenolic compound obtained from various extracted solvent

Type of mixed solvent**	TPC		
	ADS*	RSS3*	STR20*
ChA	89.13 ± 5.89 ^{Cb}	185.12 ± 8.47 ^{Ca}	197.18 ± 7.84 ^{Aa}
ChM	90.25 ± 7.50 ^{Cb}	186.05 ± 7.84 ^{Ca}	201.30 ± 17.65 ^{Aa}
CyA	128.46 ± 13.31 ^{Bc}	240.75 ± 16.95 ^{Ba}	207.03 ± 21.59 ^{Ab}
CyM	149.45 ± 15.72 ^{Ac}	292.59 ± 15.28 ^{Aa}	221.26 ± 25.17 ^{Ab}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

Table 2. Ferric Reducing/Antioxidant Power obtained from various extracted solvent

Type of mixed solvent**	FRAP		
	ADS*	RSS3*	STR20*
ChA	32.12 ± 1.40 ^{Dc}	60.06± 0.86 ^{Ca}	52.92 ± 1.84 ^{Bb}
ChM	37.92 ± 0.14 ^{Cc}	59.19± 1.68 ^{Ca}	47.33 ± 2.26 ^{Bb}
CyA	41.64 ± 0.15 ^{Bb}	69.32± 3.36 ^{Ba}	47.23 ± 8.11 ^{Bb}
CyM	44.99 ± 1.10 ^{Ac}	87.87± 4.55 ^{Aa}	67.32 ± 5.88 ^{Ab}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

Table 3. cupric ion reducing antioxidant capacity (CUPRAC) assay obtained from various extracted solvent

Type of mixed solvent**	CUPRAC		
	ADS*	RSS3*	STR20*
ChA	47.71±0.97 ^{Bc}	89.78±1.23 ^{ABb}	111.20±5.86 ^{Aa}
ChM	56.77±1.46 ^{Aa}	60.99±6.08 ^{Ca}	55.08±2.67 ^{Ba}
CyA	55.21±1.15 ^{Ab}	84.02±2.09 ^{Ba}	35.69±0.85 ^{Cc}
CyM	55.39±8.57 ^{Ac}	95.29±3.64 ^{Ab}	113.59±3.91 ^{Aa}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

Table 4. ABTS assay obtained from various extracted solvent

Type of mixed solvent**	ABTS		
	ADS*	RSS3*	STR20*
ChA	23.14±1.89^{Cc}	56.20±2.63^{Cb}	61.99±4.45^{Ba}
ChM	27.61±1.37^{Bc}	58.54±3.58^{Cb}	66.93±1.33^{ABa}
CyA	27.23±2.14^{Bb}	68.66±1.61^{Ba}	71.29±6.92^{Aa}
CyM	38.43±1.42^{Ab}	80.77±2.07^{Aa}	74.42±1.63^{Aa}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

Table 5. DPPH assay obtained from various extracted solvent

Type of mixed solvent**	DPPH		
	ADS*	RSS3*	STR20*
ChA	26.32±5.01^{Bb}	43.88±1.96^{Ba}	40.86±0.84^{Ca}
ChM	33.02±4.88^{Ab}	45.78±4.37^{Ba}	55.57±7.68^{Ba}
CyA	32.86±3.92^{Ab}	53.82±8.44^{Ba}	55.20±2.96^{Ba}
CyM	33.10±3.08^{Ab}	80.38±8.66^{Aa}	71.09±6.79^{Aa}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

Table 6. Oxygen radical absorbance capacity (ORAC) assay obtained from various extracted solvent

Type of mixed solvent**	ORAC		
	ADS*	RSS3*	STR20*
ChA	175±82^{Ab}	441±16^{Ca}	475±12^{ABa}
ChM	152±74^{Ab}	563±73^{Ca}	462±37^{ABa}
CyA	134±5^{Ac}	802±78^{Ba}	368±43^{Bb}
CyM	262±9^{Ac}	1,164±83^{Aa}	539±12^{Ab}

*$^{A-C}$Values in each column marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

*$^{a-c}$Values in each row marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

**ChA, ChM, CyA and CyM mean the solvent mixtures in the volume ratio of 4:1 of chloroform:acetone, chloroform:methanol, cyclohexane:acetone and cyclohexane:methanol, respectively

other substances such as tocotrienols and proteins may also act as antioxidant which affects the FRAP values can have the ability to reduce ferric ions. (The donating electron to ferric Fe (III) of an antioxidant causes the reduction step into blue ferrous Fe (II) complex.). In addition, the wood smoke antioxidant (Kjällstrand and Petersson 2001), contaminated in RSS3 process may also effect on the amount of high antioxidant detected by the FRAP method.

For CUPRAC assay, the trend of the results was quite different from the other methods. The possible reason may be due to CUPRAC reagent can react with polyphenols, flavonoids and other substrates. Moreover, cupric chloride causes the destruction of carotene[29] which is one of the non-rubber contaminations in natural rubber. In addition, antioxidants in the presence of metal ions such as copper can create the pro-oxidative effect which exhibit new free radical after the reaction. Therefore, CUPRAC assay had shown fewer differences in results compared to the other antioxidant methods.

For oxygen radical absorbance capacity assay, standard of trolox was used to compare. Fluorescein was used to be the substrate. 2, 2'- azobis (2-amidino-propane) dihydrochloride was used to generate peroxyl radical. After mixing both substances with the sample solution, antioxidant in sample will react with radical, then fluorescein. The automated plate reader FLUO Star Omega (BMG Labtech, German) detected that fluorescein remained in the system. In case of high antioxidant, the delayed drop peak was obtained. The integral area of sample peak was compared to the standard curve of trolox. The signal curves of standard trolox were shown in figure 1.

From the results shown in table 6, it was found that the extraction using the mixture of cyclohexane:methanol shows a higher value of antioxidant by ORAC method than that of the others solvents which obviously is seen in higher quantity of antioxidant in RSS3 results than that of other rubbers. These results supported the results from the method of electron transfer mechanism (Dasgupta and Klein 2014). (TPC, FRAP, CUPRAC, ABTS and DPPH results) which most antioxidants were obtained from the solvent mixtures of cyclohexane: methanol in the volume ratio of 4:1 with the same previous reason.

The repeatability of each technique was performed by calculating the % relative standards deviations (%RSD). The maximum %RSD of TPC, FRAP, CUPRAC, ABTS, DPPH and ORAC were 11.4, 17.2, 15.5, 20.6, 19.03 and 23.0%, respectively. The correlations (Table 7) between TPC and values for antioxidant activity (FRAP, ABTS, DPPH and ORAC) were high except CUPRAC. According to the previous reason, the trend of the CUPRAC results

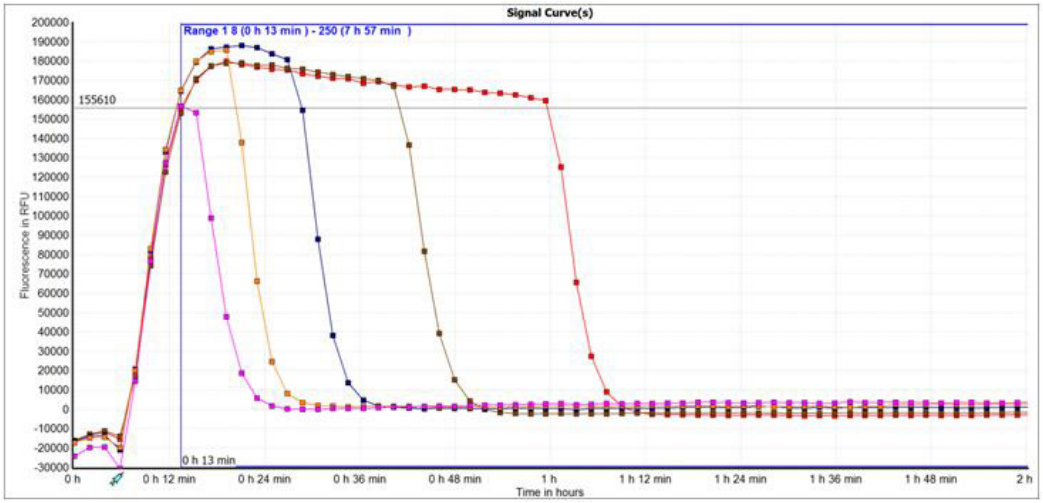

Figure 1. Signal curve of standard trolox obtained from automated plate reader FLUO Star Omega (BMG Labtech, Germany)

Table 7. Correlation obtained from various antioxidant test method

	Correlation					
Methods	TPC	FRAP	CUPRAC	ABTS	DPPH	ORAC
TPC	-	0.91	0.55	0.95	0.90	0.91
FRAP	0.91	-	0.68	0.80	0.85	0.95
CUPRAC	0.55	0.68	-	0.51	0.50	0.55
ABTS	0.95	0.80	0.51	-	0.90	0.81
DPPH	0.90	0.85	0.50	0.90	-	0.83
ORAC	0.91	0.95	0.55	0.81	0.83	-

were quite different form the other methods due to CUPRAC reagent can react with polyphenols, flavonoids and other substrates. Moreover, pro-oxidative effect may occur.

Individual phenolic compounds of ADS with different solvent extractions

The standard curve of individual phenolic compounds; gallic acid, 4-hydroxy benzoic acid, caffeic acid, p-coumaric acid and ferulic acid were plotted and shown in figure 2. The obtained equations from the curves were used to calculate quantity of individual phenolic compound in the ADS extracted solution samples from different solvents; chloroform:acetone (ChA), chloroform:methanol (ChM), cyclohexane:acetone (CyA) and cyclohexane:methanol (CyM). It was found that all 5 phenolic compounds can be extracted from every mixed solvent types. Each phenolic compound can be extracted by different solvents with different quantity trend but the trend of cyclohexane base solvent affected the quantity of phenolic compound more than that of chloroform base solvent. The summation of all 5 types of phenolic compounds was also shown in figure 2. The presence of cyclohexane in the extraction medium may be the main reason for higher phenolic acids content since these compounds are more extractable in cyclohexane than the chloroform solvents because the solubility parameter of cyclohexane (δ=16.8) is more closer to natural rubber (δ=16.8) than that of chloroform (δ=18.7).

Individual flavonoid compound of ADS, RSS3 and STR20 with different solvent extraction

The standard curves of individual flavonoid compounds; Myricetin, Quercetin, Luteolin, Kaempferol and Apigenin were plotted and shown in figure 3. The obtained equations from the curve were used to calculate quantity of individual flavonoid compound (table 8) in the ADS, RSS3 and STR20 solution samples extracted from different solvents; chloroform:acetone (ChA), chloroform:methanol (ChM), cyclohexane:acetone (CyA) and cyclohexane:methanol

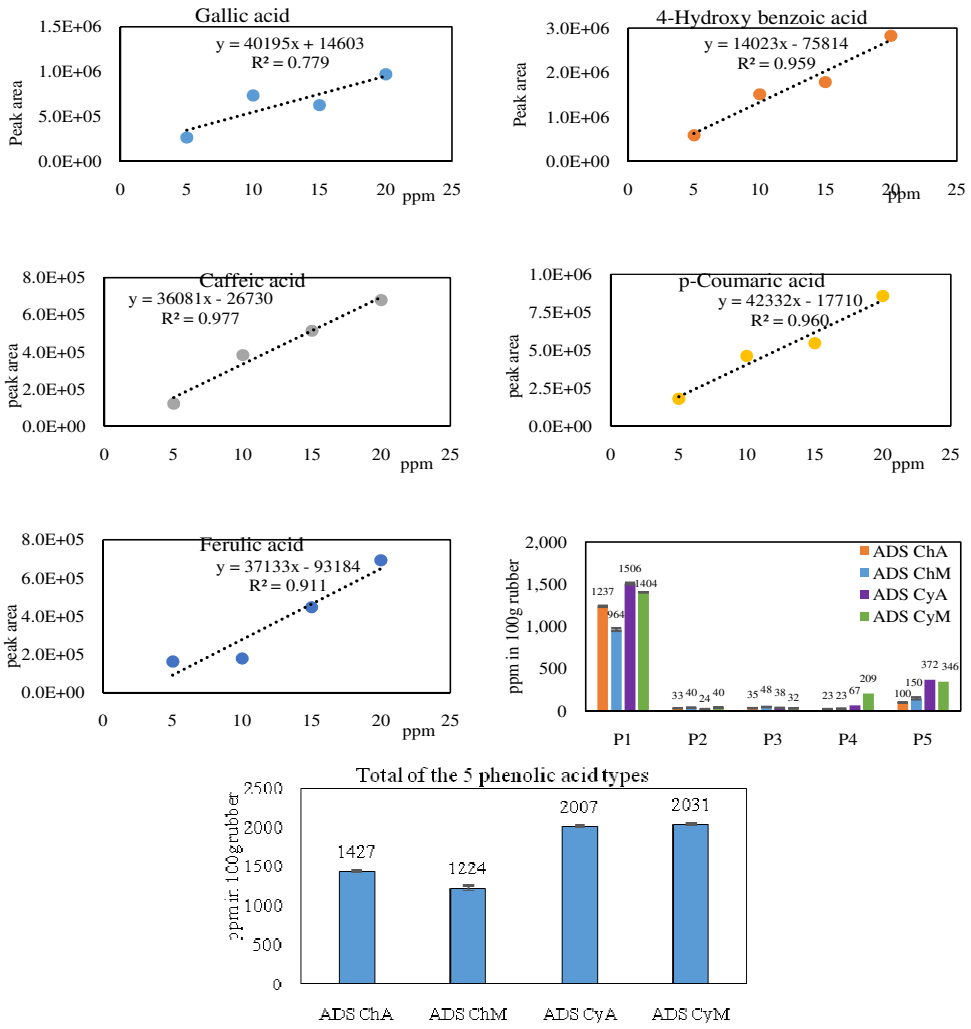

Figure 2. Individual phenolic compounds; gallic acid (P1), 4-hydroxy benzoic acid (P2), caffeic acid (P3), p-coumaric acid (P4), ferulic acid (P5) standard curve and quantity of individual phenolic compound in the ADS solution samples extracted from different solvents; chloroform:acetone (ChA), chloroform:methanol (ChM), cyclohexane:acetone (CyA) and cyclohexane:methanol (CyM).

(CyM). It was found that all 5 flavonoid compounds can be extracted from every mixed solvent types with different quantity, but most of the data of cyclohexane base solvent affected the quantity of flavonoid compound more than that of chloroform base solvent. The trend of each flavonoid type shows some different trend. It might be because the solubility of each flavonoid is different. Myricetin has 3 adjacent hydroxyl groups in ring that can be soluble in various organic solvents. Myricetin was well dissolved in tetrahydrofuran, dimethylformamide and dimethylacetamide and moderately soluble in acetone, methanol and ethanol but almost insoluble in chloroform, petroleum ether, methylbenzene and n-hexane. Quercetin is a highly polar (water-soluble) compound. Therefore, the fewer amount detected than that of other individual flavonoid types was obtained. Luteolin is soluble in alcohol, acetone, ether and alkalies and slightly soluble in chloroform. Kaempferol is slightly soluble in water and highly soluble in hot ethanol, ethers and dimethyl sulfoxide. Apigenin is insoluble in water and moderately soluble in hot alcohol.

The summation of all 5 types of flavonoid compounds is also shown in table 8 and figure 4. It is obviously seen in ADS and RSS3 that the presence of cyclohexane in the extraction medium shows higher flavonoid content than that of chloroform but it was not significantly different in STR20 results. For ADS and RSS3, the results were supporting the results from the previous experiment which the most flavonoid obtained were from the solvent mixtures of cyclohexane:methanol. Conversely, STR20 generally come from different shelf life and contamination. Therefore, some antioxidant and flavonoid might be changed with time

Table 8. Individual flavonoid compounds; Myricetin, Quercetin, Luteolin, Kaempferol and Apigenin obtained from ADS, RSS3 and STR20 in various extracted solvent types; chloroform:acetone (ChA), chloroform:methanol (ChM), cyclohexane:acetone (CyA) and cyclohexane:methanol (CyM).

*unit = mg in 100g rubber

Type of rubber and mixed solvent	Myricetin	Quercetin	Luteolin	Kaempferol	Apigenin	Total
ADS ChA	0.04±0.01^{B}	0.02±0.00 ^{A}	0.09±0.00 ^{A}	0.10±0.00 ^{B}	0.08±0.01 ^{B}	0.34±0.00 ^{B}
ADS ChM	0.05±0.00^{B}	0.02±0.00 ^{A}	0.09±0.00 ^{A}	0.10±0.00 ^{B}	0.08±0.00 ^{B}	0.33±0.00 ^{B}
ADS CyA	0.06±0.03^{B}	0.05±0.03 ^{A}	0.13±0.05 ^{A}	0.12±0.01 ^{A}	0.09±0.00 ^{A}	0.44±0.12 ^{A}
ADS CyM	0.12±0.02^{A}	0.06±0.03 ^{A}	0.13±0.01 ^{A}	0.11±0.00^{AB}	0.08±0.00 ^{B}	0.50±0.06 ^{A}
RSS ChA	0.08±0.03^{B}	0.04±0.00 ^{B}	0.10±0.01 ^{B}	0.10±0.01^{AB}	0.08±0.00 ^{B}	0.40±0.04 ^{C}
RSS ChM	0.08±0.00^{B}	0.04±0.00 ^{B}	0.11±0.00 ^{B}	0.09±0.00 ^{B}	0.08±0.00 ^{B}	0.41±0.00 ^{C}
RSS CyA	0.15±0.01^{A}	0.09±0.04^{AB}	0.18±0.07^{AB}	0.11±0.02^{AB}	0.09±0.02 ^{B}	0.61±0.16 ^{B}
RSS CyM	0.17±0.00^{A}	0.12±0.01 ^{A}	0.23±0.00 ^{A}	0.12±0.00 ^{A}	0.10±0.00 ^{A}	0.75±0.01 ^{A}
STR ChA	0.11±0.00^{D}	0.04±0.01^{AB}	0.15±0.00 ^{A}	0.09±0.01 ^{A}	0.09±0.01 ^{A}	0.48±0.02 ^{B}
STR ChM	0.15±0.00^{C}	0.05±0.00 ^{A}	0.15±0.00 ^{A}	0.08±0.01 ^{A}	0.09±0.00^{A}	0.53±0.00^{AB}
STR CyA	0.23± 0.01^{A}	0.03±0.00 ^{B}	0.14±0.00 ^{A}	0.08±0.00 ^{A}	0.08±0.00 ^{B}	0.55±0.01 ^{A}
STR CyM	0.17± 0.01^{B}	0.04±0.01^{AB}	0.13±0.04 ^{A}	0.08±0.00 ^{A}	0.08±0.00 ^{B}	0.51±0.07^{AB}

$^{A-D}$Values in each column in the group of rubber marked by the same letter are not significantly different at P <0.05. The results showed mean ± SD.

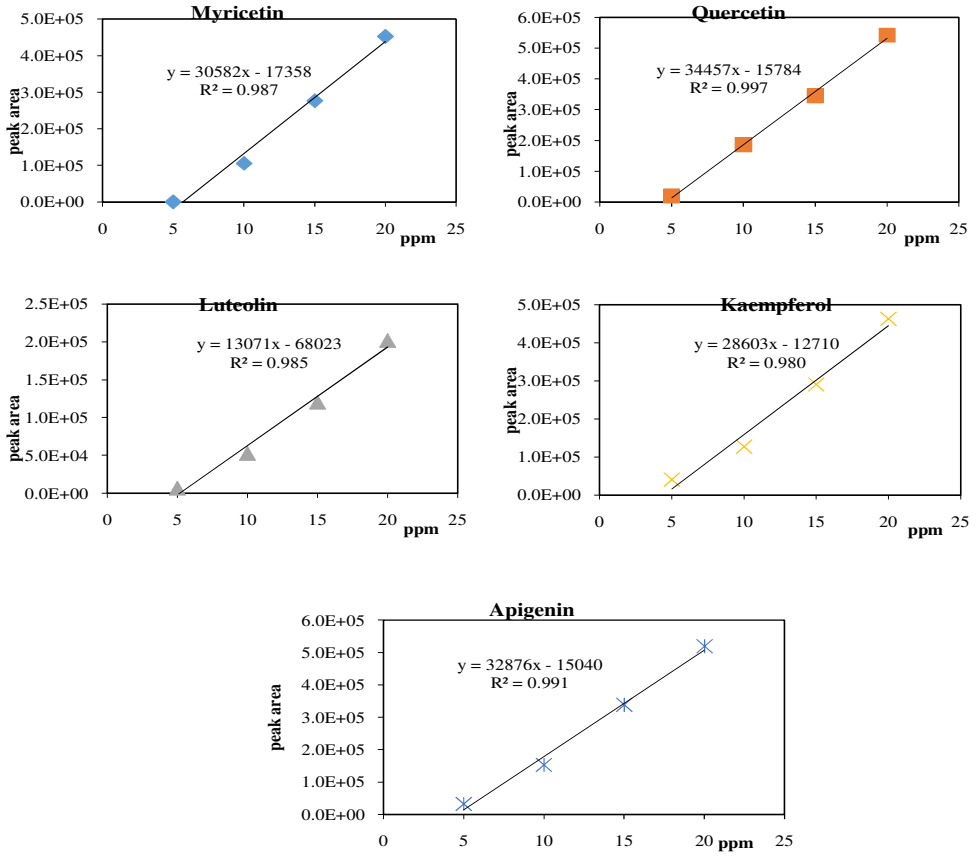

Figure 3. Individual flavonoid compounds; Myricetin, Quercetin, Luteolin, Kaempferol and Apigenin standard curve.

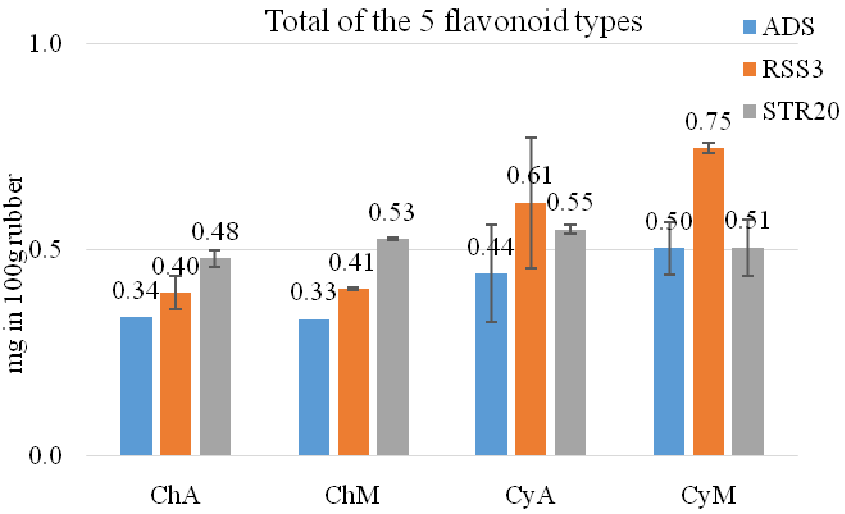

Figure 4. Total of 5 flavonoid compounds; Myricetin, Quercetin, Luteolin, Kaempferol and Apigenin obtained from ADS, RSS3 and STR20 in various extracted solvent types; chloroform:acetone (ChA), chloroform:methanol (ChM), cyclohexane:acetone (CyA) and cyclohexane:methanol (CyM).

and environment before the STR20 production. It might be the reason why some flavonoid in STR20 can be more extracted by chloroform such as quercetin and luteolin.

CONCLUSIONS

The best method to extract antioxidant from solid natural rubber; ADS, RSS3 and STR20 is by using the mixture of cyclohexane:methanol in the volume ratio of 4:1 which gave the highest antioxidant detected by TPC, FRAP, ABTS, DPPH and ORAC method. Moreover, when compared between natural rubber types, RSS3 showed higher antoxidant activity than that of STR20 and ADS when using TPC, FRAP and ORAC methods. High correlations between various methods of antioxidant determination (TPC, FRAP, ABTS, DPPH and ORAC) were obtained except CUPRAC. In addition, from the results of individual phenolic acid and flavonoid by HPLC method confirmed the TPC, FRAP, ABTS, DPPH and ORAC results that cyclohexane base solvent can extract more quantity of antioxidant from natural rubber than that of chloroform base solvent.

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