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ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND FLAVONOID CONTENT FROM LEAVES AND SEED EXTRACTS OF *Hevea brasiliensis* CLONE

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Abstract

Rubber tree or *Hevea brasiliensis* is an industrial crop from Euphorbiaceae family that grows in tropical climates and commonly known as an important economical commodity to Malaysia. However, there is a little information available on its phytochemicals in each part of rubber tree as well as their antioxidant activity. The aims of this study are to determine the extraction yield of different clone of rubber tree as well as to determine the antioxidant activity, total phenolic and flavonoid content from leaves and seeds extracts. The leaves and seeds extraction process were optimized by varying different parameter's such as drying temperature, types of solvent used and temperature of extraction. The drying process was done at temperature of 70℃ for 24 hours. The dried samples were macerated with methanol and the extract was concentrated using rotary evaporator at 60℃. The highest percentage of inhibition was based on the weight of dried plant materials. The determination of antioxidant was assessed by using the DPPH assay method. The total phenolic and flavonoid content was determined by using Folin-Ciocalteau assay and aluminium chloride colorimetric assay respectively. From this study, the result indicates that, the highest extraction yield was seeds extract (29.77%) from clone RRIM 2020. The highest amount of antioxidant was (79.16) from clone RRIM 2025. Therefore, the highest amount for total phenolic and flavonoid content were clone RRIM 2020 (0.020) and RRIM 3001 (0.200) respectively. As a conclusion, it showed that each clone gives a different result on each of the analysis. The result will be benefit to others for their reference in order to produce the product.

Keywords: *Hevea brasiliensis*, extraction yield, antioxidant, phenolic, flavonoid content

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Introduction

Rubber tree (*Hevea brasiliensis*) from Euphorbiaceae family is tropical crop that was origin from Brazil. Rubber tree is a tropical crop that is the perennial crop. The rubber tree cultivation has been introduced in Malaysia since early independence. Nowadays, the rubber tree was planted in many countries. In a well-known industrial process the rubber latex usually was processed into the natural rubber (Widyarani *et al*., 2017). The government institution that is responsible for the development of the Malaysian rubber industry is Malaysian Rubber board (MRB). Latex is the main product come from rubber tree. Other than that, tree can be use as timber and seeds. However, the seeds consist of oily shell and kernel contains 30% to 50% of oil (Verheye, 2010). The oil can be used as ingredient in paints, soap after processing of oil in the shell. The wood can be used as domestic and industrial fuel, manufacture of pulp for paper industry, produce charcoal and others.

Antioxidants is a substance that will delay and prevent the oxidation of the cell content when present at low concentrations such as the carbohydrates, proteins, lipids, and DNA (Gupta & Sharma, 2006). Polyphenols are secondary metabolites of plants and normally involved in resistance against the ultraviolet radiation and also the aggression by any pathogens (Pandey & Rizv, 2009). Polyphenols

form a complex group of molecules associated with most plant cell walls. They range in chemical complexity from simple phenolic acids (e.g., caffeic acid) to high-molecular-weight tannins (Kondratyuk & Pezzuto 2004). There are abundant of flavonoid in plants where they perform numerous functions such as important pigments to produce the necessary colors to attract the insect pollination. Flavonoids are always present in plants and also in one group of the polyphenolic compound (Pandey & Rizv, 2009).

The plant usually produces a large variety of secondary metabolites which are used directly as lead compounds in the pharmaceutical industry or as a precursor. The plant extracts were expected to showed the target sites which will be active against drug resistant microbial pathogen. Natural rubber has certain substances which usually present a small amount that can afford to protect the plant from oxidation. These natural antioxidants will decrease the useful life of rubber whenever it gets removed from the plant (Verheye, 2010). Polyphenols in rubber may be passed into the aqueous phase of waste effluent. These valuable natural antioxidants may be utilized to optimize resources from the rubber industry. Such utilization has been explored in other studies of other industrial effluents (Baysan *et al*., 2020). Thus, understanding the polyphenol composition of the effluent from rubber processing is valuable knowledge that can generate possible financial benefits through its isolation, rather than otherwise being a waste product. To the best of our knowledge , this is the first report on antioxidant on the selected clone. From the literature review, there is limited study conducted on antioxidant from plant extract from *Hevea brasiliensis.*

Materials and Methods

Plant materials

The studies have been designed by using random sampling method to collect the sample of leaves and seeds from three different clone of rubber tree which are RRIM 2020, RRIM 2025 and RRIM 3001. The sampling was conducted at Lau Leong Estate, Kampung Asahan, Melaka. The experiment has been done by collecting the six samples of leaves and seeds manually by hand picking to determine the extraction yield and their phytochemical.

Antioxidant activity

DPPH assay was done by method of Brand-Williams *et al*., (1995) with slightly modification. The antioxidant assay of the sample extracts was carried out by dissolving 100 µL of extracts in 2.9 ml 0.1 mm DPPH. This solution should be given vibration by using vortex to ensure all mixed up and allow to stand for 30 min at room temperature. The absorbance of the standard were measured against the reagent blank (MeOH 70%) at 517 nm under a UV/Visible spectrophotometer.

The determination of antioxidant activity is using DPPH assay. DPPH assay is based on the ability of 2, 2-diphenyl-1-picrylhydrazyl, a stable free radical to decolourize from purple to yellow colour to show the presence of antioxidants. The DPPH contains an odd electron, which responsible for the absorbance at 517nm and a visible deep purple colour. The DPPH would decolourized when DPPH accepts an electron donated by an antioxidant compound (Bag & Devi, 2015). The percentage inhibition (%) was calculated using the following formula:

$$\text{Percentage inhibition } \% = [(A_o \text{ (blank)} - A_1 \text{ (sample)}) / A_o] \times 100$$

Total phenolic content (TPC)

This analysis followed the method used by Swain & Hills (1959) with slight modification. Folin-Ciocalteu method was used to determine the total phenolic content of the plant extracts using gallic acid as an internal standard. Briefly, 0.5 ml of the extract was mixed with 8 ml of distilled water in a test tube and should be given vibration using vortex. After 5 min, 1 ml of 20% of sodium carbonate was added. A set of standard solutions of gallic acid (20, 40, 80 and 100 mg/ml) were prepared in the same manner as described for the extracts. The absorbance of the extracts and standard solutions were read against the reagent blank (ddH_2O) at 725 nm using UV/Visible spectrophotometer (UV-2450, Shimadzu, Japan).

Total flavonoid content (TFC)

This analysis was done by method of Kiranmai *et al.* (2011). Aluminium-chloride colourimetric assay was used to determine the total flavonoid content in the sample extracts. Then, 1 ml of the extract was mixed with 4 ml of distilled water in a test tube. 0.3 ml of 5% sodium nitrite was added to the test tube. After 5 min, 0.3 ml of 10% $AlCl_3$ was added to the mixture and followed by addition of 2 ml of 1.0 M NaOH after 1 min and dilute to the mark of 10 ml with distilled water. The solution was vibrated by using vortex to ensure its mixed well. A set of catechin (20, 40, 60, 80 and 100 mg/ml) were prepared as describe for the extract. The absorbance of the sample extracts and standard catechin were measured against the reagent blank (ddH_2O) at 510 nm using UV/Visible spectrophotometer.

Data analysis

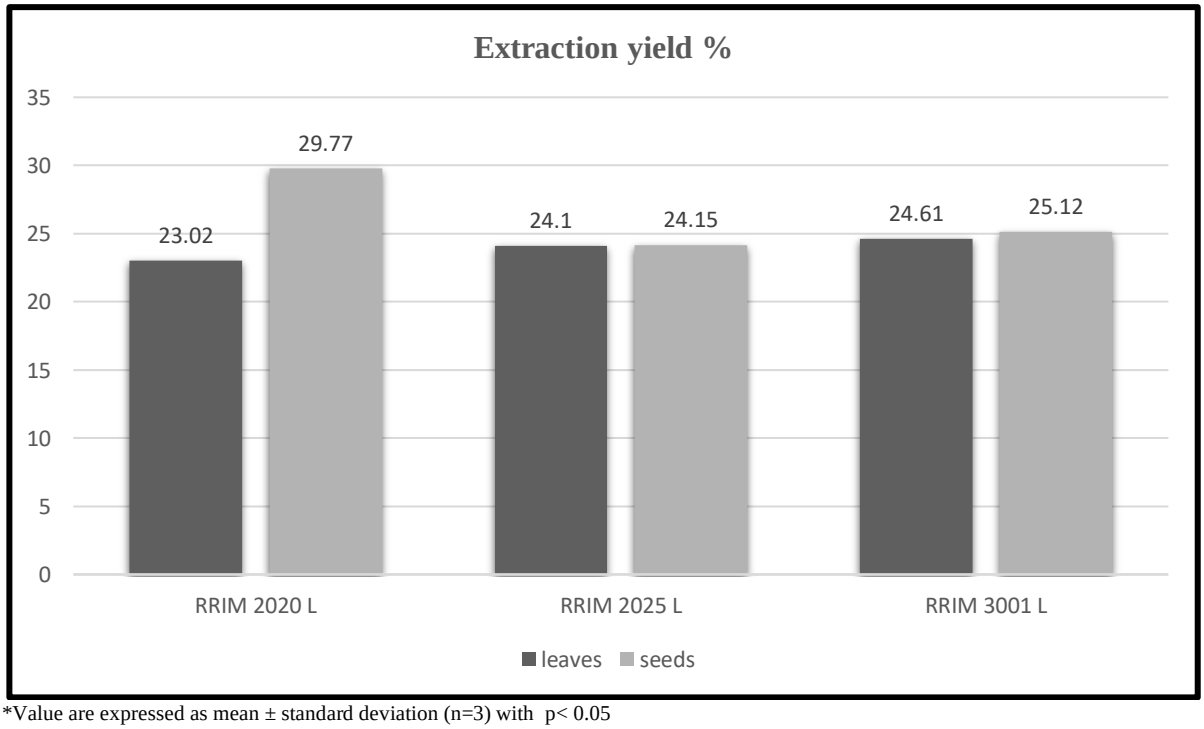
The result was expressed as mean of three replicates. Data on plant extract analysis were analysed using the SPSS (Statistical Package for Social Science) software version 22 to solve the research problems which has a very versatile data processing capability. The data analyse by using UV/Visible spectrophotometer and conducted at the laboratory to identify the phytochemical on the leave and seed samples that collected from three different clone.

Analysis of variance (ANOVA) and differences among means were determine for significance at $P < 0.05$ using Tukey's test. Then, to analyse the relationship degree of the statistic between linear the Pearson correlation was used to show the correlation. In this analysis, there are several relationships that can be classified between this study. The relationship between antioxidant activity, phenolic and flavonoid content also can be determined by using Pearson's correlation.

Results and Discussion

The extraction yield of samples.

There was 30 gram of samples extract of *Hevea brasiliensis* were dried in an oven as a method of preservation after which the samples were ground to powder. Then the 5-gram dried sample was allowed to have a maximum contact with the 10 ml of methanol. The solvent used in this study is 100% methanol which come from polar solvent to enable the extraction and to separate the components that widely present in the sample extract.



*Value are expressed as mean ± standard deviation (n=3) with $p< 0.05$
*Y axis = extraction yield (%)
*X axis= three different types of rubber clone

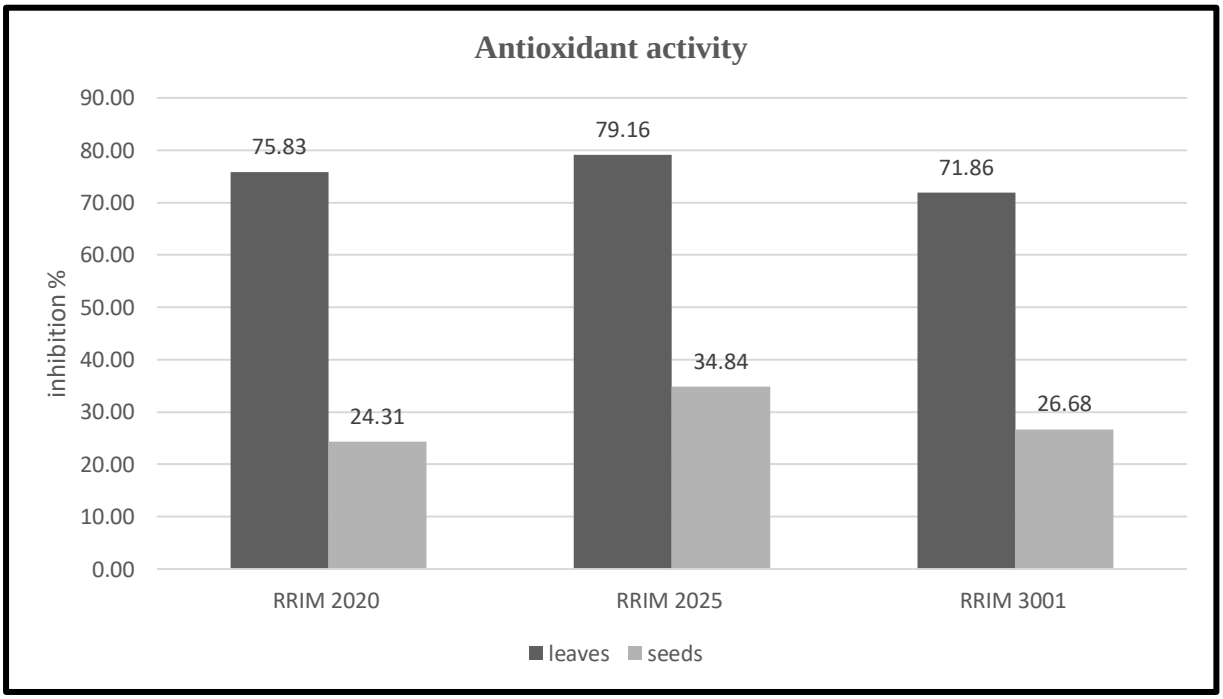
Figure 1. Extraction yield

The percentage of extraction yields was shown in Figure 1. It based on the weight of dried and ground plant materials. The extraction yield of seeds extract RM2020 L was the highest (29.77%) among the six samples whereas extraction yield of leaves extract was the lowest compared to the others.

There are several factors that can contribute to different extraction yield. The temperature was maintained not exceeding 70°C to avoid any degradation of targeted compound (Sembiring *et al.*, 2017). Methanol was classified as high polarity solvent that can extract amino acids, sugar and glycosides from the samples. Usually, 80 % methanol and 70 % ethanol are most used to extract the compound in the plant (Apak *et al*., 2007).

The antioxidant activity of extracts

The free radical can be stabilized and deactivate by antioxidants before they cause oxidative damage to the cellular structures. Free radical scavenging is by donating hydrogen to the free radical. Thus, the free radical scavenging activity can be used to determining the antioxidant activity of plant extract. In this studies, DPPH free radical scavenging activity of plant leaf and seed extracts were evaluated to determine their antioxidant activities.



*Value are expressed as mean ± standard deviation (n=3) with $p < 0.05$
*Y axis= extraction yield (%)
*X axis= three different types of rubber clone

Figure 2: Antioxidant activity

Figure 2 showed that the leave extract has the highest ability to scavenge free radicals than the seed parts. The clone RRIM 2025 showed the highest percentage of 79.16% than the other two clones for the leaf's extracts. However, the clone RRIM 2020 showed the lowest percentage which is 24.31%. The highest antioxidant activity of *Hevea brasiliensis* among the clone may be due to higher presence of total flavonoid content.

According to Altemimi *et al*., (2017), discovered highly polar solvent, such as methanol, have a high effectiveness as antioxidants. On the other hand, the antioxidant activities will be different according to the different type and amount of antioxidant compound present in plants (Pramai *et al.*, 2018). The different type and amount of antioxidant compounds present in the plants can be affected by the environmental condition. Factors such as increased intensity of sunlight, deficiency soil nutrient, infestation of pest and drought stress will increase the synthesis and accumulation of secondary products in the plant (Selmar & Kleinwächter, 2013). Different age of plant growth also has the effect on the

bioactivity and bioactive compounds of the plants (Ismail *et al*., 2012).

This result supported the finding by Sreelatha *et al*., 2003 which test on antioxidant enzymes which indicated the leaves significantly higher antioxidant compared to barks. These enzymes play a crucial role in protecting the tissues from acute oxidative damage. Stressful environment caused by conditions such as drought is another factor of rubber tree growth also in producing antioxidant metabolite. Under conditions of abiotic stress such as over exploitation, the plants experience oxidative stress. Oxidative stress is defined as the cumulative and accumulated affects od the potentially lethal reactions initiated by various forms of active oxygen spesies (AOS). AOS which cause inactivation of enzymes, clhlorophyll, lipid peroxidation or protein degradation which damage cellular membrane systems and trigger early senescences.

The total phenolic content (TPC) and Total Flavonoid Content (TFC) of extracts

The total phenolic content of the leaf and seed of each of the plants are shown in Table 1. The leaf has the highest number of phenolic compounds while the seed has the lowest number of phenolic compounds. The total phenolic content of the plant leaf extracts varied from 0.003 to 0.020 mg GAE/ml. The leaf of RRIM 2025 contained 0.003, followed by RRIM 3001 and RRIM 2020 at 0.016 and 0.020 mg GAE/ml, respectively. The same result can be observed for the seeds extract varied from not detected (nd) to 0.010 mg GAE/ml. The seed of RRIM 2020 not detected, followed by RRIM 3001 and RRIM 2025 at 0.006 and 0.010 mg GAE/ml respectively. From this study, the leaves of *Hevea brasiliensis* had higher amount of phenolic compound than the seeds. This could be explained by the reason that leaves are the site of biosynthesis of these phenolic compounds and they moved from leaves to the site of storage via the phloem or xylem tissues through long distance translocation. According to Abu Bakar *et al*., (2009), even the different parts of the fruits such as *Mangifera pajang* and *Artocarpus odoratissimus* has a different level of the bioactivities and the chemical compounds present. Therefore, it is not surprising for the leaf and seed in this studied has a different level of phenolic compounds. According to Selmar & Kleinwächter (2013), the location of the plant grew also play the roles for different amount of phenolic compound. It is because there is different level of stressor such as soil composition, high temperature and deficiency of nutrition in the plant.

The total flavonoid content of the different plant extracts measured by spectrophotometer by using the aluminium chloride colorimetric assay also was shown in Table 1. The flavonoid content of sample extracts was expressed as mg catechin equivalent per gram of the extract. Colorimetric reactions are used to determine flavonoid content in food or plant samples. Colorimetric assay is easy to perform, rapid and applicable in routine laboratory use and low cost (Blainski *et al*., 2013). The total flavonoids content in the extracts were only present in the leaves. The result demonstrated that flavonoid compound highly present in RRIM 3001 (0.200) and the least quantity of total flavonoid was not detected in RRIM 2025. As reported by Rizk, (1987), the Euphorbiacaea mainly contain triterpenoids, diterpenoids, tannin, flavonoids and polyphenol. Although seeds extract from this plant is not rich in flavonoid compound, as shown by negative result in flavonoid content, it may be containing another phytochemical constituent. On top of that, in this study catechin was used as a reference, studies by Bag & Devi (2015), have reported quercetin to be suitable references to determine total flavonoid content in plant sample extract. Quercetin, a plant pigment is a potent antioxidant flavonoid and more specifically a flavonol that has a high amount of active flavonoid. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and freeradical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity (Vessal *et al.*, 2003).

Table 1: Total phenolic and Total Flavonoid content (mg GAE/ml)

Samples	TPC (mg GAE/ml)	TFC (mg CAE/ml)
RRIM 2020 L	0.020	0.113
RRIM 2025 L	0.003	0.086
RRIM 3001 L	0.016	0.200
RRIM 2020 S	nd	nd
RRIM 2025 S	0.010	nd
RRIM 3001 S	0.006	nd

*nd: not detected.

*Value are expressed as mean ± standard deviation (n=3) with $p < 0.05$

Conclusion

Based on the study that had been done, it clearly stated that each clone gives a different result on extraction yield, antioxidant activity, total phenolic and flavonoid content. The value of seed extraction yields was showed the highest percentage which is 29.77% than the percentage of leaves. The result of chemical analysis shows that phytochemicals in leaves was the highest. The most antioxidant activity was found in clone RRIM 2025 while phenolic and flavonoid content were found in clone RRIM 2020 and RRIM 3001 respectively. This study showed that the leaves has the highest antioxidant activity and this was strongly correlated with high total flavonoid content. As a recommendation, the further study should carry out on the isolation and eluicidation of the secondary metabolite in the rubber tree. Thus, it will help to analyses the correlation between compounds and antioxidant activity

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