Total phenolic content, total flavonoid content and antioxidant activity of ethanolic extract of *Rafflesia kerri* Meijer, Lojing Highlands, Peninsular Malaysia

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Total phenolic content, total flavonoid content and antioxidant activity of ethanolic extract of *Rafflesia kerri* Meijer, Lojing Highlands, Peninsular Malaysia

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Abstract. *Rafflesia kerri* Meijer belongs to genus Rafflesia, recognized as the biggest flower in the world. This species found distributed in tropical rainforest of Thailand and Peninsular Malaysia. The flower believed to have medicinal values and used as traditional herbs by aborigine ethnic group and locals. Herein, this study was conducted to evaluate the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of the flower by using several antioxidant assays, which includes 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH) and ferric reducing antioxidant power (FRAP). The extract of female ethanolic extract exhibited highest antioxidant activity in all of the assays (FRAP = 4642.844±10.777 mM/mg) and median inhibition concentration (IC_{50} = 49.850±2.179). The same extract showed the presence of phenolic and flavonoid content with respective value of (Gallic acid equivalent, GAE) of 1634.731±20.465 mg/100g and Quercetin equivalent (QE = 817.000±69.282 mg/g) respectively with significantly differences ($p < 0.05$) compared to male ethanolic extract. Thus, the flower is highly valuable not only for ecotourism industry but also has potent medicinal value for human health improvement.

1. Introduction

The genus Rafflesia belongs to parasitic flowering plants from the Rafflesiaceae family. The flower found distributed in Southeast Asia's tropical rainforests, primarily in Malaysia, Brunei, Indonesia, Thailand and the Philippines. The flower known as *Pakma* in Malaysia is also known as *Puspa langka* in Indonesia, *Bua-phut* in Thailand, and *Uruy* in the Philippines [1, 2]. The list of Rafflesia species reported from Malaysia was recently revised by [3]. *R. cantleyi*, *R. azlanii*, *Rafflesia kerri*, *R. sumeiae*, *R. parvimaculata*, *R. sharifah-hapsahiae*, *R. tuanku-halimii* and *R. tiomanensis* were discovered in Peninsular Malaysia, while another five species were discovered in Sabah and Sarawak (*R. pricei*, *R. tuan-mudae*, *R. keithii*, *R. tengku-adlinii* and *R. hasseltii* [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 1].

R. kerri is Peninsular Malaysia's biggest species, having an average diameter of 58.42 – 107.95 cm [4, 16]. Thailand and Peninsular Malaysia are home to this species [17]. The findings of this species was first recorded in 1929 by A.F.G Kerr in Ranong, Province, and continued till the flower was identified in the Lojing Highlands, Kelantan, Malaysia by [18]. In Lojing Highlands, 26 populations of *R. kerri* were mapped in 2008, covering three localities, Kg. Jedip, Kg. Cedau and Kg. Kuala Rengi [10].

According to locals, Rafflesia not only well known for unique characteristics; as the largest flower grown without the visible vegetative organs but also believed to have medicinal values [1]. The flower consumed as traditional herbs to cure internal organ after giving birth and for aphrodisiac treatment [13]. Previous studies regarding antioxidant activities and bioactive compounds on this species reported by [19, 20, 21]. Recent studies by [22] identified the presence of secondary metabolites such as: alkaloids, triterpenoids/steroids, flavonoids and tannins, believed to play roles in contributing to the biochemical properties of Rafflesia. The *cocktails* of diverse chemical compounds and other miscellaneous compounds had contributed parts of the information regarding *R. kerri*. However, scientific findings reported on pharmacology importance on this species in Peninsular Malaysia is limited, and thus more research activities needed to be conducted. Therefore, this study was aimed to investigate the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of *R. kerri* in Lojing Highlands, Peninsular Malaysia.

2. Materials and methods

2.1. Plant materials collection and ethanolic extract preparation

Fresh samples of *R. kerri* obtained from Rafflesia Conservation Area (RCA), Lojing Highlands, Kelantan. The location is located between latitude of 40º 32' to 40º 47' N and longitude of 101º 20' to 101º 34' E at an elevation of approximately 1, 400 m above sea level (a.s.l.) [10]. The collected samples weighed, cut into small pieces and kept into a sampling bottle contained 100 % ethanol, macerated at room temperature for three days. Then, the solution filtered and evaporated using rotary evaporator (Heildolph, Germany). MCD for male ethanolic extract and FCD for female ethanolic extract are labels on the concentrated ethanolic extract maintained in an airtight container.

2.2. Chemicals and reagents

Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox, 2,4,6-tris(2-pyridyl)-8-triazine (TPTZ), acetate buffer, iron chloride ($FeCl_3$), hydrochloric acid (HCl), Folin-Ciocalteu's reagent, sodium bicarbonate, potassium iodide, sodium nitrate ($NaNO_3$), gallic acid, aluminium chloride ($AlCl_3$), sulphuric acid, sodium hydroxide (NaOH), anhydrous sodium sulphate, mercury dichloride, acetic acid, acetic anhydride and glacial acetic acid were from Sigma-Aldrich (St. Louis, MO, USA). Green tea leaf reference extract from Chromadex, USA. Organic solvents (AR grade)- methanol, absolute ethanol, ammonia, and chloroform were purchased from HmbG (Orioner Hightech Sdn.Bhd, M'sia). Water was deionised and purified by Milli-Q system.

2.3. Determination of total phenolic content (TPC)

The test conducted as described by [23] with a slightly modifications to suit microplate system. Briefly, 1.0 mg of the ethanolic crude extract dissolved in 1 mL of distilled water (1 mg mL^{-1}) and vortex until stock solution homogenized. Then, the Folin-Ciocalteu reagent added to the solution and allowed to incubate for 5 min. Next, 7.5 % (w/v) sodium carbonate added to the solution and incubated for 2 h at room temperature before the absorbance measured using microplate reader UV/vis spectrophotometer (FLUOstar® Omega, Germany) at 765 nm. The TPC calculated based on calibration curve from gallic acid standard solution and expressed as mg gallic acid equivalents (GAE) per gram of sample (mg/g). All the tests conducted in triplicates.

2.4. Determination of total flavonoid content (TFC)

The test determined according to [24] with slightly modification to high-throughput microplate system. Briefly, 30 µL of 10% NaNO2 solution added to 100 µL of sample extract (1 mg mL^{-1}) in 2 mL 96 – deep well. After 5 min, 30 µL of 5% $AlCl_3$ solution added followed up by the addition of 1% NaOH solution (200 µL) to the deep well. The solution homogenised before being transfer to microplate. The absorbances measured at 510 nm by using microplate reader UV/vis spectrophotometer (FLUOstar® Omega, Germany). The standard curve obtained by using Quercetin as standard and expressed as mg QE/g.

2.5. Determination of antioxidant activity

2.5.1. DPPH assay. The assay conducted based on methods described by [25] with slightly modifications. The workflows start by pipetting 50 µL of the sample, 50 µL of DPPH solution (1 mM; 0.4 mg/ mL) and 150 µL of absolute ethanol into 96-well microtiter plate, in triplicates. Ascorbic acid served as positive control in this assay. The scavenging effect was determined using a microplate reader UV/vis spectrophotometer (FLUOstar® Omega, Germany) at 520 nm after 30 min of incubation. The following equation was used to compute the DPPH scavenging activity:

$$\text{DPPH scavenging activity (\%)} = [\ (Abs_{control} - Abs_{sample})/\ Abs_{control}] \times 100 \quad (1)$$

Where:

$Abs_{control}$ = Absorbance of DPPH + absolute ethanol

Abs_{sample} = Absorbance of DPPH radical + sample or standard

The y-axis refers to scavenging activity (%) whereas the x-axis refers to the concentration of extract and ascorbic acid (AA). The IC_{50} values calculated based on the graph.

2.5.2. FRAP assay. The Ferric Reducing Antioxidant Power assay conducted referring to [26] protocol with minor modifications. Briefly, 10 mL of 300 mM acetate buffer (pH 3.6), 1 mL of 10 mM 2,4,6-tris (2-pyridyl)-8-triazine(TPTZ) and 1 mL of 20 mM $FeCl_3.6H_2O$ were mixed with the ratio 10:1:1 to generate FRAP reagent. The 200 µL of FRAP reagent was added to 20 µL of sample extract (1 mg mL^{-1}) in microtiter plate and allowed to stand for 30 min at room temperature. The readings of absorbance measured using microplate reader UV/vis spectrophotometer (FLUOstar® Omega, Germany) at 595 nm. Trolox used to obtain standard curve and all tests conducted in triplicates.

3. Statistical analysis

All of the assays performed in triplicates of individual experiments. The data expressed as mean values ± standard deviation (SD). The ANOVA and post-hoc-t-test analysed using Microsoft Excel 2010 software version 14.0.4734.1000. The 'p-value' less than 0.05 ($p<0.05$), indicates significant difference in the data obtained.

4. Results and discussion

According to the current investigation, 1000 mg of fresh R. kerri central disc samples extracted with ethanol solvent yielded extract yields of around 50 to 60 mg. The extract's physical form discovered to be thick solid resin-like with a dark-brown colour.

4.1. Total phenolic content (TPC) and total flavonoid content (TFC)

The result of quantitative phytochemical testing of TPC and TFC presented in Table 4.1, the result expressed in Gallic acid equivalent, GAE (mg/g) and Quercetin equivalent, QE (mg/g), respectively. The GT was used as reference extract due to the effectiveness of GT as antioxidant agent, able to scavenge oxygen free radicals, lipid radicals, supress lipid peroxidation, supress tumorgenesis and also anti-aging [27, 28]. Table 4.1 showed the FCD has the highest value of TPC and TFC compared to

MCD, with value of 16.347 ± 0.204 mg GAE g^{-1} and value of 817.000 ± 69.282 mg QE g^{-1}, respectively. Similarly, a study by [19] in Thailand reported *R. kerri* flower extract, *R. cantleyi* [29] and *R. hasseltii* [30] has the high content of TPC. Additionally, [30] in her report listed three phenolic compounds identified in *R. hasseltii*, namely catechin, proanthocyanidin and phenolic acid. A study by [21] mentioned *R. kerri* to contain four tannin compounds together with phenylpropanoid glucoside. Current findings indicate, phenolics and flavonoids may be potential active compounds in *R. kerri* flowers that scavenge harmful free radicals and exhibited anti-oxidative properties.

Table 4.1. Quantitative phytochemical screening on TPC with gallic acid equivalent (mg/g) and TFC with Quercetin equivalent (mg/g) on the sample extract.

Samples	Phytochemical content	
	TPC GAE (mg/g)	TFC QE (mg/g)
MCD	8.762 ± 0.049[a]	560.333 ± 55.076[ab]
FCD	16.347 ± 0.204[b]	817.000 ± 69.282[ba]
GT (reference extract)	7.125 ± 0.1097[c]	903.667 ± 75.719[c]

Value presented are *mean ± SD of triplicate independent experiments. Mean with different letters (a-c) in the same column indicates significantly different ($p<0.05$).

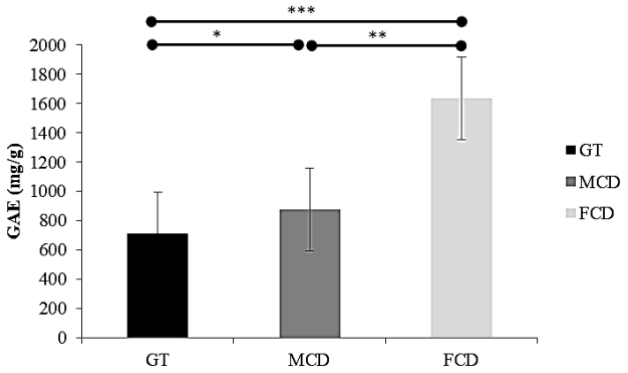

Figure 4.1.A. Comparison of total phenolic content (TPC) between green tea (GT), male central disc (MCD) and female central disc (FCD) ethanolic extract. * Indicates the significant different ($p < 0.05$) between the sample tested as measured with One-way ANOVA and post-hoc-t-test.

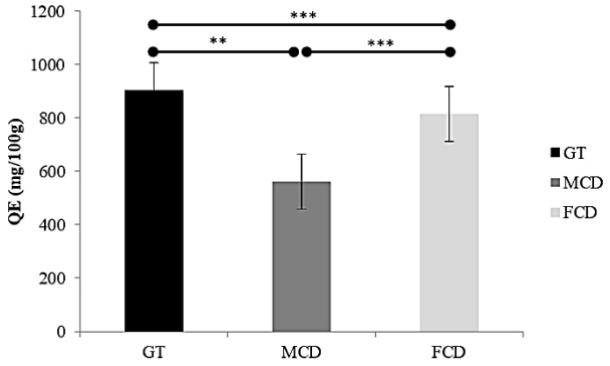

Figure 4.1.B. Comparison of total flavonoid content (TFC) between green tea (AA), male central disc (MCD) and female central disc (FCD) ethanolic extract. * Indicates the significant different ($p < 0.05$) between the sample tested as measured with One-way ANOVA and post-hoc-t-test.

[31] stated the plant phenolics and flavonoids are the main substances that had potent antioxidant property, thus able to treat the health issue. Figure 4.1.A indicates there were differences in the TPC values of the sample tested ($p<0.05$), whereas Figure 4.1.B indicates no significant different ($p>0.05$) between FCD and MCD in TFC values. The differences in the value obtained were dependent on the chemical constituents exist in the plant parts. Besides, the properties of antioxidant activities were likely influenced with the content of TPC and TFC. [32] explained the characteristics of flavonoids as metal-chelating and radical scavenging property has showed the substances had the potential antioxidant agent. The phenolic hydroxyl in phenolic compound resulting the compound possess radical scavenging activities and gives the character as antioxidant enzymes activators, hydrogen donors, metal ion chelators and oxidases inhibitor [33]. Studies has been reported on these substances and discovered these substances react with the active oxygen radicals, for instances, hydroxyl radicals [34], superoxide anion radicals [35], lipid peroxy radicals [36]. [37] reported on phenolic substance that capable in inhibiting cyxlooxygenase and lipoxygenase of platelets and macrophages, thus reduce the potential of thrombotic in vivo.

4.2. Antioxidant activity

Antioxidant is a "redox active compound that supress the oxidation stress, protect the body, cells and tissues from damage due to free radical activities and reactive oxygen resulting from normal oxygen metabolism process or produced by exogenous damage [38]. Antioxidant has been thought to be important in preventing variety of diseases and arresting disease progression.

Table 4.2. Antioxidant activity based on FRAP assay (TEAC) and DPPH scavenging activity (IC_{50}) of the extracts.

Samples	Antioxidant activity	
	TEAC (mM/mg)	IC_{50} (ug/ml)
MCD	2942.844 ± 27.756[a]	57.81 ± 3.628[a]
FCD	4642.844 ± 10.777[b]	49.850 ± 2.179[b]
GT (reference extract)	4459.289 ± 32.560[c]	-
AA	-	76.174 ± 1.837[c]

Value presented are *mean ± SD of triplicate independent experiments. Mean with different letters (a-c) in the same column indicates significantly different ($p<0.05$).

The data in Table 4.2 showed the FCD ethanolic extract exhibited the highest potential of ferric ion reducing power with FRAP values of 4642.844 ± 10.777 mM/mg and IC_{50} at 49.850 ± 2.179 ug/mL compared to MCD. The MCD ethanolic extract has the lowest value among other tested extracts such as the GT and FCD with FRAP value 2942.844 ± 27.756 mM/mg with IC_{50} value of 57.810 ± 3.628 µg/ml. The lower IC_{50} value indicates the higher its effectiveness to reduce ferric ion. It can be concluded the Rafflesia has higher effectiveness to reduce ferric ion compare to standard (ascorbic acid) used. In literature, [19] reported *R. kerri* flower has high antioxidant activity that supported the current findings. In addition to that, [39] reported on *R. cantleyi* to have high radical scavenging activities. Figure 4.2.A and B shows all the samples are significantly different from each other with the values ($p<0.05$). Although the MCD and FCD were from the same plant species, the analyzed data showed both was significantly different ($p< 0.05$). The differences in the value indicate presence of different in chemical constituents in plant part, thus exhibit different antioxidant property.

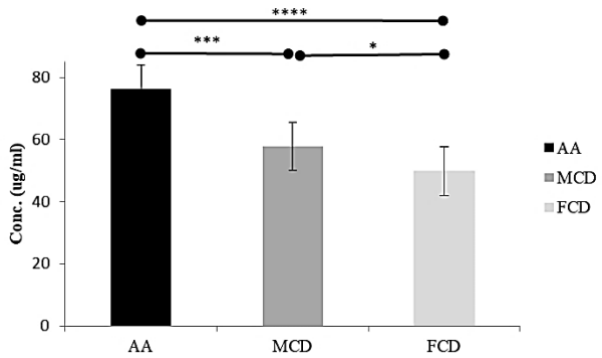

Figure 4.2.A. Comparison of antioxidant activity IC_{50} between ascorbic acid (AA), male central disc (MCD) and female central disc (FCD) ethanolic extract. * Indicates the significant different ($p < 0.05$) between the sample tested as measured with One-way ANOVA and post-hoc-t-test.

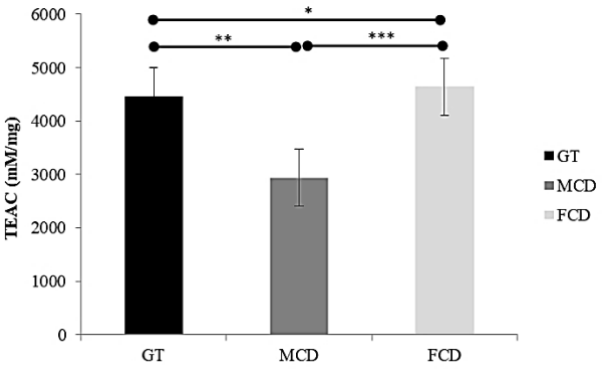

Figure 4.2.B. Comparison of antioxidant activity using FRAP assay (trolox equivalent antioxidant capacity, TEAC) between green tea (GT), male central disc (MCD) and female central disc (FCD) ethanolic extract. * Indicates the significant different ($p < 0.05$) between the sample tested as measured with One-way ANOVA and post-hoc-t-test.

5. Conclusions

In conclusion, the antioxidant assays conducted showed Rafflesia could serve as a new resource of antioxidants for human health improvement. However, the overdose intake might cause toxicity effect to the consumer. Therefore, more test recommended determining the exact/other potential of the flower as medicinal flowering plant. Thus, the conservation of this flower should be strengthened not only because of its uniqueness as the biggest flower in the world, rare to be found but also due to its potential as one of the medicinal herbs. The support from the government through reinforcement of laws and acts and the non-government bodies (NGOs) are needed to protect this flower from encroachment activities, exploited by locals, illegal harvested without concerned to the survival of population, may contributed to the extinction.

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