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Total phenolic content, flavonoid content, and antioxidant capacity of S*yzygium. cumini* (L.) Skeels leaves grown in Wonosobo, Java, Indonesia and comparison against current findings of *Syzygium cumini* leaves and *Syzygium polyanthum* (Wight) Walp leaves

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Abstract

Objective: To investigate the total phenolic and flavonoid content and antioxidant capacity of *Syzygium cumini* grown in Wonosobo, Central Java, Indonesia. The findings will be used to compare similarities and differences with other current findings for *Syzygium cumini* leaves and *Syzygium polyanthum* leaves.

Methods: *Syzygium cumini* was extracted using sequential cold percolation method. Total phenolic and flavonoid content were quantified using Folin-Ciocalteu and down colorimetric method, respectively. Antioxidant capacity was quantified by measuring scavenging activity of extracts towards DPPH radicals (DPPH assay), as well as FRAP assay to measure the extract's reducing power.

Results: In the present study, *Syzygium cumini* water extract had the best percentage yield (4.83 ± 0.32 %), total phenolic content (40.94 ± 9.79 mg GAE/g DW), and antioxidant capacity (both DPPH and FRAP test) compared to other extracts (petroleum ether, toluene, ethyl acetate, and acetone). Toluene extract was found to contain the highest total flavonoid content with value of 42.90 ± 8.64 mg QE/g DW. Comparing with other current findings, various similarities and differences were found between intra species (*Syzygium cumini* from different locations) and interspecies (*Syzygium cumini* and *Syzygium polyanthum*).

Conclusion: Total phenolic, flavonoid contents and antioxidant capacity of *Syzygium cumini* collected in Indonesia were successfully investigated. There are also a lot of similarities and differences between species of *Syzygium cumini* and *Syzygium polyanthum*.

Keywords: *S. cumini*, antioxidant, phenolic contents, DPPH, FRAP, *S. polyanthum*

INTRODUCTION

S. cumini is a popular herb used by folklores in previous centuries for the treatment of various diseases such as diabetes and as an antimicrobial [1]. It is also known as *Eugenia Jamboolana* Lam., *Syzygium jambolana DC*., and *Eugenia cumini* (Linn.). *S. cumini* (*S cumini*) is known to be found across South and South East Asia such as Malaysia, Indonesia, India, and others [2]. In previous centuries, before the discovery of insulin, many traditional practitioners used herbs including *S. cumini* to treat diabetes [3]. Other activities of *S. cumini* include antibacterial, anti-inflammatory, antifungal, anti-ulcerogenic, antioxidant, radical scavenging, and others [4]. *Syzygium polyanthum* (*S. polyanthum*) is another species of Syzygium and is from the same family as *S. cumini*, which is Myrtaceae. *S. polyanthum* is also known as *Eugenia polyantha* or Salam [5], and widely used in folklores as additives in cuisine and as treatment for diarrhea, diabetes, and skin infections [6].

Reactive Oxygen Species, known as ROSs such as hydroxyl ions, superoxide ions, and hydrogen peroxide are reactive species that negatively impact health. They are toxic in high amounts and are generated by our own cells throughout our daily activities [7]. Specific cellular sites such as the mitochondria, peroxisomes, microsomes, cytochrome P450, and some enzymes including NADPH oxidase, cytochrome P450 reductase, and xanthine oxidase were found at the site of ROS production [8-10]. ROS are harmful agents; nevertheless, they are required in small and balanced quantities in order to serve and maintain good cellular regulatory functions and prevent oxidative stress. Oxidative stress might cause damage in DNA and negatively affect our health. Therefore, a balanced and low ROS level needs to be maintained for a healthy physiological environment [11]. The oxidative stress that arises due to high ROS levels produced in a flawed redox mechanism can be re-stabilized with high level of antioxidants [12]. That is why consumption of herbs or nutraceuticals rich in antioxidants is important to maintain a healthy body system.

Phytochemical content and antioxidant capacity of plants are expected to differ according to between different plant parts used [13], different extraction methods [14], different environmental stress [15] and different type of plants. Therefore in this study, we aimed to study the total flavonoids, phenolics, and antioxidant capacity of *S. cumini* grown in Wonosobo Central Java Indonesia and compare the data with current findings regarding *S. cumini* and *Syzygium polyantahum*. This will enable us to identify the similarities and differences between intra species, and inter species of *S. cumini* and *S polyanthum.*

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals used in sequential cold percolation extraction were petroleum ether, toluene, ethyl acetate, acetone as well as methanol, purchased from R&M Chemicals (Essex, United Kingdom). Folin-Ciocalteu's reagent used for total phenolic content test was purchased from Merck, Milipore (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent used for DPPH assay as well as 2,4,6-tripyridyl-S-triazine (TPTZ) used for FRAP antioxidant assay both were purchased from Sigma-Aldrich (St Louis, USA).

Plant Sources

S. cumini (L.) Skeels leaves were collected from a local farm in Sukorejo, Wonosobo Central Java Indonesia in January 2017, wrapped and directly couriered to Kulliyyah of Pharmacy, International Islamic University Malaysia Kuantan campus. Upon arrival, the leaves were unpacked, and washed under running tap water for 15 minutes to wash away all the possible dirt and debris. Once cleaned, the leaves were oven-dried at 50 ºC (Memmert, Germany) for 3 days until crisp and dry. All the leaves were then ground to powder size and weight of the powdered dried leaves were recorded [16].

Plant Extraction

Extraction of plant was carried out using sequential cold percolation extraction method, since this method was found to be capable of extracting high amounts of phenolics and flavonoids from *S. cumuni* leaves [17]. 5 solvents were used sequentially, starting with petroleum ether, toluene, ethyl acetate, acetone, and lastly water [18]. Firstly, the dried leaf powder was soaked in petroleum ether at concentration of 10 % w/v, equal to 25 g of powder in 250 mL of petroleum ether in a conical flask. The conical flasks were shaken at 120 rpm, 27 ºC using an incubator shaker. After 1 day, the solvents were filtered using Whatmann no. 1 filter paper and vacuum filtered to separate the leaf powder from the solvents. The filtrate was centrifuged at 5000 rpm for 10 min, at 27 ºC to completely separate the supernatant from the fine dried leaves powder. The supernatant was then pipetted out, leaving the residues at the bottom of the centrifuge tube. The residue and dried leaf powder was successively extracted using the next solvent (toluene) and the same procedure was repeated. This was followed by ethyl acetate, acetone and lastly water.

The solvents collected were then dried using rotary evaporator (model IKA RV8) until dryness. The crude extract collected from the round bottom flask was left under the fume hood for another 2 days until complete dryness from any remaining solvents. In this process, the extracts were covered with aluminum foil to protect it from light. The crude extract was then collected and kept at -20 ºC before further analysis [17, 19].

Determination of Percentage yield

Percentage yield (% w/w) was determined by comparing the weight of the dried leaves with the weight of the crude extract produced. The extraction was done in replicates of 3, and results were expressed as mean ± standard deviation.

Determination of Total Phenolic Contents

Total phenolic content was quantified using Folin-Ciocalteu reagent in 96-well microplate as explained by Ahmed et al. [20]. Folin-Ciocalteu reagent was diluted at concentration of 20% w/v in water, while extract samples of 1 mg/mL concentration were prepared for each extract. 100 µL of the diluted Folin-Ciocalteu reagent was pipetted into the 96-well microplate. 20 µL of the extract sample was added and the mixture was incubated for 5 minutes. Next, 100 µL of 40 % w/v sodium carbonate in water was added into the sample mixture and further incubated for 2 hours at room temperature. The 96-well microplate was covered with aluminum foil to protect the sample mixture from exposure to light to prevent degradation. The absorbance of the samples were read at 725 nm against blank using TECAN Infinite 200 PRO (Mannedorf, Switzerland) controlled by Tecan i-control software version 1.6.19.2. The blank sample consisted of all the reagents except the extract. Standard curve of gallic acid was prepared to quantify the total phenolic content of all the extracts. Results were expressed as mean ± SD (n=3) and the values recorded were in mg of gallic acid equivalent per gram of dried leaves weight (mg GAE/g DW).

Determination of Total Flavonoid Contents

Total flavonoid content was quantified using down colorimetric method [21, 22]. 2% w/v aluminum chloride was diluted with methanol, and a volume of 100 µL was pipetted into a 96 well microplate. 100 µL of extract diluted in methanol to 1 mg/mL was added into the microplate well. The sample mixtures were incubated for 10 min before absorbance reading at 415 nm. All the sample mixtures were subtracted with blank containing the extract and methanol only (without aluminum chloride). A standard calibration curve was prepared using quercetin diluted in methanol at concentration of 1 µg/ml to 50 µg/ml. The results were expressed as mean ± SD (n = 3) and the value recorded was in mg quercetin equivalent per 100g of dried leaves weight (QE/100g DW).

FRAP Assay

FRAP assay was conducted to quantify the reducing power of the extract, indicating antioxidant activity. The reagents for FRAP assay were prepared by mixing 40 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (diluted in 40 mM hydrochloric acid at 50 ºC), 20 mM ferric chloride hexahydrate ($FeCl.6H_2O$), and 300 mM acetate buffer (pH 3.6) at 10:1:1 ratio. The 300 mM acetate buffer (pH 3.6) was prepared by mixing 3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid in 1 L of deionized water. The assay was initiated by introducing 150 µL FRAP reagent into 96 well microplate wells, added with 5 µL extract at concentration of 1 mg/mL and 15 µL deionized water. The extracts were diluted with methanol for petroleum ether, toluene, ethyl acetate and acetone extract, or water for the water extract. Absorbance of the reaction mixtures were read at 593 nm using TECAN Infinite 200 PRO (Mannedorf, Switzerland) microplate reader controlled by Tecan i-control software version 1.6.19.2. The readings were subtracted with blank consisting of 150 µL FRAP reagents, 5 µL methanol, or water without the extract, and 15 µL deionized water. Standard calibration curve was prepared using ascorbic acid at concentration of 1 µg/mL to 500 µg/mL. Results were expressed as mean ± SD (n=3) and values were recorded as mg ascorbic acid equivalent per gram of dried leaves (mg AAE/g DW) [16, 23].

Determination of DPPH free radical scavenging activity.

The DPPH free radical scavenging activity was quantified using DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay with slight modifications [19, 24]. All the reagents were tested in 96-well microplate where a volume of 50 µL of DPPH (0.3 mM) was mixed with 50 µL of sample extracts. Each of the sample extracts were diluted with methanol at various concentrations starting from 1 µg/mL up to 2 mg/mL, except for the water extract which was diluted with water (since it does not dissolve in methanol). Lastly, 50 µL methanol was added to each well, and the mixtures were incubated for 10 min, wrapped in aluminum foil to protect the sample mixtures from light photo-degradation. The absorbance of each sample mixture was UV scanned at 517 nm using TECAN Infinite 200 PRO (Mannedorf, Switzerland) microplate reader controlled by Tecan i-control software version 1.6.19.2. All the readings were subtracted with blank containing 50 µL diluted DPPH and 100 µL methanol without sample extract. Ascorbic acid was used as positive control for the calculation of IC_{50}, and as standard to quantify the antioxidant capacity. The results were expressed as mg ascorbic acid equivalent antioxidant capacity per gram of dried leaves (mg AEAC/g DW) as well as concentration of extract successfully inhibiting 50% of DPPH radical (IC_{50}).

Statistical Analysis

The data was analyzed and processed using one way ANOVA. Significant differences in the expressed data (mean values stated) was determined by p value, where the data is considered significantly different at p value ≤ 0.05.

RESULTS AND DISCUSSIONS

Table 1: Percentage yield, total phenolic content, total flavonoid content, and FRAP assay values

Extract	Percentage Yield (%)	Total Phenolic Content (mg GAE /gDW)*	Total Flavonoid Content (mg QE/100g DW) *	FRAP assay (mg AAE/g DW) *
Petroleum Ether	0.83 ± 0.10[a]	3.98 ± 1.22[a]	1.11 ± 0.69[a]	0.54 ± 0.08[a]
Toluene	6.66 ± 0.42[b]	24.27 ± 8.29[b]	42.90 ± 8.640[b]	4.33 ± 0.49[b]
Ethyl Acetate	1.92 ± 0.25[c]	5.82 ± 2.33[a]	27.03 ± 5.83[c]	1.62 ± 0.23[c]
Acetone	1.31 ± 0.15[d]	6.07 ± 0.59[a]	26.85 ± 7.24[c]	1.90 ± 0.33[c]
Water	4.83 ± 0.32[e]	40.94 ± 9.79[c]	2.78 ± 1.13[d]	4.52 ± 0.66[b]

*Values are mean ± SD (n=3)
Means with different letter indicates significant difference ($p< 0.05$)

Table 2: Scavenging activity of DPPH radicals (mg AEAC/g DW) and IC_{50}

Extract	DPPH(mg AEAC/g DW)*	IC_{50} DPPH (µg/ml)
Petroleum Ether	0.20 ± 0.04[c]	864.23
Toluene	0.25 ± 0.03[c]	2898
Ethyl Acetate	0.91 ± 0.18[b]	412
Acetone	0.92 ± 0.15[b]	418
Water	16.39 ± 1.56[a]	44
Ascorbic Acid	-	19.8

*Values are mean ± SD (n=3)
Means with different letter indicates different significantly ($p < 0.05$)

Tables 1 and 2 show the antioxidant capacities and phytochemical properties of *S. cumini* (L.) Skeels leaves collected from Wonosobo, Central Java. Based on table 1, the percentage yield was highest for toluene extract, followed by water, ethyl acetate, acetone, and petroleum ether extracts. The findings are different compared to the work of Kaneria et al. [19], using *S. cumini* leaves from Gujarat, India. ($p< 0.05$). In the Kaneria et al. study, water extraction via sequential cold percolation was found to produce the highest yield with value of 11.35 ± 0.74%, followed by acetone extraction (3.59 ± 0.27%), petroleum ether extraction (1.61 ± 0.03%), ethyl acetate extraction (1.05 ± 0.04%), and finally toluene extraction (0.63 ± 0.04%). Another study done by Hidayati et al. [25] on *S. polyanthum* leaves found that methanol yielded 7% of extract, ethyl acetate produced 5.6%, dichloromethane produced 2.08%, and n-hexane produced 1.32% percentage yield. The water extract in Kaneria et al. study had highest percentage yield compared to all extracts in the present study and the study by Hidayati et al. ($p< 0.05$). Acetone extract in the present study had comparable percentage yield to *S. cumini* n-hexane extracts ($p> 0.05$) in the study by Kaneria et al.

Total phenolic content assay in the present study revealed that *S. cumini* water extract had the highest total phenolic content, followed by toluene, acetone, ethyl acetate, and lastly petroleum ether extracts. However, acetone, ethyl acetate and petroleum ether extracts total phenolic content were not significantly different ($p> 0.05$) According to Wong et al. [26] water extract of *S. polyanthum* leaves collected in Singapore contained 11 mg GAE/g DW total phenolic content. In contrast, toluene (24.27 ± 8.29 GAE/g DW) and water (40.94 ± 9.79 GAE/g DW) extracts of *S. cumini* leaves in the present study contained higher total phenolic content. The difference could be attributed to the high levels of total phenolic content in the *S. cumini* leaves, or due to the different method of extraction employed. A study conducted by Kaur et al.[1] on *S. cumini* leaves collected from Punjab, India found that the water extracts contained a total of 36.05 ± 1.47 mg GAE/g DW, and ethyl acetate contained 20.86 ± 1.15 mg GAE/g DW. Comparing all three studies, the water extract of *S. polyanthum* leaves from Singapore had the lowest total phenolic content compared to water extract in the present study and the Kaur et al. study ($p< 0.05$). In the present study and study by Kaur et al., comparable phenolic content was observed for water extracts but ethyl acetate extract of the present study had much lower total phenolic content compared to values reported by Kaur et al. ($p< 0.05$). The differences were expected due to the different plant sources and different methods of extraction. The present study employed sequential percolation method, while Kaur et al. used aqueous or ethyl acetate extract per se without sequential successive use of the leaves.

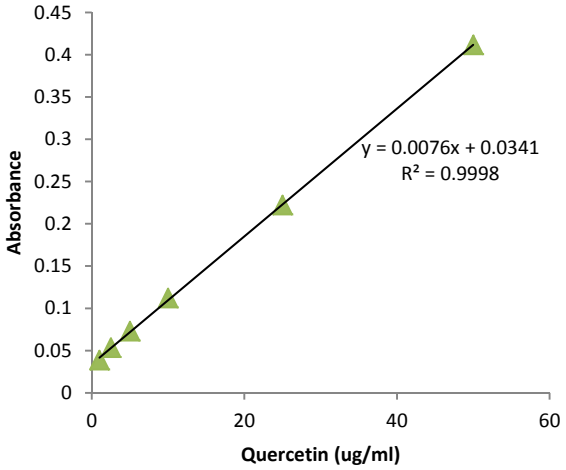

Fig 1: Standard curve for Quercetin

Total flavonoid content in the extract was ranked as follows; toluene > ethyl acetate > acetone > water > petroleum ether. Ethyl acetate and acetone extract total phenolic content were significantly different from each other ($p> 0.05$), and toluene, water, and petroleum ether total phenolic content were significantly different from each other ($p< 0.05$). Flavonoid content was not necessarily linked to total phenolic content; extracts with high total flavonoid content had low total phenolic content (such as ethyl acetate and acetone extract). Fig 1 shows the standard curve of quercetin when tested with down colorimetric method to detect quercetin content. R^2 value equals to 0.9998, and the regression equation is $y = 0.0076x + 0.0341$. A standard curve was prepared for gallic acid to measure total phenolic content, and ascorbic acid standard curve for DPPH scavenging activity and FRAP assay. According to a study done by Mohamed et al. [27], *S. cumini* leaves from Egypt contained higher flavonoid content, 6.22 ± 0.10 mg QE/g DW in methanol extracts, and 2.04 ± 0.11 mg QE/g DW in methylene chloride extracts ($p< 0.05$). This was expected due to different harvesting locations of the plants. Different weather and climate produces different phytochemical profiles. As explained by Fini et al. [28], excessive stress induced by light (including various light wavebands) results in increased production of dihydroxy B-ring substituted flavonoid glycosides to counteract the excessive ROS produced. It is known that Egypt is a middle eastern country with very intense solar radiation [29], so this explains the significantly higher flavonoid content in *S. cumini* leaves. A study done on methanolic *S. polyanthum* leaf extracts found the total flavonoid content of the leaves to be 148.7 µg QE/g DW [22]. In the present study, all the extracts had lower total flavonoid content compared

to *S. polyanthum* ($p< 0.05$). *Sygyzium cumini* leaves from Egypt were found to contain the highest total flavonoid content ($p< 0.05$) [27].

FRAP assay is carried out to measure antioxidant power and was first introduced by Benzie et al., (1996) [23]. It uses the principal of reduction from Fe^{3+} to Fe^{2+} at acidic pH in the presence of Tripyridyl-s-Triazine (TPTZ), forming a blue-colored ferrous tripyridyl triazine complex. UV absorbance is used to quantify the intensity of violet blue colored complex, and can be read optimally at 593 nm [30-32]. Based on table 1, water and toluene extracts had the highest reducing power, followed by acetone/ethyl acetate ($p> 0.05$), and finally petroleum ether extract. This means toluene and water extracts had the highest antioxidant capacity in terms of reducing power. Analyzing the data, similarly we found total phenolic content and percentage yield of toluene and water extract to be the highest compared to other extracts. Based on the study by Chanda et al. [17] on *S. cumini* leaves from India extracted using sequential cold percolation method, reducing capacity of extract was as follows: water < ethyl acetate < toluene, < acetone extract. The reported antioxidant power of *S. cumini* leaves collected from Indonesia and India were widely different. The difference is contributed by the different location of the plant, as well as different harvest seasons. The Indonesian *S. cumini* leaves were harvested in January, while the Indian *S. cumini* were collected in October. A study conducted on *S. polyanthum* leaves collected from Medan, Indonesia, found that methanol extracts possessed higher reducing power compared to petroleum ether and chloroform extracts ($p< 0.05$). The extract reducing power obtained from FRAP assay test were ranked as follows: methanol extract > water extract > chloroform, and water extract [33]. Comparing with the present study, both *S. cumini* and *S. polyanthum* leaves had the same reducing power (illustrated as antioxidant capacity) pattern where petroleum ether was found to have the weakest reducing power, and water extract had the highest reducing power in FRAP assay.

Table 2 illustrates the scavenging activity of DPPH radicals, which is expressed as mg ascorbic acid equivalent antioxidant capacity per g of dried leaves (mg AEAC/g DW), as well as IC_{50}. In the present study, water extract contained the highest antioxidant capacity compared to other extracts ($p< 0.05$). This was followed by acetone and ethyl acetate extracts which were not significantly different ($p> 0.05$), followed by toluene, and lastly petroleum ether extract which was not significantly different ($p> 0.05$). Water extract possessed the highest scavenging activity, indicating its highest antioxidant capacity, supporting the FRAP assay, and total phenolic content results. The concentration of leaf extract that inhibits (scavenges) 50% of the DPPH radical (IC_{50}) is shown in table 2; water extract had the least concentration compared to other extracts ($p< 0.05$). This was followed by ethyl acetate, acetone, toluene, and lastly petroleum ether extract. Water extract had the highest antioxidant capacity, with the lowest concentration to achieve IC_{50}. In *S. polyanthum* leaves studied by Perumal et al. (2012) harvested in Malaysia and extracted using methanol, IC_{50} was 20.90 µg/ml, illustrating higher antioxidant capacity than *S. cumini* leaves in Indonesia ($p< 0.05$) [34]. The IC_{50} antioxidant capacity of *S. polyanthum* was comparable to positive control (ascorbic acid) (IC_{50} = 19.80 µg/ml). Another study done by Belapukar et al. [35] on *S. cumini* leaves collected from Madhya Pradesh, India found that at concentrations of 100 µg/ml to 500 µg/ml, the extracts showed scavenging activity exceeding the activity of positive control (ascorbic acid) ($p< 0.05$). The antioxidant capacity of the leaf extract was very high ($p< 0.05$) compared to the present study on *S. cumini* leaves collected in Wonosobo, Central Java, Indonesia and compared to *S. polyanthum* leaves collected in Malaysia.

CONCLUSION

In conclusion, it was clear that the water extract of *S. cumini* leaves extracted using sequential cold percolation had the best yield, phytochemical properties and antioxidant capacities compared to other extracts (petroleum ether, toluene, ethyl acetate, and acetone extract). However the water extract did not contain the highest total flavonoid content among all extracts. Comparison with other studies reporting the phytochemical and antioxidant capacity of *S. cumini* and *S. polyanthum* leaves enabled us to find similarities and differences between intra species of *S. cumini*, and interspecies between *S. cumini* and *S. polyanthum* leaves originating from the same genus (Syzygium) and family (Myrtaceae).

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