Phytochemical screening and antioxidant activities of Methanol extracts from eight Syzygium species

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INTRODUCTION

The Myrtaceae is in the major group of flowering plants which comprised about 100 genera and 3000 species throughout the tropics and subtropics especially in Australia. In Malaysia, Myrtaceae consists of about 9 genera and 210 species which are distributed from the lowland to mountain forests [1]. The leaves are simple, stipulate, opposite or alternate, and finely dotted with oil glands. The flowers are small to medium sized, regular, pinkish leaves while their flowers are pollinated by flies, beetles, and butterflies [1]. All the Malaysia’s species are evergreen but they flush new leaves and flowers seasonally. There are eight other genera in Myrtaceae family of Malaysia such as Syzygium species (kelat) is the largest genus in Malaysia and it occurs in all parts of the country from high tide level to the summit of Gunung Tahan. Kelat or Kayu kelat is the local name for most wild species which have small or inedible fruits and it refers to its peculiarly rigid bark [1]. Malaysia’s Syzygium is an evergreen tree with pale green or pinkish leaves while their flowers are pollinated by flies, beetles, and butterflies [1]. The Syzygium plants have been reported to have many phenolic compounds such as alkaloids, flavonoids, anthocyanins, glucosides, ellagic acid, isouqueretin, kaempferol, and myricetin which have been proven to have significant antioxidant activities [2]. Previous work on this genus reported significant bioactivities especially antimicrobial and antioxidant [3,4]. Traditionally, this genus is used for the treatment of numerous ailments such as fever, diarrhea, stomachache, dysentery, catarrh, diabetes, asthma, and as a stimulant for the nerves [4,5]. To the best of our knowledge, there is no report on phytochemicals and bioactivity studies towards these eight Syzygium species (S. filiforme var. filiforme, S. papillosum, S. griffithii, S. pseudoformosum, S. claviflorum var. claviflorum, S. glaucum, S. syzygioides, and Eugenia Sp. 57). Hence, the study aimed to analyze the phytochemical of methanol extracts from the aforementioned Syzygium species and their antioxidant activities against DPPH, FRAP, and ABTS assays.

EXPERIMENTAL

Plant materials

The leaves, twigs, and heartwoods of eight Syzygium species S. filiforme var. filiforme (SK2946/16), S. papillosum (SK2947/16), S. griffithii (SK2948/16), S. pseudoformosum (SK2951/16), S. claviflorum var. claviflorum (SK2950/16), S. glaucum (SK2952/16), S. syzygioides (SK2953/16), and Eugenia Sp. 57 (SK2949/16) were collected from Gunung Cakah, Taman Botani Negara Shah Alam, Selangor in August 2015. The plant samples were authenticated by Dr. Shamsul bin Khamis, a botanist from Forestry Department, Universiti Putra Malaysia. The specimens were deposited in the Biodiversity Unit,
Herbarium, Institute of Bioscience, Universiti Putra Malaysia. All parts of the plants were dried for a few weeks.

Extraction method

The dried leaves, twigs, and heartwoods of Syzygium species were ground into powdered form and extracted using cold extraction method with MeOH solvent. The extracts were filtered and the solvent was removed under vacuum using a rotary evaporator to obtain the MeOH extracts.

Phytochemical screening

The MeOH extracts of the samples were analysed to identify the presence of flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoids, and tannins according to standard method [6,7].

Alkaloid test

0.3 g of MeOH crude extracts of each parts of Syzygium species were dissolved in 15 mL of ammoniacal chloroform and the obtained solutions were filtered. About five to ten drops of sulphuric acid (2M) were added to the filtrate and shake well, then the entire solution was allowed to stay for a while. Two layers were formed. An acidic upper layer and an organic lower layer were formed. The acidic layer was pipetted out into a test tube (5×10 mm) and two drops of Mayer reagent were added. Any changes in the turbidity of the solution were observed.

Flavonoid test

0.3 g of each crude extract was dissolved in MeOH and the solution was filtered into two test tubes (5×10 mm) labelled as A and B. Test tube A was referred as blank. A magnesium plate was added into test tube B followed by 0.5 mL of concentrated hydrochloric acid. Colour changes of the solution were observed. Appearance of reddish pink or dirty brown colour indicated the presence of flavonoid.

Steroid test

About 100 mg of dried MeOH crude extract was dissolved in 2 mL of chloroform. About two to three drops of sulphuric acid were carefully added to form a lower layer. A reddish-brown colour at the interface was an indicative of the presence of steroidal ring.

Saponin test

A drop of sodium bicarbonate was added in a test tube containing about 50 mL of the MeOH extract. The mixture was shaken vigorously and kept for 3 mins. A honey comb-like froth was formed, indicating the presence of saponins.

Phenol test

In a test tube containing 2 mL of MeOH crude extract, 2 mL of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green colour indicated the presence of phenols.

Tannin test

A few drops of 1% solution of ferric chloride was added to the test tube containing 5 mL of MeOH extract. The mixture was observed to note the formation of blue or green colour which indicated the presence of tannins.

Terpenoid test

2 mL of chloroform and 1 mL of concentrated sulphuric acid was added to the test tube containing 1 mg of MeOH extract. The solution was observed for reddish brown colour formation which indicated the presence of terpenoids.

Antioxidant activity

The antioxidant activity was determined using three assays, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, ferric reducing antioxidant potential (FRAP), and 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Solvents and chemicals

DPPH, ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butyalted hydroxytoluene (BHT), and butyalted hydroxyanisole (BHA) were obtained from Sigma-Aldrich. Analytical grade methanol, glacial acetic acid, dilute hydrochloric acid (HCl), and dimethyl sulfoxide (DMSO) were purchased from Merek. Sodium acetate trihydrate, ferric sulphate solution, 2,4,6-tripyridyl-s-triazine (TPTZ), and potassium persulfate were purchased from Fluka.

DPPH free radical scavenging assay

The free radical scavenging assay was conducted based on method described by Najihah et al. [8] with minor modification. Briefly, 100 µM DPPH (1 mL) dissolved in MeOH was added to the MeOH solution (3 mL) of the tested samples at different concentrations. An equal volume of MeOH was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. BHA and ascorbic acid (AA) were used as standard references. Then, the absorbance at 517 nm was measured with Epoch microplate reader. The percentage of scavenging of DPPH was calculated using the following equation:

\[
S\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

where \(A_{\text{blank}}\) is the absorbance value of the control reaction (containing all reagents except the test compound) and \(A_{\text{sample}}\) is the absorbance value of the test compound. The sample concentration providing 50% scavenging (SC50) was calculated by plotting scavenging percentages against concentrations of the sample. All tests were carried out in triplicate and scavenging values were reported as means (scavenging percentage).

FRAP assay

The FRAP assay was carried out according to Channarong et al. and Shahwar et al. with minor modification [9,10]. FRAP reagent was freshly prepared, consisted of stock solution with ratio 10:1:1 of 300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl3.6H2O solutions. 5 µL of sample, 15 µL of MeOH, and 150 µL of FRAP reagent were added to the 96-well plates. The absorbance at 573 nm was measured after 10 min of incubation at 37 °C. FeSO4.7H2O solution (0.1 mM–1.0 mM) was used to build up calibration curves of standard antioxidant.

ABTS assay

The ABTS assay was performed as described by Zou et al. [11] with minor modification. ABTS and potassium persulfate were dissolved with distilled water to obtain concentrations of 7 mM and 4.9 mM, respectively. The two solutions were mixed and incubate in the dark for 12–16 hours at room temperature. After incubation time, the ABTS radical was added with distilled water until the absorbance was 0.7 at 734 nm was measured after 10 min of incubation at 37 °C. FeSO4.7H2O solution (0.1 mM–1.0 mM) was used to build up calibration curves of standard antioxidant.

Scavenging Percentage = \[\frac{\text{Abs (ABTS)} - \text{Abs (ABTS+Sample)}}{\text{Abs (ABTS)}} \times 100\]

RESULTS AND DISCUSSION

Phytochemical screenings

The preliminary phytochemical screening of the MeOH crude extracts were conducted in order to determine the presence of secondary metabolites in all parts of eight Syzygium species (S. filiforme var. filiforme, S. papillosum, S. griffithii, S. pseudoformosum, S. claviflorum var. claviflorum, S. glaucum, S. syzygioides, and Eugenia Ap. 57). Analysis of different parts of the plant extracts (leaves, twigs, and heartwoods) revealed the presence of alkaloids, flavonoids, phenols, steroids, terpenes, and tannins except saponins. The result of phytochemical analysis of eight Syzygium species is shown in Table 1 while Table 2 shows the observation scale for the phytochemicals.
analysis. The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences such as flavonoids and alkaloids. Flavonoids and phenols are known to exhibit good bioactivities such as antioxidant, antifungal, cytotoxicity, and antibacterial in Syzygium species [12-32]. Alkaloids have been associated with medicinal uses for centuries owing to their bioactivities such as cytotoxicity, antibacterial, and antioxidant [33]. Plant steroids and terpenes are known for their importance as antimicrobial and insecticidal activities [19, 34]. Tannins are reported to exhibit antiviral, antibacterial, and anti tumor activities [34].

Table 1 Result of phytochemical analysis of eight Syzygium species.

| TEST     | Leav es | heartwoods | twigs |
|----------|---------|------------|-------|
| Alkaid  | Flavonoid | Steroid | Phenol | Tannin | Terpenoid |
| S1LM     | +1       | +2        | +1     | +2     | +2      | +1       |
| S2LM     | +3       | +2        | 0      | +2     | 0       | 0        |
| S3LM     | +1       | +1        | 0      | +2     | +2      | 0        |
| S4LM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S5LM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S6LM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S7LM     | +1       | +1        | +1     | +2     | +2      | +1       |
| S8LM     | +1       | +1        | 0      | +1     | +2      | 0        |
| S1HM     | +3       | +2        | 0      | +2     | +2      | +1       |
| S2HM     | +2       | +2        | 0      | +2     | +2      | +1       |
| S3HM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S4HM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S5HM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S6HM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S7HM     | +2       | +2        | +1     | +2     | +2      | +1       |
| S8HM     | +2       | +2        | +1     | +2     | +2      | +1       |

Table 2 Observation scale for phytochemical test [6-7].

| Observations                  | Scale | Results |
|------------------------------|-------|---------|
| Alkaloid Test No changes     | 0     | Negative |
| Mostly transparent solution with faint trace of precipitate | +1 | Positive |
| Translucent solution with definite precipitate visible | +2 | Positive |
| Immediate precipitate formation | +3 | Positive |
| Large amount of precipitate formed immediately | +4 | Positive |
| Flavonoid Test No changes    | 0     | Negative |
| Formation of slightly brown or reddish pink colour | +1 | Positive |
| Formation of dirty brown or reddish pink colour | +2 | Positive |
| Steroid and Terpenoid Tests No changes | 0 | Negative |
| Thin layer of reddish brown colour at the interface | +1 | Positive |
| Thick layer of reddish brown colour at the interface | +2 | Positive |
| Saponin Test No changes      | 0     | Negative |
| Formation of honey comb like froth | +1 | Positive |
| Phenol and Tannin Tests No changes | 0 | Negative |
| Formation of slightly blue or green colour | +1 | Positive |
| Formation of dark blue or green colour | +2 | Positive |

Table 3 Scavenging percentage of eight Syzygium species.

| Heartwoods | Leaves | Twigs | S (%) |
|------------|--------|-------|-------|
| S1HM       | 76.15  | 95.55 |       |
| S2HM       | 94.34  | 76.55 | 95.92 |
| S3HM       | 96.22  | 94.87 | 89.06 |
| S4HM       | 95.29  | 87.71 | 93.13 |
| S5HM       | 94.85  | 95.99 | 95.10 |
| S6HM       | 95.12  | 95.88 | 95.39 |
| S7HM       | 90.73  | 94.13 | 87.39 |
| S8HM       | 93.92  | 95.44 | 91.66 |
| Standards  | BHA    | AA    |       |
|            | 84.25  | 83.95 |       |
|            |        |       |       |

Antioxidant activities

Antioxidant assay was done to screen the antioxidant property of the MeOH crude extracts from the leaves, twigs and heartwoods of eight Syzygium species (S. filiforme var. filiforme, S. papillosum, S. griffithii, S. pseudoformosum, S. claviflorum var. claviflorum, S. glaucum, S. syzygioides and Eugenia Sp. 57). It can be measured using three different methods; DPPH, FRAP, and ABTS assays. The antioxidant activity cannot be evaluated by only a single method due to the complex nature of phytochemicals and the antioxidant activity determination is reaction-mechanism dependent [11]. Therefore, it is important to employ multiple assays to evaluate the antioxidant activity of plant extracts and pure compounds.

DPPH assay

In this study, ELISA microplate reader was used to determine the radical scavenging activity of the crude extracts of Syzygium species against free radical of DPPH. ELISA microplate reader measured the reduction in optical absorbance of each pure compound due to the scavenging of stable free radical DPPH at 517 nm [8]. BHA and AA were used as standard controls. Table 3 shows the percentage of scavenging activity at 100 ppm of the leaves, twigs, and heartwoods of eight Syzygium species. From the results, all of the plant extracts show good DPPH scavenging activity with the percentage inhibition more than 70 % at 100 ppm. Previous study on plant extracts of S. camini, S. paniculatum, and S. aromaticum also exhibited strong DPPH scavenging activity [22,24,30].

FRAP assay

Determination of the ferric reducing antioxidant power towards the crude extracts of eight Syzygium species were tested in order to measure the reductive ability of antioxidant by evaluating the transformation of Fe (III) to Fe (II) in the presence of reductants in the samples [9]. The reducing power of the samples was monitored at the absorbance of 573 nm using ELISA microplate reader. BHT, Trolox, and AA were used as standard controls. Table 4 shows the FRAP results of the leaves crude extracts of eight Syzygium plants. S. griffithii (S3LM) exhibits the most potent ferric ion reducer ranging from 1.03 ± 0.03 to 7.52 ± 0.49 mM FRAP equivalent among all the tested leaves extracts followed by S. pseudoformosum (1.17 ± 0.07 to 7.31 ± 1.48 mM) and S. glaucum (1.09 ± 0.18 to 6.54 ± 0.24 mM).

The crude extracts of the twigs of S. griffithii (S3TM) also exhibit good FRAP activity with the value range from 1.43 ± 0.12 to 9.68 ± 0.38 mM FRAP equivalent. Crude extracts of the twigs of S. syzygioides (S8TM) also show the potential as antioxidant activity with the FRAP equivalent value ranging from 0.93 ± 0.05 to 8.48 ± 0.25 mM as shown in the Table 5.
Table 4 FRAP assay of the leaves extracts of eight Syzygium species.

| Samples | mM FRAP EQUIVALENT to FeSO₄·7H₂O |
|---------|-----------------------------------|
|         | 0.1 mM | 0.6 mM | 1.0 mM |
| Leaves  |         |        |        |
| S1LM    | 0.99 ± 0.04 | 2.37 ± 0.09 | 3.04 ± 0.21 |
| S2LM    | 1.29 ± 0.06 | 4.16 ± 0.03 | 5.58 ± 0.25 |
| S3LM    | 1.03 ± 0.03 | 3.23 ± 0.48 | 4.72 ± 0.49 |
| S4LM    | 1.34 ± 0.05 | 4.09 ± 0.15 | 5.02 ± 0.26 |
| S5LM    | 1.16 ± 0.01 | 4.00 ± 0.64 | 5.57 ± 0.31 |
| S6LM    | 1.17 ± 0.07 | 3.65 ± 0.10 | 7.31 ± 1.48 |
| S7LM    | 1.09 ± 0.18 | 4.38 ± 0.20 | 6.54 ± 0.24 |
| S8LM    | 0.73 ± 0.06 | 2.70 ± 0.31 | 4.46 ± 1.04 |

Standards
- Trolox: 0.43 ± 0.08
- BHT: 0.33 ± 0.09
- AA: 0.07 ± 0.08

S1= S. filiforme var. filiforme; S2= S. papillosum; S3= S. griffithii; S4= Eugenia Sp. 57; S5= S. claviflorum var. claviflorum; S6= S. pseudofomosum; S7= S. glaucum; S8= S. syzygioides; BHT= Butylated hydroxytoluene; AA= Ascorbic acid; (*p < 0.05).

Table 5 FRAP assay of the twigs extracts of eight Syzygium species.

| Samples | mM FRAP EQUIVALENT to FeSO₄·7H₂O |
|---------|-----------------------------------|
|         | 0.1 mM | 0.6 mM | 1.0 mM |
| Twigs   |         |        |        |
| S2TM    | 1.07 ± 0.01 | 2.80 ± 0.40 | 4.74 ± 0.45 |
| S3TM    | 1.43 ± 0.12 | 4.61 ± 0.28 | 9.68 ± 0.38 |
| S4TM    | 1.46 ± 0.08 | 4.44 ± 0.32 | 6.04 ± 0.82 |
| S5TM    | 1.28 ± 0.01 | 4.05 ± 0.24 | 6.86 ± 0.55 |
| S6TM    | 0.84 ± 0.02 | 3.34 ± 0.16 | 6.02 ± 1.09 |
| S7TM    | 0.96 ± 0.15 | 2.68 ± 0.14 | 4.48 ± 1.33 |
| S8TM    | 0.93 ± 0.05 | 4.03 ± 0.44 | 8.48 ± 0.25 |

Standards
- Trolox: 0.43 ± 0.08
- BHT: 0.33 ± 0.09
- AA: 0.07 ± 0.08

S1= S. filiforme var. filiforme; S2= S. papillosum; S3= S. griffithii; S4= Eugenia Sp. 57; S5= S. claviflorum var. claviflorum; S6= S. pseudofomosum; S7= S. glaucum; S8= S. syzygioides; BHT= Butylated hydroxytoluene; AA= Ascorbic acid; (*p < 0.05).

For the heartwoods crude extracts, S. syzygioides (S8HM) showed the most potent ion reducer ranging from 1.20 ± 0.01 to 10.47 ± 0.12 mM FRAP equivalent as shown in Table 6. The crude extracts of the Eugenia Sp. 57 also have a potential as antioxidant activity with the FRAP equivalent value range from 1.56 ± 0.19 to 8.81 ± 0.46 mM.

Table 6. FRAP assay of the heartwoods extracts of Eight Syzygium species.

| Samples | mM FRAP EQUIVALENT to FeSO₄·7H₂O |
|---------|-----------------------------------|
|         | 0.1 mM | 0.6 mM | 1.0 mM |
| Heartwoods |         |        |        |
| S1HM    | 1.12 ± 0.04 | 3.94 ± 0.54 | 4.70 ± 0.13 |
| S2HM    | 1.02 ± 0.08 | 3.03 ± 0.43 | 5.96 ± 0.58 |
| S3HM    | 1.40 ± 0.06 | 4.84 ± 0.14 | 6.62 ± 0.27 |
| S4HM    | 1.56 ± 0.19 | 5.94 ± 0.16 | 8.81 ± 0.46 |
| S5HM    | 1.46 ± 0.09 | 3.03 ± 1.38 | 6.17 ± 0.50 |
| S6HM    | 1.56 ± 0.44 | 5.00 ± 0.34 | 6.78 ± 0.74 |
| S7HM    | 0.95 ± 0.01 | 2.80 ± 0.04 | 4.83 ± 0.35 |
| S8HM    | 1.20 ± 0.01 | 5.39 ± 0.81 | 10.47 ± 0.12 |

Standards
- Trolox: 0.43 ± 0.08
- BHT: 0.33 ± 0.09
- AA: 0.07 ± 0.08

S1= S. filiforme var. filiforme; S2= S. papillosum; S3= S. griffithii; S4= Eugenia Sp. 57; S5= S. claviflorum var. claviflorum; S6= S. pseudofomosum; S7= S. glaucum; S8= S. syzygioides; BHT= Butylated hydroxytoluene; AA= Ascorbic acid; (*p < 0.05).

ABTS assay

The determination of the ABTS radical was tested towards all the crude extracts of Syzygium species in order to evaluate the antioxidant activity by delocalization the ABTS**+** through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm [11]. BHA, BHT, and Trolox were used as standard controls. Table 7 shows the ABTS radical scavenging activity from the leaves, twigs, and heartwoods of eight Syzygium species which is expressed as Scavenging Concentration at 50 % (SC50). From the results, the crude extracts of S. syzygioides (S8HM) exhibit good antioxidant activity among the heartwoods extracts with the SC50 value of 96.20 mM. All leaves crude extracts show the potential as antioxidant activity towards ABTS assay. S. glaucum (S7LM) has obtained good SC50 value (116.90 mM) followed by S. papillosum (S2LM, SC50 value = 132.70 mM) and Eugenia Sp. 57 (S4LM, SC50 value = 143.10 mM). The twigs crude extracts of S. syzygioides (S8TM) and S. griffithii (S3TM) show excellent antioxidant activity towards ABTS assay with the SC50 values of 130.60 mM and 145.40 mM, respectively.

| Sample | SC50 values (mM) |
|--------|------------------|
| Heartwoods |         |
| S1HM    | 120.00 |
| S2HM    | 145.70 |
| S3HM    | 152.50 |
| S4HM    | 130.70 |
| S6HM    | 161.00 |
| S7HM    | 218.30 |
| S8HM    | 96.20 |
| Twigs   |         |
| S2TM    | 221.20 |
| S2LM    | 132.70 |
| S3LM    | 176.30 |
| S4LM    | 143.10 |
| S5LM    | 144.40 |
| S6LM    | 186.70 |
| S7LM    | 116.90 |
| S8LM    | 226.90 |

| Sample | SC50 values (mM) |
|--------|------------------|
| Standards |         |
| Trolox  | 385.00 |
| BHT     | 175.00 |
| BHA     | 100.00 |

STATISTICAL ANALYSIS

Data obtained from biological activities were expressed as mean values. The statistical analyses were carried out by employing one way ANOVA (p < 0.05). A statistical package (SPSS version 16.0) was used for the data analysis.

CONCLUSION

In summary, the present paper reports for the first time on the phytochemical screening of eight Malaysian Syzygium species (S. filiforme var. filiforme, S. papillosum, S. griffithii, S. pseudofomosum, S. claviflorum var. claviflorum, S. glaucum, S. syzygioides, and Eugenia Sp. 57) on their antioxidant activities. All plant extracts showed good DPPH scavenging activity with the percentage inhibition of more than 70 % at 100 ppm. The crude extracts of S. syzygioides showed the most potent ion reducing activity ranging from 1.20 ± 0.01 to 10.47 ± 0.12 mM FRAP equivalent. The plant also showed the most potential antioxidant activity towards ABTS assay with the SC50 value of 96.20 mM. These findings are important to search for novel phytochemicals from Malaysian Syzygium species with bioactivities and to provide scientific evidence that can be used in future for the development of new active agents.
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