Growth and phenolic constituents production of roselle (Hibiscus sabdariffa var. UKMR-2) in response to soil media

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Abstract. Plant phenolic content frequently varies in response to the changes in the environment and genetic factors. However, studies on the response of phenolic contents when cultivating under different soil media is still scarce. The present study investigates the phenolic constituents’ production and growth quality of Hibiscus sabdariffa var. UKMR-2 in response to different soil media formulation ratio of topsoil, organic matter and sand. The cultivation using two different media; Soil Media 1 (SM1) with 2:1:1 v/v and Soil Media 2 (SM2) with 2:1:2 v/v. The UKMR-2 calyx extract was analysed for total phenolic (TPC), total anthocyanin (TAC), antioxidant activity (in IC₅₀) and evaluated based on HPLC-PDA. The result showed the mean value for SM1 and SM2 treatment, respectively: TPC was 2.54±0.34 and 2.47±0.34 mg GAE/g DW; TAC was 8.06±1.11 and 7.86±1.99 mg cyanidin-3-glucoside equivalents/g DW; IC₅₀ value was 0.146±0.018 and 0.210±0.063 mg/mL. The HPLC-PDA showed the presence of delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside. The cultivation using SM1 tends to increase plant growth, calyx yields, phenolic constituents’ production and antioxidant activity. However, the different soil media ratio has no significant influence on the growth and phenolic constituent production (p > 0.05). In general, SM1 (2:1:1 v/v) soil media ratio may produce better growth, higher percentage yield and phenolic constituents’ for UKMR-2 cultivation.

1. Introduction
Roselle is a subtropical plant from Malvaceae family is commonly known as asam kumbang or asam paya in Malaysia and has been traditionally used as a medicinal plant used to treat several diseases [1-2]. The red calyces of this plant have a pleasant acidic taste, used for making a refreshing beverage, ice cream, syrup, jams and pudding [3]. Phytochemistry studies so far found that Roselle mainly consists of alkaloids, steroids, anthocyanins, flavonoids, tannins, saponins and sterols [4]. This herbaceous shrub produced numerous secondary metabolites that have been reported contain biological activities including anti-oxidant, antimicrobial agent, antidiabetic, anticancer, antihypertensive and many other properties [5-6]. Several compounds have been reported from this plant which includes cyanidin-3-O-sambubioside, delphinidin-3-O-sambubioside, delphinidin-3-
glucoside, cyanidin-3-glucoside, chlorogenic acid, quercetin, ascorbic acid, luteolin, caffeic acid, rutin and kaempferol [4,7].

Numerous studies have shown the effect of dietary phenolics due to their anti-oxidative and possible anti-carcinogenic activities. Therefore, various studies related to environmental stress have the potential to enhance the production of anthocyanin (powerful antioxidants) without affecting other fruit quality attributes in Roselle. Any ecological factor is capable to induce a potentially injurious strain in plants and it can be caused or prevented using different cultivation methods. In principle, phenolic content differs between species in the same family or even among cultivars. Phenolic content in plants frequently varies in response to genetic factors and changes in the environment, such as temperature, UV radiation, harvest conditions, soil nutrients, seasonality and herbivores [8-9]. Among these factors, soil nutrients clearly affect plant development [10]. The soil media influence on the plant growth are much dependent on the relationship between water and air in the soil pores. However, the nutrients effects on the secondary metabolism is still controversial [10].

Roselle was introduced into Malaysia as a new commercial crop, thus the information about its agronomic practice for sustainable production is still scarce. Therefore, it is timely to investigate the most successful practices to enhance fruit crop growth characteristics and active metabolites in Roselle. Proper practices of container-grown plants in nursery and greenhouse production are mostly dependent on the physical and chemical properties of the growing media. These media should be resisted compacting for root growth, well drained, proper aeration and retain sufficient water to reduce the irrigation frequency. Other parameters to consider include media availability, batch consistency and media stability. Proper media component selection is critical to the success for plant growth [11]. Roselle will grow well on fertile soils, but can also tolerate moderately fertile sandy and loamy soils. However, adaptation to the soil types as well as fertilization is the primary concern which influences the growth, quality and metabolite production of Roselle. Research on the impact of growth media on the production of secondary metabolites, especially of the phenolic compounds is still scarce. Therefore, this present study is aimed to investigate the influences of different soil media formulation ratio of topsoil, organic matter and sand on the plant growth parameters, yield component, phenolic constituents’ production and antioxidant activity in H. sabdariffa var. UKMR-2 cultivated under controlled conditions.

2. Materials and methods

2.1. Chemicals and reagents

Folin-Ciocalteu reagent, gallic acid, formic acid, methanol, ascorbic acid, potassium chloride, sodium carbonate, sodium acetate acquired from Merck (Darmstadt, Germany). Delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside purchased from Extrasynthese (France). Acetonitrile HPLC grade obtained from Fisher Scientific (USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) purchased from Sigma Aldrich (USA).

2.2. Plant samples and treatments

H. sabdariffa var. UKMR-2 cultivation was conducted using the method as described by Siti Aishah et al. [12] at the Kompleks Ramah Tumbuhan greenhouse, Universiti Kebangsaan Malaysia (UKM), Bangi from March until July 2017 where the seeds and soil media obtained. Different soil media formulation ratio of top soil, organic matter and sand as follows; Soil Media 1 (2:1:1 v/v) and Soil Media 2 (2:1:2 v/v) were used in this study. The soil media were prepared and then left for two weeks to allow for mineralization before sowing. All the plants received similar fertilizer and irrigation treatment per polyethylene bags. UKMR-2 plant growth parameters and sample preparation for extraction was determined using the method as described by Siti Aishah et al. [12]. Meanwhile the extraction analysis was determined using the method as described according to Chumsri et al. [13].
2.3. **Chemical analysis**

Total phenolic contents (TPC) were determined using Folin-Ciocalteu assay as described by Waterhouse [14]. The gallic acid standard curve was prepared with linear correlation co-efficient, \( R^2 = 0.999 \). The TPC expressed as mg gallic acid equivalent /g of dry weight. The total anthocyanin content (TAC) was measured by a pH-differential method as described by Giusti & Wrolstad [15]. TAC were calculated as described in equation (1). All samples and standard absorbance (gallic acid) was measured using Epoch Microplate Spectrophotometer (BioTek, USA) and analysed in triplicates.

\[
\text{mg C3G/g DW} = \frac{A \times MW \times DF \times V \times 10^3}{\varepsilon \times I \times SW}
\]

where A = \((A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH4.5}\); DF = dilution factor, SW = sample weight used for extraction (in g); MW = molecular weight for cyanidin-3-glycoside (449.2 g/mol); V = total volume of sample solution after extraction (in L); \( \varepsilon \) (molar absorptivity) = 26,900 Lmol\(^{-1}\) cm\(^{-1}\) for C3G; I = cell path length (in cm); and \( 10^3 \) = factor conversion from g to mg.

2.4. **DPPH radical scavenging assay**

The modified method was used for the DPPH radical scavenging activity as described by Kouakou et al. [3]. The samples and ascorbic acid (standard reference) absorbance was measured at 517 nm using the Epoch Microplate Spectrophotometer (BioTek, USA). The radical scavenging activity or inhibition percentage was calculated according to equation (2).

\[
\% \text{ RSA} = \frac{A_a - A_b}{A_a} \times 100
\]

where A\(_a\) is the absorbance of DPPH without an extract solution and A\(_b\) is the absorbance of DPPH with the sample.

2.5. **High-Performance Liquid Chromatography (HPLC) method**

Quantitative analysis was carried out using an HPLC Waters e2695, using Purospher STAR RP-18e LichroCART column (250 mm × 4.6 mm × 5 um) and photodiode array detector. HPLC-PDA method analyses and sample preparation were performed as described by Siti Aishah et al. [12]. Two solvents were used for gradient elution: A. 0.1% formic acid in water; B. 0.1% formic acid in acetonitrile. The injection volume was 30 μL with 1 mL/min flow rate. The chromatograms were monitored at 520 nm.

2.6. **Statistical analysis**

The mean value ± standard deviation collected data were subjected to analysis of variance using Statistical Package for Social Science for Windows version 25.0 software (SPSS Inc., Chicago, USA) to determine the significant differences between treatments (defined at the 5% level). The correlations between chemical and biological activities was calculated using Pearson correlation.

3. **Results and discussion**

3.1. **Growth characteristics**

Each plant species vary remarkably with their nutritive and soil requirements. Fresh and dry weights of calyces, plant heights (cm), stem diameter (mm), number of branches and leaves were taken as a UKMR-2 plant growth indicator. The effect of different soil media’s treatment for every growth parameter for a seven day interval on Roselle were presented in figure 1.

There is no significant effect was recorded on the growth parameter with different soil media treatment (p > 0.05). In general, all plant growth indicators showed an increasing trend throughout 70 days after transplanting for both treatments. However, SM1 treated plant growth-tend to be higher compared with SM2 treatments. These results are similar to Nur Amirah et al. [16] on H. sabdariffa var. UMKL-1 and Azza et al. [17] on Jatropha curcas. Nur Amirah et al. [16] reported that no significant differences were found in UMKL-1 growth, cultivated in Beach Ridges Interspersed with...
Swales (BRIS) soil concerning the leaf area index, plant height, stem diameter or the post-harvest quality of UMKL-1. Azza et al. [17] also showed that clay media significantly increased all growth parameters compared with the sandy soil. This effect may attribute to the physical properties of the soil. According to Seghatoleslami et al. [18] the non-significant change in plant growth showed that the stress was not severe enough to inhibit the growth of Roselle stem cells. SM2 treated plant showed significantly reduced plant height and stem diameter by 7.1% and 20.2% respectively as compared to SM1 treatment. The reduction in plant height attributed to water loss which diminishes nutrient uptake thus causing a disturbance in physiological processes of plant growth [19]. However, Khalil & Abdel-Kader [20] stated that sandy soil increased all growth characters significantly for *H. sabdariffa* L. originated from Egypt compared with clay media. The root system may penetrate more in-depth and be extending wider in sandy soil more than clay media and make the plant established well [17].

![Graphs showing plant growth parameters](image)

**Figure 1.** The effect of soil media’s treatment on plant growth with different parameter: a) Plant height; b) Stem diameter; c) Number of branches and d) Number of leaves. Data points are means of biological replicates (*n* = 6). The vertical bar represents the standard deviation.

Metwally et al. [21] stated that the soil aeration, plant nutrient availability, water movement and microbiological activities are much dependent on soil pores. Sandy soil may be leach or move downward the nutrients and other chemicals with water more rapidly [22]. Thus, higher sand content in soil media SM1 might improve the formation of water stable aggregate, increase nutrient and water holding capacity and thus will improve soil permeability and aeration. SM1 and SM2 produced similar fresh weight of the UKMR-2 calyx (with seed) harvested with 123.8 g and 129.8 g, respectively. For the number of branches and leaves, SM2 treated plant showed significantly reduced as compared to SM1 by 33.3% and 53.3%, respectively. Ali et al. [23] stated that soil drying will decrease leaf growth by reducing the leaf water status and accumulate organic solutes to osmotic adjustment. Therefore, it will inhibit the incorporation of small substrate molecules into the polymers needed to grow new cell.
In addition, Kathiravan et al. [24] stated that clay soil media was superior compared to a sandy soil in stimulating *Jatropha curcas* morphological growth. According to Adzemi et al. [25] plant growth performance of UKMR-2 cultivated in BRIS soil was statistically lower because BRIS soil is too sandy, nutrient deficient and low water holding capacity. Khalil & Abdel-Kader [20] on Roselle originated from Egypt shows different result, whereas sandy soil media increased all growth parameters significantly compared with clay and sandy clay loam soil. These results are in agreement with those previously reported by Abou-Leila et al. [26] and Russell [27].

In this study, fresh Roselle calyces were air dried at room temperature until it achieved constant weight. It was observed that both soil media treatments produced similar percentage of fresh (without seed) and dried yield. The fresh and dried percentage yield for SM1 treated plants were 57.2% and 9.3%, while for SM2 were 56.8% and 9.2%, respectively. Dried Roselle calyx with 10% moisture content will provide better extraction and enhances the resistance of high humid products against degradation by decreasing their water activity [13, 28]. Therefore, this air-dried Roselle calyx should be a stable product with low degradation.

### 3.2. Biological and chemical analysis

There is scarce information regarding on chemical differences in Roselle calyces when cultivated under different soil media treatments. In this study, the phenolic contents in UKMR-2 were determined by measuring TPC, TAC and their antioxidant activity (table 1). The overall range of TPC and TAC from all treated plants from both treatments was 1.71 to 3.21 mg/g DW and 5.57 to 10.13 mg C3G equivalent/g DW respectively. Meanwhile the overall range of antioxidant activity in IC50 was 0.112 to 0.285 mg/mL.

| Media Treatment | TAC (mg C3G/g DW) | TPC (mg GAE/g DW) | IC50 (mg/mL) |
|-----------------|-------------------|-------------------|--------------|
| SM1             | 8.06 ± 1.10a      | 2.54 ± 0.34a      | 0.146 ± 0.018b |
| SM2             | 7.86 ± 2.09a      | 2.45 ± 0.55a      | 0.210 ± 0.063a |

Values represent the mean value ± standard deviation, n = 6. Mean denoted by same letter indicate no significant differences between the treatments (p > 0.05). DW, dry weight

TPC and TAC were not significantly affected by the media's treatment applied (p > 0.05), but both results on the SM1 treated plant tended to be slightly higher as compared to SM2 treatment. This may be due to the leaching of the nutrients since SM2 contain more sand compared to SM1. Soils with a high proportion of sand can drain easily and the nutrients easily wash through the soil [17]. According to Zlati’c & Stankovic [29], TPC values in *C. intybus* varied depending on the type of soil locality from which the plant material was taken. Similarly, species *Nigella sativa* [30] and *Mentha pulegium* [31] demonstrated the difference in the TPC due to saline content in ground substrate. However, Khalil & Abdel-Kader [20] stated that Roselle grown in sandy media have a higher TAC compared to clay media. This result may due to easier water uptake in sandy media for photosynthesis process which also influenced the formation of colour pigments.

Azza et al. [17] stated that clay media increased the average proline content, chlorophyll a, b, a + b and carotenoids of *Jatropha curcas* L. leaves compared with sandy media. Similarly, El-Sallami [32] on *Luecaena leucocephala* found that clay medium gave the highest total carbohydrates, chlorophyll, and N, P, K percentages compared with sandy soil media. According to Johnston [33], a complex interactions between the physical, biological and chemical properties of the soil would affect the soil fertility. A productive and fertile soil is the fundamental resource for the plant growth and the ecosystem. In addition, Isherwood [34] stated that a good aeration, good physical structure, an optimal nutrient status, organic matter and adequate moisture content are needed to maintain a good
productivity of the soil. The soil productivity can either be improved or reduced by cultivation techniques employed.

The TPC is closely related to TAC. The Pearson correlation test on the values revealed a range coefficient, $r$ of $0.650 < r < 0.843$ (p < 0.01) for both media treatments, which indicates a significant strong linear positive correlation between TAC and TPC in Roselle water extracts. This result suggested that there is an association between total phenolics and anthocyanins content in Roselle calyx’s growth. This correlation expected because anthocyanins consist of a phenolic moiety in their structure.

In this study, the antioxidant activities from different soil media’s treatment were investigated using the DPPH scavenging assay and compared with ascorbic acid as a reference standard. The antioxidant activity was expressed as DPPH inhibition percentage and IC$_{50}$ (mg/mL). According to Kouakou et al. [3], high antioxidant activity of studied extract corresponds to lower IC$_{50}$ values. Figure 2 shows that both soil media treatments have a similar DPPH inhibition percentage, but their value is lower than ascorbic acid. At the concentration of 0.6 mg/mL, both UKMR-2 calyx extracts has reached the optimum level of free radical scavenging activity. For IC$_{50}$ values, SM1 shows a lower value of $0.146 \pm 0.018$ mg/mL compared to SM2 with $0.210 \pm 0.063$ mg/mL. For reference, the IC$_{50}$ value for ascorbic acid is $0.071$ mg/mL. In addition, antioxidant activity also shows significant differences between the two soil media treatments (p < 0.05). Total anthocyanin content is closely related with antioxidant activity. A Pearson correlation was run to determine the relationship between the antioxidant activities and TAC values for both media treatments. There was a very strong, negative linear correlation between IC$_{50}$ and TAC ($r = -0.897$, N = 6, p < 0.01) only for SM2 treatment. The relationship is negative because, as one variable increase, the other variable will decrease.

Figure 2. The antioxidant activities of UKMR-2 extracts and ascorbic acid (standard reference). Data represent the mean value ± standard deviation (vertical bar).

### 3.3. Chromatographic profiling

Phenolic contents, especially for anthocyanin measurements have long been utilized as an indicator to determine the quality and processing of Roselle products. According to Deshmukh et al. [35], the anthocyanins production and distribution in plants are believed influenced by several factors such as type of cultivation, the degree of fruit maturation, post-harvest storage condition, environmental conditions, plant variety and genotypes. In this study, the anthocyanin compounds were identified and quantified based on their retention times and compared with the standard reference. The representative HPLC-PDA chromatograms of UKMR-2 anthocyanins were monitored at 520 nm are given in figure
3. Generally, two predominant anthocyanins are detected in UKMR-2 calyx with retention time of 6.12 min and 9.11 min. The peaks were identified as delphinidin-3-O-sambubioside (1) and cyanidin-3-O-sambubioside (2), respectively.

Delphinidin-3-O-sambubioside concentration ranged from 1.42 to 3.12 mg/g DW, whereas for cyanidin-3-O-sambubioside varied from 0.53 to 0.97 mg/g DW for both media treatments. In this study, no interaction was observed (p > 0.05) between the soil media and the contents each anthocyanin of UKMR-2. SM1 plant showed a slightly higher concentration of both anthocyanin compounds compared to SM2 treated plants (figure 4). Since soil media’s treatment showed non-significant change in UKMR-2 growth, which showed that the stress was not severe enough to inhibit the carbon that was fixed during photosynthesis to be used to form more secondary compounds such as phenolic and triterpenes [36]. The presence of cyanidin-3-O-sambubioside and delphinidin-3-O-sambubioside in UKMR-2 were in line with those previously reported by other Roselle varieties around the world [3] [6] [37-38].

**Figure 3.** HPLC-PDA profile of anthocyanin contents (520 nm) in UKMR-2 water extracts. The two lanes represent a) SM1 (red) and b) SM2 (green).

**Figure 4.** Anthocyanin compounds in UKMR-2 cultivated in different soil media ratio. The data and standard deviation (vertical bar) represent the mean of three replicates.

4. Conclusion
This study presents the data on the proximate phenolic compositions and antioxidant attributes of *H. sabdariffa* var. UKMR-2 using different soil media ratio. The results showed that different soil mixtures on UKMR-2 cultivation have no significant influence on the growth, phenolic constituents and antioxidant activity, p > 0.05. Our finding also showed that UKMR-2 calyces in SM1 treatment has higher concentrations of phenolic compositions and antioxidant activity compare to SM2. It can be concluded that the 2:1:1 v/v soil media ratio may produce better growth, higher percentage yield and phenolic constituents’ for UKMR-2 cultivation.

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