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FULL PAPER

Qualitative phytochemical analysis, enzymatic and non-enzymatic antioxidant activities in stems and leaves of *Vanilla planifolia* (Orchidaceae)

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Abstract

Vanilla planifolia, commonly known as vanilla orchid, is an epiphytic plant native to Mexico. This plant is used in the food and pharmaceutical industries due to its flavour and antioxidant properties. Vanilla bean is becoming more expensive due to current market demand. Consequently, less than 1% of the vanillin used in the market is derived naturally from vanilla plants. The present study was conducted to identify the phytoconstituents and the antioxidative activities (enzymatic and non-enzymatic) in the stems and leaves of *V. planifolia* collected from Temerloh, Pahang. Phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, tannins, terpenoids, cardiac glycoside, carbohydrate and reducing sugar in both *V. planifolia* stems and leaves. Vanilla stem contains more ascorbic acid (94.49±2.92 µg/mL) and carotenoids (2.61±0.08 µg/mL) as compared to that of leaves (71.79±2.05 µg/mL and 0.82±0.07 µg/mL, respectively). However, there was no significant difference ($P>0.05$) in α-tocopherol concentration. Enzymatic activities showed that ascorbate peroxidase (APX) and catalase (CAT) were more distinct in leaves while more peroxidase (POD) activities were observed in the stems. High concentrations of phytochemical elements with high antioxidant activities in stems and leaves showed that *V. planifolia* is a promising source of antioxidants.

1. Introduction

Antioxidants are substances that may protect cells from the damage caused by free radicals. They are commonly derived from plant sources, and the efficacy is determined by plant species, variety, extraction and/or processing methods, and the growing environment. Antioxidants can be categorized into two groups, which are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidases (PODs) work in a coordinated fashion with low-molecular-weight non-enzymatic antioxidants i.e., ascorbate, glutathione, α-tocopherol, phenolic compounds, flavonoids, alkaloids to inhibit overproduction of reactive oxygen species (ROS) (Mehta and Gowder, 2015; Hasanuzzaman *et al*., 2020). SOD is directly related to stress, which initiates the first line of defence, converting superoxide radicals ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). The generated H_2O_2 can be further converted into water (H_2O) by enzymes e.g. CAT, APX, PODs, or catalyzed by the powerful non-enzymatic antioxidants in the Halliwell-Asada Pathway (Laxa *et al.*, 2019).

The genus Vanilla, which belongs to the Orchidaceae family includes 90 to 110 species which is distributed in tropical parts of the world. Most of these species are wild, with only two of them; *V. planifolia* and *V. tahitensis* are grown for commercial vanilla production. *V. planifolia* comprises about 95% of world vanilla production and is widely cultivated due to its fragrance flavoured pods (Bory *et al*., 2008). *Vanilla planifolia* is categorized as Crassulacean Acid Metabolism (CAM) plant. The leaves uptake carbon dioxide during the night and form malate. Then, sugar was generated during the day after entering the Calvin cycle. The cycle indeed explains the process of carbohydrates produced especially in leaves that have a

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high number of chloroplasts as compared to the stems. These findings can be explained by the function of carbohydrates as the 'main vessel' in all plants which acts as a storage and transportation of energy in the form of starch and works as a structural component in a plant called cellulose which keeps the composition of plant in a place (Maton *et al*., 1993).

Vanilla is widely used in the production of perfume, food flavouring agents, cosmetics products, medicinal and pharmaceutical as well as aromatherapy to relieve fatigue, nausea, vomiting, and increase mood and food intake (Okigbo *et al*., 2009). It is also used to treat fever, ulcers, congestion and respiratory pain. Furthermore, *V. planifolia* possesses anticancer, anti-inflammatory and antimicrobial properties. The medicinal value of vanilla may be contributed by its bioactive phytoconstituents (Katary and Salahuddin, 2017).

Vanilla is valued most because of its pod quality and yield. However, the chemical composition of vanilla leaves and the stem has yet to be explored. Hence, this study aimed to screen for phytochemical constituents, the total phenolic and flavonoid contents, as well as antioxidative activities (enzymatic and non-enzymatic) of the stems and leaves extract of *V. planifolia*.

2. Materials and methods

2.1 Plant materials and sample preparation

The fresh stems and leaves of *V. planifolia* were collected from Amani Vanilla, Temerloh, Pahang, Malaysia. The stems and leaves were rinsed with tap water and shade dried at room temperature. Then, they were cut into small pieces and ground into powder form. The powdered samples were soaked in methanol at room temperature for three days. The extracts were filtered (Whatman paper no. 1) and concentrated under reduced pressure to give the crude extract of stems and leaves, respectively. Both crude extracts were stored at -20^{o}C until further use.

2.2 Phytochemical screening

The methanolic extracts of stems and leaves were evaluated for the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, carbohydrates, reducing sugars and anthraquinones (Nurul *et al*., 2020).

2.3 Quantitative analysis

Each extract of *V. planifolia* (stems and leaves) was dissolved in 100 mL methanol and was further diluted for the determination of total antioxidants, total phenolic and total flavonoid assays.

2.3.1 Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu colourimetric method with a few modifications. An aliquot of 0.5 mL (2 mg/mL) of crude extract was mixed with 2.25 mL Folin-Ciocalteu phenol reagent for 5 mins. Then, 2.25 mL of 6% sodium carbonate was added to the mixture and allowed to stand for 90 mins at room temperature. The absorbance was read at 725 nm using a spectrophotometer (Shimadzu UV 1601). Gallic acid was used as standard calibration in the range of 0 to 200 µg/mL. Total phenolic content was expressed as mg Gallic acid (GA) per gram extract (Iqbal *et al*., 2015).

2.3.2 Total flavonoids content

Total flavonoids were analyzed using the aluminium colourimetric with a slight modification (Iqbal *et al*., 2015). A total of 0.3 mL (2 mg/mL) crude extract and 0.5 mL standard solution were placed in different test tubes. Each test tube was added with 0.1 mL of 10% aluminium chloride ($AlCl_3$), 0.1 mL of 1 M potassium acetate ($KC_2H_3O_2$), 1.5 mL of 80% methanol and 2.8 mL of distilled water. All tubes were incubated for 30 mins at room temperature. Absorbance was read at 415 nm using a spectrophotometer (Shimadzu UV 1601). Distilled water was used as a blank. Quercetin was used as standard calibration in the range of 0 to 200 µg/mL. Total flavonoids were expressed as mg quercetin per gram extract.

2.3.3 Total antioxidants

Each extract (0.15 mL) at the concentration of 2 mg/mL was mixed with linoleic acid ($C_{18}H_{32}O_2$) emulsion (2 mL, 10 mM, pH 6.5) in the test tubes and placed in the dark incubator at 37±2°C for 15 hrs to accelerate oxidation. About 6 mL of 60% methanol was added into the test tubes and the absorbance of the mixture was measured at 234 nm using a spectrophotometer (Shimadzu UV 1601). The linoleic acid ($C_{18}H_{32}O_2$) emulsion was prepared by dissolving 0.2804 g linoleic acid ($C_{18}H_{32}O_2$) and 0.2804 g Tween 20 ($C_{58}H_{114}O_{26}$) in 0.2 M sodium phosphate buffer (50 mL, pH 6.5). The analyses were done in triplicate (Yang *et al*., 2000) and were calculated according to Lingnert *et al.* (1979).

2.4 Enzymatic antioxidant assays

2.4.1 Ascorbate peroxidase assay

Ascorbate peroxidase (APX) was assayed according to Nakano and Asada (1981). The reaction mixture consists of 1.5 mL of 100 mM phosphate buffer (pH 7.0), 0.5 mL of 3 mM ascorbate, 0.1 mL of 3 mM of EDTA, 0.3 mL of distilled water and 0.2 mL of hydrogen peroxide (H_2O_2). Enzyme extract (100 µL) was

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added and changes in absorbance were monitored at 290 nm for 3 mins. APX specific activity was expressed as μmoles ascorbate oxidized per hour per mg protein.

2.4.2 Catalase assay

Catalase (CAT) was assayed according to the method by Claiborne (1985). The reaction mixture contained 3 mL of reaction buffer (19 Mm H_2O_2 in 50 mM phosphate buffer, pH 7.0) and 100 μL of enzyme extract was added. Changes in absorbance were monitored at 240 nm for 3 mins. CAT specific activity was expressed in μmoles of hydrogen peroxide (H_2O_2) per minute per mg protein.

2.4.3 Peroxidase assay

Peroxidase (POD) was extracted based on the method by Agrawal and Patwardhan (1993). The reaction mixture consists of 3 mL of a solution that contained 1 mL 50 mM phosphate buffer (pH 7.5), 1 mL 20 mM guaiacol ($C_7H_8O_2$), 1 mL 30 mM H_2O_2 and 100 μL of enzyme extract. Changes in absorbance were monitored at 470 nm for 3 mins. Specific activity for POD was expressed in μmoles of H_2O_2 per minute per mg protein.

2.4.4 Protein determination

The protein was determined using the method of Bradford (1976). The protein concentration was measured according to the standard curve prepared with 0.2 to 1.0 mg/mL of Bovine Serum Albumin (BSA).

2.5 Non-enzymatic antioxidant assays

2.5.1 Determination of α-tocopherol

α-Tocopherol was extracted as described by Hodges *et al.* (1996). The assay mixture was prepared as described by Kanno and Yamauchi (1997). The absorbance of the mixture was measured at 554 nm. The blank was prepared in the same manner except the absolute ethanol was used instead of hexane extract. A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 μg/mL).

2.5.2 Determination of ascorbic acid

The extracts were added into 1.0 mL of 10% trichloroacetic acid (TCA) and centrifuged (Shimadzu UV 1601) at 10 000 rpm for 10 mins at 4°C. A total of 300 μL of the obtained supernatant was added into 1700 μL distilled water and 200 μL of 10% Folin reagent. The mixture was gently swirled and left on a bench under dim light for 10 mins. The absorbance of the mixture was measured at 760 nm. A standard curve was plotted from the various concentrations of ascorbic acid (0-60 μg/mL) (Jagota and Dani, 1982).

2.5.3 Determination of carotenoids

Plant extracts were mixed with 3 mL of 80% (v/v) acetone. The homogenate was centrifuged (Shimadzu UV 1601) at 10 000 rpm for 10 mins. The absorbance of the supernatant obtained was measured at 663, 646 and 470 nm. The 80% acetone was used as a blank. Carotenoid content was calculated based on Lichtenthaler (1987).

2.6 Statistical analysis

The phytochemical screening and antioxidant activities were carried out in triplicates. Mean±SE (standard error of the mean) was calculated using SPSS software and a T-test was performed using Graphpad Prism (Version 5.0). Differences were considered significant at $p<0.05$.

3. Results and discussion

3.1 Phytochemical screening analysis

The qualitative phytochemical analysis of stem and leaves extracts of *V. planifolia* is illustrated in Table 1. Phytochemical analysis showed the presence of alkaloids, saponins, flavonoids, tannins, terpenoids, cardiac glycosides, carbohydrates and reducing sugar in methanol extracts of *V. planifolia* stems and leaves. There were similar studies reported on the presence of terpenoids (Shanmugavalli *et al.*, 2009; Jamal Uddin *et al*., 2015), glycosides (Shanmugavalli *et al*., 2009), tannins (Jamal Uddin *et al*., 2015), carbohydrates and reducing sugar (Palama *et al*., 2014) in *V. planifolia* stem and leaves. In contrast, Shanmugavalli *et al*. (2009) reported the presence of anthraquinone glycosides in *V. planifolia* leaves.

Table 1. Phytochemical constituent of stem and leaves extracts of *V. planifolia*

Phytochemical Test	(MeOH extract)	
	Stem	Leaves
Alkaloids	+	+
Saponins	+	+
Flavonoids	+	+
Tannins	+	+
Terpenoids	+	+
Cardiac glycoside	+	+
Carbohydrate	+	+
Reducing sugar	+	+
Anthraquinone	-	-

+: Presence, -: Absence

3.2 Quantitative analysis

3.2.1 Total phenolic content and total flavonoid content

TPC of methanolic extract from *V. planifolia* was determined in terms of mg of gallic acid per mL (mg

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GA/mL). The results are displayed in Figure 1(a). The present study found that the TPC of *V. planifolia* leaves was significantly higher (P<0.05) as compared to the stems. Phenolic compounds play a significant structural role in plants, as lignin is the dominant phenolic component of the cell wall (Whetten and Sederoff, 1995). In contrast, *Dendrobium thyrsiflorum* from the same family as *V. planifolia* exhibited the highest TPC value in methanolic stem extracts for ISO-derived plants, followed by DSO-derived plant and mother plant as compared to the leaves. The highest TPC was in methanolic extract followed by acetone and chloroform with the lowest TPC value (Bhattacharyya *et al*., 2014). The differences between the leaves and the stems TPC can be attributed to the diversity of phenolic contents and their different distribution in plant tissues (Mitsopoulos *et al*., 2016). Phenolic contents are synthesized mostly in chloroplasts or the cytoplasm via the phenylpropanoid pathway (Khoddami *et al.,* 2013), thus higher TPC was observed in the vanilla leaves compared to stem in this current study. This phenomenon is also consistent with the assumption that phenolic compounds play a part in protecting leaves from photo-oxidative damage by limiting reactive oxygen species (ROS) in chloroplasts. They directly neutralize ROS and thus, protect the major photosynthetic organs in plant tissues (Zhang *et al.,* 2018).

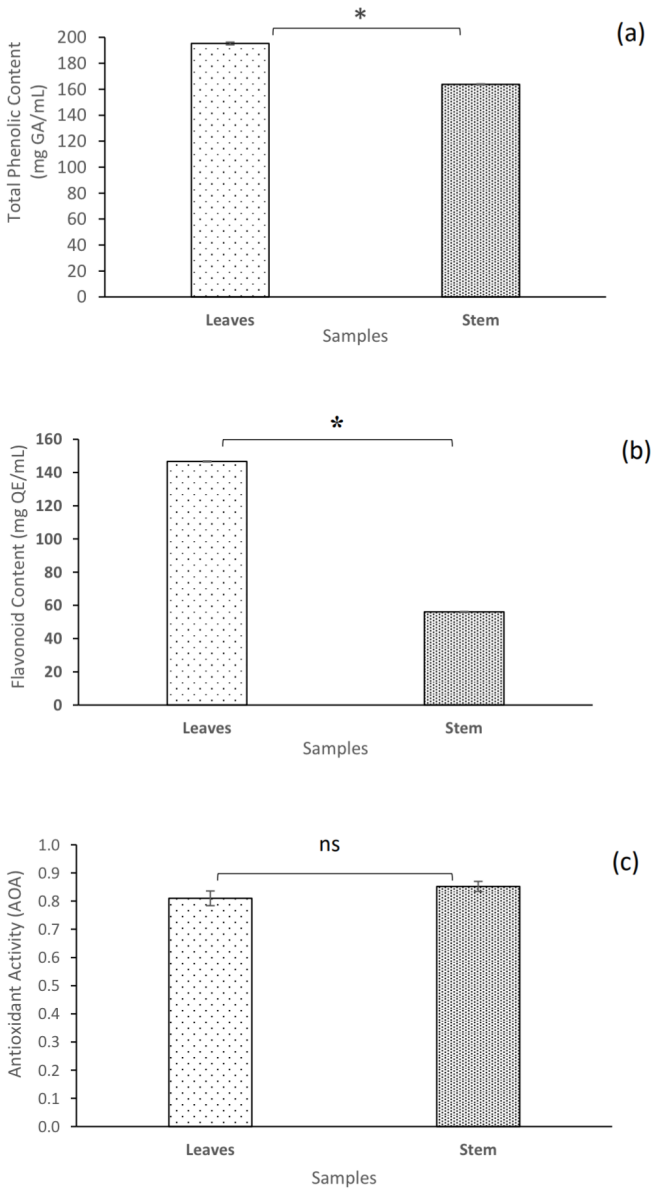

Figure 1. Total phenolic (a), total flavonoid (b) and total antioxidants (c) in *Vanilla planifolia* leaves and stems. Values are the mean±SEM (n = 3). *indicates significant difference at p<0.05, ^{ns}no significant difference at p<0.05.

The TFC of the methanolic extract of *V. planifolia* was determined in terms of mg of Quercetin per mL (mg Quercetin/mL). The results are shown in Figure 1(b). The TFC of *V. planifolia* leaves was found to be significantly higher (P<0.05) as compared to the stems. These findings are in accordance with the report by Bhattacharyya *et al.* (2014) in which *D. thyrsiflorum* orchid recorded the highest yield of TFC in the methanolic leaves extracts of the ISO-derived plant, followed by DSO-derived plant and mother plant compared to stems. The highest TFC was also recorded in methanolic leaves extracts of propagated plants and *V. coerulea* orchid at 4 mg/L chitosan treatment as compared to the stems (Bhattacharyya *et al*., 2016; Nag and Kumaria, 2018).

According to Rebaya *et al*. (2016), the utilization of polar solvents for extraction gives an outstanding volume of phenolic and flavonoid compounds. The types of plant species, organs and growth stages can be influenced by the concentration and effectiveness of polyphenol synthesis in plant organs (Bystricka *et al*., 2010). The biosynthetic pathways of phenolic constituents may be affected due to the developmental stage of the plant itself and eventually affect the total phenolics and flavonoid content (Krizman *et al*., 2007).

3.2.2 Total antioxidants

Total antioxidant capacity (TAC) is a technique to determine the antioxidant level of biological samples and to evaluate the antioxidant activity against free radicals (Rubio *et al*., 2016). Figure 1(c) shows the total antioxidants of methanolic extracts of *V. planifolia* stems and leaves. Total antioxidants in stems were generally higher compared to the leaves. However, there was no significant difference (P>0.05) between them.

Yen *et al*. (2002) proposed the possibility of higher antioxidant activity in leaves and stem extracts is due to the existence of polyphenolic compounds. Chand *et al*. (2016) also suggested that the extracts of various parts of plants showed different levels of antioxidant response depending on the accumulation of polyphenols in the respective parts.

3.3 Enzymatic antioxidant activities

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Antioxidant enzymes such as APX, POD and CAT function as defence mechanisms of the plants by scavenging the ROS production in different cell compartments. The rate of enzymatic specific activities is varied in different parts of plants. Figure 2(a) demonstrates the APX specific activities in leaves and stem extracts of *V. planifolia*. The APX specific activities in leaves were 12.5 folds higher as compared to the stem.

Higher APX activities in leaves might be associated with photosynthetic pigment. APX is localized predominantly in the stroma and thylakoid of a higher plant chloroplast. Chloroplast is involved in photosynthesis reaction, which generates the reducing power and molecular oxygen by the process of splitting the water under illumination, thus making them significant sources of ROS. This situation enhances the activities of APX in chloroplast to eliminate the ROS in the leaves. APX was also reported to maintain a high rate of photosynthesis under unfavourable environmental conditions (Foyer and Shigeoka, 2011). Accumulation of APX activities might also be related to the large leaf size of *V. planifolia* obtained in this study, where the abundance of chloroplast correlates with high photosynthetic reaction, thus increasing the APX specific activities in the leaves.

POD specific activities in this study were significantly ($P<0.05$) higher in stem (16.84±1.50 units/mg protein) as compared to leaves (8.29±1.16 units/mg protein) of *V. planifolia* (Figure 2(b)). POD was reported to be involved in the polymerization of the precursors of lignin in *Arabidopsis thaliana* (Liu *et al*., 2018). Lignin is a polymer that is localized in the cell wall and is the key structural resource in the support tissues and functions to make the plant rigid and stronger. The lignification process of vascular plants involving POD that mainly occurs in vessels, fibrous tissue and tracheid could be the reason why the POD activities were higher in the stem of *V. planifolia.*

CAT specific activity was comparable in stem (2.11±0.32 units/mg protein) and leaves (3.01±0.32 units/mg protein) of *V. planifolia* [Figure 2(c)]. CAT has usually located in leaves' peroxisomes and is involved in photorespiration. *V. planifolia* is an epiphytic orchid that is usually categorized as a CAM plant and depends on the other tree trunks in subtropical and tropical forests where water deficits normally occur (Silvera *et al.*, 2005). CAM plants have moist and thick leaves where they always guard their stomata opening time to prevent water loss during transpiration (Barbante *et al*., 2012), lowering the plant's photorespiration rate. Thus, low photorespiration might contribute to the low amount of CAT specific activity in the plants.

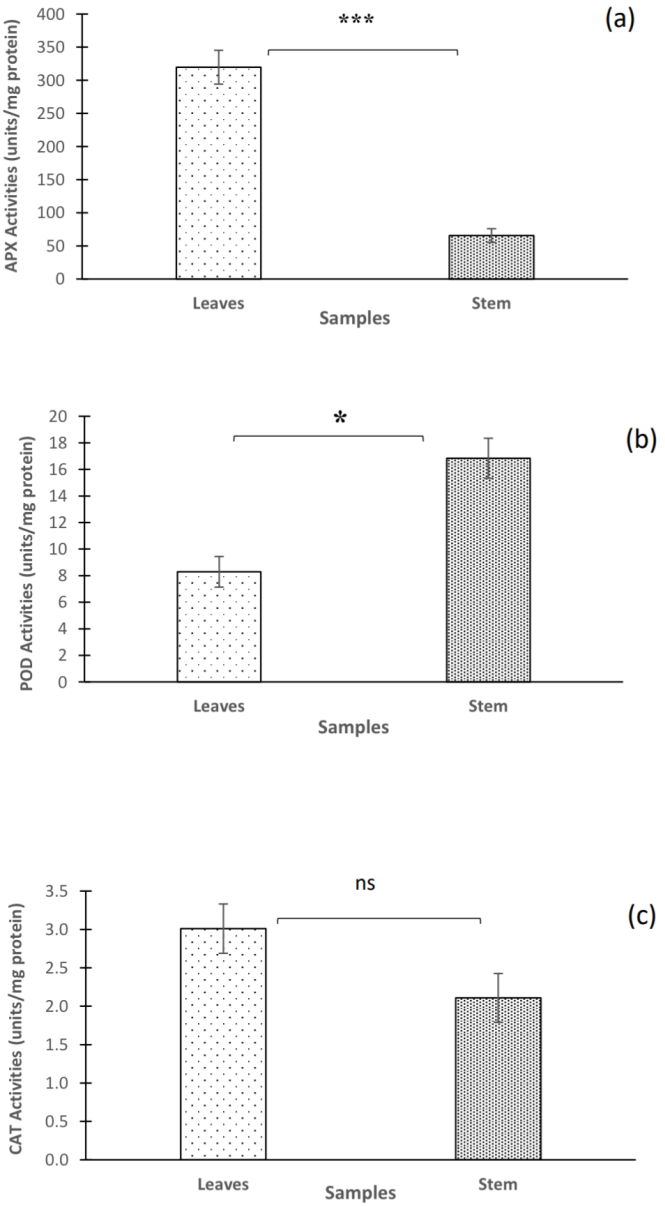

Figure 2. APX specific activities (a), POD specific activities (b) and CAT specific activities (c) of *V. planifolia* leaves and stems. Values are the mean±SEM (n = 3). ***highly significant difference at $p<0.05$, *indicates significant difference at $p<0.05$, ^{ns}no significant difference at $p<0.05$.

In general, APX activities were higher in both stem and leaves as compared to other enzymatic antioxidants such as CAT and POD. APX is one of the most important H_2O_2 scavenging enzymes and always participates in many biochemical reactions. APX can break down H_2O_2 into H_2O and hydrogen similar to CAT. But, APX has a higher substrate affinity than CAT (Hassan *et al*., 2017). In addition, APX is dominant in the stroma and thylakoid of plant leaves compared to other enzymes. POD has excellent activities in the stem compared to CAT. PODs are produced by a plant to protect the cells from the destructive influence of H_2O_2 and derived ROS produced by environmental stresses and are also involved in the biosynthesis process of

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lignin in the stem. This study indicated that the leaves and stem of *V. planifolia* have good enzymatic antioxidant activities for APX and POD which can be used in industries, mostly in dietary supplement production as free radical neutralisation in the human body to prevent chronic diseases.

3.4 Non-enzymatic antioxidants

Antioxidant molecules can prevent oxidation (chemical reactions of electrons or hydrogen transfer to oxidizing agents) which produces free radicals (Hasanuzzaman *et al.*, 2019). Figure 3(a) shows that there is no significant difference ($p<0.05$) between α-tocopherol concentration in stems and leaves of 3.52±0.04 µg/mL and 3.46±0.02 µg/mL, respectively. Figures 3(b) and (c) illustrate the ascorbic acid and carotenoid concentration of *V. planifolia* leaves and stem. The ascorbic acid concentration was almost 32-fold higher in the stem compared to the leaves. A similar pattern was observed in carotenoid concentration about 2.6-fold higher in stem compared to leaves.

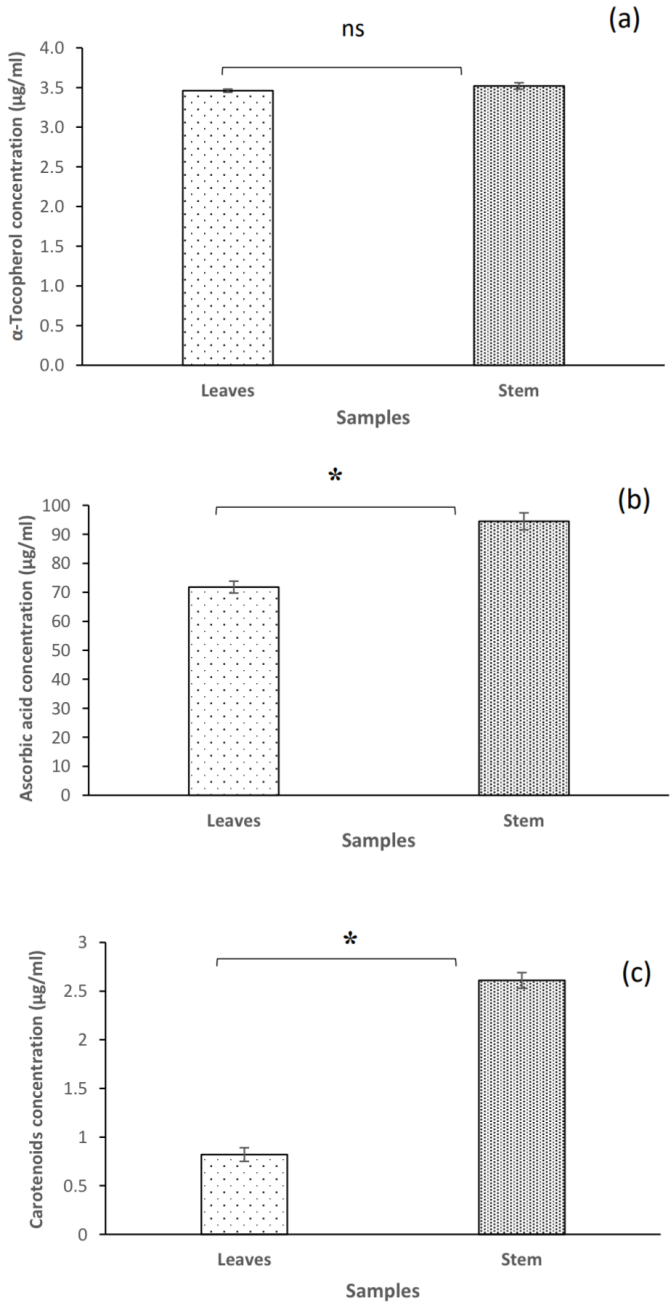

Figure 3. α-Tocopherol (a), ascorbic acid (b) and carotenoids (c) concentration in stems and leaves of *V. planifolia*. Values are the mean±SEM (n = 3). *Significant difference at $p<0.05$, [ns]no significant difference at $p<0.05$.

Antioxidant capacity in vanilla had been previously reported by Calva-Estrada *et al*. (2018). Higher ascorbic acid and carotenoid concentration in stems indicate that stems preferred these low molecular weight antioxidants for protection against ROS as compared to leaves. In addition, more concentrated ascorbic acids and carotenoids in the stem might be due to their special adaptations to environmental stresses. Stems of *V. planifolia* can be formed in the aerial part, above the water level, or on the flooded stems. Therefore, stems have special adaptations to deal with the additional burden of O_2^- and derived ROS such as $O_2^{\cdot-}$ radical and H_2O_2. Due to these special adaptations, stems are suggested to play a significant role in ROS detoxification in *V. planifolia*.

4. Conclusion

The above results indicated that *V. planifolia* can be a good source of natural antioxidants due to the significant amount of various phytochemical constituents including TPC, TFC, carbohydrates, reducing sugar and total antioxidant activities in stems and leaves of *V. planifolia*. Different parts contain different amounts of biochemical compounds thus contributing to reducing the amount of this orchid waste in industrial production, as its stems and leaves can be exploited for pharmaceutical and nutraceutical purposes.

Conflict of interest

The authors declare no conflict of interest.

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