ANTIOXIDANT ACTIVITY AND NITRATE, FLAVONOID AND PHENOL CONTENTS OF LEAF, STALK AND ROOT OF *Apium graveolens* L. (TAYOKE NAN-NAN)

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Abstract

Celery (*Apium graveolens* L.) is used in folk medicine as antihypertension agent and it has also been known to possess antioxidant activity. The plant is also known to be rich in nitrate which is antihypertension. Therefore the plant has been selected to study its nitrate content, antioxidant activity as well as total phenolic and flavonoid contents. It was found that crystals of potassium nitrate abundantly fell out of the ethanol extract on standing. By the first derivative UV spectrophotometric method based on nitration of salicylic acid, nitrate content in stalk of celery from Kalaw (S2) is 6,314.398 mg/kg FW (fresh weight). This is one of the highest among the different parts of celery. Total flavonoids (27.170 and 29.757 mg QE/g FW) and phenols (28.566 and 32.769 mg GAE/g FW) contents in leaf from Nyaung Hnit Pin (S1) and (S2) and antioxidant activity IC_{50} value (45.219 μg/mL) in stalk from (S2) are also high.

Keywords: Celery (*Apium graveolens* L.), potassium nitrate, flavonoids, phenols, antioxidant activity

Introduction

Most of the people use medicinal herbal plants in all over the world, including Myanmar especially in village tracts. All medicinal plants have primary and secondary metabolites which have biological activities and which are used for curing of various human diseases and also play an important role in the healing and illnesses. Among the phytochemical compounds of celery, one can mention carbohydrates, phenols such as flavonoids and alkaloids. Presence of compounds such as limonene, selinene, frocoumarin glycosides, flavonoids, and vitamins A and C are the reason that celery is the most widely used plant in traditional medicine. Celery can prevent cardiovascular diseases, jaundice, liver and lien diseases, urinary tract obstruction, gout, and rheumatic disorders (Kooti and Daraei, 2017). Tayoke Nan-nan is a plant from *Apiaceae* family which contains many phenols and antioxidant compounds which have many health benefits and that can be easily bought in the markets. *Apium graveolens* L. (celery) includes the anticancer potency of popular vegetables consumed in Indonesia (Octaviani *et al.* 2013).

Moreover, celery (*Apium graveolens* L.) is a leafy plant with significant levels of nitrites and nitrates; it is a possible natural source of these compounds for use in cured meat products. Nitrates content in celery is greater than 2500 mg/100 g fresh weight, which is very high (Santamaria, 2006).

Inorganic nitrites or nitrates therapy has potent anti-inflammatory, antioxidant properties, reduces circulating biomarker levels of oxidative stress and cardiac hypertrophy, improves rental function and lowers the blood pressure (Münzel and Daiber, 2018). Nitrates and nitrites as preservatives can be used to the cured meat flavor such as bacon, ham, sausages and hot dogs by helping to prevent the growth of microorganisms, particularly *Clostridium botulinum*, and to control rancidity by inhibiting lipid oxidation (Govari and Pexara, 2015).

An Acceptable Daily Intake (ADI) of nitrates is 0- 3.7 mg kg^{-1} of body weight (b. w) (equivalent to 222 mg nitrates per day for a 60 kg adult) (Gorenjak *et al*., 2014). The increase

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amount of nitrates and nitrites in food can cause gastrointestinal cancer and in infants, methemoglobinema (Hord *et al.,* 2009).

Nitrates in plant tissue have been quantitatively determined by several methods. Some are potentiometric (Olmos *et al.,* 2013), chromatographic (Liu *et al.,* 2016), tirimetric (Digregorio and Moris, 1970), Cadmium reduction (Huffman and Barbarick, 1981) and spectrophotometric (Muresan *et al*., 2012) methods. The first four methods have some disadvantages, such as lower sensitivity, interferences, technician exposure to carcinogenic chemicals (Cataldo *et al.*, 1975; Vendrell and Zupancic, 1990) and time-consuming (Cataldo *et al.,* 1975), whereas the principal problem in the classical spectrophotometric method is the presence of pigments and colloidal materials (Gaya and Alimi, 2006). But nitration of salicylic acid is rapid, free of interference from other ions present in plant tissue (Cataldo *et al.,* 1975), a method was designed for nitrate–N determination in plant tissue using first-derivative spectrophotometry (Lastra, 2003). The aim of this research is to isolate some active compounds such as potassium nitrate crystals from *Apium graveolens* L. and assess some of their biological activities.

Materials and Methods

Celery samples were purchased from Kalaw in Shan State and Hmawbi Township in Yangon Region. After collection, the scientific name of the sample was verified by authorized botanist at Botany Department, Dagon University. The leaf, stalk, and root were washed with distilled water to remove dust. The samples were separately dried at 90 ˚C in Electronic Oven SEO 2260. The dried samples were cut into small pieces and then ground into powder by means of the Panasonic MX- GM 1011 (green) Blender. The dried powder samples were separately stored in the air tight containers.

Isolation of Nitrate Crystals from the Stalk Celery

Dry stalk tissue (5 g) was accurately weighed and placed in a sealed vessel, mixed with 100 mL of 95 % ethanol and then the vessel was placed in an ultrasonic cleaning bath (model number Power Sonic 410) for 90 min at 25 ˚C. After extraction, the ethanol extract was dried on a water bath. At this time, the crystals came out from the solution. The crystals were purified with methanol several times until clear crystals were obtained. The crystals, free from chlorophyll were dried on water bath.

Identification of Potassium Nitrate Crystals in the Stalk of Celery

(A) Analysis of acid radical, nitrate ions

Action of concentrated sulphuric acid and copper turnings

Concentrated sulphuric acid and copper chips were added to the crystals from stalk ethanol extract and then heated. Reddish brown fumes were observed. This observation indicated the presence of nitrate (Vogel, 1979).

By using Brown ring test

Dilute sulphuric acid was added to the crystals from the ethanol extract of stalk till the effervescence ceased. Freshly prepared ferrous sulphate solution was added to the above solution and then concentrated sulphuric acid was poured through the sides of the test-tube. Brown ring was formed at the junction of the two liquids. This observation indicated the presence of nitrate (Vogel, 1979).

(B) Analysis of the basic radical, potassium and sodium ions by AAS method

Crystals from ethanol extract of stalk were analyzed by Atomic Absorption spectroscopic method in the National Analytical Laboratory, Department of Research and Innovation.

(C) Identification of potassium nitrate

Crystals from ethanol extract of stalk were identified by FT IR spectroscopy by comparison with the spectra of $NaNO_3$ and KNO_3.

Determination of Nitrate Content from Different Parts of Celery

Nitration of salicylic acid

Apparatus

(a) Spectrophotometer- UV-1800 UV-Vis spectrophotometer (UV double beam UV-Vis with 1 cm quartz cells) from Shimadzu Company attached to a printer was used. The spectra were obtained with a spectral bandwidth of 2 nm. The derivative spectra were obtained by instrumental electronic differentiation (Lastra, 2003).

(b) Power Sonic 410- Maintain the extract of stalk tissue at 45 ˚C.

Reagents

All reagents were of analytical grade.

Nitrate-N stock standard solution

Solution of 500 mg/L was prepared from KNO_3. Working standard solutions of 10, 20, 30, 40, 50, 60, 80, and 100 mg/L were prepared by diluting the standard with distilled water and were stored at 4 ˚C.

Salicylic acid solution 5 % (m/v) in concentrated H_2SO_4

Salicylic acid (5.00 g) was dissolved in concentrated sulphuric acid and diluted to volume (100 mL) with the same acid; prepared at least once each 48 h, and stored in an amber bottle at 4 ˚C.

2 N Sodium hydroxide solution

Sodium hydroxide (40 g) was dissolved in 500 mL of distilled water and was stored at room temperature.

Calibration

Aliquots (0.1 mL) of working standard solutions (10–100 mg/L nitrate-nitrogen) in a 30 mL tube were mixed thoroughly with 0.4 mL salicylic acid solution. After 20 min at room temperature, 9.5 mL 2N NaOH solution was slowly added to obtain 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mg/L nitrate-nitrogen solutions. This method is based on the formation of nitrosalicylic acid that shows, in highly basic solution, a maximum absorption at 412 nm in zero-order absorption spectrum, and 388 and 440 nm in first-order spectrum ($1D_{388–440}$). The sum of the heights of both peaks (388 and 440 nm) positive and negative (peak-to-peak) of the first derivative was used. The wavelength range selected to obtain the spectrum was 356–500 nm; data interval, 4 nm; scaling factor 18.

Extract Preparation from Plant Tissues

Portions (0.1000 g) of vegetable tissues (each celery ground sample) was suspended in 10 mL distilled water, kept at 45 ˚C for 1 h under sonication, and then filtered through filter

paper. Samples were extracted and analyzed immediately or within 24 h after extraction when stored at 4 ˚C (Catald *et al.*,1975).

Determination

A 0.1 mL volume of the preceding extract was thoroughly mixed in a 30 mL tube with 0.4 mL salicylic acid solution. After 20 min at room temperature, 9.5 mL 2 N NaOH solution was slowly added.

Calculation

Nitrate-N in plant tissue express as mg NO_3^--N per kg dry weight:

$$C_x = \frac{(1D_{388\text{-}440} - a) \times 1000}{b \times w}$$

where $1D_{388–440}$ corresponds to the spectrophotometric measurement of the sample (UD derivative unity); "b" and "a" correspond to the slope and the intercept of the calibration curve $1D_{388–440}$ (UD) vs NO_3^--N concentration (mg/L) and w = tissue weight (g) respectively.

Determination of total flavonoids and phenols Contents

Preparation of plant extract

One gram powder was accurately weighed and placed in sealed vessel by adding 70 mL of 96 % ethanol solvent, and then the vessel was placed in an ultrasonic bath (model number Power Sonic 410) for extraction for 60 min at (40 ˚C). After extraction the extract was stored in glass vials at refrigerated temperature for further analysis (Bharti and Ray, 2014). This extract was used to determine the total flavonoids and phenols contents.

Determination of total flavonoids content

Total flavonoids content (TFC) of the extract was measured by the aluminium chloride colorimetric assay. The sample (2 mL) was mixed with 0.2 mL of 5 % sodium nitrite. After 5 min, 0.2 mL of 10 % aluminum chloride was added to the mixture and mixed. After 6 min, 2 mL of 1 M sodium hydroxide was added to the mixture. The end volume of the reaction mixture was made up to 5 mL with aqueous ethanol and mixed thoroughly. Absorbance of the reaction mixture was measured at 510 nm against a blank. The flavonoid content was determined using a standard curve of Quercetin at 1.56- 100 μg/ mL and the results were expressed as μg/mL quercetin equivalents (QE) (Bharti and Ray, 2014).

Determination of total phenols content

Total phenols were determined by using Folin-Ciocalteu assay (Bharti and Ray, 2014). The extract preparation was the same as in the determination of total flavonoids content. The sample (0.2 mL) was taken in a test tube and then 0.5 mL FC reagent (1:1 diluted with distilled water) and 1 mL of saturated sodium carbonate solution were added. The final volume was made to 5 mL with water. Then the mixture was allowed to stand for 15 min. The absorbance was read at 746 nm using UV-Vis Spectrophotometer. The standard solutions of Gallic acid in water 0- 50 mg/mL were prepared to construct the calibrating curve. Total phenols content were expressed in terms of Gallic acid equivalent (GAE) (mg/g dry mass) (Bharti and Ray, 2014).

Determination of Antioxidant Activity

The ethanol extract (0.004 g) was dissolved in 10 mL to get 400 μg/mL solution. The DPPH radical scavenging activity (DPPH SA, %) is often used to evaluate the antioxidant capacity of ethanol extract (Brand-Williams *et al*., 1995). The extract solution (1.5 mL) was mixed with 1.50 mL of freshly prepared DPPH (0.05 mM) in ethanol. The mixture was shaken vigorously and kept at room temperature for 30 min. Then the absorbance was measured at 517 nm against a blank (without extract) in a UV-Vis spectrophotometer. The scavenging activity of DPPH radical (DPPH SA, %) was calculated using the following formula:

$$\text{DPPH SA (\%)} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of the control (DPPH solution without sample) and the absorbance of the test sample, respectively (Dinç *et al.*, 2017).

Results and Discussion

Identification of Potassium Nitrate crystals in the Stalk of Celery (*Apium graveolens* L.)

(A) Analysis of acid radical, nitrate ions

Qualitative analysis of nitrate ions in celery (*Apium graveolens* L.) showed evolution reddish brown vapour by the action of concentrated sulphuric acid and copper; and the appearance of a brown ring by the brown ring test (Table 1). From the positive results of acid radical, the negative part of crystal is nitrate ion.

Table 1 Qualitative Analysis of Nitrate Ions in the Stalk of celery (*Apium graveolens* L.)

No.	Test	Observation
1.	Action of H_2SO_4 (conc.) acid and Cu	Reddish brown vapour was evolved.
2.	Brown ring test	Brown ring was formed at the junction of the two liquids.

(B) Analysis of basic radical, potassium and sodium ions by AAS method

The amounts of potassium and sodium ion are 27.943 % and 0.013 % respectively (Table 2). Therefore, potassium nitrate is the main component in celery.

Table 2 The Result for Crystals from Ethanol Extract of Stalk by Atomic Absorption Spectroscopy

No.	Parameters	Analysis Result (%)
1.	Sodium (Na)	0.013
2.	Potassium (K)	27.943

(C) Identification of Potassium Nitrate

Crystals from stalk ethanol extract were identified by FT IR spectroscopy (Table 3, Figure 1).

Table 3 Assignment of Bands in the FT IR Spectrum of the crystals from ethanol extract of stalk

Wavenumber (cm^{-1})		Vibrational mode	Functional group
Experimental value	**Reference Value***		
1762	1767	Stretching of N=O	NO_3^-
1366	1340- 1369	Antisymmetric stretching of NO_3^-	NO_3^-
823	830	symmetric stretching of NO_3^-	NO_3^-

* (Miller and Wilkins, 1952, Trivedi *et al.*, 2015)

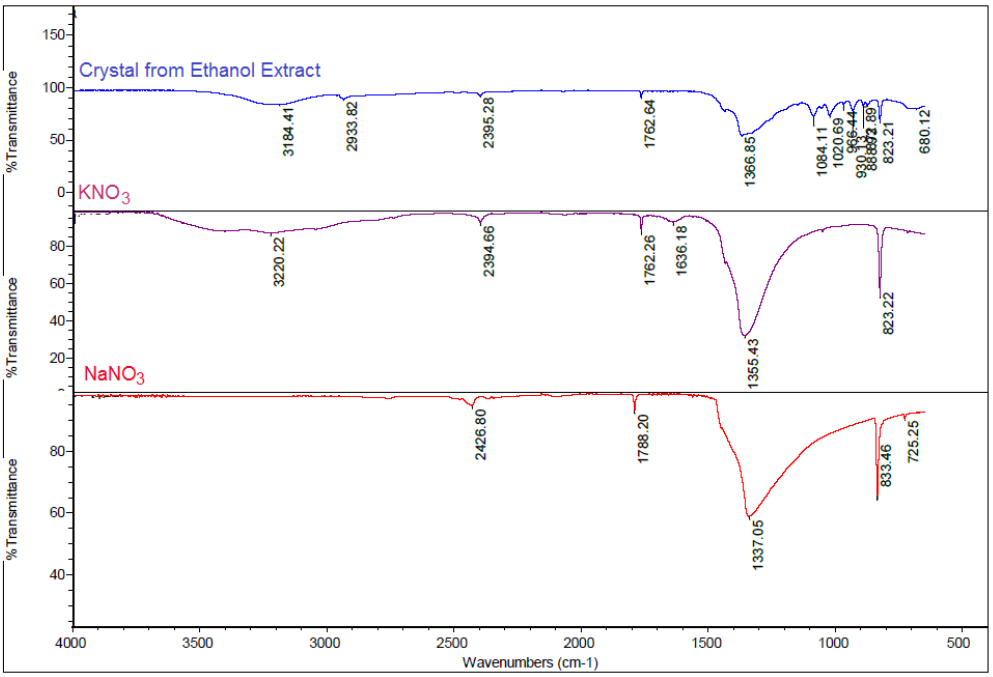

Figure 1 Comparison of The FT IR spectra of the crystals from ethanol extract of stalk, potassium nitrate and sodium nitrate

Determination of Nitrates Content from Different Parts of Celery

Construction for the calibration curve of standard potassium nitrate solution

Zero- and first-order spectra for nitro salicylic acid obtained with standard nitrate-N solutions (0.1- 1.0 mg/mL) are shown in Figures 2 and 3, respectively. The optimum conditions were first-order derivative, wavelength range of 356–500 nm, and $\Delta\lambda$ 4 nm. In this derivative method, the measurement selected to prepare the analytical calibration graphs was peak-to-peak (sum of the heights of 388 and 440 nm peaks), which exhibits good linearity to nitrate–N concentration. The intercept is near zero. The calibration graph and statistical results are given in Figure 4 and Table 4, respectively. In this experiment the highest nitrate-N and nitrate were observed in stalk from Kalaw (Table 5 and Table 6).

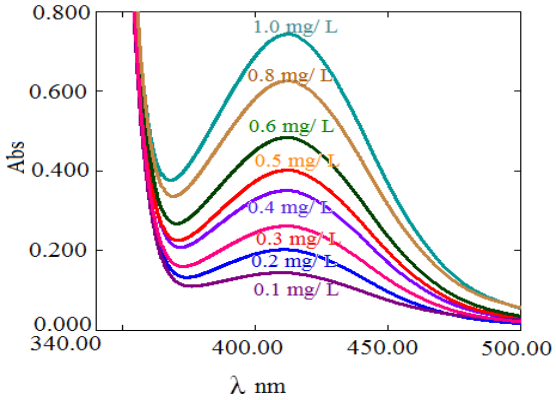

Figure 2 Zero-order overlaid spectra of nitro salicylic acid in highly basic solution obtained using standard potassium nitrate solutions

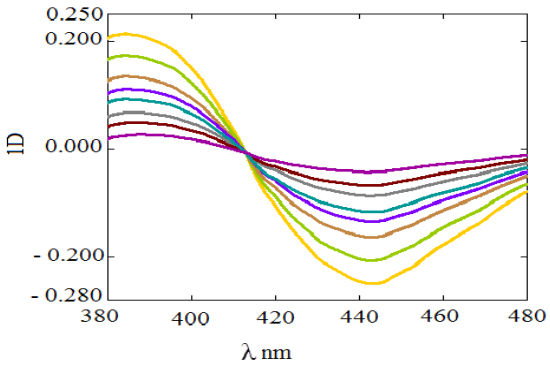

Figure 3 First-order derivative overlaid spectra of nitrosalicylic acid in highly basic solutions using standard potassium nitrate solution Δ λ 4 nm

Table 4 Concentrations of Standard Nitrate-N with their Respective Spectrophotometric Values in the First-derivative Spectrum between λ_{max} 388 - 440

Concentration (μ g/ mL)	1D 388-440		mean	STDEV
	1st	2nd		
0.1	0.089	0.057	0.073	0.023
0.2	0.101	0.117	0.109	0.011
0.3	0.157	0.154	0.156	0.002
0.4	0.187	0.206	0.197	0.013
0.5	0.222	0.243	0.233	0.015
0.6	0.278	0.293	0.286	0.011
0.8	0.363	0.375	0.369	0.008
1.0	0.449	0.455	0.452	0.004

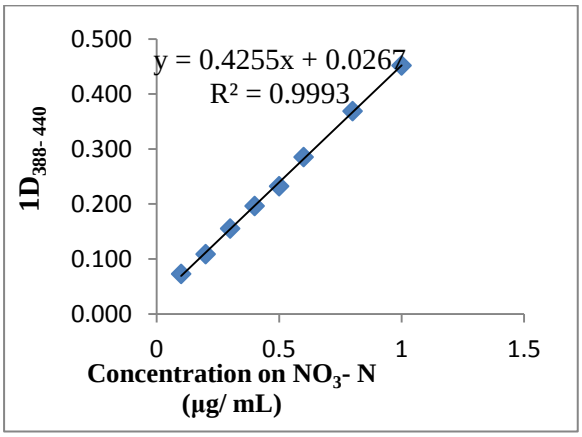

Figure 4 Calibration curve for nitrate-N determination using first-order derivative spectrophotometry

Table 5 Concentration of Nitrate-Nitrogen in Dry Leaf, Stalk, and Root

Sample	1D 388-440	Concentration mg/kg dry weight
L- S1	0.079 ±0.010	1,229.142
L- S2	0.088 ± 0.007	1,440.658
S- S1	0.068 ± 0.004	958.872
S- S2	0.154 ± 0.033	11,967.098
R- S1	0.185 ± 0.008	7,417.156
R- S2	0.199 ± 0.001	4,037.603

S1 = Nyaung Hnit Pin (Hmawbi Township)
L- S1 = Leaf from S1
S- S1 = Stalk from S1
R- S1 = Root from S1

S2 = Kalaw (Shan State)
L- S2 = Leaf from S2
S- S2 = Stalk from S2
R- S2 = Root from S2

Table 6 Concentration of Nitrate-N and Nitrate in Dry and Fresh Weight

Sample	Conc. of NO_3^- -N mg/ kg dry weight	Conc. of NO_3^- mg/ kg dry weight	Conc. of NO_3^- mg/ kg fresh weight
L- S1	1,229.142	5,443.343	680.027
L- S2	1,440.658	6,380.057	436.047
S- S1	958.872	4,246.433	513.013
S- S2	11,967.098	52,997.146	6,314.398
R- S1	7,417.156	32,847.406	3,675.234
R- S2	4,037.603	17880.812	1,896.450

Determination of Total Flavonoids Content

Flavonoids are a group of polyphenolic compounds naturally present in most yellow edible vegetables. There is evidence of a potential role for flavonoids as free radical scavenging and in lowering the risk of coronary heart disease, cardiovascular diseases, neurodegenerative diseases, osteoporosis, lung cancer and other biological disorders (Bharti and Ray, 2014). The calibration curve of the determination of flavonoids content was constructed between at 0- 100 μg /mL concentration and the results are expressed as milligram quercetin equivalent/g (Table 7 and Figure 5). The highest total flavonoids were observed in leaf from Kalaw, lowest flavonoids in root from Nyaung Hnint Pin (Table 8 and Figure 6).

Table 7 Absorbance and Concentration (μg/mL) of Total Flavonoids Content and STDEV

Concentration (μg mL^{-1})	Absorbance			Mean	STDEV
	1st	2nd	3rd		
100.000	0.355	0.350	0.352	0.352	0.003
50.000	0.170	0.166	0.170	0.169	0.002
25.000	0.084	0.085	0.085	0.085	0.001
12.500	0.045	0.042	0.042	0.043	0.002
6.250	0.023	0.025	0.023	0.024	0.001
3.125	0.017	0.018	0.019	0.018	0.001
1.560	0.008	0.009	0.008	0.008	0.001

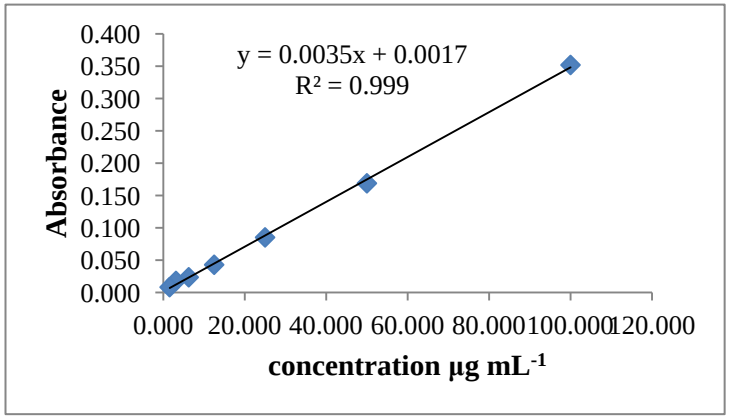

Figure 5 Calibration curve for total flavonoids content

Table 8 Total Flavonoids Content in Leaf, Stalk and Root of Celery from Two Different Sources

Sample	TFC content mg QE/g dry weight	TFC content mg QE/g fresh weight
L- S1	217.486	27.170
L- S2	263.453	29.757
S- S1	24.473	2.957
S- S2	28.743	3.425
R- S1	27.600	3.088
R- S2	37.886	4.018

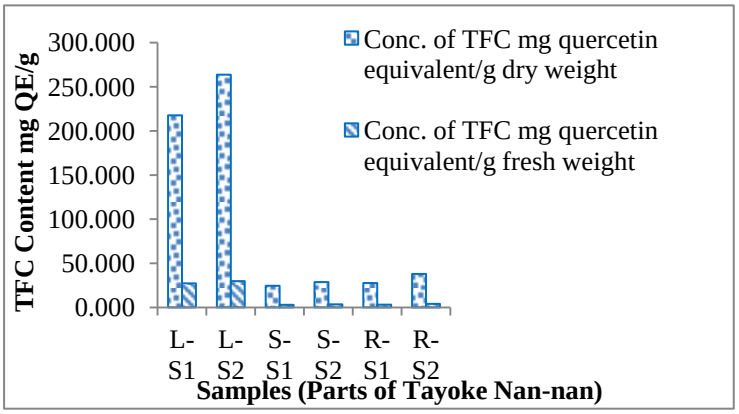

Figure 6 Total flavonoids content in dry and fresh leaf, stalk, and root of celery from S1 and S2

Total Phenols Content in Leaf, Stalk and Root of Celery

The total phenols content was determined by a modified Folin-Ciocalteu reagent method. Using the standard curve of Gallic acid (R^2 = 0.9997) (Table 9 and Figure 7), the phenols content was expressed as mg Gallic acid equivalent per g. The highest amount of total phenols content was observed in the leaf from Kalaw, the lowest in the root from Nyaung Hnint Pin (Table 10 and Figure 8).

Table 9 Absorbance Values of Standard Gallic Acid Solutions

Concentration (µg/ mL)	Absorbance
50.000	0.798 ± 0.008
25.000	0.430 ± 0.005
12.500	0.240 ± 0.006
6.250	0.135 ± 0.007
3.125	0.096 ± 0.006

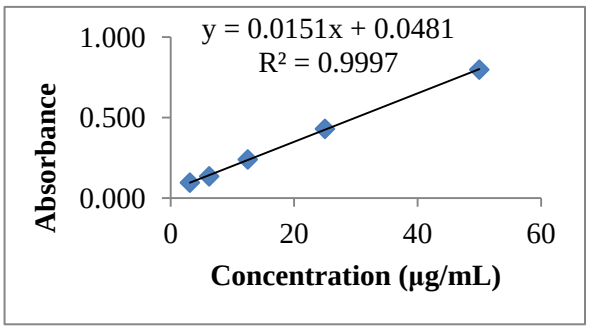

Figure 7 Calibration curve for total phenols content determination using gallic acis as standard

Table 10 Total Phenols Content in Leaf, Stalk and Root of Tayoke Nan-nan from Two Different Sources

Sample	TPC content mg GAE/g dry weight	TPC content mg GAE/g fresh weight
L- S1	228.662 ± 0.002	28.566
L- S2	290.119 ± 0.004	32.769
S- S1	42.331 ± 0.004	5.114
S- S2	51.338 ± 0.002	6.117
R- S1	41. 272 ± 0.001	4.618
R- S2	53. 987 ± 0.001	5.726

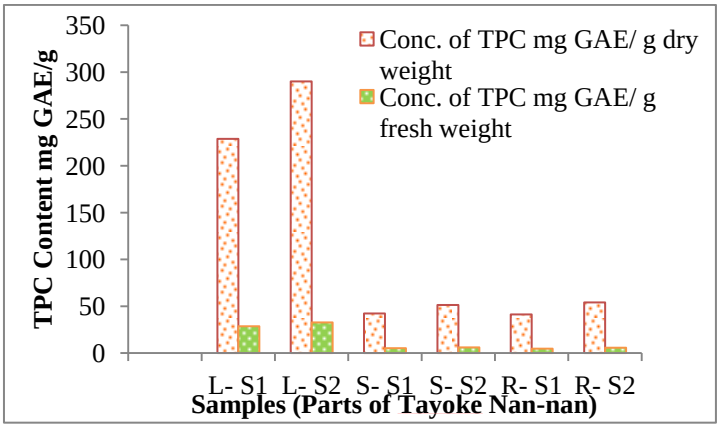

Figure 8 Bar graph showing the Total phenols content in dry and fresh leaf, stalk, and root of Tayoke Nan-nan from S1 and S2

Antioxidant Activity of Crude Extract

The radical scavenging activity of Gallic acid for 50 % scavenging (IC_{50}) of the DPPH radicals is shown in Table 11 and Figure 9. The percent inhibition and IC_{50} of DPPH by the extract of different parts of celery are recorded in Table 12 and Figures 10 and 11. In DPPH assay, the ethanol stalk extract showed most antioxidant activity when compared to ethanol leaf and root extract. DPPH scavenging activity was ranging from 2.721 ± 0.013 to 62.585 ± 0.006 % in the case of ethanol stalk extract, whereas in the case of leaf and root, they were 4.195 ± 0.004 to 56.689 ± 0.011 % and 19.728 ± 0.008 to 89.229 ± 0.010 %. In case of ethanol stalk extract, the highest scavenging activity was found at a concentration 200 μg/mL and the lowest was found at a concentration of 12.5 μg/mL .

Table 11 % Radical Scavenging Activity and IC_{50} Value of Standard Gallic acid by DPPH Assay Method

Concentration (μg/mL)	% Inhibition	IC_{50} (μg/mL)
0.3125	24.603 ± 0.004	0.492
0.625	68.481 ± 0.005	
1.250	87.642 ± 0.002	
2.500	88.776 ± 0.003	
5.000	90.249 ± 0.030	
10.000	86.508 ± 0.045	

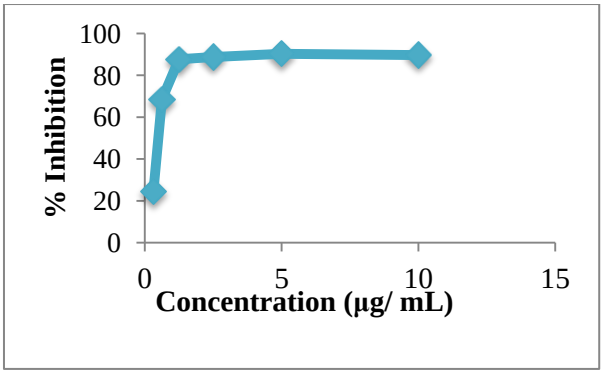

Figure 9 % Radical scavenging activity of standard gallic acid

Table 12 % Inhibition for Different Concentrations of Leaf, Stalk and Root of Celery from S2

Concentration (µg/mL)	% Inhibition Leaf	% Inhibition Stalk	% Inhibition Root
12.5	4.195 ± 0.004	2.721 ± 0.013	19.728 ± 0.008
25.0	8.617 ± 0.004	18.821 ± 0.003	23.810 ± 0.017
50.0	18.481 ± 0.004	57.37 ± 0.003	30.863 ± 0.005
100.0	50.227 ± 0.007	59.249 ± 0.001	41.950 ± 0.005
200.0	55.409 ± 0.003	62.585 ± 0.006	73.696 ± 0.055
400.0	56.689 ± 0.011	62.358 ± 0.146	89.229 ± 0.010
IC_{50}	99.646 µg/mL	45.219 µg/mL	125.342 µg/mL

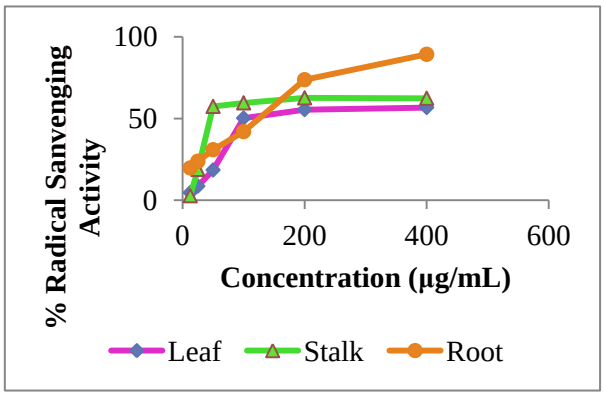

Figure 10 % Radical scavenging activity of different concentrations of ethanol crude extracts of leaf, stalk and root of celery from S2

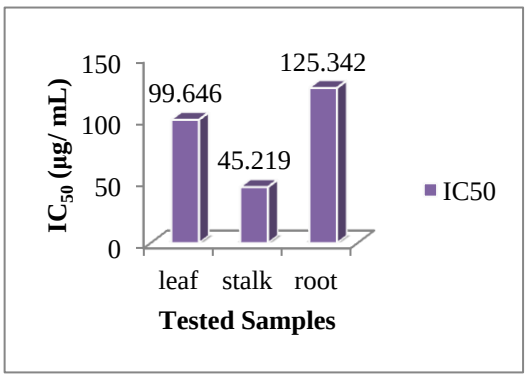

Figure 11 A bar graph of IC_{50} values of gallic acid and ethanol crude extracts of leaf, stalk and root of celery from S2

Conclusion

Nitrate contents of celery (*Apium graveolens*) (leaf, stalk and root from Kalaw and Nyaung Hint Pin) were analysed by nitration of salicylic acid using UV-1800 UV-Vis spectrophotometer. The trend of nitrate contents are stalk > root> leaf. Potassium nitrate crystals are mostly abundance in the ethanol extract of celery stalk.

Identification of acid radical on these crystals were confirmed nitrate ion, and for the basic radical, potassium ion by Atomic Absorption Spectroscopy method.

Crystals from stalk ethanol extract were identified by FT IR spectroscopy by using potassium and sodium nitrate.

In addition, the total flavonoids and phenols contents in leaf from Kalaw (S2) are the highest. Therefore the trend of total flavonoids and phenols contents are leaf > root > stalk and leaf > stalk > root.

IC_{50} values of antioxidant activities showed stalk > leaf > root (45.219 μg/mL > 99.646 μg/mL> 125.34 μg/mL). In this result, the antioxidant activity of stalk is the highest because of total phenols and nitrate contents.

Acknowledgements

The authors would like to express our gratitude to the Department of Higher Education, Ministry of Education, Myanmar, for their permission to do this research and also to the Myanmar Academy of Arts and Science for allowing to read this paper.

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