Original Article

Correlation Analysis between Total Acid, Total Phenolic and Ascorbic Acid Contents in Fruit Extracts and Their Antioxidant Activities

Weerasak Samee[1]*, Mongkol Engkalohakul[2], Nakharin Nebbua[2], Paradorn Direkrojanavuti[2], Chayanid Sornchaithawatwong[1] and Narisa Kamkaen[1]

[1] Natural Product Evaluation Center (NPEC), Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Srinakharinwirot University, Nakhon Nayok, Thailand
[2] 5th year pharmacy student, Faculty of Pharmacy, Srinakharinwirot University, Nakhon Nayok, Thailand
* Corresponding author: weerasak@swu.ac.th

ABSTRACT

The purpose of this work was to study relationships between the total acid (TA), total phenolic (TP) and ascorbic acid (AA) contents of 140 fruit extracts from 28 plants and their antioxidant activities. Total acid was determined by potentiometric titration and expressed as citric acid equivalent (CAE). Ascorbic acid was determined by method provided by the Association of Official Analytical Chemists (AOAC). The content of total phenolic compounds was determined by Folin-Ciocalteu method and expressed as gallic acid equivalent (GAE). Antioxidant activity has been determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and expressed as Trolox equivalent antioxidant capacity (TEAC). Statistical study has been performed using multiple regression analysis. The correlation between TEAC and ascorbic acid content (r^2 = 0.772) was higher than that of total phenolic content (r^2 = 0.549) and total acid (r^2 = 0.344). The multi-linear regression line indicated that the antioxidant activity of fruit extracts correlated well with the combination of total acid, total phenolic and ascorbic acid contents (r^2 = 0.973). In conclusion, this result suggested that ascorbic acid, total phenolic and total acid contents of fruit extracts should be considered together when determining their antioxidant activity.

Key words: total acid, total phenolic contents, ascorbic acid, antioxidant, fruit, multiple linear regressions

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Introduction

Reactive oxygen species are involved in a number of degenerative diseases such as coronary heart disease, cancer and diabetes.[1-3] Many epidemiological researches have shown an inverse relationship between the intake of fruit and the incidence of cardiovascular disease and some types of cancer.[4-8] The beneficial effects of these fruits are attributed largely to substances proven to have antioxidant properties, such as ascorbic acid (vitamin C), vitamin E, carotenoids and polyphenolic compounds. Many fresh fruits have been found to contain natural antioxidants, mainly ascorbic acid and phenolic compounds.[9-14] Ascorbic acid is easily oxidized, and the majority of its functions *in vivo* rely on this property. It plays a key role in the body's synthesis of collagen and norepinephrine by keeping the enzyme responsible for these processes in their active reduced forms.[15-17] Ascorbic acid also plays a role in detoxifying by-products of respiration. Occasionally during

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respiration, oxygen (O_2) is reduced to a superoxide ion (O_2^-) instead of being reduced completely to its -2 oxidation state (as in H_2O). Normally an enzyme called superoxide dismutase converts O_2^- to hydrogen peroxide (H_2O_2) and O_2. However, in the presence of Fe^{2+}, the hydrogen peroxide is converted into the highly-reactive hydroxyl radical ($^{\bullet}OH$). The hydroxyl radical can initiate unwanted and deterious chemistry within a cell when it removes a hydrogen atom ($H^{\bullet}$) from an organic compound to form H_2O and a new, potential more reactive free radical. Ascorbic acid can donate a hydrogen atom to a free radical, and thus prevent these reactions from occurring.[18-20] The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen.[21-23] Ascorbic acid and phenolic contents differ depending on the particular origin and growth conditions of original fruits.[24-27] The purpose of this study was to evaluate tropical fruits as potential sources of natural antioxidants and phenolic compounds. Our study also demonstrated the possible relationships between contents of total acid, total phenolic and ascorbic acid, and their antioxidant activities.

Materials and Methods

Chemicals and reagents

Chemical reagents were purchased from various sources; 1,1-diphenyl-2-picrylhydryzyl (DPPHŸ) radical from Fluka, standard L-ascorbic acid and gallic acid from Sigma, 2,6-dichloroindophenol (DCIP) and Trolox from Aldrich, sodium bicarbonate and Folin-Ciocalteu's reagent from Carlo Erba. All chemicals used were of analytical grade.

Plant materials

Fruit samples used in this study were those cultivated in a tropical region, which is the main fruit growing area in Thailand. Crop variation of a given fruit was taken into account by obtaining 5 samples (each purchased on 5 different days). This resulted in a total of 140 samples for 28 kinds of fruit. These fruits included banana (*Musa sapientum* Linn., Musaceae), chebulic myrobalans (*Terminalia Chebula* Retz., Combretaceae), durian (*Durio zibethinus*, Bombacaceae), emblic myrabolan (*Phyllanthus emblica* Linn., Euphorbiaceae), gandaria (*Bouea Burmanica* Griff., Anacardiaceae), garcinia (*Garcinia schomburgkiana* pierre., Guttiferae), green apple (*Pyrus malus* Linn., Rosaceae), green grape (*Vitis vinifera* Linn., Vitidaceae), guava (*Psidium guajava* Linn., Myrtaceae), hog plum (*Spondias pinnata* Kurz., Anacardiaceae), jujube (*Ziziphus mauritiana* Lamk, Rhamnaceae), lime (*Citrus aurantifolia* Swing. Rutaceae), mango (*Mangifera indica* var. namdokmai, Anacardiaceae), manila tamarind (*Pithecellobium dulce*, Benth., Leguminosae), ma-uek (*Solanum stramonifolium* Jacq., Solanaceae), musk melon (*Cucumin melo* Linn., Cucurbitaceae), pineapple (*Ananus comosus* Merr., Bromeliaceae), pomelo (*Citrus maxima* Merr., Rutaceae), red apple (*Pyrus malus* Linn., Rosaceae), red grape (*Vitis vinifera* Linn., Vitidaceae), sandy pear (*Pyrus lindteyi* Rehd., Rosaceae), spanish plum (*Ellaeocarpus hygrophilus* Kurz., Elaecocarpaceae), star fruit (*Averhoa carambola* Linn., Oxalidaceae), sweet orange (*Citrus sinensis* Osb., Rutaceae), tamarind (*Tamarindus indica* Linn., Leguminosae), tangerine (*Citrus reticulata* Blanco., Rutaceae), water melon (*Citrullus vulgaris* Eckl &Zeyh., Cucurbitaceae) and wax jambu (*Eugenia javanica* Lamk., Myrtaceae). All samples were stored at 5 °C and processed into fruit juice within 1 week.

Instruments

The instruments for analysis included potentiometric titrator (Metrohm® 751 GPD Titrino, Switzerland), microplate spectrophotometer (Anthos ADAP Zynyth 200®, Austria), blender (Moulinex T574, France), centrifuge (Hettich Universal 32R Ver.302, Germany), and UV-VIS spectrophotometer (Shimadzu UV-1601, Japan).

Experimental procedures

Sample preparation for total acid and antioxidant capacity analysis

Fruits were washed with tap water and cut in half. Ten grams of sliced fruit was extracted by 50 mL water using a laboratory-scale extractor (Moulinex T574, France). After extraction, raw juice was heated at 96 °C for 1 min to inactivate enzymes. Then, the juices were rapidly cooled down to room temperature, and filtered through 8-folded cheesecloth to eliminate particulates. The volume of each juice was adjusted by water to 100 mL. These 140 fruit extract samples were stored at -18 °C for analyses of phenolic content and antioxidant activity.

Analysis of antioxidant activity

Each of the 140 fruit extract samples was measured for antioxidant activity using the stable DPPH radical. In a 96-well plate, 50 μL of each sample was added to 250 μL of 100 μM $DPPH^{\bullet}$ dissolved in methanol. The plate was covered with aluminum foil and left to stand at room temperature for 30 min. The reduction in absorbance was measured at 517 nm with a microplate spectrophotometer. The amount of reduction in absorbance was compared to that of Trolox as a standard. All determinations were repeated three times. The result was expressed as Trolox equivalent antioxidant capacity (TEAC) in μM per gram of fresh weight fruit.

Analysis of total acid

Each of the 140 fruit extract samples was measured for total acid level by potentiometric titration. A 10.0 mL of the fruit extract sample each was added with 100 mL distilled water, and then titrated with 0.1 N NaOH. All determinations were repeated three times and the results were expressed as citric acid equivalence (CAE) in μg per gram of fresh weight material.

Determination of ascorbic acid content[28] using the Association of Official Analytical Chemists (AOAC) method

In determining ascorbic acid content, specific fruit samples were prepared. For each of 140 fruit samples, ten grams of sliced fruit was mixed with 70 mL of cold extracting solution. This cold extracting solution consisted of 30 mg metaphosphoric acid, 80 mL of acetic acid and distilled water added to 1000 ml, stored at 4 °C. The mixture was homogenized in a blender for 1 min and then filtered through 8-folded cheesecloth to eliminate particulates, centrifuged at 9,000 g (at 4 °C) for 20 min and adjusted to a final volume of 100.0 mL. Several precautions were taken in order to perform all the operations under reduced light and at 4 °C. The result was ascorbic acid extract. To quantify ascorbic acid content, ten milliliters of ascorbic acid extracts were titrated with 2,6-dichloroindophenol solution (25 mg DCIP and 21 mg $NaHCO_3$ in 100 mL water) until light but distinct rose pink color appeared and persisted for more than 5 seconds. The 2,6-dichloroindophenol solution was standardized daily with ascorbic acid solution. All determinations were repeated three times and the results were expressed as μg ascorbic acid per gram of fresh weight material.

Determination of total phenolic content

Total phenolics were determined using the Folin-Ciocalteu reagent. In determining total phenolic content, specific fruit samples were prepared. A total 10 grams of each of the fruit samples was homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10000 g for 15 min and the supernatant was saved. The residue was re-extracted twice with ethanol and supernatants were collected, put into evaporating dishes and evaporated to dryness at room temperature. Residue was dissolved in 5 mL of distilled water. One-hundred microlitres of this extract was diluted to 3 mL with water and 0.5 mL of Folin-Ciocalteu reagent was

added. After 3 min, 2 mL of 20% sodium bicarbonate was added and the contents were mixed thoroughly. The color was developed and absorbance measured at 750 nm in a spectrophotometer after 2 h using gallic acid as a standard. All determinations were repeated three times and the results were expressed as gallic acid equivalent (GAE) in μg per gram of fresh weight material.

Statistical Analysis

For each of the 140 fruit samples, three replicate values were averaged. A total of 140 values for each parameter measured was used for statistical analysis. Coefficient of determination (r^2) was used to determine relationships between TEAC and each of other parameters, i.e., TEAC and content of total acid, TEAC and content of phenolic compounds, and TEAC and content of ascorbic acid. These coefficients were calculated using MS Excel software (CORREL statistical function). To examine the relationships between TEAC and these various parameters simultaneously, multiple linear regression analyses were carried out using SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). Concentrations of total acid, total phenolic compounds and ascorbic acid, and antioxidant activity of each of 28 kinds of fruits were also reported.

Table 1 Concentrations of total acid, total phenolic, ascorbic acid and antioxidant activity in fruit extracts per gram of fresh weight fruit

Fruit	Total acid[a] (mg CAE/g)	Total phenolics[a] (mg GAE/g)	Ascorbic acid[a] (mg /g)	Antioxidant activity[a] (mM TEAC/g)
Hog plum	26111.5 ± 912.0	414.6 ± 33.0	1561.9 ± 107.9	77.53 ± 1.29
Emblic myrabolan,	29514.9 ± 2356.6	361.5 ± 35.2	1536.0 ± 93.1	73.95 ± 2.92
Gandaria	14363.4 ± 697.7	332.3 ± 49.4	1008.9 ± 58.9	59.74 ± 4.69
Guava	10429.9 ± 671.7	32.0 ± 5.5	1707.2 ± 47.3	45.95 ± 1.21
Lime	28269.9 ± 1730.8	186.4 ± 21.3	761.2 ± 47.7	43.85 ± 1.67
Ma-uek	20533.6 ± 1390.7	334.6 ± 57.3	456.7 ± 40.8	40.06 ± 2.26
Spanish plum	8103.3 ± 302.1	359.3 ± 11.2	448.3 ± 47.6	39.00 ± 0.64
Pomelo, Shaddock	5315.0 ± 285.7	292.2 ± 53.0	516.7 ± 52.7	36.42 ± 3.62
Durian	1146.6 ± 262.8	350.6 ± 78.4	309.1 ± 17.0	35.97 ± 3.66
Manila tamarind	9945.6 ± 681.4	230.1 ± 31.8	463.7 ± 26.8	31.89 ± 5.91
Mango	15837.8 ± 933.5	213.6 ± 34.0	454.7 ± 55.2	30.88 ± 3.36
Sweet orange	7020.0 ± 351.6	194.3 ± 18.0	508.1 ± 43.8	30.23 ± 1.61
Tangerine	14712.7 ± 2681.2	240.0 ± 44.5	366.2 ± 86.3	29.56 ± 4.37
Jujube	12197.5 ± 1253.4	148.0 ± 36.6	488.3 ± 7.3	27.56 ± 2.53
Musk Melon	6836.0 ± 198.4	57.8 ± 16.2	615.4 ± 26.4	26.91 ± 1.63
Chebulic myrobalans	21337.0 ± 1208.2	173.7 ± 55.2	306.6 ± 39.4	22.86 ± 3.33
Star fruit	16046.2 ± 528.5	181.3 ± 14.4	350.5 ± 25.8	21.03 ± 5.34
Water melon	12835.8 ± 1889.0	13.9 ± 5.7	547.6 ± 37.8	20.37 ± 1.62
Wax jambu	16430.3 ± 755.6	119.2 ± 16.2	270.4 ± 9.7	19.21 ± 1.41
Pineapple	7061.1 ± 225.3	122.7 ± 31.5	252.5 ± 13.2	18.68 ± 2.02
Green apple	7479.2 ± 274.3	176.5 ± 15.1	137.0 ± 16.6	17.04 ± 1.38
Green grape	4766.7 ± 31.8	164.0 ± 19.1	68.8 ± 7.9	14.93 ± 1.15
Red apple	4671.8 ± 285.4	139.8 ± 16.8	63.9 ± 13.9	11.92 ± 0.86
Red grape	7807.1 ± 136.4	133.3 ± 23.8	60.5 ± 15.4	11.35 ± 1.88
Garcinia	14956.4 ± 1759.1	76.7 ± 16.6	108.5 ± 13.2	9.14 ± 1.34
Banana	2496.2 ± 303.9	23.4 ± 10.7	154.3 ± 19.0	8.23 ± 0.80
Tamarind	12762.4 ± 729.7	62.7 ± 9.5	87.0 ± 11.2	7.41 ± 0.85
Sandy pear	4905.5 ± 81.4	17.7 ± 6.6	40.8 ± 8.5	2.67 ± 0.55

[a] Values present as mean ± SD (n = 15)

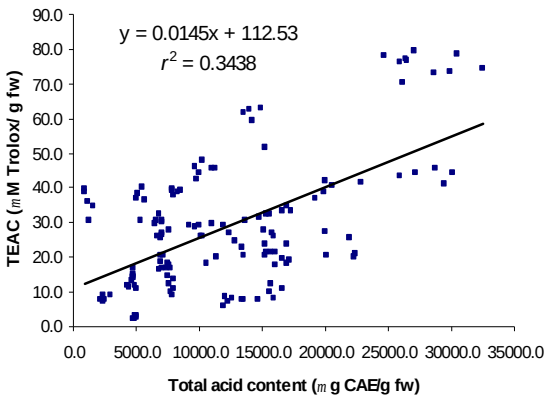
Note: CAE = citric acid equivalence, GAE = gallic acid equivalence, Trolox equivalent antioxidant activity (TEAC)

Results and Discussions

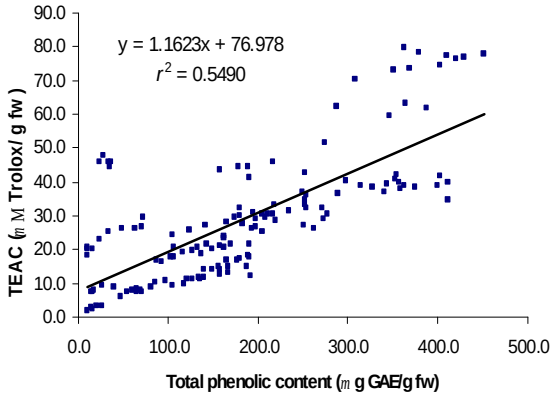
Data on a total of 140 fruit samples were available for analysis. Total acid (TA), total phenolic (TP), ascorbic acid (AA) contents and their Trolox equivalent antioxidant activity (TEAC) were measured in all samples. The antioxidant content was determined using a standard curve of Trolox (0 - 100 μM) and percent reduction of DPPH$^{\bullet}$ absorbance (r^2 = 0.9998).

The results of total acid, total phenolic, ascorbic acid contents and TEAC of 28 kinds of fruits are summarized in Table 1 in an ascending order of their antioxidant activity. The fruit with the highest antioxidant activity was hog plum or *Spondias pinnata* Kurz. (77.53 ± 1.29 μM TEAC/g fw) and the lowest one was sandy pear or *Pyrus lindteyi* Rehd. (2.67 ± 0.55 μM TEAC/g fw).

In order to determine the total acid concentration, each extract was titrated using NaOH together with phenolphthalein. However, the results are not always satisfactory particularly in color fruit extracts. Potentiometry was thus commonly used as an alternative method. For all samples, total acid content (μg of citric acid/g fw) largely varied from 1,146.6 ± 262.8 μg in durian (*Durio zibethinus*) to 29,514.9 ± 2356.6 μg in emblic myrabolan (*Phyllanthus emblica* Linn.) using a standard curve of citric acid (r^2 = 0.9999). It could be observed that the amounts of total acid in fruit extracts were not highly correlated with the TEAC (r^2 = 0.3438) (Figure 1a).



(a)



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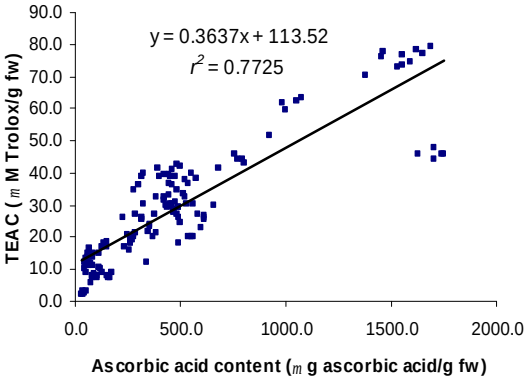

(c)

Figure 1 Linear correlation of TEAC (Y-axis) versus the total acid content (a), total phenolic content (b) and ascorbic acid content (c) (X-axis) of 140 extracts, using MS Excel software (CORREL statistical function). Note: fw = fresh weight, CAE = citric acid equivalence, GAE = gallic acid equivalence

Using the standard curve generated by gallic acid (r^2 = 0.9997), the total phenolic content of fruit sample (μg of gallic acid/g fw) was shown to vary from 13.9 ± 5.7 (in water melon) to 414.6 ± 33.0 μg in hog plum (*Spondias pinnata Kurz.)*. It was shown that the contents of phenolics in the fruit extracts were slightly correlated with their antioxidant activity (r^2 = 0.5490) (Figure 1b).

The amount of ascorbic acid varied in different fruits and ranged from 40.8 ± 8.5 to 1,707.2 ± 47.3 μg ascorbic acid/g of fresh fruit (r^2 = 0.9993 for ascorbic acid calibration curve). The highest content of ascorbic acid was detected in guava (*Psidium guajava* Linn.). It can be observed that the contents of ascorbic acid in the fruit extracts were fairly correlated with their antioxidant activity (r^2 = 0.7725) (Figure 1c).

In multiple regressions, antioxidant activity presented as TEAC value, was found to have higher correlations when more parameters (i.e., total acid, total phenolic and ascorbic acid contents) were included in the model (Table 2). The results strongly suggested a given component, either total acid, total phenolic, or ascorbic acid content, had a low correlation with antioxidant activity. On the other hand, when all three components were considered simultaneously (TA + TP + AA model), they had the highest correlation with the antioxidant activity (r^2 = 0.973, F = 1660.721, *SEE* = 3.0696, P < 0.001). With the highest coefficient of determination and lowest standard error of the estimate (*SEE*), this model was considered the best one and its regression model is expressed as follow:

$$TEAC = 1.527 \times 10^{-4}(\pm 4.0 \times 10^{-5})TA + 7.360 \times 10^{-2}(\pm 2.41 \times 10^{-3})TP + 2.776 \times 10^{-2}(\pm 7.1 \times 10^{-4})AA + 0.131(\pm 0.567) \quad \text{(eq. 1)}$$

$$n = 140,\ r^2 = 0.973,\ F = 1660.721,\ SEE = 3.0696,\ P < 0.001$$

Table 2 Correlation between antioxidant activity and parameters evaluated from various linear regression analyses

Model with parameter(s)	r^2	*SEE*	*F*-value	*P*-value
TA content	0.344	15.1435	72.298	< 0.001
TP content	0.549	12.5541	167.997	< 0.001
AA content	0.772	8.9171	468.508	< 0.001
TA + TP content	0.671	10.7674	139.488	< 0.001
TA + AA content	0.791	8.5803	259.034	< 0.001
TP + AA content	0.971	3.2050	2278.961	< 0.001
TA + TP + AA content	0.973	3.0696	1660.721	< 0.001

Note: r^2 = coefficient of determination, *SEE* - standard error of the estimate, TA = total acid, TP = total phenolic, AA = ascorbic acid

Based on the model with 3 components, we calculated the predicted TEAC value for each sample. High correlation between the measured TEAC and the predicted values of 140 samples was obtained with a slope of 0.9742 and r^2 of 0.9734 (Figure 2).

In conclusion, the antioxidant activity of fruit extract could be predicted by total acid, total phenolic and ascorbic acid contents, which also suggested that these fruit extracts acted as antioxidant food by multiple components.

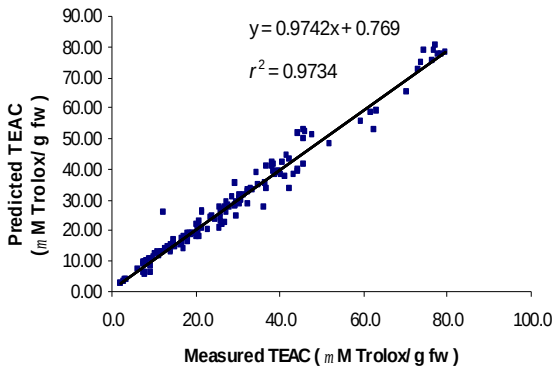

Figure 2 Scatter plot of measured and predicted TEAC values, based on linear regression equation (eq. 1) of 140 fruit extract samples

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