Phytochemical Composition, Antioxidant and Antityrosinase Activities, and Quantification of (+)-Dihydrokaempferol of Different Parts of *Manilkara zapota*

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Chunhakant *et al.*: Quantification of (+)-Dihydrokaempferol in *Manilkara zapota*

Free radicals can cause oxidative damage in biomolecules and lead to pathological diseases. Antioxidants can protect against the oxidation of living cells. The aim of this study was to evaluate the total phenolic and flavonoid contents and the antioxidant and antityrosinase activities of different parts of *Manilkara zapota* and measure the quantity of (+)-dihydrokaempferol in different plant parts. The bark, flowers, fruit, leaves, roots, seeds and wood of *Manilkara zapota* were extracted with methanol and water. All the crude extracts were evaluated for biological activities and measured the quantity of (+)-dihydrokaempferol using high performance liquid chromatography. The methanol crude extract of the flowers showed the highest total phenolic (743±29 milligrams of gallic acid equivalent per gram crude extract) and flavonoid contents (133.8±3.1 milligrams of quercetin per gram of crude extract). Moreover, it exhibited the strongest antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl (half maximal inhibitory concentration of 22.74±0.67 µg/ml), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (half maximal inhibitory concentration of 20.89±0.17 µg/ml) and ferric reducing antioxidant power values (790.22±0.81 milligrams of trolox equivalent per gram of crude extract). The methanol crude extract of the bark displayed the strongest monophenolase inhibitory activity (half maximal inhibitory concentration of 85.2±2.1 µg/ml), while the methanol crude extract of the roots exhibited the highest diphenolase inhibitory activity (half maximal inhibitory concentration of 33.52±0.68 µg/ml). (+)-Dihydrokaempferol was found in the bark, flowers, leaves, roots and wood. The highest content of (+)-dihydrokaempferol was detected in the methanol crude extract of the bark. These results suggested that the flowers of *Manilkara zapota* may be used as a source of natural antioxidants and the bark and roots may be beneficial sources of tyrosinase inhibitors.

Key words: *Manilkara zapota*, dihydrokaempferol, antioxidant, antityrosinase activity, high performance liquid chromatography

Reactive oxygen species (ROS) are chemically reactive molecules and free radicals containing oxygen. ROS are formed as natural by-products of the normal metabolism of oxygen[1]. ROS have roles in cell signaling, including in apoptosis, gene expression and the activation of cell signaling cascades[2]. The effect of ROS on cellular processes is a function of the strength, duration and context of exposure. High levels of ROS lead to cellular damage, oxidative stress and the damaging of lipids, proteins and nucleic acids[3]. Thus, elevated ROS are linked to myriads of pathologies, such as skin photoaging and photocarcinogenesis leading to rheumatoid arthritis, asthma, cancer, diabetes, inflammation and hypertension, as well as neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases[4,5]. Antioxidants protect the body from oxidative stress and damage caused by free radicals. They either protect from the oxidation of radical chain reactions or inhibit the processing of oxidation. Natural antioxidants, such

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as phenolic compounds and flavonoids were reported to protect against oxidative stress, act as reducing agents for ROS production and delay skin aging[6]. Moreover, they reduce chelated metal ions and inhibit the increasing of melanin synthesis. The antioxidant activity of phenolic compounds and flavonoids depends on the number of hydroxyl groups in the structure[7]. Furthermore, phenolic compounds protect against free radical processes and reduce the production of browning[8]. Phenolic compounds are secondary metabolites in plants that possess several biological activities, such as anticancer, antidiabetic, antihyperlipidemic, antimicrobial, antioxidant and antityrosinase activities[9,10]. Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that is distributed in microorganisms, animals and plants[11]. Tyrosinase catalyzes both the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to (2S)-2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl)propanoic acid (dopaquinone) in the melanin biosynthesis pathway[12]. A serious esthetic problem of melanin products is hyperpigmentation, such as melasma, age spots and freckles. Commercial skin whitening agents such as arbutin and kojic acid inhibit tyrosinase from protecting against skin hyperpigmentation. However, they cause long-term side effects such as allergic reactions, photosensitivity and skin irritation[13]. Thus, effective and safe tyrosinase inhibitors from medicinal plants are still being investigated[13,14]. *Manilkara zapota* (*M. zapota*) belongs to the Sapotaceae family. It is a native plant of South America and widely cultivated throughout the tropics. Ripe fruits are edible with a sweet taste[15,16]. Previous reports indicated that the phytochemicals of *M. zapota* exhibited various biological activities. It was reported that triterpenoids and flavonoids from its bark and seeds exhibited antioxidant, anti-inflammatory, antipyretic, antidiabetic, antiaging, antilipidemic and acaricidal activities[17,18]. The fruit of *M. zapota* is a rich source of polyphenolic compounds that has significant antioxidant, antihyperglycemic and hypercholesterolemic activities[19,20]. Several triterpenoids were isolated from the bark and fruit of *M. zapota* and they exhibited antitumor activity[21,22]. Its seeds showed antibacterial activity[18] and the seed coats exhibited tyrosinase inhibitory activity[23]. (+)-Dihydrokaempferol (fig. 1), a flavonoid, was isolated from the bark of *M. zapota* and it exhibited potent *in vitro* cytotoxicity in various human tumor cell lines, such as the breast (BT474), colon (SW620), gastric (KATO-III), liver (HepG2) and lung bronchus- (Chago-K1) carcinoma cell
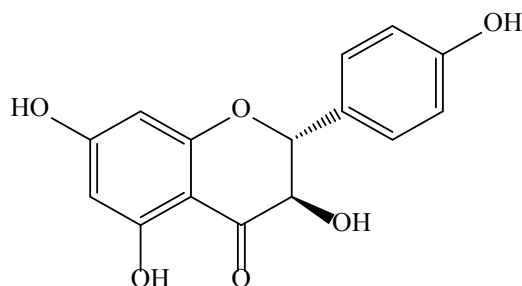

Fig. 1: Chemical structure of (+)-dihydrokaempferol

lines[24]. Additionally, (+)-dihydrokaempferol exhibited significant antityrosinase and antioxidant activities[24]. Therefore, it may be of use as a marker compound for biologically active compounds present in *M. zapota*. A standardization of the chemical profiles of plant extracts may be useful for the elucidation of the chemical components and biological properties of plant extracts.

In the present study, total phenolic and flavonoid contents of different parts of *M. zapota*, including bark, flowers, fruit, leaves, roots, seeds and wood, were evaluated. Antioxidant activity of the crude extracts was determined by *in vitro* assays, namely, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assays, with the Ferric Reducing Antioxidant Power (FRAP) assay. The antityrosinase activity of the crude extracts was also investigated *in vitro*. Furthermore, the quantification of the (+)-dihydrokaempferol content of the different parts of *M. zapota* was evaluated using High Performance Liquid Chromatography (HPLC).

MATERIALS AND METHODS

ABTS, DPPH, L-DOPA, L-tyrosine, 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and mushroom tyrosinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin–Ciocalteu reagent, absolute ethanol, arbutin, gallic acid, Dimethyl Sulfoxide (DMSO), methanol (MeOH), potassium persulfate, kojic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, acetic acid and quercetin were purchased from Merck (Darmstadt, Germany). HPLC grade solvents were purchased from Merck (Darmstadt, Germany), including Acetonitrile (ACN), water and methanol. Aluminium chloride, ferric chloride, sodium carbonate, sodium hydroxide, sodium acetate (anhydrous) and sodium nitrite were purchased from Fisher Scientific (Loughborough, UK). (+)-Dihydrokaempferol was isolated in our laboratory

from *M. zapota* bark and used as a reference compound for HPLC analysis.

Crude extracts were evaporated using a vacuum rotary evaporator (Tokyo Rikakikai, Tokyo, Japan). Ultraviolet (UV) absorbance was measured on a multimode plate reader (PerkinElmer, Hamburg, Germany). HPLC analysis was performed on an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany). HPLC equipment consisted of a G1379A model vacuum degasser, a G1311A model quaternary pump and a G1315B model diode array detector (Agilent Technologies, Waldbronn, Germany).

Plant material:

The bark, flowers, fruit, leaves, roots, seeds and wood of *M. zapota* were collected from Saraburi province, Thailand. A voucher specimen (BKF No. 187749) was deposited at the Forest Herbarium Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Plant extraction:

The bark, flowers, fruit, leaves, roots, seeds and wood of *M. zapota* were finely chopped and dried in a hot air oven at 60. Each 500 g of dried sample was extracted with methanol (2×0.5 l) and water (2×0.5 l) by maceration for 24 h and filtered through Whatman No. 1 filter paper. The filtrate was then evaporated under reduced pressure to obtain the methanol and aqueous crude extracts of the seven different parts of *M. zapota*.

Total phenolic content:

Total phenolic content was determined using a Folin-Ciocalteu assay[25]. First, 100 µl of sample (1 mg/ml) was added to 6 ml of water. After that, 500 µl of undiluted Folin–Ciocalteu reagent was added to the solution and it was incubated for 1 min at 37°. Then, 1.5 ml of 20 % w/v sodium carbonate was added and the reaction mixture was made up to 10 ml in a volumetric flask with water. Absorbance was measured at 760 nm after 2 h incubation. Total phenolic content was expressed as the milligrams of gallic acid equivalent per gram of crude extract (mg GAE/g crude extract).

Total flavonoid content:

Total flavonoid content was determined by a modified method of Tohidi *et al.*[26]. First, 125 µl of sample (1 mg/ml) was added to 75 µl of 5 % w/v sodium nitrite. The solution was incubated for 6 min at 37° and 150 µl of 10 % w/v aluminium chloride was then added. After 5 min of incubation, 750 µl of 1 M sodium hydroxide was added and the final volume of the reaction mixture was made up to 2500 µl with water. After incubation for 15 min at 37°, absorbance was measured at 510 nm. Total flavonoid content was expressed as the milligrams of quercetin per gram of crude extract (mg QE/g crude extract) by using a quercetin calibration curve.

Antioxidant activity, DPPH radical scavenging assay:

The DPPH radical scavenging activity was determined using a modification method by Sulaiman *et al.*[27]. Briefly, the reaction solution contained 50 µl of the sample (100 mg/ml) and 150 µl of the 0.05 M DPPH solution in methanol. The reaction mixture was then vortexed and incubated in the dark for 30 min at 37°. Absorbance of the reaction mixture was recorded at 517 nm. Trolox was used as a standard control. The DPPH scavenging effect was calculated using the following equation 1.

$$\text{DPPH radical scavenging activity (\%)}=[1-[(A_s-A_b)/A_d]\times100 \tag{1}$$

where, A_s is the absorbance of the sample mixed with DPPH solution, A_b is the absorbance of the sample without the DPPH solution and A_d is the absorbance of the DPPH solution without sample.

ABTS radical scavenging assay:

ABTS scavenging capacity was determined using a method modified from Wootton-Beard *et al.*[28]. The stock solution contained 100 ml of 7.0 mM ABTS solution and 100 ml of 2.4 mM potassium persulfate solution. The solution mixture was then left in the dark for 14 h at 37°. Then, 1 ml of ABTS solution was added to the reaction mixture and diluted with 60 ml of absolute ethanol to evaluate an absorbance of 0.700±0.001 absorbance units at 734 nm. After that, 500 µl of the sample (100 mg/ml) was added to 500 µl of the ABTS solution and absorbance was measured at 734 nm after 7 min incubation. The ABTS scavenging capacity of Trolox was assayed for comparison. The percentage of scavenging activity was calculated with the equation 2.

$$\text{ABTS radical scavenging activity (\%)}=[(A_c-A_s)/A_c]\times100 \tag{2}$$

where, A_c is the absorbance of the control and A_s is the absorbance of the ABTS radical with the sample.

FRAP assay:

The FRAP assay was determined using a protocol modified from Wootton-Beard *et al.*[28]. The FRAP reagent was prepared by mixing 25 ml of 0.3 M acetate buffer (pH 3.6), 2.5 ml of 20 mM ferric chloride solution and 2.5 ml of 10 mM TPTZ and total volume was adjusted to 50 ml using 40 mM HCl solution. The FRAP reagent was then put in a water bath for 30 min at 50°. Then, 600 µl of the FRAP reagent was reacted with 25 µl of the sample (100 mg/ml). After 4 min of incubation at 37°, absorbance was measured at 595 nm. Trolox was used as a positive control. Ferric reducing capacity was expressed as the milligrams of trolox equivalent per gram of crude extract (mg TE/g crude extract).

Antityrosinase activity:

The inhibition of tyrosinase activity was determined using a method modified from Dej-adisai *et al.*[29] and performed by measuring the enzymatic reaction using L-tyrosine (monophenolase) and L-DOPA (diphenolase) substrates. Briefly, the sample (1 mg/ml) was dissolved in water. The solution mixture contained 150 µl of 0.2 M sodium phosphate buffer (pH 6.8), 50 µl of the sample and 50 µl of 500 µM substrate solution. The reaction solution was blended and incubated for 10 min at 37°. Absorbance at 490 nm (t=0 min) was immediately recorded after 50 µl of tyrosinase solution (200 U/ml) was added. Finally, the reaction mixture was incubated for 20 min at 37° and absorbance was again measured at 490 nm (t=20 min). The percentage of inhibition was calculated using the equation 3.

Tyrosinase inhibitory activity (%)=[(A–B)–(C–D)/(A–B)]×100 (3)

where, A is the difference of UV absorbance of the control at t=0 and 20 min, B is the difference of UV absorbance of the blank control at t=0 and 20 min, C is the difference of UV absorbance of the test sample and the positive control at t=0 and 20 min and D is the difference of UV absorbance of the blank of the test sample and the positive control at t=0 and 20 min.

Chromatographic conditions:

Quantitative analysis by HPLC was optimized on an octadecylsilyl (ODS) Thermo Hypersil Keystones column (250×4.6 mm internal diameter (i.d.,) 5 µm, YMC Co., Kyoto, Japan) equipped with a guard column (20×3.0 mm i.d., 3.5 µm, Phenomenex Inc., Torrance,

California, USA). The gradient-elution system was carried out with 0.1 % v/v formic acid in water (A) and acetonitrile:methanol (1:4 % v/v, B) using the following gradient: 100 % A at 0-10 min, 80 % A at 11-30 min, 60 % A at 31-60 min, 40 % A at 61-100 min, 20 % A at 101-120 min and 100 % B at 121-160 min. Sample was filtered through a 0.45 µm Polytetrafluoroethylene (PTFE) membrane filter and 10 µl of the sample solution was injected into the HPLC system for analysis. The column was operated at 25°. The flow rate of the mobile phase was 1.0 ml/min. The absorbance was measured at a wavelength of 254 nm.

HPLC method validation:

Quantification of (+)-dihydrokaempferol in the aqueous and methanol crude extracts of the different parts of *M. zapota* was performed after validation of the HPLC method. Analytical method validation was achieved in terms of linearity, sensitivity, accuracy and precision.

Linearity: The (+)-dihydrokaempferol standard solutions were prepared with methanol to obtain eight concentrations of 100, 200, 400, 600, 800, 1000, 1200 and 1400 ng/ml. Solutions were filtered before analysis through a 0.45 µm PTFE membrane filter. Seven repetitions were conducted in this experiment. The calibration curve was plotted with peak area versus concentration using the method of least squares regression analysis. The values of the slope and y-intercept of the plot were calculated.

Sensitivity: Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined using a linear calibration curve of (+)-dihydrokaempferol standards. (+)-Dihydrokaempferol was prepared at different concentrations of 200, 400, 600, 800, 1000 and 1200 ng/ml. Seven repetitions of each concentration were injected into the chromatographic column. The LOD and LOQ were calculated according to the following equation 4 and equation 5.

$$LOD = [3.3 \times S_y]/A_m \qquad (4)$$

$$LOQ = [10 \times S_y]/A_m \qquad (5)$$

where, S_y is the standard deviation of the y-intercept and A_m is the average slope.

Accuracy: The percentage recovery was used to evaluate the accuracy of the method. Three different concentrations of (+)-dihydrokaempferol (n=7) were obtained from six different concentrations and analyzed. Briefly, (+)-dihydrokaempferol was prepared at

concentrations of 400, 600 and 800 ng/ml in methanol. Then, 1 ml of the methanol crude extract of barks (400 ng/ml) was added to 1 ml of (+)-dihydrokaempferol solution. All data were calculated using the following equation 6,

$$Recovery\ (\%)=[(C_1–C_2)/C_3]\times100 \qquad (6)$$

where, C_1 is the sample contents after adding standard solution, C_2 is the sample before adding standard solution and C_3 is the standard solution.

Precision: The percentage relative standard deviation (% RSD) was used to determine both intra and inter-day precision of the method. Intra-day precision was computed by evaluating with three different concentrations of (+)-dihydrokaempferol (n=7) that were obtained from six different concentrations and analyzed. (+)-Dihydrokaempferol was prepared at concentrations of 400, 600 and 800 ng/ml in methanol. Inter-day precision was measured by investigating the above concentration (n=7) on five consecutive days. All data were computed by the equation 7.

$$\%\ RSD=[SD/\bar{x}]\times100 \qquad (7)$$

where, SD is standard deviation and $\bar{x}$ is the mean of the concentration of the standard solution.

Quantitative analysis of (+)-dihydrokaempferol in different parts of *M. zapota* by HPLC:

The validated HPLC method was used for quantitative analysis of (+)-dihydrokaempferol in the aqueous and methanol crude extracts of different parts of *M. zapota*. The sample solution and (+)-dihydrokaempferol were dissolved in methanol. The (+)-dihydrokaempferol content of the crude extracts of the different parts of *M. zapota* were expressed as milligrams of (+)-dihydrokaempferol/gram of crude extract (mg/g crude extract).

Statistical analysis:

Results were expressed as mean±SD. Statistical data analysis was performed with Statistical Package for the Social Sciences (SPSS) version 24 and differences were considered significant at p<0.05.

RESULTS AND DISCUSSION

Plant extraction was done and the extraction yield of the aqueous crude extracts of *M. zapota* was in the range of 5.4–34.0 % and the extraction yield of the methanol crude extracts was in the range of 6.7–39.7 % (Table 1). The fruits gave the maximum yield for both

the aqueous and methanol crude extracts.

Total phenolic and flavonoid contents were determined. In the present study, the methanol crude extracts of the flowers and leaves, and the aqueous crude extracts of the fruit and roots showed high total phenolic contents, which were 743±29, 675±19, 727±53 and 698±46 mg GAE/g crude extract, respectively (Table 2). The wood presented a low content of phenolic compounds in both the aqueous and methanol crude extracts. The high content of flavonoids of the methanol and aqueous crude extracts of flowers and the methanol crude extract of seeds, were determined to be 133.8±3.1, 131.6±1.4 and 127.8±1.7 mg QE/g crude extract, respectively (Table 2). These values were not significantly different (p<0.05). This was followed by the methanol crude extract of bark, which was 116.1±4.2 mg QE/g crude extract. The wood displayed a low content of flavonoids in both the aqueous and methanol crude extracts. Phytochemicals of *M. zapota* were reported as alkaloids, saponins, sterols, tannins, triterpenoids and phenolic compounds[16,24,30-32]. Previous studies reported that the acetone crude extract of *M. zapota* leaves, the ethanol crude extracts of *M. zapota* fruit and pulp, and the methanol crude extracts of *Manilkara hexandra* (*M. hexandra*) fruit and seeds contained a high amount of phenolic compounds[32-35]. In contrast, the aqueous crude extracts of *M. zapota* fruit pulps and peels showed low total phenolic content[36]. In addition, the acetone crude extract of *M. zapota* leaves and the ethyl acetate crude extract of *M. zapota* seed coats were previously reported to show a high amount of flavonoids[33,37]. The methanol extracts of *M. hexandra* fruit and seeds also consisted of flavonoids[34]. The phenolic compounds; methyl-4-O-galloylchlorogenate and 4-O-galloylchlorogenic acid were isolated from *M. zapota* fruit and 3,4-dihydroxybenzoic acid, (+)-dihydrokaempferol and 6-hydroxyflavanone

TABLE 1: EXTRACTION YIELD OF DIFFERENT PARTS OF *M. zapota*

Plant part	Extraction yield (% w/w dry weight)	
	Aqueous crude extract	Methanol crude extract
Bark	12.5	14.5
Flowers	9.4	6.7
Fruit	34.0	39.7
Leaves	20.7	20.1
Roots	11.6	11.0
Seeds	5.4	7.3
Wood	10.6	18.8

TABLE 2: TOTAL PHENOLIC AND FLAVONOID CONTENTS OF DIFFERENT PARTS OF *M. zapota*

Plant part	Solvent extract	Total phenolic content (mg GAE/g crude extract)	Total flavonoid content (mg QE/g crude extract)
Bark	Methanol	495±40[f]	116.1±4.2[e]
	Water	220±34[bc]	64.5±2.8[b]
Flowers	Methanol	743±29[j]	133.8±3.1[g]
	Water	227±17[bc]	131.6±1.4[fg]
Fruit	Methanol	633±47[g]	76.7±1.8[c]
	Water	727±53[hi]	74.7±4.1[c]
Leaves	Methanol	675±19[gh]	97.8±1.5[d]
	Water	545±36[f]	75.6±2.1[c]
Roots	Methanol	307.5±6.7[d]	74.5±2.5[c]
	Water	698±46[hi]	101.2±1.0[d]
Seeds	Methanol	398±46[e]	127.8±1.7[f]
	Water	249±10[c]	65.4±1.3[b]
Wood	Methanol	178±8[ab]	56.7±4.0[a]
	Water	144±31[a]	63.6±5.1[b]

Note: Values are mean±SD (n=3). Statistical comparison between values of different extracts was done using Duncan's multiple range test (p<0.05)

were isolated from *M. zapota* bark[23,24]. Gallic acid, a phenolic acid, was reported to be a major component of *M. hexandra* fruit and seeds[34]. The contents of phenolic compounds and flavonoids of the extracts depend on extraction procedures, temperature and solvent polarity in extract preparation[9]. Furthermore, the content of flavonoids is correlated with the content of phenolic compounds[38,39] because flavonoids are one of the major phenolic compounds in *Manilkara* species.

The DPPH radical scavenging activity of the aqueous and methanol crude extracts of *M. zapota* was expressed as the Half Maximal Inhibitory Concentration or 50 % Inhibitory Concentration (IC_{50}) as shown in Table 3. The methanol crude extract of flowers showed the highest DPPH radical scavenging capacity with an IC_{50} value of 22.74±0.67 µg/ml. The aqueous crude extracts of the fruit and roots also displayed potent DPPH radical scavenging capacity with IC_{50} values of 33.08±0.26 and 44.24±0.49 µg/ml, respectively, whereas, the wood exhibited weak DPPH radical scavenging activity. Trolox exhibited greater DPPH radical scavenging capacity than that of the crude extracts of *M. zapota* with an IC_{50} value of 6.06±0.01 µg/ml (24.21±0.04 µM). The results of ABTS radical scavenging activity of the aqueous and methanol crude extracts of *M. zapota* are presented in Table 3. The methanol crude extract of the flowers exhibited the highest ABTS radical scavenging activity with an IC_{50} value of 20.89±0.17 µg/ml, followed by the aqueous crude extracts of the fruits and roots with IC_{50} values of 35.29±0.58 and 41.34 ±0.65 µg/ml, respectively. The methanol crude extracts of the roots, leaves and fruit exhibited

similar ABTS radical scavenging activity with IC_{50} values of 54.59±0.22, 55.28±0.24 and 55.43 ±0.55 µg/ml, respectively. The wood showed low ABTS radical scavenging activity. Trolox exhibited ABTS radical scavenging activity with an IC_{50} value of 6.04 ±0.01 µg/ml (24.13±0.04 µM). FRAP activity of the aqueous and methanol crude extracts of *M. zapota* was expressed as statistical trolox equivalents (Table 3). The methanol crude extracts of the flowers and leaves, and the aqueous crude extracts of the fruit and roots, showed high reducing capacity with FRAP values of 790.22±0.81, 718.89±0.77, 728.67±0.75 and 725.33±0.75 mg TE/g crude extract, respectively. This was followed by the methanol crude extract of the fruit with a FRAP value of 624.22±0.64 mg TE/g crude extract, while the wood showed low FRAP activity. This plant has previously been reported that the acetone crude extract of *M. zapota* leaves exhibited stronger DPPH radical scavenging activity than that of ascorbic acid[33]. The extract of the fresh pulps of *M. zapota* exhibited stronger DPPH radical scavenging activity than that of the dry pulp extract[30]. The methanol extracts of *M. hexandra* fruit and seeds also showed potent antioxidant activity[34], similar to the methanol extract of *M. zapota* leaves[20]. In contrast, the crude extract of *M. zapota* fruits showed low DPPH radical scavenging activity and the 60 % ethanol crude extract of *M. zapota* seeds exhibited no effect on DPPH radical scavenging activity[30,40]. Usually, phenolic compounds act as reducing agents and react with free radicals via the hydroxyl groups in hydrogen atom or single electron transfer mechanisms[41,42]. Phenolic compounds and

TABLE 3: ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF *M. zapota*

Plant part/Standard	Solvent extract	IC_{50} (µg/ml)		FRAP (mg TE/g crude extract)
		DPPH	ABTS	
Bark	Methanol	66.42±0.70[h]	70.54±0.16[h]	438.22±0.52[e]
	Water	77.99±0.53[k]	95.9±1.1[l]	183.78±0.35[bc]
Flowers	Methanol	22.74±0.67[b]	20.89±0.17[b]	790.22±0.81[h]
	Water	77.84±0.20[k]	94.93±0.78[k]	257.33±0.35[cd]
Fruit	Methanol	58.0±1.5[f]	55.43±0.55[e]	624.22±0.64[g]
	Water	33.08±0.26[c]	35.29±0.58[c]	728.67±0.75[h]
Leaves	Methanol	55.4±1.2[e]	55.28±0.24[e]	718.89±0.77[h]
	Water	63.20±0.33[g]	67.95±0.63[g]	530.22±0.60[f]
Roots	Methanol	69.89±0.39[j]	54.59±0.22[e]	307.56±0.41[d]
	Water	44.24±0.49[d]	41.34±0.65[d]	725.33±0.75[h]
Seeds	Methanol	68.2±1.2[i]	64.85±0.53[f]	411.78±0.51[e]
	Water	77.10±0.33[k]	72.93±0.63[i]	276.00±0.34[d]
Wood	Methanol	84.61±0.24[l]	93.89±0.52[j]	164.44±0.29[b]
	Water	94.59±0.52[m]	98.34±0.44[m]	66.67±0.18[a]
Trolox		6.06±0.01[a]	6.04±0.01[a]	

Note: Values are mean±SD (n=3). Statistical comparison between values of different extracts was done using Duncan's multiple range test (p<0.05)

flavonoids were reported to have significant DPPH scavenging and metal chelating activities[32]. The number and substitution pattern of hydroxyl groups in phenolic compounds and flavonoids are responsible for free radical scavenging potential[43,44]. Regarding DPPH scavenging activity, antioxidants transfer hydrogen atoms to free radicals and ABTS radical scavenging activity revealed an electron transfer system. In terms of FRAP, antioxidants reduced ferric ions to ferrous ions[45-47]. Phenolic compounds and flavonoids are well known in protection against skin aging, cancer, diabetes and neurodegenerative diseases[48,49].

The seven different parts of *M. zapota* were investigated for tyrosinase inhibitory activity, including both monophenolase and diphenolase inhibitory activities (Table 4). Results indicated that the methanol crude extracts of the bark (IC_{50} 85.2±2.1 µg/ml) and roots (IC_{50} 87.9±1.2 µg/ml) showed stronger monophenolase inhibitory activity than that of other crude extracts. The methanol crude extract of the fruit (IC_{50} 466.3±1.9 µg/ml) showed the weakest monophenolase inhibitory activity. The aqueous crude extracts of the leaves (IC_{50} 128.54±0.42 µg/ml) and roots (IC_{50} 107.24 ±0.41 µg/ml), and the methanol crude extracts of the bark (IC_{50} 85.2±2.1 µg/ml), leaves (IC_{50} 108.0±1.1 µg/ml) and roots (IC_{50} 87.9±1.2 µg/ml) showed stronger monophenolase inhibitory activity than that of arbutin (IC_{50} 134.23±0.53 µg/ml, 493.0±2.0 µM). However,

kojic acid showed the strongest monophenolase inhibitory activity with an IC_{50} value of 1.19±0.02 µg/ml (8.37±0.14 µM). For diphenolase inhibitory activity, the methanol crude extracts of the roots (IC_{50} 33.52 ±0.68 µg/ml), leaves (IC_{50} 48±10 µg/ml) and flowers (IC_{50} 51.01±0.33 µg/ml) exhibited strong antityrosinase activity. The weakest diphenolase inhibitory activity was found in the aqueous crude extract of the bark (IC_{50} 437.6±1.7 µg/ml). Kojic acid and arbutin displayed potent diphenolase inhibitory activity with IC_{50} values of 2.48±0.02 µg/ml (17.45±0.14 µM) and 8.62 ±0.11 µg/ml (31.66±0.41 µM), respectively. A previous study reported that the ethyl acetate crude extract of *M. zapota* bark exhibited moderate diphenolase inhibitory activity and the methanol crude extract of *M. zapota* leaves showed moderate monophenolase inhibitory activity[23,24]. Moreover, 3,4-dihydroxybenzoic acid, (+)-dihydrokaempferol, 6-hydroxyflavanone and myricetin-3-O-α-L-rhamnoside that were isolated from *M. zapota* showed moderate antityrosinase activity[23,24]. Furthermore, antityrosinase activity is based on the number and position of the substitution of hydroxyl groups of phenolic compounds that bind to active sites of tyrosinase and result in low enzymatic activity[44,50]. (+)-Dihydrokaempferol was reported to be a strong antioxidant and tyrosinase inhibitor[24]. It was described that its hydroxyl group may be correlated with both high ABTS and FRAP activities[51]. Thus, quantification

TABLE 4: ANTITYROSINASE ACTIVITY OF DIFFERENT PARTS OF *M. zapota*

Plant part/Standard	Solvent extract	IC$_{50}$ (µg/ml)	
		Monophenolase inhibitory activity	Diphenolase inhibitory activity
Bark	Methanol	85.2±2.1[b]	66.18±0.82[d]
	Water	175.90±0.76[i]	437.6±1.7[l]
Flowers	Methanol	181.7±1.2[j]	51.01±0.33[c]
	Water	171.0±3.3[h]	203±18[i]
Fruit	Methanol	466.3±1.9[n]	68.2±5.3[d]
	Water	266.8±1.4[m]	262.2±6.9[j]
Leaves	Methanol	108.0±1.1[d]	48±10[c]
	Water	128.54±0.42[e]	337.5±9.0[k]
Roots	Methanol	87.9±1.2[c]	33.52±0.68[b]
	Water	107.24±0.41[d]	83.3±1.6[e]
Seeds	Methanol	166.79±0.37[g]	133.6±1.7[g]
	Water	236.3±2.6[l]	82.7±2.7[e]
Wood	Methanol	269.24±0.61[m]	174.8±1.9[h]
	Water	218.0±1.8[k]	117.64±0.32[f]
Arbutin		134.23±0.53[f]	8.62±0.11[a]
Kojic acid		1.19±0.02[a]	2.48±0.02[a]

Note: Values are mean±SD (n=3). Statistical comparison between values of different extracts was done using Duncan's multiple range test (p<0.05)

of (+)-dihydrokaempferol in the aqueous and methanol crude extracts of the different parts of *M. zapota* was performed via a validated HPLC method.

Analytical method validation of (+)-dihydrokaempferol was achieved and showed good linearity, sensitivity, accuracy and precision. The linear relationship between concentration of the (+)-dihydrokaempferol solution and peak area was within the range of 200-1200 ng/ml. As a result, the regression equation was y=0.6339x–40.869, with correlation coefficient (R²) 0.9991. LOD and LOQ were estimated for (+)-dihydrokaempferol at a concentration of 200-1200 ng/ml. LOD and LOQ values were found to be 214.5 and 650.0 ng/ml, respectively. Result of accuracy was calculated and reported in terms of percentage recovery (Table 5). The percentage recovery of (+)-dihydrokaempferol was found to range from 99.80 to 102.35 %. Intra and inter-day assays were used to determine the precision of HPLC method (Table 6). The intra and inter-day % RSD were found to be in the range of 0.04-0.07 % and 0.05-0.06 %, respectively.

Quantitative analysis of (+)-dihydrokaempferol in different parts of *M. zapota* by HPLC is shown here. Chromatograms of the different parts of *M. zapota* are presented in fig. 2 and quantification

TABLE 5: ACCURACY RESULTS OF (+)-DIHYDROKAEMPFEROL

Concentration (ng/ml)	Recovery (%)	RSD (%)
400	99.80	0.22
600	101.55	0.13
800	102.35	0.08

of (+)-dihydrokaempferol is listed in Table 7. (+)-Dihydrokaempferol showed retention time of 54.198 min (fig. 2O) and it was detected in decreasing order of concentration, in: bark>roots>wood>flowers>leaves. The methanol crude extract of the bark (33.62±0.01 mg/g of crude extract) showed the highest content of (+)-dihydrokaempferol, followed by the aqueous crude extract of the bark (27.94±0.01 mg/g of crude extract), the methanol crude extract of the roots (23.20±0.01 mg/g of crude extract) and the methanol crude extract of the wood (22.96±0.01 mg/g of crude extract). However, (+)-dihydrokaempferol was not detected in the fruit or seeds. In the present study, the flowers contained more phenolic compounds and flavonoids than the bark. Moreover, the methanol crude extract of the flowers exhibited strong antioxidant and moderate diphenolase inhibitory activities. Therefore, a high content of (+)-dihydrokaempferol leads to strong antityrosinase activity. The presence of bioactive compounds, such as

TABLE 6: PRECISION RESULTS OF (+)-DIHYDROKAEMPFEROL

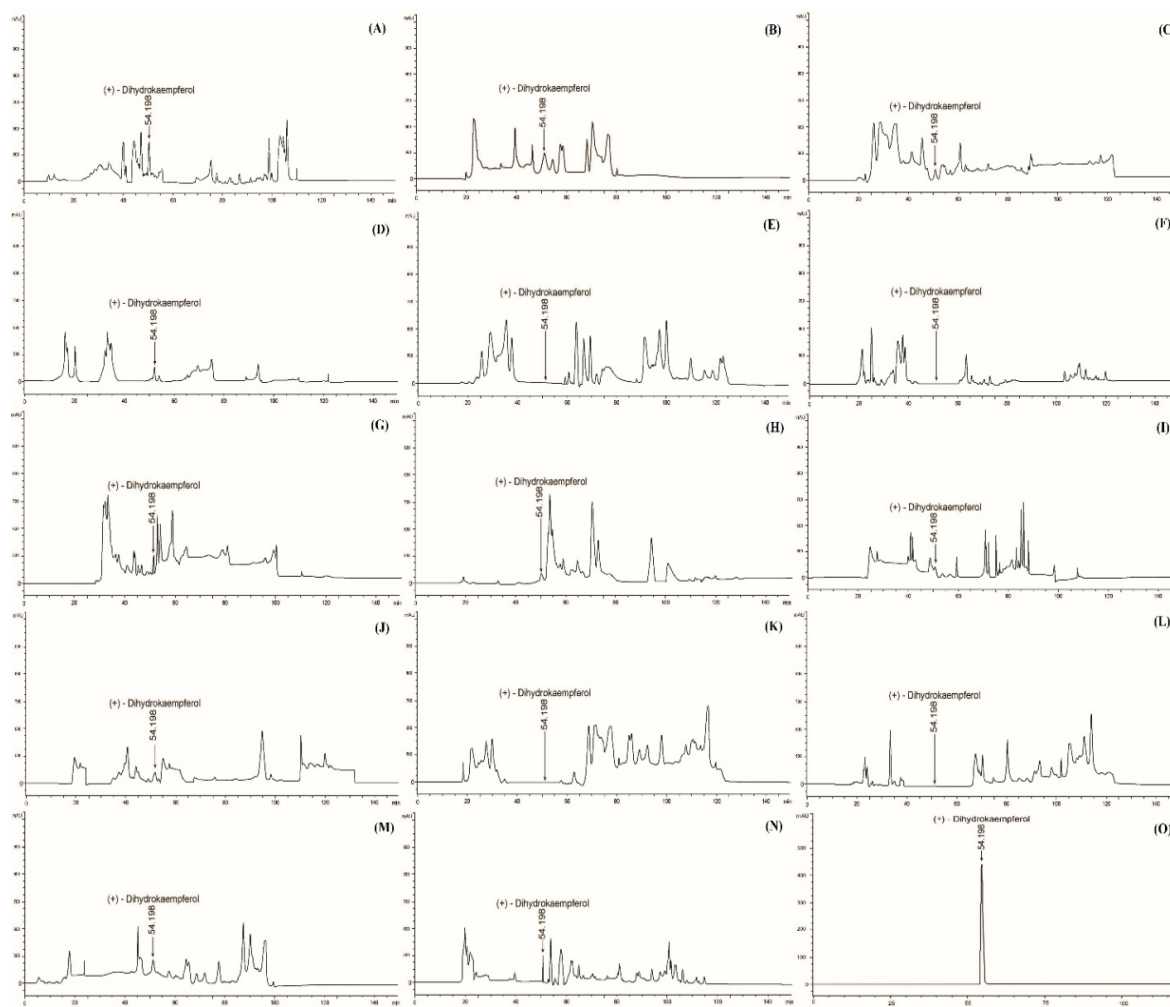| Concentration (ng/ml)	Intra-day		Inter-day	
	Mean±SD (ng/ml)	RSD (%)	Mean±SD (ng/ml)	RSD (%)
400	398.97±0.26	0.07	398.96±0.24	0.06
600	602.07±0.31	0.05	602.06±0.31	0.05
800	798.97±0.35	0.04	798.96±0.36	0.05

Values are mean±SD (n=7)

Fig. 2: HPLC chromatograms of different parts of *M. zapota*. (A) Aqueous crude extract of bark; (B) methanol crude extract of bark; (C) aqueous crude extract of flowers; (D) methanol crude extract of flowers; (E) aqueous crude extract of fruit; (F) methanol crude extract of fruit; (G) aqueous crude extract of leaves; (H) methanol crude extract of leaves; (I) aqueous crude extract of roots; (J) methanol crude extract of roots; (K) aqueous crude extract of seeds; (L) methanol crude extract of seeds; (M) aqueous crude extract of wood; (N) methanol crude extract of wood and (O) (+)-dihydrokaempferol

phenolic compounds and flavonoids, in the extracts of *M. zapota* relates to their antioxidant and antityrosinase activities.

In conclusion, the analysis of seven different parts of *M. zapota* indicated that the flowers contained the highest amounts of phenolic compounds and flavonoids and exhibited the strongest antioxidant activity, and

showed moderate diphenolase inhibitory activity. The bark presented the strongest monophenolase inhibitory activity and the roots exhibited the strongest diphenolase inhibitory activity. Quantification of (+)-dihydrokaempferol using HPLC analysis showed that this compound was presented in the bark, flowers, leaves, roots and wood, but was absent in the fruit and

TABLE 7: QUATIFICATION OF (+)-DIHYDROKAEMPFEROL IN DIFFERENT PARTS OF *M. zapota*

Plant part	Solvent extract	Content of (+)-dihydrokaempferol (mg/g of crude extract)
Bark	Methanol	33.62±0.01[i]
	Water	27.94±0.01[h]
Flowers	Methanol	11.30±0.01[e]
	Water	9.12±0.02[c]
Fruit	Methanol	ND
	Water	ND
Leaves	Methanol	8.46±0.01[b]
	Water	5.70±0.01[a]
Roots	Methanol	23.20±0.01[g]
	Water	12.44±0.01[f]
Seeds	Methanol	ND
	Water	ND
Wood	Methanol	22.96±0.01[g]
	Water	10.10±0.01[d]

Note: Values are mean±SD (n=3); ND: Value not detected

seeds. The methanol crude extract of the bark presented the highest amount of (+)-dihydrokaempferol. Therefore, the flowers of *M. zapota* may be used as a natural antioxidant source and the bark and roots may be beneficial for sources of tyrosinase inhibitors.

Conflicts of interest:

The authors declare that they have no conflicts of interests.

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