Asian Journal of Chemistry; Vol. 33, No. 11 (2021), 2608-2614

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2021.23355

Isolation and Characterization of Antioxidant Active Compounds from the Leaves and Flowers of *Manilkara hexandra* (Roxb.)

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Received: 4 June 2021; *Accepted*: 7 July 2021; *Published online*: 20 October 2021; AJC-20537

Fractionation and chromatographic purification of the crude methanol extract of air-dried leaves of *Manilkara hexandra (*Roxb) yielded five compounds *p*-methoxy methyl cinnamate (**1**), *p*-hydroxymethylcinnamate (**2**), 2,5-dihydroxybenzaldehyde (**3**), gallic acid methyl ester (**4**) and quercetin-3-O-β-D-glucoside (isoquercitrin) (**5**), whereas air-dried flowers yield phenylethylpalmitate (**6**). The structures of these compounds were confirmed using spectroscopic data, comparison with literature data and co-TLC comparison with authentic samples. To the best of our knowledge compounds **1**, **3**, **5** and **6** were isolated for the first time from this plant. Constituents of the plant were known to be active against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Comparative antioxidant activity of compounds along with the crude extract and fractions were assessed using four different assay protocols. These compounds show moderate activity in the antioxidant assay systems. Present results rationalize the usage of the leaves and flower paste as bathing aid as suggested in the folklore literature to maintain a dynamic balance of skin microflora.

Keywords: Antioxidant activity, *Manilkara hexandra*, Secondary metabolites.

INTRODUCTION

Balancing microbiota is an emerging field of research and essential understandings are still emerging. Skin infections such as atopic dermatitis, rosacea and acne are known to harbor more amounts of certain selective microorganisms over others [1]. The critical balance of skin microflora (Eubiosis) is essential to maintain healthy skin. Various factors disturb the eubiosis. Some of these factors such as UV radiation, pH of the skin, skin moisture level, skin dryness and excessive oiliness are known to tilt the microbial balance. Disturbances in these factors are the result of undesirable alterations in various chemical reactions happening inside and outside the skin [2].

Antioxidants applied on the skin are reported to work synergistically to protect the skin against UV damage [3]. Antioxidants can play a broader role in maintaining the balance of the skin microbiome. There are atleast two reasons behind such claims, as explained above, their ability to act synergistically with other known actives and their inherent ability to undergo diverse varieties of chemical reactions to counter the undesirable chemical reactions happening over the skin surface. The topical application of antioxidants is reported to reduce skin wrinkles. Maintenance of skin barrier properties strongly depends on the individual's ability to repair the challenges faced by their skin [4-7].

Folklore knowledge helps in identifying useful interventional strategies to maintain proper skin barrier function. Indian system of medicines proposes the usage of herbal paste as an aid in taking bath and usage of flower/flower essence as after bath to get the lovely fragrance. The reasons behind these practices were taught to be that of getting good cosmetic effects such as cleaning and fragrance. However, *Manilkara hexandra (*Roxb.) an evergreen tree species belongs to *Sapotaceae* family; native of south Asia carries different reasoning for this practice [8]. Prince *et al*. [9] reported that the presence of methyl-*p*-coumarate and 3,4-dihydroxybenzaldehyde in this plant suggests that usage of this plant as daily bathing aid can help in maintaining a balance of skin microbiome. Since, there is less report on the isolated secondary metabolites of *Manilkara hexandra (*Roxb.), we planned to investigate more potent metabolites as well as to estimate the antioxidant properties of the compounds.

EXPERIMENTAL

Melting points were uncorrected and measured on a Stuart™ melting point apparatus SMP3. The IR spectra were recorded on Prestige 21 FT IR (Shimadzu) model using KBr pellets. ^{1}H & ^{13}C NMR spectra of the compounds in DMSO-d_6/$CDCl_3$ were recorded on Bruker 400 MHz NMR spectrophoto-meter. Mass spectral data of the prepared compounds were recorded on Jeol SX 102/DA 600 mass spectrometer. The absorbance measurements were carried out using UV-VIS Spectrophotometer (Shimadzu, Model no: UV-1900) with 1 cm quartz cuvettes.

Column chromatography was carried on a silica gel column (100-200 mesh). All the chemicals and solvents were procured as analytical grade and used without further purification. Sodium phosphate buffer, phosphate buffered saline of required pH were prepared by the standard procedures. The purity of the samples was checked by TLC on pre-coated aluminium sheets, silica gel 60 F_{254} (20 cm × 20 cm, 0.2 mm thickness, Merck) and compounds were detected under UV light (254 & 366 nm) and spraying with 5% sulphuric acid in methanol followed by heating the plates at 110 ºC for 5 min.

Plant material: A shadow-dried aerial parts of *Manilkara hexandra (*Roxb.) leaves 2.5 kg and flowers 1.2 kg were collected from Red-Hills, Chennai and identified by a taxonomist, at Durva Herbal Centre, Chennai, India. A voucher specimen of the species was deposited in the Department of Chemistry, RK.M. Vivekananda College, Chennai, India.

Extraction and isolation: Extraction, fractionation and vacuum liquid chromatographic purification of fractions of the leaves and flowers of the plant were performed as reported earlier [9]. Fractionation of crude methanol extract (300 g) of leaves yielded four fractions, chloroform fraction (CF-L) 18.79 g, ethyl acetate fraction (EF-L) 9.4 g, *n*-butanol fraction (BF-L) 74.16 g and aqueous fraction (AF-L) 81.71 g. Vacuum liquid chromatography (VLC) of chloroform fraction yielded three fractions, Fr.1C (3.51 g), Fr.2C (0.85 g) and Fr.3C (4.3 g). VLC of ethyl acetate fraction yielded five major fractions Fr. 1E (1.11 g), Fr. 2E (0.85 g), Fr.3E (0.32 g), Fr. 4E (2.81 g), Fr. 5E (2.16 g). Similarly, fractionation of crude methanol extract of the flowers yielded four fractions, chloroform fraction (CF-F) 11.4 g, ethyl acetate fraction (EF-F) 5.4 g, *n*-butanol fraction (BF-F) 14.16 g and aqueous fraction (AF-F) 65.4 g.

Fr.1C was further purified over silica gel column repeatedly to get compound **1** (yield: 0.36 g). Based on TLC Fr.3C was combined with column chromatography fractions remaining after isolation of cinnamic acid from Fr.1E (less polar than cinnamic acid) to get 5.1 g of a crude fraction. This fraction adsorbed over silica gel and performed repeated chromatographic purification to get compound **2** (yield: 0.05 g). Similarly, fractions containing less polar compounds compared to 3,4-dihydroxybenzaldehyde obtained from Fr.5E were combined based on TLC to get a yield of 1.03g of UV254 active fraction. Repeated quick chromatographic purification of this fraction over silica gel column yields compound **3** (yield: 0.29 g).

A BF-L (25 g) was further purified over LH-20 column using varying proportions of water and methanol 100:0 to 0:100 to get four fractions. Fr.1B (yield: 12 g), Fr.2B (yield: 1.9 g), Fr.3B (yield: 5.2 g) and Fr.4B (yield: 5.7 g). Fr.4B was found to contain a two UV active compound, one of which was yellow coloured and adsorbed over 230-400 mesh silica gel and performed repeated chromatographic purification using chloroform, and chloroform:methanol 9:0.5 to 1:1 to get four fractions. Fr.1 (4B-1) (yield: 1.2 g), Fr.2 (4B-2) (yield: 0.6 g), Fr.3 (4B-3) (yield: 1.9 g) and Fr.4 (4B-4) (yield: 1.4 g). Fraction-2 (4B-2) on crystallization with methanol yielded compound **4** (yield: 0.31 g). Fr.4 (4B-4) was purified by LH-20 column using water: methanol 100:0 to 0:100, crystallization of fraction rich in UV active yellow compound with methanol yielded compound **5** (yield: 0.25 g).

A CF-F fraction was found to be waxy solid, adsorbed over silica gel 100-200 mesh and VLC performed using hexane, hexane:chloroform 9:1 to 1:9, chloroform and chloroform: methanol 8:2. Based on TLC, combined closely related fractions to get six fractions as CF-F-1 (yield: 0.6 g), CF-F-2: (yield: 0.9 g), CF-F-3: (yield: 1.6 g), CF-F-4: (yield: 1.2 g), CF-F-5: (yield: 2.5 g) and CF-F-6: (yield: 3.9 g). CF-F-1 purified over silica gel column using chloroform followed by 2% silver nitrate impregnated silica gel column to get 0.06 g of compound **6**. All the isolated compounds were studied against *Staphylococcus epidermidis* and *Staphylococcus aureus.* In this study, four different antioxidant assay methods were adopted to quantify the antioxidant activity. The crude methanol extract, four fractions and seven active principles isolated from the leaves and flowers were analyzed.

Antioxidant assay

Superoxide radical ($O_2^{\bullet-}$)scavenging activity: Super-oxide radical scavenging activity of test samples and standard were determined based on reported method with required modifications [10]. Experiments were performed using two sets of assemblies with a lining of aluminum foil; one set was maintained in dark throughout the process it served as blank in the UV absorbance measurement at 590 nm. In each set of assembly, for every sample, 3 mL reaction mixture was placed, containing sodium phosphate buffer (pH 7.6), 12 mM EDTA, 20 µg ribo-flavin, 0.1 mg NBT and 1 mL sample solution and the reaction was started in one of the assemblies by illuminating the reaction mixture using 100 W fluorescent lamp for 90 s. Absorbance at λ_{max} = 590 nm was recorded immediately for samples main-tained in both assemblies. The inhibition percentage of super-oxide anion generated was calculated using the formula given below:

$$\text{Inhibition (\%)} = \frac{A_o - A_1}{A_o} \times 100$$

where A_o = absorbance measurement of the control and A_1 = absorbance measurement of the sample/standard.

β-carotene bleaching assay (β-carotene-linoleic acid system): β-Carotene bleaching inhibition activity of the samples and standard were determined based on reported method [11] with some modifications. A solution of β-carotene in chloroform (2 mL of 0.02 %) was transferred into a 250 mL round bottom flask. Chloroform was removed completely by drying in rota-vapor under a vacuum. After this, 100 mL of aerated water

containing 40 mg of linoleic acid 400 mg of Tween-40 was added and vigorously shaken to form an emulsion. A 4.8 mL of this emulsion was used to determine the efficacy of the test samples. A 4.8 mL of emulsion and 0.2 mL of test samples were mixed and recorded the absorbance at 470 nm. The sample vials were incubated at 50 ºC for 2 h and again recorded the absorbance. A blank experiment was performed using emulsion without β-carotene and used as a control solution for UV analysis. Synthetic antioxidant, butylated hydroxyltoluene (BHT) was used as a positive control.

Antioxidant activity was calculated using the following expression:

$$\text{Antioxidant activity} = \frac{\text{β-Carotene content after 2 h of assay}}{\text{Initial β-carotene content}} \times 100$$

Metal ion chelating activity: Metal ion chelating potential of the samples and the standard were determined based on the reported method [12]. Various concentrations of test samples and standards were taken in 10 mL screw-capped vials. A $FeCl_2$ soultuion (50 µL of 2 mmol/L) was mixed well with these solutions followed by the addition of 200 µL of 5 mmol/L ferrozine. The mixture was shaken vigorously and left at room temperature for 10 min. The UV absorbance of the solution was measured at 562 nm. A blank solution was prepared without test samples/standard and used as control. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Inhibition of ferrozine-Fe}^{2+}\text{ complex }(\%) = \frac{A_o - A_1}{A_o} \times 100$$

where A_o = absorbance of the control and A_1 = absorbance of the extract/standard.

Phosphomolybdenum complex assay: The Mo(VI) to Mo(V) reducing the power of test samples and standard were determined based on the reported method [13] with slight modification. Reagent solution was prepared using 28 mM sodium phosphate, 0.6 M sulfuric acid and 4 mM ammonium molybdate were mixed. This reagent solution (3 mL) was combined with 300 µL of sample/standard solution. Blank solution was prepared using distilled water instead of sample solution. The screw-capped culture tubes were maintained at 95 ºC for 90 min. Then, the reaction mixture cooled to room temperature and UV absorbance at 695 nm for each solution was measured. The percentage of reducing power was calculated using the formula given below:

$$\text{Increase in reducing power }(\%) = \frac{A_o - A_1}{A_o} \times 100$$

where A_o is the absorbance measurement of control and A_1 is the absorbance measurement of the sample.

RESULTS AND DISCUSSION

Folklore information-based exploratory phytochemical investigation has yielded five more compounds. All the five compounds were screened for the microbiology assay to study against *Staphylococcus aureus* and *Staphylococcus epidermidis.* All the five compounds and the two active compounds reported earlier [9] were evaluated for their antioxidant activity using four different assay systems. Compound **2** was moderately active against the studied organisms, which was already studied by us [9], except this compound other compounds did not show considerable activity against these organisms. To our surprise, only 3,4-dihydroxybenzaldehyde is active while 2,5-dihydroxybenzaldehyde was not much active. However, the literature survey reveals that there is a structure-activity relationship that exists in the benzaldehyde derivatives, 3,5-derivatives of benzaldehyde and 3,4-dihydroxybenzaldehyde gives comparatively better antimicrobial action. The yield and content of these compounds (3,4-dihydroxybenzaldehyde and methyl *p*-coumarate) were very less, however, the aerial parts of the plant were suggested as bathing aid ingredients. Hence, it is hypothesized that the plant material may encompass the potential antioxidant principles to deliver topical benefits. The antioxidant potential of the seven compounds using a set of four antioxidant assay systems was also studied.

Structure elucidation

Compound 1 (*p*-methoxymethylcinnamate): White solid with a tint of pinkish colour, m.p.: 97-99 ºC. UV max (MeOH, nm): Characteristic of an aromatic compound UV λ_{max} 309 nm. FTIR (KBr) cm^{-1}: 2839 (-O-CH_3), 1720 (ester), 818 (*para* disubstituted benzene), 1705 (-C=O) and 1635 (-C=C-). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 3.83 (s, 3H), 3.89 (s, 3H,), 6.42 (d, 1H, 16 Hz), 7.00 (dt, 2H, 4 Hz, 12 Hz, 4 Hz), 7.58 (dt, 2H, 4 Hz, 8 Hz, 4 Hz), 7.68 (d, 1H, 16 Hz). ^{13}C NMR (100 MHz, CD_3OD) δ ppm: 168.13, 161.76, 144.72, 129.56, 126.89, 115.24, 114.02, 54.46, 50.64. MS (ESI): 193 *m/z* $[M+H]^+$.

The molecular ion at *m/z* 193 in the mass spectrum, it was considered to have a molecular formula of $C_{11}H_{12}O_3$. Based on UV, it was found to be aromatic. IR spectrum confirmed the absence of hydroxyl groups, as well as the presence of carbonyl, ester and methoxy groups. ^{13}C NMR indicates the presence of eleven carbon atoms with three oxygen attached carbons. One oxygen attached carbon present at above 165 ppm and two oxygen attached carbon showing a peak above 50 ppm indicates the presence of a carbonyl (ester) and ether functional group respectively. Based on IR and ^{13}C NMR two of these carbons (168.13 and 50.64 ppm) were considered as constituting an ester functional group and one of the carbon at 54.46 ppm was considered as belonging to an aromatic ether. The presence of *trans* carbon-carbon (-C=C-) double bond was confirmed based on characteristic ^{1}H NMR signals two signals, with one proton each, both having with J = 16 Hz and presence ^{13}C NMR signals at 144.72 and 114.02 ppm. The presence of two doublets with J value of 16 Hz can be attributed to a carbon-carbon double bond, with one end of it attached with a carbonyl group. ^{1}H NMR indicates the presence of a symmetric aromatic group by the presence of two sets of doublet of triplets. This indicates the presence of *para* substitution. Proton NMR also confirmed the presence of two singlets each of three protons, confirming an ester and ether functionality. Based on all these information and comparing the ^{1}H NMR and ^{13}C NMR and IR spectral data of compound **1**, the structure of the compound was deduced as *p*-methoxymethyl cinnamate, the NMR data is in agreement

with reported data [14]. Isolation of this compound is the first report from the plant.

Compound 2 (*p*-hydroxymethylcinnamate): White amorphous solid, m.p.: 134-138 ºC. UV_{max} (MeOH, nm): Characteristic of an aromatic compound UV λ_{max} 312 nm. FTIR (KBr) cm^{-1}: 3379 (-OH), 1682 (ester), 833 (*p*-disubstituted benzene) and 1635 (C=C-). ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 3.77 (s, 3H), 6.26 (d, 1H, 16Hz), 6.86 (d, 2H, 8 Hz), 7.39 (d, 2H, 8 Hz), 7.61 (d, 1H, 16 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 167.91, 159.67, 144.99, 129.83, 125.63, 116.07, 116, 114.02, 51.42. MS (ESI): 174 *m/z* $[M+H]^+$.

The molecular ion at *m/z* 174, which was considered to have a molecular formula of $C_{10}H_{12}O_3$. Based on UV, it was found to be aromatic. IR spectrum confirmed the presence of an aromatic hydroxyl group, as well as the presence of a carbonyl group. ^{13}C NMR indicates the presence of ten carbons with three oxygen attached carbons. DEPT 135 indicates the presence of two quaternary carbon (159.67 & 125.63 ppm) and one oxygen attached carbon as carbonyl (167.91 ppm). This observation along with the presence of one oxygen attached carbon at above 51.42 ppm in ^{13}C NMR and a singlet at 3.77 ppm for three protons in ^1H NMR indicates the presence of an ester functional group. The other two quaternary carbons present in ^{13}C NMR were assigned for the substituted benzene. The presence of *trans* carbon-carbon double bond (-C=C-) was confirmed based on characteristic ^1H NMR signals two signals, with one proton each, both having with J = 16 Hz and presence of ^{13}C signals at 144.99 and 114.02 ppm. The presence of two doublets with J value of 16 Hz can be attributed to a *trans* carbon-carbon double bond, with one end of it attached to carbonyl. ^1H NMR indicates the presence of a symmetric aromatic group by the presence of two sets of doublet of triplets. This indicates the presence of *para* substitution. Based on all these information and by comparing the ^1H NMR and ^{13}C NMR and IR spectral data of compound **2**, with that of compound **1**, the structure of the compound was deduced as *p*-hydroxymethyl cinnamate. The ^1H NMR of compound **1** shows the presence of two methyls, compound **2** shows only one methyl. Presence of alcohol functional group and absence of aromatic-aliphatic mixed ether group in the IR spectra (presence of 3379 cm^{-1} and the absence of 2839, 1257, 1010 cm^{-1}) indicates replacement of methoxy group in compound **1** by a hydroxyl group in compound **2**, the same compound was isolated and characterized earlier [9].

Compound 3 (2,5-dihydroxybenzaldehyde): Pale yellow solid, m.p.: 94-96 ºC. UV_{max} (MeOH, nm): UV λ_{max} 279, 297 and 313 nm. FTIR (KBr) cm^{-1}: 1652 (C=O) & 1590, 1455 (C=C). ^1H NMR (400 MHz, CD_3OD) δ ppm: 6.97 (d, 1H), 7.29 (d, 1H), 7.39 (dd, 1H), 9.75 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ ppm: 191.06, 151.63, 145.42, 129.42, 124.87, 115.27, 114.79. MS (ESI): 139 *m/z* $[M+H]^+$.

The molecular ion at *m/z* 139 is considered to have a molecular formula of $C_7H_6O_3$. The TLC of the compound is closely related to 3,4-dihydroxybenzaldehyde isolated and characterized from the same plant earlier [9]. This compound also exhibit an aldehyde functionality, confirmed based on a peak at 191.06 ppm in ^{13}C NMR. Comparison of ^1H & ^{13}C NMR of compound **3** with 3,4-dihydroxybenzaldehyde reveals similarities. IR spectra of both the compounds were also found to be the same. The presence of hydroxyl functionalities is confirmed based on IR. However, TLC spots have difference in R_f 0.44 (compound **3**) *vs.* 0.35 in 7.5:2.5 $CHCl_3$:ethylacetate). Compound **3** produces three signals in ^1H NMR for three protons, indicating the trisubstitution in the benzene ring. In 3,4-dihydroxybenzaldehyde earlier reported as a constituent of this plant produces two signals for three protons. A comparison of ^1H NMR spectral value of these two compounds is given in Table-1. Along with these differences in the ^1H NMR characteristics; the melting point of compound **3** is far less than that of the earlier compound (101 *vs.* 155 ºC). There is an absence of 1,2,3 or 1,2,6-substitutions in the benzene ring, as evident from ^1H NMR. The other possibility is either 2,4-dihydroxy or 2,5-dihydroxybenzaldehyde. However, in case of 2,4-dihydroxybenzaldehyde, the proton designated as C, was expected to resonate more downfield, (since it was the only *ortho* substi-tution to aldehyde group), however, it is not so, (Table-1), hence this possibility was ruled out. The ^1H NMR values of the comp-ound are in agreement with the reported value [15]. Based on all these facts, the structure of the compound was deduced as 2,5-dihydroxybenzaldehyde.

Compound 4 (Methyl gallate): White amorphous solid, m.p.: 198-201 ºC. UV_{max} (MeOH, nm): UV λ_{max} 218, 275 nm. FTIR (KBr) cm^{-1}: 3526 (-OH), 3364 (-COOH), 1697 (ester). ^1H NMR (400 MHz, CD_3OD) δ ppm: 3.80 (s, 3H), 7.02 (s, 2H). MS (ESI): 185 *m/z* $[M+H]^+$.

Compound **4** is a white amorphous solid and identified as an ester-based on IR spectrum. ^1H NMR spectra show the presence of two singlets. One in the aromatic region 7.0 ppm and the other as an oxygen attached singlet at 3.8 ppm for three protons. The singlet with three protons was assigned to a methyl group. The ^1H NMR spectrum of this compound is similar to compound **2** (gallic acid) reported as one of the constituents of the bark of this plant [16] with just one methyl group extra.

TABLE-1
COMPARISON OF ^1H NMR SPECTRAL DATA OF ISOLATED COMPOUND **3** WITH 3,4-DIHYDROXYBENZALDEHYDE

Proton label	Compound **3**		3,4-Dihydroxybenzaldehyde		Changes with respect to 3,4-dihydroxybenzaldehyde
	Chemical shift (ppm)	*J* value (Hz)	Chemical shift (ppm)	*J* value (Hz)	
A	7.29 (d)	8	7.38 (d)	4	Chemical shift moderately shifted upfield, more than expected meta coupling (equivalent to ortho coupling value) due to placement of A&B in between electron donating hydroxyl group.
B	7.39 (dd)	8	7.36 (dd)	2	Almost similar chemical shift, however, more than expected meta coupling *J* value, equal to ortho coupling value) due to placement of A&B in between electron donating hydroxyl group.
C	6.97 (d)	8	6.97 (d)	8	No change in chemical shift and *J* value

TLC of this compound is less polar compared to gallic acid. ^{1}H NMR and melting point (198-201 ºC) of the compound matches with the reported value [17]. All these features enable to assign the structure of the compound as methyl ester of gallic acid.

Compound 5 (Isoquercitrin): Yellow crystalline solid, m.p.: 228 ºC (decomp.). UV_{max} (MeOH, nm): UV λ_{max} 257, 369 nm. FTIR (KBr) cm^{-1}: 3410 (-OH), 1666 (-C=C-). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 7.70 (1H, d, *J* = 2.1 Hz, H-2′), 7.58 (1H,dd, *J* = 8.5 Hz, 2.2 Hz, H-6′), 6.86 (1H, d, *J* = 8.5 Hz, H-5′), 6.37 (1H, d, *J* = 2.19 Hz, H-8), 6.19 (1H,d, *J* = 2.05 Hz, H-6), 5.25 (1H, d, *J* = 7.5 Hz, H-1″), 3.69 -3.29 (5H, m); ^{13}C NMR (CD_3OD, 100 MHz) δ: 157.48 (C-2), 134.09 (C-3), 177.98 (C-4), 161.54 (C-5), 98.35 (C-6), 164.5 (C-7), 93.185 (C-8), 156.95 (C-9),104.17 (C-10), 121.55 (C-1′), 116.03 (C-2′), 144.39 (C-3′), 148.33 (C-4′), 114.47 (C-5′), 121.67(C-6′), 102.76 (C-1″), 74.21 (C-2″), 76.60 (C-3″), 69.69 (C-4″), 76.88 (C-5″), 61.03 (C-6″). MS (ESI) 465 *m/z* $[M+H]^+$.

The molecular ion at *m/z* 465 is considered to have a molecular formula of $C_{21}H_{20}O_{12}$. IR spectrum indicate the presence of sp^2 hybridized hydroxyl groups. ^{1}H NMR of the compound indicates the presence of doublet at 5.25 pm for one proton (probably an anomeric proton). The presence of a bunch of peaks from 3.29 to 3.70 ppm indicates the presence of many protons attached to a hydroxyl group. In the aromatic region, five peaks belonging to two different sets are present from 6.18 to 7.89 ppm. Indicating the presence of tri and tetra-substituted benzene rings.^{13}C NMR indicates the presence of 21 carbon. Based on this primary information, one can conclude that the compound is a glycoside, linked to the aglycone by a β-glycosidic bond. The compound (2 mg) was hydrolyzed using β-glucosidase enzyme at 40 ºC for 1 h and the aglycone formed was found to be quercetin. Based on this information the nature of the compound was confirmed as quercetin glycoside. There are three popular quercetin glycosides known in the literature *viz*. hyperoside, isoquercitrin and quercitrin based on the glycoside counterparts. Based on ^{13}C NMR, compound is not quercitrin (absence of rhamnose as glycoside). ^{13}C NMR further clearly indicates the glycoside (galactose or glucose), based on the resonance of the 3rd, 4th and 5th carbon of the glycosides at 74.2, 76.6, 76.9 ppm, respectively the nature of the glycoside as glucose was confirmed, thus the structure of the compound was deduced as quercetin-3-O-β-glucoside (isoquercitrin). The ^{1}H and ^{13}C NMR of the compound was in complete agreement with the reported value [18].

Compound 6 (phenylethylpalmitate): White waxy solid, m.p.:46-48 ºC. UV_{max} (Chloroform, nm): UV λ_{max} 276 nm. FTIR (KBr) cm^{-1}: 2978 (alkyl), 1712 (ester), 771, 1450 (aromatic ring with mono substitution). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 7.28 (m, 5H), 4.26 (m, 2H), 2.93 (t, 2H), 2.27 (t, 2H), 1.59 (m, 2H), 1.25 (m, 24H), 0.88 (t, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ: 173.85, 137.9, 128.9, 128.47, 126.52, 64.69, 35.16, 34.35, 31.94, 25.78, 24.95, 22.7, 14.13. MS (ESI) 361 *m/z* $[M+H]^+$.

The IR spectrum indicate the presence of alkanes 2978 cm^{-1}, ester functional group 1712 cm^{-1}, an aromatic ring with mono substitution 771, 1450 cm^{-1}. ^{1}H NMR shows the presence of a large number of methylene protons indicating the presence of fatty acid ester. Since monosubstituted aromatic is expected based on IR and the presence of a multiplet for five protons, the ester was expected to be a monosubstituted aromatic fatty acid ester. In ^{1}H NMR, methylene groups α and β to the carboxylic group were resonating at 2.27 and 1.59 ppm, respectively each having two protons, a bunch of peaks resonating at 1.25 ppm for 24 protons were considered as derived from a fatty acid, with the terminal methyl group presenting at 0.88 ppm, all these features confirm the presence of a fatty acid. Two more proton signals present at 2.93 ppm and 4.26 ppm are assigned to an ethylene ester groups with one end (4.26 ppm peak) connecting the fatty acid with the benzene, thus the compound was tentatively assigned as a phenyl ethyl fatty acid ester. ^{13}C NMR shows the presence of ester 173.85 ppm, aromatic ring 137.9-126.52 ppm, one oxygen-connected methylene, non-oxygenated methylene groups and a terminal methyl group. The presence of methylene groups, α and β to the carboxylic group were indicated by the presence of peaks at 31.94, 34.35 ppm. The terminal methylene of the fatty acid denoted as ω_1 was resonating at 14.13 ppm, methylene α and β to ω_1 denoted as ω_2 and ω_3 are resonating at 24.95 and 22.7 ppm, respectively. Oxygen attached methylene of the connecting ethylene moiety was presenting at 64.69 ppm with methylene attached to the benzene ring present at 35.16 ppm, these results together with the presence of ten symmetrical methylenes at 29.13-29.7 ppm conform to $C_{24}H_{40}O_2$. Hence, the structure of the compound was deduced as phenylethyl palmitate [18].

The present work aims to understand and document the reason behind the suggested use of the flower and leaves of the plant as bathing aids. The active secondary metabolites were present in the range 0.004-0.02%. About 30 g of fresh leaves paste (25 g fresh leaves + 5 g water made into a paste) as a bathing aid, then based on the isolated yield of compounds methyl-*p*-coumarate (MPC) and 3,4-dihydroxybenzaldehyde (3,4-DHB), one can expect the presence of 60 μg/g of MPC and 20 μg/g of 3,4-DHB. Which are several folds less than the MIC of these compounds 250 and 500 μg/mL against *Staphylococcus epidermidis* and *Staphylococcus aureus*, respectively. Hence, to deliver any usefulness as a bathing aid, the combined ingredients of the plant (all put together, organic, inorganic components, insoluble solids, *etc.*) must have a broad spectrum of activity. The antioxidant potential of the crude extract, four fractions and seven active metabolites of the plant using four assay systems was also investigated. The assay systems selected were expected to play a role in determining the efficacy of the test samples for skincare application.

The results of the antioxidant assay of the crude methanol extract (CME), chloroform fraction (CF), ethyl acetate fraction (EAF), *n*-butanol fraction (nBF), an aqueous fraction (WF) and seven compounds isolated using above described four protocols are summarized in Table-2. The IC_{50} value estimation based on carotene bleaching activity of the crude extract and the fractions was also discussed. The carotene bleaching activity (OD values) of the crude extract and fractions were determined from 150 μg/mL to 15000 μg/mL. Then % of carotene bleaching by the test samples was determined. Based on these values,

the % inhibition *vs.* concentration graph is drawn. From the graph, regression equation and R^2 (correlation coefficient) value for the test samples was estimated. Using the regression equation IC_{50} values were calculated.

Based on the regression equation IC_{50} value of carotene bleaching activity of the crude extract & fractions were calculated. The IC_{50} value of the CME, CF, EAF, nBF and WF were found to be as 64.8, 4.6, 4.2, 16.6 and 21.9 mg/mL, respectively. Similarly, IC_{50} values of pure compounds were calculated, approximated to nearest tens and reported in Table-2. The overlay diagram of % inhibition *vs.* concentration graph of the crude methanol extract and fractions is given in Fig. 1.

The chloroform and ethyl acetate fractions are more active compared to other fractions and crude extract. These two fractions are the most efficient in scavenging both electron and hydrogen atom transfer mechanisms. Thus, it gives hope that lipid peroxidation, synergistic UV protection and cumulative reduction in oxidative stress are expected out of this plant. All the extracts and fractions (except ethyl acetate fraction) were more active in metal ion chelation assay. This may enable the binding of the actives from the leaves with certain metal ions present in the enzymes of harmful microorganisms. Simultaneously, this property assures the chelation and inactivation of reactive oxygen species (ROS) generated under oxidative stress within the sebaceous gland of antioxidant deprived patients. The actual quantities of these lipid-soluble fractions in the crude methanol extract are very less compared to the water soluble fractions. The water-soluble extractive of the leaves is 7.25%. The experimental details revealed that 2500 g of the fresh plant gives 1430 g of air-dried leaves. Extraction of 1430 g of the plant provides 303 g of methanol extract. Fractionation of 300 g of crude methanol extract provides 18.79 g of chloroform fraction, 9.4 g of ethylacetate fraction, 74.16 g of *n*-butanol fraction and aqueous fraction 81.71 g.

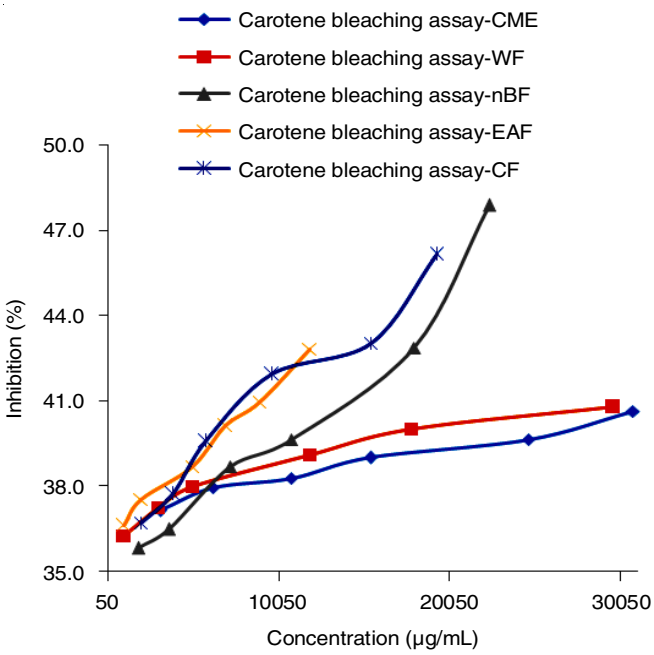

Fig. 1. Carotene bleaching assay overlay diagram

Thus 21% of extract was obtained from the dried leaves, while 79% of plant material is present as a non-extractable mass and the expected constituents of this major portion of plant material are insoluble fibers. It is understood that the antioxidant delivery of crude methanol extract, *n*-butanol and the aqueous fraction is well guaranteed since the IC_{50} value of all the four assay systems were within these values. The chloroform and ethyl acetate fraction-derived antioxidant benefits are expected to be delivered as per the IC_{50} value given in Table-2. Thus, the usage of the plant paste is expected to deliver at least four known benefits, antioxidant, antimicrobial, gentle cleaning and scrubbing to remove dead cells from the skin surface.

The phytochemical exploration provided six compounds, of these six compounds; four compounds are the first report from the plant species. The constituents such as *p*-methoxy-

TABLE-2
COMPARATIVE DATA OF ANTIOXIDANT ACTIVITY OF ACTIVES FROM LEAVES AND FLOWER OF *Manilkara hexandara*

Description	(1)	(2)	(3)	(4)
	Type of method			
	ET BASED	HAT BASED		Other mechanisms
	Concentration (mg/mL)			
Crude methanol extract	61	65	25	31
Chloroform fraction	11	5	14	9
Ethyl acetate fraction	9	4	6	12
n-Butanol fraction	20	17	18	19
Aqueous fraction	25	22	17	22
	Concentration (µg/mL)			
p-Methoxy methyl cinnamate (1)	185	125	148	154
p-Hydroxymethylcinnamate (2)[9]	150	150	152	124
2,5-Dihydroxy benzaldehyde (3)	75	84	147	141
3,4-Dihydroxy benzaldehyde (7)[9]	75	75	25	140
Gallic acid methyl ester (4)	500	500	125	65
Quercetin-3-O-β-D-glucoside (Isoquercitrin) (5)	215	120	10	65
Phenylethylpalmitate (6)	500	147	258	5

(1) = Superoxide radical scavenging activity; (2) = β-catotene bleaching assay; (3) = Metal ion chelating activity; (4) = Phospho molybdenum reducing power

methyl cinnamate, 2,5-dihydroxybenzaldehyde, phenylethyl palmitate and isoquercitrin are the first report from this plant. Certain flavonoids and gallic acid derivatives are already reported from this plant species, however, to the best of our knowledge; isolation of isoquercitrin is not published so far. This flavonoid is an excellent metal ion chelator, it could be the reason for the potent metal ion chelation demonstrated by the n-butanol and aqueous fraction of the plant. Isoquercitrin, methyl gallate and 3,4-dihydroxybenzaldehyde were present in the range of 0.004 to 0.008%. The other four compounds were present at least 0.02% level in the plant leaves. Thus, the reported work [9] together with the current findings supports the present hypothesis that the leaves and flowers of *Manilkara hexandra* are suitable as daily bathing aid. Daily usage of these plant parts can deliver efficient skin barrier function.

Conclusion

Daily usage of leaves paste of this plant and flower is a known folklore information. Present research shows that fresh leaves paste of the plant *Manilkara hexandra (*Roxb.*)*, is expected to deliver 21% of soluble extractives and 79% of insoluble fibers. The soluble extractive encompasses the bioactive metabolites against pathogenic organisms such as *Staphylococcus aureus* and certain constituents are active against skin commensal organism *Staphylococcus epidermidis*. Present research study indicates at least four benefits of the folklore practice, gentle cleansing and scrubbing using the insoluble fibers. Anti-microbial action to maintain skin microbiome and strong antioxidant benefits by potent antioxidants such as flavonoids, aldehydes and cinnamic acid esters. Thus based on the studies, a cosmetic formulation can be developed using the paste of the leaves and flowers of the plant. A stable and standardized herbal cosmetic formulation delivering all the desired skin-friendly benefits can be explored based on the scientific understanding established in the present study.

ACKNOWLEDGEMENTS

The authors thank Dr. P. Santhan, Taxonomist, Durva Herbal centre, Chennai, India for the identification of plant species. This work is the part of the Ph.D. work of T. Annamalai. The authors also thank Dr. Meenakshi Narayanan, Vice-president R&D, CavinKare Research Centre for her encouragement and support. The authors wish to acknowledge the Principal, Head of the department of chemistry and the management of the Ramakrishna Mission Vivekananda College. Chennai-4, for their encouragement and support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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