Characterization of Chemical Isolates of *Caryopteris odorata*

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Summary: The phytochemical investigation of the chloroform soluble fraction of *Caryopteris odorata* (Ham. ex Roxb.) led to the isolation of one new constituent namely; *n*-pentadecyl[3-(4-hydroxyphenyl)] propanoate (**1**). The compounds 2-ethyl-*n*-hexyl benzoate (**2**), 2-ethyl-*n*-hexyl *trans*-cinnamate (**3**) *bis*-dodecylbenzene-1,4-dicarboxylate (**4**) were reported synthetically but these have been isolated for the first time from a natural source. Similarly, the known constituent like *trans*-cinnamic acid (**5**) and *cis*-cinnamic acid (**6**), henicosanoic acid (**7**), methyl tricontanoate (**8**) and octacosanyl tricontanoate (**9**) were also obtained for the first time from this plant source. The structures of these compounds were determined by IR, HR-MS, ^{1}H-NMR, ^{13}C-NMR spectroscopy and by comparison with the published data of the closely related compounds [8-15]. These isolates were screened for their antioxidant, anti-urease and anti-tyrosinase activities and results revealed that *n*-Pentadecyl [3-(4-hydroxyphenyl)] propanoate (**1**) exhibits good antioxidant potential with low to zero enzyme inhibition activities.

Key words:*Caryopteris odorata* (Ham. ex Roxb.), fatty constituents, aromatic esters, antioxidants, ^{13}C-NMR spectroscopy.

Introduction

Caryopteris odorata (Ham. Ex Roxb.) belongs to the family Verbenaceae which is a family of ca. 100 genera and nearly 2600 species, mostly tropical and subtropical in distribution [1]. In Pakistan it is represented by 17 genera and ca. 35 species [2]. The family is characterized mostly by woody habit (shrubs or trees, zygomorphic flowers, androcecium didynamous, 2 carpels, ovary usually 4 loculed with terminal or subterminal style. Fruit is usually drupe or nutlets. The family is important for teak timber i.e. *Tectona grandis* and some ornamental plants [3]. *Caryopteris odorata* is a shrub distributed in Subtropical or outer Himalayas from Pakistan to Bhutan, India and Bangladesh. It is common in the low hills of Punjab, and has also been reported from Baluchistan. Sometimes it is also cultivated as an ornamental [2]. Its powdered leaves and flowers are used in diabetic foot ulcer, tumors and wounds [4].

Reactive oxygen species, such as superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\blacksquare}$), and singlet oxygen ($^1O_2$), are of the greatest biological significance. They are extremely reactive and potentially damaging transient chemical species [5]. Lipid peroxidation is one of the major causes of deterioration in foods that results in the formation of potentially toxic compounds. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone (TBHQ) and propylgallate (PG), are widespread food additives used to preserve against deterioration; however, their use is increasingly restricted, due to their potential health risks and toxicity. Moreover, there is a growing awareness among consumers regarding food additive safety [6]. There is currently an upsurge of interest in phytochemicals as potential new sources of natural antioxidants. The goal is to use them in foods and pharmaceutical preparations to replace synthetic antioxidants [7-8].

Results and Discussion

The chloroform soluble fraction of *Caryopteris odorata* (Ham. ex Roxb.) yielded nine compounds (**1-9**) and their structures were determined on the basis of spectroscopic studies and by comparison with the published data of the closely related compounds [9-16]. We are reporting **1** as a new natural product however the compounds **2**-**4** have already been reported [9-11] but in the present investigation these are reported from a natural source

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for the first time. To the best of our knowledge, the known constituents **5-9** have also been isolated for the first time from this plant source.

The ester (**1**) was obtained as a white powder and its molecular formula was determined through HR-MS showing a $[M]^+$ ion peak at *m/z* 376.5196, corresponding to the molecular formula $C_{24}H_{40}O_3$ which indicated 05 degrees of unsaturation. The absorption bands at 3373 cm^{-1} and 1715 cm^{-1} in IR spectrum revealed the presence of hydroxyl and ester functionality, respectively, in the molecule. The ^{1}H-NMR spectrum of **1** showed two *ortho*-coupled doublets at δ 7.06 (2H) and δ 6.74 (2H) with a coupling constant of 8.7 *Hz* exhibiting an AA′BB′ spin system. The spectrum also showed three triplets of two proton integration each at δ 4.25 (*J* = 7.2 *Hz*), δ 2.84 (*J* = 7.2 *Hz*) and δ 2.25 (*J* = 7.2 *Hz*). These values were assigned to H-1″, H-3 and H-2, in agreement with reported data of related compounds [17,18]. An upfield triplet at δ 0.86 (*J* = 6.9 *Hz*) was due to a terminal methyl group and a quintet at δ 1.56, integrated for two protons, was assignable to H-3″. The broad clustered singlet at δ 1.24 was accounted for 24 protons revealing the presence of twelve additional methylene groups in the molecule. The ^{13}C-NMR spectrum displayed apparently sixteen resonances some overlapped) which were resolved through DEPT experiment into three quaternary carbons, two methines (one for C-2′ and C-6′ and other for C-3′ and C-5′) while all remaining signals were of methylene carbons. The most downfield quaternary carbon at δ 173.81 was attributed to carbonyl of ester and the others, which appeared at δ 154.18 and δ 130.14 were assigned to C-4′ and C-1′, respectively. The methine resonance at δ 130.05 was assignable to two symmetrical carbons (C-2′ and C-6′) while the signal at δ 115.33 was characteristic of magnetically equivalent C-3′ and C-5′ for such 1,4-disubtituted aromatic moiety. Hence from the above spectral evidences, the structure of **1** was identified as *n*-pentadecyl[3-(4-hydroxyphenyl)] propanoate.

The ester **2**, isolated as colourless solid, had a molecular formula; $C_{15}H_{22}O_2$, deduced from HR-MS. The intense IR absorption band at 1725 cm^{-1} revealed the presence of a carbonyl group and its ^{1}H-NMR signals at δ 7.70 (d, *J* = 7.4 *Hz*; C-2′ and C-6′), δ 7.68 (t, *J* = 7.5 *Hz*; C-4′) and δ 7.49 (t, *J* = 7.6 *Hz*; C-3′ and C-5′) indicated that it was a benzoate ester. The ^{1}H-NMR spectrum also displayed two overlapped triplets at δ 0.90 (*J* = 7.1 *Hz*) and at δ 0.87 (*J* = 7.1 *Hz*) for two terminal methyl groups. A doublet at δ 4.20 (2H, *J* = 6.5 *Hz*) was assignable to H-1 while a multiplet integrated for one proton was assigned to H-2. A broad singlet, integrated for eight protons, revealed four more methylene groups in the molecule. The typical signals in ^{13}C-NMR spectrum also confirmed **2** as a benzoate ester [19] but attached with a branched chain. The DEPT spectrum, in addition to benzoate ester, also revealed two methyl, five methylenes and a methine group in the molecule. The downfield chemical shift of δ 68.14 was attributed to an oxymethylene group (C-1) while an upfield signal for a methyl group at δ 23.73 (C-1″) along with a methylene signal at δ 10.94 (C-2″) , indicated an ethyl group branched to the main chain in agreement with the reported data [20]. A cross peak in the COSY spectrum between a multiplet at δ 1.66 (H-2) and a broad doublet at δ 4.20 (H-1) further confirmed the branching at this point. So, on the basis of above spectral evidence the structure of **2** was elucidated as 2-ethyl-*n*-hexyl benzoate which has been reported synthetically [9] earlier to this investigation.

The ester **3** was also purified as a colourless solid. Its molecular formula $C_{17}H_{24}O_2$ was established through HR-MS exhibiting $[M]^+$ ion peak at 260.3554. After analyzing the signals in ^{1}H-NMR and ^{13}C-NMR spectra, it was inferred that it had a similar chain but esterified with cinnamate moiety. Further, the larger coupling constant for doublets at δ 7.76 (1H, *J* = 15.9 Hz; H-7′) and δ 6.43 (1H, *J* = 15.9 Hz; H-8′) confirmed it to be a *trans*-cinnamate moiety and this assignment was also supported by its ^{13}C-NMR data [21]. Consequently, the structure of **3** was established as 2-ethyl-*n*-hexyl *trans*-cinnamate. This ester also has been reported as a synthetic molecule [10] but we are reporting for the first time from a plant source.

Derivatives of phthalic acid isomer have been widely used in various industries and have also been detected in sea water, rivers and in the tissues of marine animals, and are generally accepted as pollutant [22]. The compound **4** was isolated from our investigated source as a *bis* ester of terephthalic acid. Its molecular formula was established as $C_{32}H_{54}O_4$ through HR-MS having $[M]^+$ ion peak at 502.7579 and showing six degrees of unsaturation. Its ^{1}H-NMR spectrum exhibited a singlet at δ 8.08 which was integrated for four protons and was characteristics of terephthalate moiety [21]. The spectrum also displayed a triplet at δ 4.66 (4H, *J* = 6.9 Hz) for two symmetrical oxymethylene groups, a quintet at δ 1.52 (4H, *J* = 6.7 Hz) was integrated for four protons assignable to two (O-CH_2-CH_2-CH_2) groups and broad clustered singlet at δ 1.24 was due

to 18 protons, revealing the presence of 16 more methylene groups in the molecule. The 13C-NMR spectrum revealed only 15 carbon resonances and the DEPT experiment suggested that in these, two were quaternary, one for each methyl and methine and remaining were of methylene carbons. These lesser number of carbon resonances as compared to calculated molecular formula $C_{32}H_{54}O_4$ were attributed to the symmetry and dimeric nature of the molecule. In the ^{13}C-NMR spectrum a quaternary carbon at δ 167.58 was obvious for two symmetrical ester carbonyls and this assignment was also supported by an IR absorption band 1735 cm^{-1}. Hence, on the basis of above cumulative evidences the structure of **4** was deduced as *bis*-dodecylbenzene-1,4-dicarboxylate. This molecule is reported synthetically [11] however in the present study we are reporting it as a contaminant from this plant species.

From our investigated source other known compound like *trans*-cinnamic acid (**5**) [12] and *cis*-cinnamic acid (**6**) [13], henicosanoic acid (**7**) [14], methyl tricontanoate (**8**) [15] and octacosanyl triacontanoate (**9**) [16] were isolated for the first time. Fig. 1 shows all the compounds.

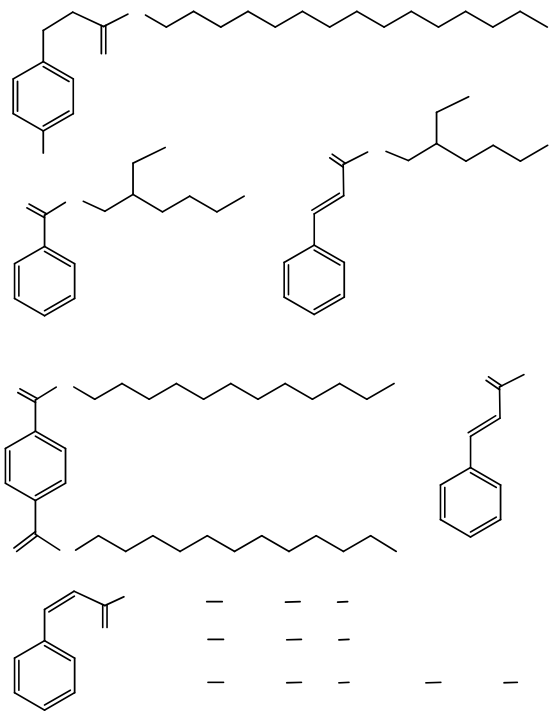

Fig. 1: Structures of compounds **1-9.**

Antioxidant activity of isolated compounds was studied by ferric reducing antioxidant power (FRAP) and phosphomolybdenum method. FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ [2,4,6-tri(2-pyridyl)-s-triazine] and $FeCl_3.6H_2O$. The absorbance is measured spectrophotometrically at 595 nm [23]. Our results revealed that **6** exhibited well FRAP value as compared to other compounds [Table-1]. Phenolic compounds usually posses biological properties such as: antioxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-artherosclerosis, cardiovascular protection, improvement of the endothelialfunction, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilities [24]. The constituent **1** showed a good total antioxidant activity as compared to other compounds **2-9** [Table-1]. Its therapeutic potential was obvious from the fact that it total antioxidant activity was even close to standard BHT. The good total antioxidant activity of this molecule was probably due to donation of phenolic proton which might reduce Mo (VI) to Mo (V). This hypothesis was also supported from the observation that compounds **8** and **9** due to non-availability of such proton did not show total antioxidant activity. All the isolated compounds were also checked for their anti-urease and anti-tyrosinase activity but they were not found to be active against these enzymes.

Table-1: The FRAP values and Total Antioxidant Activity of plant isolates.

Sample	FRAP value TE (μM/ml) ± S.E.M[a)]	Total antioxidant activity (abs. at 695 nm) ± S.E.M [a)]
***n*-pentadecyl[3-(4-hydroxy-phenyl)]propanoate**	**110.5 ± 1.54**	**0.638 ± 0.008**
***trans*-cinnamic acid**	**148 ± 0.001**	**0.328 ± 0.002**
***cis*-cinnamic acid**	**154 ± 0.91**	**0.243 ± 0.01**
methyl tricontanoate	**129.5 ± 1.41**	**-**
octacosanyl triacontanoate	**131.5 ± 1.01**	**-**
BHT [b)]		**0.818 ± 0.01**
Blank		**0.159 ± 0.003**

[a)] Standard mean error of three assays.
[b)] Standard antioxidant
Note:- Antioxidant activity could be performed only for those compounds which were present in sufficient amount

Experimental

General

For column chromatography (CC), silica gel (Merck) (70-230 mesh) and for flash chromatography

(FC), silica gel (230-400 mesh) was used. TLC was performed on pre-coated silica gel G-25-UV_{254} plates. Detection was carried out at 254 nm, and by ceric sulphate (Merck) reagent. Purity was checked on TLC with different solvent systems using acetone, $CHCL_3$ and *n*-hexane giving single spot. The IR spectra were recorded Jasco-320-A spectrophotometer. ^{1}H-NMR, ^{13}C-NMR and COSY Spectra were run on Bruker spectrometers operating at 300 MHz. The chemical shifts are given in δ in ppm and coupling constants in Hz. HR-MS spectra were recorded on a JMS-HX-110 spectrometer, with a data system.

Plant material

The plant *Caryopteris odorata* (Ham. ex Roxb.) having common name of path geri was collected from hills of Azad Kashmir in March 2009, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen no. (G.C. Herb. Bot. 862) has been deposited in the herbarium of the same university.

Extraction and Isolation

The shade-dried ground whole plant (7.5 kg) was exhaustively extracted with methanol (12L × 4) at room temperature. The extract was evaporated to yield the residue (650 g), which was dissolved in distilled water (1.5L) and partitioned with *n*-hexane (1L × 4), chloroform (1L × 4), ethyl acetate (1L × 4) and *n*-butanol (3L × 4) respectively. These fractions were concentrated separately on rotary evaporator. The chloroform extract (352 g) was subjected to column chromatography over silica gel (70-230 mesh) using hexane with gradient of $CHCl_3$ (from 100: 0, 90: 10, 80: 20 – 10:90, 0:100) (4000 ml for each gradient) and followed by methanol up to 100 %. Ten fractions (Fr. 1-10) were collected, having elution volumes as 2000, 2825, 3450, 3400, 3250, 2500, 2000, 3300, 3225, and 3100 ml respectively. The Fr. 2 (2825 ml) from the first column as loaded on silica gel (70-230 mesh) (50 g) and eluted with $CHCl_3$: *n*.hexane (2:98) (2400 ml) to get two sub-fractions (Fr_{sb}. 2.1-2.2), having elution volumes as 800 ml and 600 ml respectively. The Fr_{sb}. 2.1 (800 ml) was subjected to CC and eluted with $CHCl_3$: *n*.hexane (2:98) (750 ml) to isolate 8 (38mg). The Fr_{sb}. 2.2 (600 ml) was subjected to CC and eluted with $CHCl_3$: *n*.hexane (5:95) (650 ml) to isolate 9 (7.5mg). The Fr. 3 (3450 ml) was loaded on flash silica gel (70-230 mesh) (45 g) and eluted with $CHCl_3$: *n*.hexane (15:85) (2000 ml). This fraction contained two compounds **2** (8.5mg) and **3** (7.69mg) as a mixture, which was separated by preparative TLC $CHCl_3$: *n*.hexane (20:80) (30ml x 4). The fraction 5 (3250 ml) was loaded on flash silica (65g) and eluted with $CHCl_3$:*n*.hexane (20:80) (900ml) to isolate **4** (8.5mg). The fraction 6 (2500 ml) was loaded on flash silica gel (70g) and eluted with $CHCl_3$:*n*.hexane (15:85) (750ml) to isolate **1** (36.5mg). The fraction 8 (3300 ml) was loaded on flash silica gel (60g) and eluted with $CHCl_3$:*n*.hexane (90:10) (750ml) to purify **7** (8.32mg). The Fr. 9 (3220 ml) was loaded on flash silica gel (70 g) and eluted with $CHCl_3$: *n*.hexane (95:5) (2500 ml) to get three sub-fractions (Fr_{sb}. 8.1-8.3), having elution volumes as 1200 ml, 900 ml, and 750 ml respectively. The Fr_{sb}. 8.2 (900 ml) was subjected to preparative TLC $CHCl_3$: *n*.hexane (98:2) (30ml x 4) to purify **5** (38.2 mg) and **6** (45.5 mg) [25].

n-Pentadecyl [3-(4-hydroxyphenyl)]propanoat **1**

White powder (35.5 mg). IR ν_{max} (KBr): 3373 (OH), 2923 (C-H), 1715 (C=O, ester), 1609, 1495 (C=C, Ar), 1163,1079, (C-O) 813, 717, 624 cm^1;^{1}H-NMR ($CDCl_3$) (300MHz) (δ ppm): 7.06 (d, 2H, *J*=8.7Hz, H–2′ & H–6′), 6.74 (d, 2H, *J* = 8.7 Hz, H–3′ & H–5′), 4.25 (t, 2H, *J* = 7.2 Hz, H–1″), 2.84 (t, 2H, *J*=7.2 Hz, H–3), 2.25 (t, 2H, *J* = 7.2 Hz, H–2), 1.56 (quint., 2H, *J* = 6.89 Hz, H–2″), 1.24 (br s, 24H, H-3″ – H-14″), 0.86 (t, 3H, *J* = 6.90 Hz, CH_3–15″). ^{13}C-NMR ($CDCl_3$) (75 MHz) (δ ppm): 173.81 (C-1), 154.18 (C-4′), 130.14 (C-1′), 130.05 (C-2′ and C-6′), 115.33 (C-3′ and C-5′), 64.89 (C-1″), 64.89 (C-1″), 34.37 (C-3), 34.31 (C-2), 31.93 (C-13″), 29.70 (C-5″), 29.62 merged (C-7″- C-11″), 29.47 (C-6″), 29.36 (C-12″), 29.27 (C-4″), 29.15 (C-3″), 24.97 (C-2″), 22.69 (C-14″), 14.10 (C-15″). HRMS: $[M]^+$ 376.5196 Calcd. for $C_{24}H_{40}O_3$; 376.5732).

2-Ethyl-n-hexyl benzoate **2.**

Colourless powder (45.9 mg). IR ν_{max} (KBr): 2919 (C-H), 1725 (C=O, ester), 1616, 1499 (C=C, Ar), 1172,1083, (C-O) 817, 713, 628 cm^{-1}; ^{1}H-NMR ($CDCl_3$) (300 MHz) (δ ppm): δ 7.70 (d, 2H, *J* = 7.4 Hz; C-2′ and C-6′), 7.68 (t, 1H, *J* = 7.5 Hz; C-4′), 7.49 (t, 2H, *J* = 7.6 Hz; C-3′ and C-5′), 4.20 (br d, 2H, *J*=6.5Hz, H–1), 1.66 (m, 1H, H–2), 1.41–1.24 (m, 8H, H–3–H–5 & H–1″), 0.90 (t,overlapped), 3H, *J* = 7.1 Hz, CH_3–6), 0.87 (t (overlapped), 3H, *J* = 7.1 Hz, CH_3–2″). ^{13}C-NMR ($CDCl_3$) (75 MHz) (δ ppm): 167.74 (C-7′), 132.44 (C-4′), 131.64 (C-1′), 130.86 (C-2′ and C-6′), 128.79 (C-3′ and C-5′), 68.14 (C-1), 38.72 (C-2), 30.35 (C-3), 28.91 (C-4), 23.73 (C-1″),

22.97 (C-5), 14.04 (C-6), 10.94 (C-2″),). HRMS: $[M]^+$ 234.3293 Calcd. for $C_{15}H_{22}O_2$; 234.3347).

2-Ethyl-n-hexyl trans-cinnamate **3**.

Colourless powder (35.6 mg). IR ν_{max} (KBr): 2933 (C-H), 1714 (C=O, ester), 1623, 1511 (C=C, Ar), 1166,1094, (C-O) 824, 719, 635 cm^{-1}; ^{1}H-NMR ($CDCl_3$) (300 MHz) (δ ppm): δ 7.76 (d, 1H, *J* = 15.9 Hz; H-7′), 7.54 (m, 1H; C-4′), 7.52 (m, 2H; C-3′ and C-5′), 7.39 (m, 2H; C-2′ and C-6′), 6.43 (d, 1H, *J* = 15.9 Hz; H-8′), 4.22 (br d, 2H, *J* = 6.5 Hz, H–1), 1.65 (m, 1H, H–2), 1.40–1.23 (m, 8H, H–3–H–5 and H–1″), 0.92 (t, overlapped), 3H, *J* = 7.1 Hz, CH_3–6), 0.86 (t, overlapped), 3H, *J* = 7.1 Hz, CH_3–2″). ^{13}C-NMR ($CDCl_3$) (75 MHz) (δ ppm): 171.11 (C-9′), 147.04 (C-7′), 134.08 (C-1′), 130.71 (C-4′), 128.96 (C-3′ and C-5′), 128.33 (C-2′ and C-6′), 117.13 (C-8′), 68.19 (C-1), 38.79 (C-2), 30.41 (C-3), 29.70 (C-4), 23.79 (C-1″), 22.98 (C-5), 14.14 (C-6), 11.02 (C-2″),). HRMS: $[M]^+$ 260.3697 Calcd. for $C_{17}H_{24}O_2$; 260.3714).

bis-Dodecylbenzene-1,4-dicarboxylate **4**.

Colourless powder (30.1 mg). IR ν_{max} (KBr): 2929 (C-H), 1735 (C=O, ester), 1628, 1514 (C=C, Ar), 1159,1081, (C-O) 833, 724, 637 cm^{-1}; ^{1}H-NMR ($CDCl_3$) (300 MHz) (δ ppm): δ 8.08 (br s, 4H; H-2′,H-3′,H-5′,H-6′),4.66 (br.s, 4H; H–1 and H–1″), 1.54 (m, 4H, H–2 & H–2″), 1.24 (br.s, 36H, H–3 – H–11 and H–3″- H–11″), 0.84 (t, overlapped), 6H, *J* = 7.2 Hz, CH_3–12 and CH_3–12″). ^{13}C-NMR ($CDCl_3$) (75 MHz) (δ ppm): 167.58 (C-7′ and C-8′), 133.81 (C-1′ and C-4′), 129.72 (C-2′, C-3′, C-5′, C-6′), 62.73 (C-1 and C-1″), 31.91 (C-10 and C-10″), 29.67 (C-2-C-8 and C-2″-C-8″), 29.33 (C-9 and C-9″), 22.66 (C-11 and C-11″), 14.07 (C-12 and C-12″). HRMS: $[M]^+$ 502.7713 Calcd. for $C_{32}H_{54}O_4$; 502.7692).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was done according to Benzie and Strain [26] with some modifications. The stock solutions included 300 mM acetate buffer (3.1g $CH_3COONa.3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2, 4, 6-Tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. The solutions of plant extracts and that of trolox were formed in methanol (500 µg/mL). Ten µL of each of sample solution and BHT solution were taken in separate test tubes and 2990 mL of FRAP solution was added in each to make total volume up to 3 mL. The plant extracts were allowed to react with FRAP solution in the dark for 30 minutes. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The FRAP values were determined as micromoles of trolox equivalents per mL of volatile oil by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

Total Phenolic Contents

Total phenolics of various fractions of plant were determined by the method [27]. 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10% Na_2CO_3 and 0.1 mL of 2N Folin-Ciocalteu reagent. After 40 minutes absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. The standard curve was linear between 50 µg/mL to 500 µg/mL of gallic acid. Results were expressed in GAE µg/mL.

Anti-Urease Assay

The assay was carried out by the method of Weatherburn [28]. A total mixture of 85 µl contained 10 µl of 1 M phosphate buffer, pH 7, followed by the addition of 10 µl test compound (to a final concentration of 0.5 mM in methanol) and 25 µl (0.134 units) of urease enzyme (jack beans, Sigma). Contents were mixed and preincubated at 30ºC for 5 min. After due time, 40 µl of urea (20 mM stock) dissolved in phosphate buffer was added as substrate and contents incubated for 10 min at 30ºC. After the completion of the reaction, liberated ammonia was quantified by the addition of 115 µl phenol hypochlorite reagent (1 % w/v phenol and 0.005% w/v sodium nitoprusside: 0.5% w/v NaOH and 0.1% active NaOCl, in 45: 70 ratio). The reaction mixture was incubated at 40ºC for 10 min and absorbance was measured at 625 nm by 96-well plate reader (Synergy HT BioTek, USA). Thiourea was used as standard inhibitor. Percentage inhibition was measured by the following formula:

$$\text{Inhibition (\%)} = 100 - (A_{\text{test comp}} / A_{\text{control}}) \times 100$$

Anti-tyrosinase Assay

The tyrosinase assay was used by the method as given in Kim *et al.,* [29-31]. A total volume of 200 µl contained 130 µl of 100 mM

phosphate buffrer pH 6.8, 10 µl test compound (0.5 mM final concentration) and 30 µl (30 units) of tyrosinase (from mushroom, Sigma). Contents were mixed and preincubated at 37ºC for 10 min. After given time, 30 µl of 2.5 mM L-DOPA was added as a substrate. Reaction mixture was incubated for 10 min at 37ºC. Absorbance was measured at 490 nm by 96-well plate reader (Synergy HT BioTek, USA). Kojic acid was used as standard inhibitor. Percentage inhibition was measured by the following formula:

$$\text{Inhibition (\%)} = 100 - (A_{\text{test comp}} / A_{\text{control}}) \times 100$$

Statistical Analysis

All the measurements were done in triplicate and statistical analysis was performed by Microsoft excel 2003. Results are presented as average ± SEM.

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