Research Article

Chemical Analysis, Antioxidant Activity and Antimicrobial Activity of Isolated Compounds and Essential Oil from *Callistemon citrinus* Leaf

Manal M. Hamed[1]*, Mosad A. Ghareeb[1], Abdel-Aleem H. Abdel-Aleem[2], Amal M. Saad[1], Mohamed S. Abdel-Aziz[3], Asmaa H. Hadad[1]

[1]Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza12411, Egypt.

[2]Department of Organic Chemistry, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt.

[3]Microbial Chemistry Department, Genetic Engineering and Biotechnology Division, National Research Center, El-Bohouth Street 33, Dokki-Giza 12622, Egypt.

***Corresponding author's E-mail:** manalayman90@yahoo.com

Received: 26-02-2017; **Revised:** 28-04-2017; **Accepted:** 15-05-2017.

ABSTRACT

Natural products derived from medicinal plants provide unlimited opportunities for a new medication leads because of the unmatched accessibility of chemical variation. Six compounds were isolated from the n-butanol extract of *Callistemon citrinus* (Family Myrtaceae), they were identified as; nepetolide (1), callislignan A (2), 6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (3), 3-methyl-7-*O*-benzoyl-*β*-D-glucopyranoside (4), 5, 7, 3', 5'-tetrahydroxy-6, 8-di-*C*-methyl flavanone (5), and (2R,3R,4S,5S)-2,4-bis(4-hydroxyphenyl)-3,5-dihydroxy-tetrahydropyran (6). The isolated compounds were evaluated as antioxidant and antimicrobial agents. The antioxidant activities of the compounds were determined using DPPH-radical scavenging and total antioxidant capacity (TAC) assays. The results indicated that compound (**5**) was most active in its capacity to scavenge free radicals in the DPPH assay [SC_{50} value, 4.65 ± 0.74µg/mL] compared to the standard ascorbic acid and exhibited the highest activity in the TAC assay (610.45 ± 1.67mg AAE/g compound). The pure isolates were tested for their antimicrobial activity against four pathogenic microbial strains including *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*. Also the GC/MS analysis of its leaves essential oil presented nine identified compounds representing 91% of the total oil constituents. The outcomes got from this study give a reasonable justification to the medicinal uses of *Callistemon citrinus* plant.

Keywords: *Callistemon citrinus,* Flavanone, Antioxidant activity, Antimicrobial activity, Essential oil, Myrtaceae.

INTRODUCTION

Plants have been utilized for a last of years to flavor and preserve food, to treat health disturbance and to prohibit diseases including pestilence[1, 2]. Medicinal plants and herbs are of great significance to the human health everywhere throughout the world, which consider an imperative source of powerful therapeutic agents to treat an extensive range of ailments and health disorders[3]. Human bodies are bared to exogenous oxidizing agents for example, toxins, smoking and chemicals so they becomes at risk. Oxidizing agents such as chemical substrates are contains reactive oxygen species (ROS) like peroxyl ($ROO^{\bullet}$) radicals, superoxide anion ($O2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), as well as reactive nitrogen species (RNS) like nitric oxide ($NO^{\bullet}$) radical and peroxynitrite anion ($ONOO^{-}$); in general unsafe outcomes happened when such species assault human cell and tissues prompting to cancer[4]. *Callistemon* species belonging to family Myrtaceae are broadly utilized for forestry, ornamental agriculture, and essential recovery. The most species of *Callistemon* are well known in traditional medicine for its anti-coughs action and the essential oils have been utilized as antimicrobial and antifungal agents[5]. It was reported that *C. citrinus* possesses vital and numerous pharmacological and biological activities such as antibacterial activity[6], antioxidant activity[7] and larvicidal activity[8]. Also, the genus *Callistemon* was reported to contain diverse chemical profile; steroids, triterpenes, flavonoids[9], tannins, phenolic compounds[10, 11] and essential oils[12]. Phytochemical investigation of *C. citrinus* leaves and stems led to isolation of flavonoid and triterpenoid compounds[13].

Flavonoids plant secondary metabolites are important components of the human diet, although they are not only investigated as nutrients but also, exhibited antioxidant properties. In addition flavonoids have been reported to showed other multiple biological activities such as antiviral[14], antibacterial[15], anti-inflammatory[16] and anticancer [17]. Moreover, they are able to inhibit lipid peroxidation and platelet accumulation and enhance expanded capillary permeability and fragility[18-20]. Therefore, in our study, the isolated compounds from *Callistemon citrinus* was investigated for its free radical scavenging potential (DPPH), total antioxidant capacity (TAC) and antimicrobial activity. Moreover, the volatile oil identification from the fresh leaves of the plant by GC/MS analysis.

MATERIALS AND METHODS

Plant Materials

Leaves of *Callistemon citrinus* (Family Myrtaceae) was collected from Zoo Garden, Giza, Egypt in May 2014. The plant was identified by Dr. Threase Labib consultant of

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plant taxonomy at the Ministry of Agriculture; formerly, the Head of Taxonomist Specialists at the garden, a voucher specimen (No.C1/4/1 for *Callistemon citrinus*) was kept at the herbarium of the garden. The plant materials was air dried in shade at room temperature, powdered by an electric mill, and kept in tightly closed container in dark places until subjected to the extraction process.

Apparatus and Equipments

Portable UV lamp Vilber Louremat (VL-6LC 254 and 365 nm). UV-visible spectrophotometer, Spectronic 601 (Milton Roy, USA) was used for measuring the absorbance in UV range. Nuclear Magnetic Resonance NMR Spectrometer: Jeol Delta 2 spectrometer (300/75 MHz), NMR Department, Faculty of Science, Cairo University. Melting points, SMP3 Stuart Scientific (UK). Sensitive electric balance, BP 2215 (Germany). Microfine Cutting Grinder (IKA WERKE, Germany). The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS and TGSMS Fused Silica Capillary Column (30 m, 0.251 mm, 0.1 mm Film thickness), National Research Center, Giza, Egypt. For GC/MS detection, an electron ionization system with ionization energy for 70 ev was used as the carrier gas at a constant flow rate of 1 ml/min. The chemistry works were done at the Medicinal Chemistry Department, Theodor Bilharz Research Institute.

Chemicals for Biological Studies

All solvents and reagents used were of analytical grade. 2,2'-diphenyl-1-picraylhydrazyl (DPPH) free radical was purchased from (Sigma-Aldrich Co.). Ferric chloride, aluminum chloride, sodium bicarbonate, sodium phosphate, ammonium molybdate and ascorbic acid were purchased from (Merck Chemical Co.), D- Glucose (Merck, Germany). All solvents and acids [petroleum ether, chloroform, ethyl acetate, *n*-butanol, acetic acid, and sulphuric acid, dimethylsulphoxide (DMSO)] were purchased from (Sigma-Aldrich Co.). Paper chromatography (PC) was done on Whatmann No. 1 (57 x 46 cm) and Whatman filter paper No.3 (3 CHR 46x57 cm) Whatman Ltd., Maidstone, Kent, and England; while thin layer chromatography (TLC) was performed over pre-coated silica plates (GF_{254}, Merck). For column chromatography (CC), Silica gel (70-230 mesh) (Merck), Sephadex LH-20 (Sigma Aldrich/Germany) and Polyamide 6S (Sigma). Methanol, methylene chloride, ethyl acetate, acetone, *n*-butanol, acetic acid and petroleum ether (All purchased from El-Nasr Pharmaceutical Chemicals Co. Adwic; Egypt). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

Solvent Systems

(S1)[$CHCl_3$:MeOH, 8.5:1.5]; (S2) [AcOH:H_2O (15% AcOH), 15:85]; (S3) [BuOH:AcOH:H_2O (BAW), 4:1:5 (upper phase)]; (S4) [$CHCl_3$:Me_2CO:MeOH:H_2O, 3:3:2:1] and (S5)[Ethyl acetate: Methanol, 19:1].

Antioxidant Activity

DPPH Free Radical Scavenging Activity. The scavenging activity of the stable 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined by the method described by Marwah *et al.*, 2007. Briefly, the reaction medium contained 2 ml of 100 µM DPPH purple solution in methanol and 2 ml of sample (pure compound). The reaction mixture was incubated in the dark for 20 min and the absorbance was recorded at 517 nm, the assay was carried out in triplicate. The decrease in absorbance on addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (%IP) of DPPH radical, following the equation:

$\%IP = [A_c - A_s]/ A_c \times 100$ where A_c and A_s are the absorbencies of the control and of the test sample after 20 min, respectively. From a plot of concentration against %IP, a linear regression analysis was performed to determine the IC_{50} (compound concentration resulting in 50% inhibition) value for each sample. The scavenging reaction between ($DPPH^{\bullet}$) and an antioxidant (A-H) is expressed as:

$(DPPH^{\cdot}) + (A\text{-}H) \rightarrow DPPH\text{-}H + (A^{\cdot})$

Antioxidants react with purple colored 2, 2'-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), which is a stable free radical and is reduced to the yellow colored 2, 2'-diphenyl-1-picrylhydrazin (DPPH-H) and as consequence the absorbances decreased from the $DPPH^{\bullet}$ radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability[21].

Determination of Total Antioxidant Capacity (TAC)

The antioxidant activity (AOA) of each sample (pure compounds) was determined according to phosphomolybdenum method[22] using ascorbic acid as standard. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green colored [phosphate=Mo (V)] complex at acidic pH. In this method, 0.5 ml of each compound (100 µg/ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the sample was expressed as the number of equivalents of ascorbic acid (AAE).

Antimicrobial activity

Disc agar plate method was used to evaluate the antimicrobial activity according to the reported method[23].

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Complete Acid Hydrolysis for Compound 4

The compound (3 mg) was hydrolyzed with 10% HCl (3.5 ml) in aqueous methanol at 100°C for 2 hrs, after the removal of the solvent, hydrolysate was exhaustively extracted with ethyl acetate in separating funnel. Aglycone was identified via Co-PC with authentic sample. The aqueous phase was neutralized with 5% sodium bicarbonate and used for investigation of the sugar moieties via Co-TLC with authentic sugar markers in solvent system (S4)[24,25].

GC/MS Analysis

The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min) to 280°C was a final temperature at an increasing rate of 5°C/min (hold 5 min). The identified components were investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY Library data of the GC/MS system[26].

Statistical Analysis

All data were presented as mean ± S.D. of triplicates (n=3) according to Annegowda *et al*., 2010 using SPSS 13.0 program (SPSS Inc. USA) [27].

Extraction and Fractionation

The air-dried powder leaves (1.5 Kg) of *C. citrinus* were extracted via percolation in 85% methanol at room temperature 25±2°C, and then repeated till complete extraction process (2 liter x 5 times). The combined extracts were concentrated via Rotatory evaporator at 40°C, to afford 85% methanol extract as a dark brown residue (520 g). Then 500 g was defatted using petroleum ether (60-80°C) (2.5 L) to remove the lipoidal materials i.e., fats and sterols, to afford a semi oily residue (39.43 g). The defatted 85% methanol (400 g) was suspended in distilled water and then successively fractionated with methylene chloride (3 L), ethyl acetate (3 L), *n*-butanol (3.5 L), to afford methylene chloride (55.33 g), ethyl acetate (21.67 g), *n*-butanol (34.01 g), and aqueous (105 g) fractions. The resulting fractions were chromatographically compared via 2D-PC using solvent systems (S_2& S_3), which guide us to select the *n*- BuOH fraction for further chromatographic separation.

Chromatographic Isolation of the *n*-Butanol Fraction

Thirty grams (30 g) of the *n*-butanol fraction were subjected to chromatographic isolation using polyamide column chromatography (CC) (100 X 6 cm, 200 gm). A gradient elution was started with 5% MeOH:H_2O, and the polarity was gradually increased by methanol to pure MeOH at the end. Fractions (250 ml each) were collected, concentrated and examined [PC (S_2& S_3), TLC (S_1&S_5), 5% $AlCl_3$ and 1% $FeCl_3$, UV light for detection]. Similar fractions were pooled according to their pattern upon paper chromatography, to afford six main fractions (1-6) matching to the flavonoids & phenolic category. Fraction 1 (2 g) eluted with 15% MeOH, which was then rechromatographed over silica gel (CC) to afford compound 1 (12 mg) eluted via MeOH:CH_2Cl_2 (30:70/ v:v). Fraction 2 (0.6 g) eluted with 20% MeOH, which was then rechromatographed over Sephadex LH-20 (CC) to afford compound 2 (10 mg) eluted via H_2O:MeOH (15:85/ v:v). Fraction 3 (0.9 g) eluted with 30% MeOH, which was then rechromatographed over Sephadex LH-20 (CC) to afford compound 3 (14 mg) eluted via H_2O:MeOH (25:75/ v:v). Fraction 4 (0.6 g) eluted with 40% MeOH, which was then rechromatographed over Sephadex LH-20 (CC) to afford compound 4 (10 mg) eluted via H_2O:MeOH (30:70/ v:v). Fraction 5 (0.9 g) eluted with 50% MeOH, which was then rechromatographed over Sephadex LH-20 (CC) to afford compound 5 (10 mg) eluted via H_2O:MeOH (10:90/ v:v).Fraction 6 (0.8 g) eluted with 60% MeOH, which was then rechromatographed over Sephadex LH-20 (CC) to afford compound 6 (12 mg) eluted via H_2O:MeOH (15:85/ v:v).

Essential Oil Isolation

The fresh leaves of *Callistemon citrinus* (900 g) were fragmented into small pieces and subjected to hydro-distillation using Clavenger-type glass apparatus[2] for 3 hours, after the mixture boiled at (90°C); the obtained light yellow oil was separated, stored in a freezer in air-tight container at -20°C until analyzed. The chemical composition of the collected oil was qualitatively via GC-MS by comparing their retention times and mass spectral fragmentation patterns with the previously reported data[26].

RESULTS AND DISCUSSION

Compound 1 was obtained as gummy brown, m.p. 217°C, showed a violet spot on TLC after spraying by H_2SO_4 and heating, R_f 0.51 (S1), its ^{1}H-NMR spectra revealed the presence of two tertiary methyls at δ_H 1.23 and 0.75 (3H, *s*) and one secondary methyl at δ_H 1.02 (*d*, *J* = 7.3 Hz). A *β*-monosubstituted furan ring was indicated via resonances at δ_H 6.80 (*d*, *J* = 1.8 Hz, H-14), 7.68 (*br s*, H-16), and 7.10 (*d*, *J* = 1.8 Hz, H-15), as well as a set of aliphatic protons with a characteristic signals at δ_H 1.55 (*m*, H-1), 1.97 (*m*, H-2), 4.20 (*t*, H-3), 3.31 (*d*, H-4), 2.26 (*dt*, H-6), 2.17 (*m*, H-7), 1.46 (*m*, H-10), and 1.61 ppm (*m*, H-11) (Table 1), accordingly compound 1 was identified as nepetolide[28].

Compound 2 was obtained as a yellowish brown, m.p. 116-118°C, showed dark coloured spot on TLC, R_f 0.48 (S5), its ^{1}H- NMR spectra contained resonances assigned to δ_H 6.68 (H-4), 6.60 (H-6), methine protons 3.31 (H-3), oxy-methine protons at δ 4.96 (*d*, H-2, *J*=9.6 Hz), 1.23 (aliphatic CH_3, *d*, *J* = 6.6 Hz), 5.15 (*dq*, H-1') 5.14 (*dq*, H-2'), 1.66 (*dd*, H-3'), also a characteristic resonances for 1,2,4-trisubstituted phenyl group at δ_H 6.75 (*d*, *J* = 8.4, H-5''), 6.85 (*dd*, *J* = 8.4, 1.8, H-6'') and 7.19 (*d*, *J* = 1.8, H-2''), an aromatic methoxy group at 3.74 (3''-OCH_3) and two phenolic protons at 7.79 (7-OH), 7.60 ppm (4''-OH) (Table

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1), accordingly compound 2 was identified as callislignan A[29].

Compound 3 was obtained as a colorless needles, m.p. 251-253°C, exhibited blue fluorescent colour on TLC under long UV lamp, R_f 0.45 (S3), its ^{1}H-NMR spectra revealed the presence of a set of protons; methine proton appeared at δ_H 3.72 (1H, *q*, *J* = 7.0 Hz, H-4), aromatic proton 6.69 (*s*, H-7), two olefinic proton 4.44 (2H, *d*, *J* = 1.5 Hz, H-9), six methyl protons 1.43 (3H, *d*, *J* = 7.0 Hz, H-10), 2.0 (3H, *s*, H-11), six methoxy protons 3.59 (*s*, OCH_3-6), and 3.72 ppm (*s*, OCH_3-8) (Table 1), hence compound 3 was identified as (6,8-dimethoxy-4,5-dimethyl-3-methyleneisochr-oman-1-one)[30].

Compound 4 was obtained as a white amorphous powder, m.p. 112-114°C, it showed blue coloured spot under UV light, R_f 0.39 (S1). The ^{1}H-NMR spectra revealed the presence of glucopyranosyl moiety. The *β*-anomeric configuration for glucopyranose was determined from δ_H 5.4 (Glc-H-1'). Besides the signals of sugars from δ_H 3.72-3.50, the ^{1}H-NMR spectrum of this compound showed a set of proton signals due to a 7.50 (2H, *dd*, *J*=8.3, 1.4 Hz, H-2,6), 7.09 (2H, *dd*, *J*= 8.3, 1.4 Hz, H-3,5), 1.9 (3H, *s*, CH_3-4) (Table 2). The complete acid hydrolysis afforded D-glucose in the aqueous phase which detected by Co-thin layer chromatography (Co-TLC) with authentic sugar markers; hence compound 4 was identified as 3-methyl-7-*O*-benzoyl-*β*-D-glucopyranoside[31].

Compound 5 was obtained as a yellow amorphous powder, m.p. 200-202°C, R_f 0.07 in (S2), it showed a dark purple coloured spot under UV light. The ^{1}H-NMR spectra revealed the presence of three proton resonances at δ_H 6.16 (1H, *dd*, *J* = 12.0, 3.0 Hz, H-2), 3.34 (1H, *dd*, *J* = 17.0, 12.0 Hz, H-3a) and 2.46 (1H, *dd*, *J* = 17.0, 3.0 Hz, H-3b) were characteristic of a hydroxyl-substituted flavanone moiety structure. Furthermore, a three aromatic proton signals at δ_H 6.89 (1H, *s*, H-4') and 6.41 (2H, *s*, H-2',6') suggested the presence of 3', 5'-disubstituted B-ring. Beside a singlet at δ_H 0.83 (3H, H-6) and 1.21 (3H, H-8) for two aromatic methyl groups, and characteristic signals at δ_H 12.34 for 5-OH and 9.54 ppm for 7-OH (Table 2); therefore compound 5 was identified as 5, 7, 3', 5'-tetrahydroxy-6, 8-di-*C*-methyl flavanone[32].

Compound 6 was obtained as a colourless liquid, R_f 0.43 in (Ethyl acetate:Methanol) (19:1), it showed dark coloured spot on TLC,its ^{1}H-NMR spectra revealed the presence of a set of aromatic protons with a characteristic signals appeared at δ_H 7.49 (2H, *d*, *J*=7.5 Hz, H-2', 6'), 6.81 (2H, *d*, *J*=7.8 Hz, H-3', 5'), 7.68 (2H, *d*, *J*=8.5 Hz, H-2'', 6''), and 7.10 ppm (2H, *d*, *J*=8.5 Hz, H-3'', 5''), as well as a set of aliphatic protons at δ_H 3.76 (1H, *t*, *J*=6.4 Hz, H-4), 4.37 (1H, *d*, *J*=4.2 Hz, H-2), 4.12 (1H, *dd*, *J*=11.7, 5.4 Hz, H-6a), 3.31 (1H, *dd*, *J*=11.7, 3.7 Hz, H-6b) (Table 2); therefore compound 6 was identified as (2R,3R,4S,5S)-2,4-bis(4-hydroxyphenyl)-3,5-dihydroxy-tetrahydropyran[33].

Table 1: ^{1}H-NMR spectral data of compounds 1-3 (300 MHz, DMSO-d_6; TMS as internal standard, δ in ppm and *J* in Hz).

Compound 1		Compound 2		Compound 3	
Position	**δ_H (ppm)**	**Position**	**δ_H (ppm)**	**Position**	**δ_H (ppm)**
H-1	2H, 1.55, *m*	H-2	1H, 4.96, *d*, *J*= *9.6* Hz	H-4	1H, 3.72, *q*, *J* = 7.0 Hz
H-2	2H, 1.97, *m*	H-3	1H, 3.31, *s*	H-7	1H, 6.69, *s*
H-3	1H, 4.20, *t*	H-4	1H, 6.68, *d*	H-9	2H, 4.44, *d*, *J* = 1.5 Hz
H-4	1H, 3.31, *d*	H-6	1H, 6.60, *s*	H-10	3H, 1.43, *d*, *J* = 7.0 Hz
H-6	1H, 2.26, *dt*	H-1'	1H, 5.15, *dq*	H-11	3H, 2.0, *s*
H-7	2H, 2.17, *m*	H-2'	1H, 5.14, *dq*	6-OCH_3	3H, 3.59, *s*
H-10	1H, 1.46, *m*	H-3'	1H, 1.66, *d*	8-OCH_3	3H, 3.72, *s*
H-11	2H, 1.61, *m*	H-2''	1H, 7.19, *d*, *J* =1.8 Hz		
H-14	1H, 6.80, *d*, *J* = 1.8 Hz	H-5''	1H, 6.75, *d*, *J* =8.4 Hz		
H-15	1H, 7.10, *d*, *J* = 1.8 Hz	H-6''	1H, 6.85, *dd*, *J* =8.4, 1.8 Hz		
H-16	1H, 7.68, *br s*	3''-OCH_3	3H, 3.74, *s*		
CH_3-17	3H, 1.02, *d*, *J* = 7.3 Hz	7-OH	1H, 7.79		
CH_3-19	3H, 1.23, *s*	4''-OH	1H, 7.60		
CH_3-20	3H, 0.75, *s*				

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Table 2: ^{1}H-NMR spectral data of compounds 4-6 (300 MHz, DMSO-d_6; TMS as internal standard, δ in ppm and *J* in Hz).

Compound 4		Compound 5		Compound 6	
Position	**δ_H (ppm)**	**Position**	**δ_H (ppm)**	**Position**	**δ_H (ppm)**
H-2,6	2H, 7.50, *dd*, *J*= 8.3, 1.4 Hz	H-2	1H, 6.16, *dd*, *J*= 12.0, 3.0 Hz	H-2',6'	2H, 7.49, *dd*, *J*= 7.5 Hz
H-3,5	2H, 7.09, *dd*, *J*= 8.3, 1.4 Hz	H-3a	1H, 3.34, *dd*, *J*= 17.0, 12.0 Hz	H-3',5'	2H, 6.81, *dd*, *J*= 7.8Hz
Glc-H-1'	1H, d, *J*= 7.6 Hz	H-3b	1H, 2.46, *dd*, *J* = 17.0, 3.0Hz	H-2'',6''	2H, 7.68, *d*, *J* = 8.5Hz
Rest of sugar protons	3.72-3.50	H-4'	1H, 6.89, *s*	H-3'',5''	2H, 7.10, *d*, *J* = 8.5 Hz
H-6	1H, 2.26, *dt*	H-2',6'	2H, 6.41, *s*	H-4	1H, 3.76, *t*,*J* = 6.4Hz
H-7	2H, 2.17, *m*	CH_3-6	3H, 0.83	H-2	1H, 4.37, *d*, *J* = 4.2Hz
H-10	1H, 1.46, *m*	CH_3-8	3H, 1.21	H-6a	1H, 4.12, *dd*, *J* =11.7, 5.4 Hz
H-11	2H, 1.61, *m*	5-OH	1H, 12.34	H-6b	1H, 3.31, *dd*, *J* =11.7, 3.7 Hz
H-14	1H, 6.80, *d*, *J* = 1.8 Hz	7-OH	1H, 9.54		
H-15	1H, 7.10, *d*, *J* = 1.8 Hz				
H-16	1H, 7.68, *br s*				
CH_3-17	3H, 1.02, *d*, *J* = 7.3 Hz				
CH_3-19	3H, 1.23, *s*				
CH_3-20	3H, 0.75, *s*				

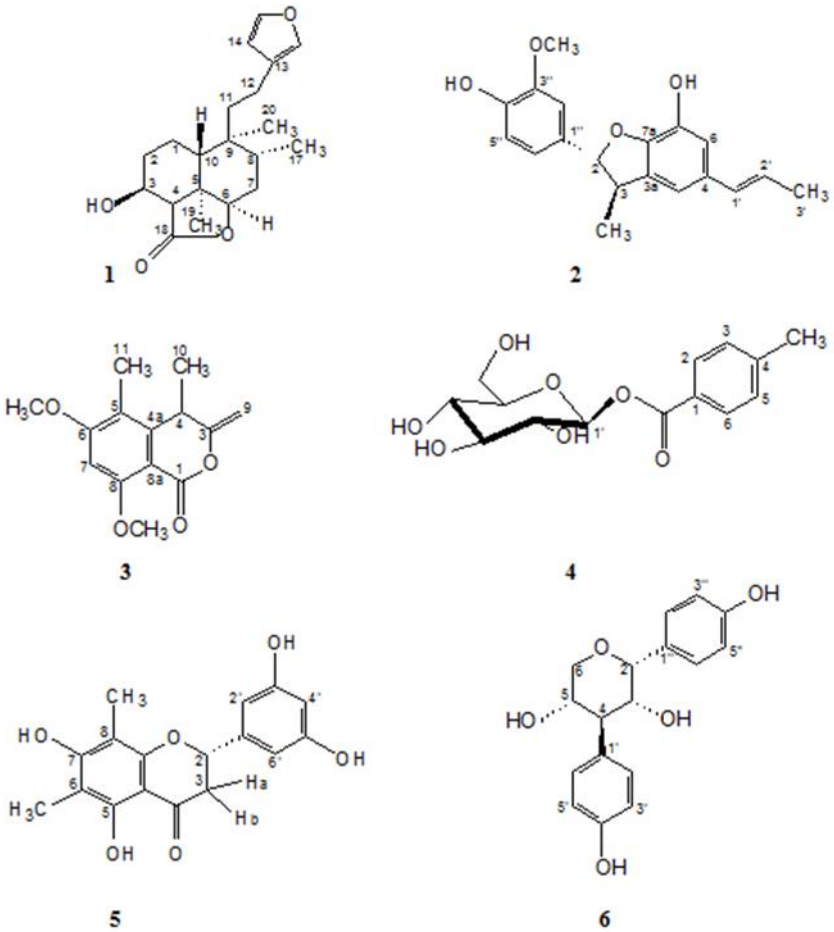

Figure 1: Chemical structure of isolated compounds 1-6

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Identification of the Essential Oil Constituents

GC/MS analysis of the essential oil of *Callistemon citrinus* leaves presented nine identified compounds representing 91% of the total oil constituents, which were major and identified based on their retention times and mass spectral fragmentation patterns into; 3-methoxyphenol (15.06%), Isofraxidin (13.96%), Hydronaphthoquinone (10.84%), Methimazole (8.82%), Sinapic acid (6.93%), as the major constituents (Table 3). Zandi-Sohani *et al.*, reported that; *Callistemon citrinus* essential oil growing in Iran was extracted by hydrodistillation and analyzed by GC-MS and GC-FID. Therefore a thirty-six compounds were elucidated and the major components showed; 1,8-cineole (34.2%), α-pinene (29.0%), α-terpineol (10.7%), α-phellandrene (9.0%) and limonene (3.4%)[34]. Moreover, Kumar *et al.*, found that; the GC and GC-MS analysis of the essential oils from *Callistemon citrinus* revealed high content of monoterpene hydrocarbons (52.1%), α-pinene (32.3%), sesquiterpenoids (14%), limonene (13.1%) and α-terpineol (14.6%) in leaf, However, the flower oil produce monoterpene hydrocarbons (44.6%), 1,8-cineole (36.6%), α-pinene (29.7%) and sesquiterpenoids (1.2%). While, the flower oil appears riches within oxygenated monoterpenes (43.5%)[35]. From the lower region of Himalayas, literature revealed that; the oil composition of *Callistemon citrinus* leaves was examined and afforded thirty two compounds, constitutes 98.1% of the oil, were summarized with major constituents of 1,8-cineole (66.3%) and α-pinene (18.7%)[36].

Table 3: Chemical constituents identified in the essential oil of *Callistemon citrinus* leaves using GC/MS.

Peak No.	R_t	Area%	M.F.	Identified Compounds
1	4.91	4.57	$C_5H_7NO_2$	Piperidene-2,5-dione
2	7.44	6.93	$C_{11}H_{12}O_5$	Sinapic acid
3	7.80	7.26	$C_{19}H_{25}NO_2$	Nylidrin
4	9.44	8.82	$C_4H_6N_2S$	Methimazole
5	10.66	9.92	$C_8H_{10}O_2$	Resorcinol, monobenzoate
6	11.65	10.84	$C_{10}H_8O_2$	Hydronaphthoquinone
7	14.69	13.66	$C_{10}H_{32}$	Isophyllocladene
8	15.01	13.96	$C_{11}H_{10}O_5$	Isofraxidin
9	16.18	15.06	$C_7H_8O_2$	3-methoxyphenol
Total %		91%		

The identification of the components was based on comparison of their mass spectral fragmentations pattern with those of the data reported in Wiley and NIST Libraries and those described by (Adams, 2001)[26]. M.F.: Molecular formula; R_t: Retention time.

***In Vitro* Antioxidant Activities of the Isolated Compounds**

Halliwell and Gutteridge (1998) suggest a definition of antioxidant material as "any substance that delays, prevents or removes oxidative harm to a target molecule"[37]. The *in vitro* antioxidant activities of the isolated compounds were evaluated via two antioxidant assays namely; DPPH free radical scavenging assay (SC_{50} values) and phosphomolybdenum assay [total antioxidant capacity (TAC)]. The results of the free radical scavenging activity showed that the SC_{50} values of the tested compounds were 14.23, 10.50, 12.15, 4.65 and 18.32 µg/ml, respectively for the compounds 2-6, compared to ascorbic acid as standard with SC_{50} of 7.60 µg/ml, and there is no any activity was recorded with the compound 1 (Table 4). Moreover, the total antioxidant capacity (TAC) values of the tested compounds were 354.0, 525.20, 448.20, 610.45 and 295.53 mg ascorbic acid equivalent AAE/g compound, respectively for the compounds 2-6, and there is no any activity was recorded with the compound 1 (Table 4). From the structural activity relationship (SAR) point of view, the tested compound exhibited a wide range of the antioxidant activities with large variation in both of SC_{50} and TAC values, this phenomena may be returns to the presence or absence of the characteristic structural criterion for effective free radical scavenging activity including; the presence of an 2, 3 unsaturated double bond, 4-oxo group at (ring-C), and 5-OH at (A-ring). Furthermore, mono and di glycosides were less active than their aglycones which may be due to steric hindrance offered by a bulky glycosidic moiety[38, 39].

***In Vitro* Antimicrobial Activities of the Isolated Compounds**

The pure isolates were tested for their antimicrobial activity against four pathogenic microbial strains including Gram (+ve) and Gram (-ve) bacteria and yeast *i.e.*, *Pseudomonas aeruginosa* with inhibition zones (10.5, 14.0, 12.5, 13.0, 12.0 and 15.0 mm; respectively for the compounds from 1-6, *Staphylococcus aureus* with inhibition zones (18.5, 17.0, 10.5, 11.0, 14.0 and 11.0 mm; respectively for the compounds from 1-6), Methicillin-

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resistant *Staphylococcus aureus* with inhibition zones (19.5, 15.0, 11.5, 14.0, 12.0 and10.0 mm; respectively for the compounds from 1-6), *Candida albicans* with inhibition zones (16.5, 13.0, 10.5, 11.0, 13.0 and 15.0 mm; respectively for the compounds from 1-6 (Table 5). Cowan, 1999 reported on the antimicrobial activity of major groups from plants *viz.,* phenolics & polyphenols, simple phenols & phenolic acids, quinones, flavones, flavonoids & flavonols, tannins, coumarins, alkaloids and terpenoids[23,40].

Table 4: *In vitro* antioxidant activities of the isolated compounds 1-6.

Compound	DPPH free radical scavenging activity SC_{50} (µg/ml)[1]	Total antioxidant capacity (mg ascorbic acid equivalent AAE /g compound)[2]
1	ND[3]	ND
2	14.23 ± 1.34	354.0 ± 1.47
3	10.50 ± 1.23	525.20 ± 1.74
4	12.15 ± 0.85	448.20 ± 1.24
5	4.65 ± 0.74	610.45 ± 1.67
6	18.32 ± 0.90	295.53 ± 1.89

[1]SC_{50}: concentration from sample required for scavenging of 50% of radical.

[2]Total antioxidant capacity (TAC) was evaluated by the phosphomolybdenum assay in mg ascorbic acid equivalent AAE /g compound.

ND[3]: Not detected.

Table 5: *In vitro* antimicrobial activities of the isolated compounds 1-6.

Compound	Clear Inhibition zone (ϕmm)			
	Pseudomonas aeruginosa	*Staphylococcus aureus*	*MRSA*	*Candida albicans*
1	10.5	18.5	19.5	16.5
2	14.0	17.0	15.0	13.0
3	12.5	10.5	11.5	10.5
4	13.0	11.0	14.0	11.0
5	12.0	14.0	12.0	13.0
6	15.0	11.0	10.0	15.0

The results of compounds (1-6) against *Staphylococcus aureus* (G+ve bacteria); Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans* (yeast).

CONCLUSION

Natural products are yet reflected as potential resources for drug discovery and therefore, play a vital role in drug development programs. This approach resulted in the isolation of six compounds from the *n*-butanol extract of *Callistemon citrinus*, they were identified as; nepetolide (1), callislignan A (2),6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (3), 3-methyl-7-*O*-benzoyl-*β*-D-glucopyranoside (4), 5, 7, 3', 5'-tetrahydroxy-6,8-di-*C*-methyl flavanone (5) and (2R,3R,4S,5S)-2,4-bis(4-hydroxyphenyl)-3,5-dihydroxy-tetrahydropyran (6). These compounds are of significant antioxidant and antimicrobial activities. Compound 5 was the most promising antioxidant among the isolated compounds. So the outright findings of this study serves remarkable contribution in proper use of *Callistemon citrinus* leaves for superior health care system of people.

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Source of Support: Nil, **Conflict of Interest:** None.

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