Anticancer and antioxidant potential of pentacyclic triterpenoids isolated from *C. carandas* a medicinally important plant

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Abstract
Carissa carandas Linn, an important medicinal plant was used for the isolation of potent bioactive compounds from its leaves. C. carandas leaf material was shade dried and macerated in solvents such as hexane, chloroform, ethyl acetate and methanol and then concentrated using a rotary vacuum evaporator. FRAP, DPPH and the reducing power assays were used to measure the antioxidant activity. Isolation and purification of compounds using column chromatography followed by spectral analysis was done for identification. Of the four solvents used hexane, chloroform, ethyl acetate and methanol, the antioxidant potential of the methanol extract of C. carandas exhibited high DPPH (76.22µg/mL), FRAP (46.00µg/mL) and reducing power (81.83µg/mL) activities respectively.

Gradient elution, followed by structural analysis using UV, IR and NMR spectral studies, resulted in the isolation of six triterpenoids, namely β-sitosterol and stigmasterol, lupeol, betulin, betulinic acid, oleanolic acid and methoxy urosolic acid. A comparative antioxidant activity of the isolated triterpenoids revealed the superiority of methoxy urosolic acid over the other triterpenes. Methoxy urosolate also exhibited anti-inflammatory (41.05%), cytotoxic (83.24%) and apoptotic potential against the lung cancer cell line A549. Of the four extracts, methanol extract exhibited the maximum antioxidant activity.

Keywords: *Carissa carandas*, leaf extract, column chromatography, anti-oxdant, anti-inflammatory, anti-cancer activities.

Introduction

Carissa carandas Linn. is an important medicinal plant widely used by tribals throughout India. It is an evergreen shrub belonging to the family Apocynaceae. More than 25 species of the genus *Carissa* are known, five of which are indigenous to India[15]. The plant is used in many traditional medical systems including unani, ayurveda and homeopathy.

It is used to treat scabies, intestinal worms, diarrhea and intermittent fever. A wide variety of phytochemicals are known to be present in *C. carandas*. Leaf decoctions are used to treat recurrent fever and unripe fruit has been shown to act as an effective appetizer, astringent, antiscorbutic and anthelmintic[24].

The highest concentrations of iron, vitamin C and other vitamins, including pyridoxine, thiamine, riboflavin, pantothenic acid, biotin and folic acid, are found in fruit extracts[2]. It is also used in most food preparations like syrups, jams, jellys and squash[22]. The demand for the plant has been increasing tremendously in the market as it contains valuable phytochemicals and they are found to be very effective in the treatment of anaemia[10,14]. The fruits of *C. carandas* are known to possess valuable phytochemicals, but the information on phytochemicals isolated from leaf extract and their biological activities is meager. Hence, the present study was carried out.

Material and Methods

Collection of plant material and prepration of plant extracts: Fresh leaf material of *C. carandas* was collected (Voucher specimen No. 22289) and shade dried. Maceration was done followed by elution of the column sequentially using non-polar to polar analytical grade refluxing solvents such as hexane, chloroform, ethyl acetate and methanol. The mixture was distilled to recover the extracts and was then filtered and dried under pressure at room temperature in a rotating vacuum evaporator. After the extracts were concentrated, the percentage yield was determined.

Antioxidant activity of leaf extracts: For the assessment of antioxidant activity, the extracts were dissolved in dimethyl sulfoxide (DMSO) as per the method of analysis.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity: The DPPH free radical scavenging activity was used to assess the antioxidant activity of various extracts[11]. 1 mL of 0.1 mM DPPH in methanol was added to 3 mL of different extracts in dimethyl sulphoxide (DMSO) at different concentrations (50, 100, 150 and 200 µg/ mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm by using a spectrophotometer (UV-VIS Shimadzu). BHT was used as a reference standard. The % DPPH scavenging activity was determined using the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of the test or standard sample.

FRAP (Ferric Reducing Antioxidant Power) assay: The assay was performed with minor modifications[7]. To prepare FRAP solution, 20 mM 2,3,5-Triphenyltetrazolium chloride (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3$ and 300 mM acetate buffer (pH 3.6) were combined in a ratio of 10:1:1. The FRAP solution was then tested against extracts by allowing them to react with it in a ratio of 1:30 for 30 minutes at 37°C in dark. After the formation of the blue-colored ferrous tripyridyl triazine complex, the absorbance was measured at 593 nm. Ascorbic acid was used as a reference standard.

Reducing Power Assay: 1 mL aliquots of different concentrations of the standard and test sample extracts (50 to 200µg/mL) were combined with 2.5ml of phosphate buffer (pH 6.6) and 2.5mL of (1%) potassium ferricyanide[25]. The mixture was incubated at 50°C in a water bath for 20min. After cooling, aliquots of 2.5mL of (10%) trichloroacetic acid were added to the mixture and then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5mL) was mixed with 2.5mL distilled water and freshly prepared 0.5mL of (0.1%) ferric chloride solution. The absorbance was measured at 700nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations (20 to100 µg/mL) was used as standard.

Isolation and purification of compounds by column chromatography: A long cylindrical glass column of 1600mL capacity was cleaned with acetone and initially packed with glass cotton at the bottom end for separation and purification. Silica gel (60-120 mesh) of about 500g was mixed with the solvent n-hexane.

To facilitate sample dispersion in a silica gel column that had already been packed, plant extract was combined with about 80g of silica gel to create a fine powder form, which was then covered with a layer of cotton. The active molecules were then separated and purified from the crude extract by passing solvents with varying polarity through the column at a constant pace while subjected to gravity.

To achieve the best elution and optimal separation of different components from the plant extract, a gradient solvent system was employed. Hexane and ethyl acetate were added to the column in the following ratios: 99:1; 98:2; 97:3; 96:4; 95:5; 94:6; 92:8; 90:10; 88:12; 86:14; 84:16; 82:18; 80:20; 75:25; 70:30. A total of 446 fractions (266.6 ml each) were collected. TLC analysis was then used to recombine the obtained fractions.

Characterization and structural elucidation of compounds: The isolated compounds were analyzed by UV-visible, FTIR, LC-MS and ^1H and ^{13}C NMR methods.

UV-Visible spectroscopy: For the isolated compounds, UV-visible spectra were captured at room temperature using a UV-visible spectrometer (Shimadzu UV-160A, Singapore). The spectrum was recorded at 200–800 nm wave length region using 1 mg of each compound diluted in 20 mL of chloroform.

Infrared Spectroscopy (IR): Thermo Nicolet Nexus FT-IR spectrometer (spectra 2000) was used to record the infrared spectra of the isolated compounds. To prepare the pellet, 1 mg of each compound was combined with spectroscopic grade KBr and well ground. The $4000cm^{-1}$ - $400cm^{-1}$ frequency range (n) was used to capture the infrared spectrum.

Liquid Chromatography-Mass Spectrometry (LC-MS): The mass spectrum of each compound was recorded using the electrospray ionization (ESI) method on an HP 1100 MSD series instrument (Palo Alto, CA). The run time was 40 minutes and the flow rate was 0.2 mL. min^{-1} on a C_{18} column. The mass spectrum was recorded by dissolving 0.1 mg of the compound in 10 mL of methanol.

NMR Spectroscopy: The new Avance III NMR spectrometer (Switzerland) was used to record the ^1H NMR for each compound (5mg in 600µL DMSO-D6). At 125 MHz, the J-modulated spin-echo for ^{13}C nuclei coupled to proton to determine the number of attached protons (SEFT) was recorded. For ^1H NMR, the spectrum width was 0–12 ppm, while for ^{13}C NMR, it was 0–220 ppm.

Antioxidant activity of isolated compounds: The antioxidant activities of individual isolated compounds were also estimated by the same procedure adopted for the different solvent extracts of leaf.

***In vitro* anti-inflammatory activity of compound 6 (CC6)**
Effect on protein denaturation: Protein denaturation assay was carried out for compound 6 according to the method described by Gambhire et al[8] with some modifications. The reaction mixture (5 mL) consists of 0.2 mL of 1% bovine serum albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4) and 0.02 mL of different concentrations of the compound (0.1mg to 0.5mg/mL). It was mixed thoroughly and incubated in water bath at 37°C for 15min and then the reaction mixture was heated at 70°C for 5 min. After cooling, the turbidity was measured at 660 nm using a UV-VIS spectrophotometer. Phosphate buffer solution was used as a control. Diclofenac sodium was used as reference standard. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

$$\text{Percentage Inhibition} = \frac{(\text{A of control} - \text{A of sample})}{(\text{A of control})} \times 100$$

***In vitro* cytotoxic activity (MTT assay) of compound 6:** The MTT assay was used to assess the cytotoxicity of compound 6[16].

Cell culture: Human lung adenocarcinoma epithelial cells (A549) were obtained from NCCS, Pune, India and cells

were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM, L-glutamine. Cells were grown at 37°C under 5% CO_2.

Preparation of A549 cell suspension: After discarding the culture medium, A549 cells were trypsinized separately. To the disaggregated cells in the flask, 25 mL of DMEM with 10% FCS was added. The cells were suspended in the medium by gentle passage with the pipette and the cells were homogenized.

Seeding of the cells: One ml of the homogenized cell suspension was added to each well of a 24-well culture plate along with different concentrations of compound (0 to 200µg/mL) and incubated at 37°C in a humidified CO_2 incubator with 5% CO_2. After 48 h of incubation, the cells were observed under an inverted tissue culture microscope. A cytotoxic assay was carried out with 80% confluent cells.

MTT was added to the wells after 48 h incubation period and they were then kept at room temperature for 3 h. After incubation, the medium was aspirated and 100µL SDS in DMSO was added and gently stirred to dissolve the formazan crystals. Absorbance was read in a Lark LIPR-9608 microplate reader at 540 nm. The IC_{50} value was determined.

DNA fragmentation assay: In order to isolate DNA, roughly 1×10^6 A549 cells were plated and treated with CC6 (~100µg/ml) for 48 h. All the cells were trypsinized and Dulbecco`s phosphate buffered saline was used for washing. Cells were lysed using lysis solution containing 10mM Tris (pH7.4), 150mM NaCl, 5mM EDTA and 0.5% Triton X-100 for 30 minutes on ice. Lysates were centrifuged at 10,000 g for 20 minutes after vortexing. An equal volume of neutral phenol, chloroform and isoamyl alcohol mixture (25:24:1) was used to extract the DNA from supernatant and electrophoresis was done on 2% agarose gels.

Statistical analysis: Data was expressed as the mean ± standard deviation of three independent replicates. Minitab Software (version 16.0, copyright 2015, Minitab Inc., State College, PA, USA) was used to analyze data. To determine whether there is a significant difference between mean values with $p < 0.05$, One-way Analysis of Variance (ANOVA) is utilized.

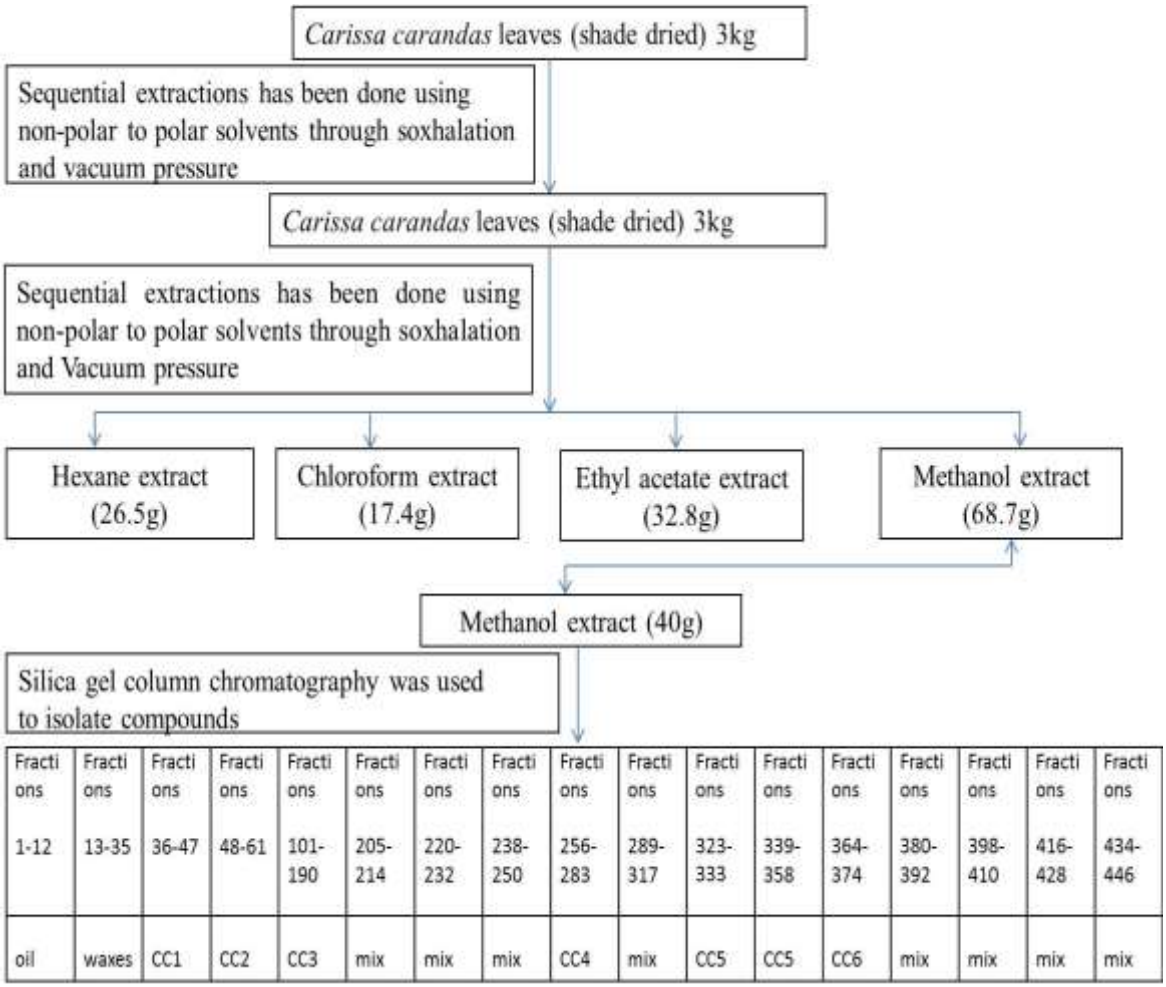
Results

Antioxidant activities of the different solvent extracts of leaf: The antioxidant activities of four different solvent extracts of leaf showed concentration-dependent activity. Among the four extractions, methanol extract showed the highest DPPH activity from 40.00±1.00 to 76.22±1.0, FRAP activity from 37.00±1.00 to 46.00±1.00 and reducing power activity from 67.50±0.50 to 81.83±0.76 µg/mL respectively (Table 1). Hence, column chromatography was employed to further isolate and purify the bioactive components using methanol extract (Figure 1).

Table 1
DPPH, FRAP and Reducing power of methanol extractions from the leaf of *C. carandas*

	DPPH				FRAP				Reducing Power			
Plant extract (µg /mL)	**Hexane**	**Chloroform**	**Ethyl acetate**	**Methanol**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Methanol**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Methanol**
50	43.66 ±0.56	30.25 ±1.09	17.05 ±1.00	40.00 ±1.00	38.66 ±6.65	20.00 ±1.00	27.00 ±1.00	37.00 ±1.00	39.16 ±1.25	4.00 ±1.00	37.00 ±1.00	67.50 ±0.50
100	53.97 ±1.00	52.25 ±1.08	23.52 ±1.50	46.22 ±1.07	40.00 ±1.00	24.00 ±1.00	35.33 ±1.52	38.00 ±1.00	46.66 ±3.21	5.50 ±0.50	38.00 ±1.00	73.33 ±4.93
150	60.09 ±1.01	56.10 ±1.01	39.09 ±1.01	63.11 ±1.01	52.00 ±1.00	26.00 ±1.00	37.00 ±1.00	39.00 ±1.00	53.83 ±1.756	10.00 ±1.00	39.00 ±1.00	74.16 ±1.25
200	69.50 ±1.50	59.11 ±1.01	43.05 ±1.00	76.22 ±1.07	52.00 ±1.00	30.33 ±1.52	40.00 ±1.00	46.00 ±1.00	69.00 ±1.00	13.83 ±0.76	46.00 ±1.00	81.83 ±0.76
BHT (100 (µg /mL))	79.04 ±1	79.04 ±1	79.04 ±1	79.04 ±1	49.00 ±1.00	49.00 ±1.00	49.00 ±1.00	49.00 ±1.00	91.95 ±0.93	91.95 ±0.93	91.95 ±0.93	91.95 ±0.93
	F= 306.53[@]	F= 462.62*	F=348.37*	F= 749.25*	F= 13.82*	F= 41.56*	F= 69.75*	F= 50.00*	F= 121.35*	F= 84.47*	F= 50.00*	F= 15.54*

Each value represents Mean±SD of three independent experiments. *The values were significant at P<0.05; [@]The values are not significant at P<0.05.



Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions	Fractions
1-12	13-35	36-47	48-61	101-190	205-214	220-232	238-250	256-283	289-317	323-333	339-358	364-374	380-392	398-410	416-428	434-446
oil	waxes	CC1	CC2	CC3	mix	mix	mix	CC4	mix	CC5	CC5	CC6	mix	mix	mix	mix

Figure 1: Schematic representation of extraction, isolation and purification of bioactive compounds from methanol extract of *Carissa carandas* leaves.

Isolation and structural elucidation of compounds from methanol extract: The collected fractions were recombined based on TLC analysis. Fractions 36-47 were named as CC1, 48-61 were named as CC2, 101-190 were named as CC3, 256-283 were named as CC4, 323-358 were named as CC5 and 364-374 were named as CC6. The remaining fractions were mixtures (Figure 1). All these compounds were characterized by spectral analysis and also tested for their biological activity.

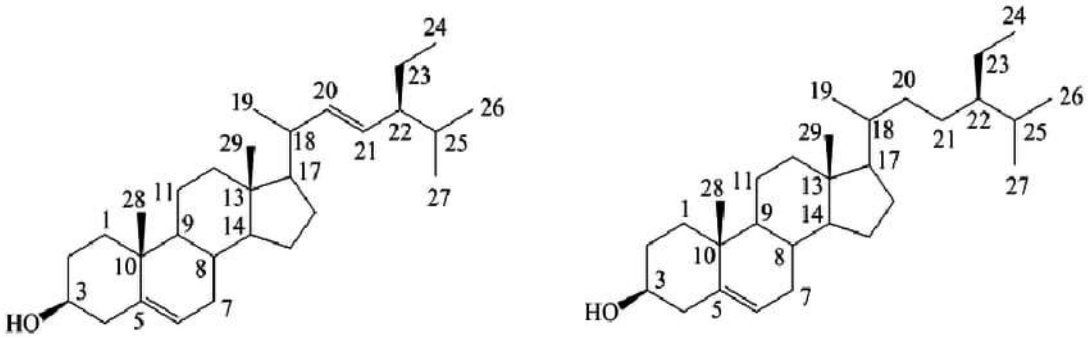
Characterization of the compounds

Compound 1 (CC1): Compound 1 was crystallised from 2% ethyl acetate in hexane as colorless fine needles and its melting point was 136-138°C. It showed ^{1}H NMR (500 MHz, $CDCl_3$) peaks at 0.80-1.25 (CH_3), 3.50(^{1}H3αH) and 5.30(^{1}H). The FTIR spectrum showed absorption peaks at IR (KBr): 3422, 2936, 2866, 1641, 1463, 1056 cm^{-1}. Based upon the spectral data, it was identified as triterpenoid consisting of β-sitosterol and stigmasterol, of which the maximum portion is stigmasterol. Molecular formulas are stigmasterol-$C_{29}H_{48}O$ and β-sitosterol - $C_{29}H_{50}O$ (Figure 2).

Compound 2 (CC2): Compound 2 was crystallized from 2% ethyl acetate in hexane as a white amorphous powder and its melting point was 215-216°C. It showed positive color reaction with the LB test for triterpenoids. It showed ^{1}H NMR (400 MHz, $CDCl_3$) characteristic peak of olefinic methylene protons at δ 4.57 (d, ^{1}H, Hα-29) and 4.69 (d, ^{1}H, Hβ-29). Its ^{13}C NMR spectrum exhibited carbon 3 bonds in the hydroxyl group and the presence of a signal at ~ δC 79.05. The UV spectrum showed maximum absorbance at 263.00 nm.

The FTIR spectrum showed absorption peaks at IR (KBr) 3321, 2942, 2860, 1641, 1461 and 1382 cm^{-1}. Based upon the above spectral data, the compound was identified as the 3β -Lup-20(29)-en-3-ol (Figure 3). Its molecular formula is $C_{30}H_{50}O$ and molecular weight from LC-MS analysis was found to be 427.200g/mol.

Compound 3 (CC3): Compound 3 was crystallized from 3% ethyl acetate in hexane as a white color compound and its melting point was 262-264°C.The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum revealed signals for five tertiary methyls at δ [0.82 (s, 3H, CH_3-25), 0.76 (s, 3H, CH_3-24), 0.98 (s, 3H, CH_3-26), 1.02 (s, 3H, CH_3-27), 0.97 (s, 3H, CH_3-23)] a vinyl methyl at 1.69 (s, 3H, CH_3-30), an oxymethine proton at δ 3.18 (t, 1H, J = 5.3 Hz, H-3), 3.79 (1H, d, J = 10.8, H-28b), 3.33 (1H, d, J = 10.8, H-28a) and an exo methylene protons at δ 4.58 (d, 1H, Hα-29) and 4.69 (d, 1H, Hβ-29).



Stigmasterol (1) **β-Sitosterol (2)**

Figure 2: Structure of Stigmasterol and β-sitosterol

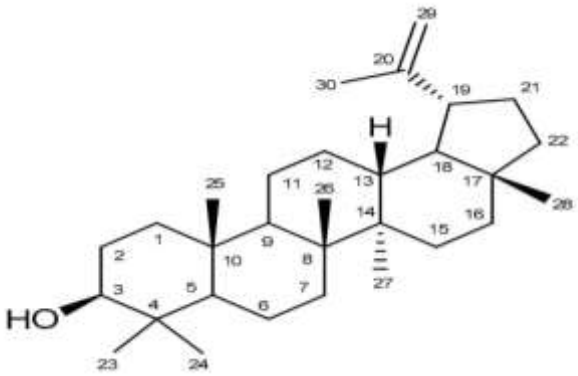


Figure 3: Structure of CC2; 3β -Lup-20(29)-en-3-ol

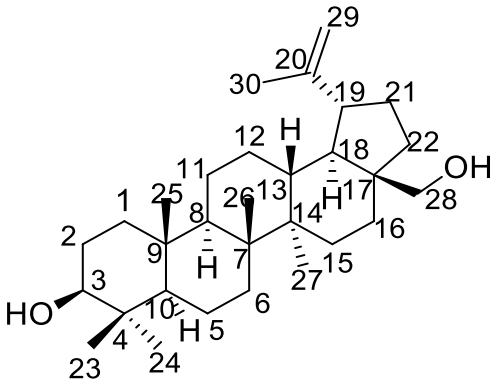


Figure 4: Structure of CC3; 3β- hydroxy-lup-20(29)-en-28-ol

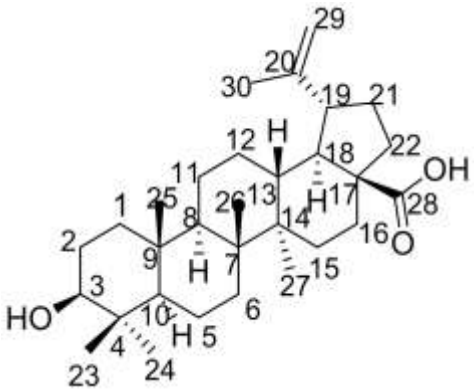


Figure 5: Structure of CC4; 3β-hydroxy-lup-20(29)-en-28-oic acid

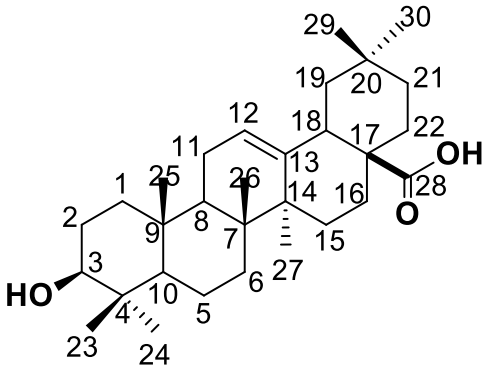


Figure 6: Structure of CC5; 3β-hydroxyolean-12-en-28-oic acid

Its ^{13}C NMR spectrum exhibited characteristic lupine disubstituted olefinic carbons at δ 109.6 (C-29), 150.4 (C-20) and an oxymethine carbon at δ 78.4 (C-3) (Figure S9). The UV spectrum showed maximum absorbance at 269.00 nm. The FTIR spectrum showed absorption peaks at IR (KBr):3385, 2937, 2865, 1586, 1481, 1032 cm^{-1}. Based on the spectral data, the compound was identified as 3β-hydroxy-lup-20(29)-en-28-ol (Figure 4). Its molecular formula is $C_{30}H_{50}O_2$. Molecular weight from LC-MS analysis was found to be 443.200g/mol.

Compound 4 (CC4): Compound 4 was crystallized from 8% ethyl acetate in hexane as a white amorphous solid and its melting point was 262-264°C. The ^1H NMR (400 MHz, $CDCl_3$) spectrum revealed signals for five tertiary methyls at δ [0.84 (s, 3H, CH_3-25), 1.03 (s, 3H, CH_3-24), 1.08 (s, 3H, CH_3-26), 1.09 (s, 3H, CH_3-27), 1.25 (s, 3H, CH_3-23)] a vinyl methyl at 1.81 (s, 3H, CH_3-30), an oxymethine proton at δ 3.48 (t, 1H, J = 8.2 Hz, H-3) and an exomethylene protons at δ 4.79 (br s, 1H, Hα-29) and 4.97 (br s, 1H, Hβ-29). Its ^{13}C NMR spectrum exhibited a carbonyl at δ 179.4 (C-28), a characteristic lupane disubstituted olefinic carbon at δ 110.5 (C-29),151.8 (C-20) and an oxymethine carbon at δ 78.7 (C-3).

The UV-spectrum showed maximum absorbance at 266.00 nm. The FTIR spectrum showed absorption peaks at IR (KBr):3452, 2941, 2869, 1690, 1455 and 1034 cm^{-1}. Based on the spectral data, the compound was identified as 3β-hydroxy-lup-20(29)-en-28-oic acid (Figure 5). Its molecular formula is $C_{30}H_{48}O_3$. Molecular weight was found to be 455.37.

Compound 5 (CC5): It was crystallized from 12% ethyl acetate in hexane as white colour sand particles and its melting point was 268-270°C.The ^1H NMR spectrum exhibited seven methyl signals at δ 0.91, 0.96, 1.02 and 1.04,1.26 and 1.30. It also showed a one-proton triplet at 5.51 (J = 3.7 Hz, H-12). Its ^{13}C NMR spectrum exhibited a carbonyl at δ 180.5 (C-28), a characteristic olean disubstituted olefinic carbon at δ 145.2 (C-13), 123.3 (C-12) and an oxymethine carbon at δ 78.4 (C-3). The UV spectrum showed maximum absorbance at 265.00 nm. The FTIR spectrum showed absorptions at FTIR (KBr): 3446, 2941, 2865, 1693, 1462, 1031 cm^{-1}. Based on the spectral data, it was identified as 3β- hydroxy-olean-12-en-28-oic acid (Figure 6). Its molecular formula is $C_{30}H_{48}O_3$. Its molecular weight from LC-MS analysis was found to be 455.2g/mol.

Compound 6 (CC6): It was crystallized from 16% ethyl acetate in hexane as light yellow colored sand particles and its melting point was 272-274°C. The ^1H NMR spectrum exhibited seven methyl signals at δ 1.34 (3H, s, Me-23), 0.93, 0.92 (d, CH_3J = 6.5 Hz), 0.86, 0.82 (d, CH_3, J = 6.5 Hz), 0.75, 0.68 (Me-27, Me-26, Me-29, Me-24, Me-30, Me-25). It showed an olefin proton at δ 5.15 (1H, s, H-12) and an oxymethine proton 3.00 (1H, q, J = 6.3 Hz, H-3). Its ^{13}C NMR spectrum exhibited a carbonyl at δ 180.2 (C-28), a characteristic olean disubstituted olefinic carbon at δ 126.0 (C-12), 139.6 (C-13) and an oxymethine carbon at δ 78.5 (C-3) and methoxy 57.4. Newly isolated compound 6 and its characteristics appear at δ 3.75 (s, 3H, OCH_3).

The UV spectrum showed maximum absorbance at 270.50 nm. The IR spectrum showed absorptions at IR (KBr):3445, 2931, 2869, 1692, 1458, 1035 cm^{-1}. Based on the spectral data, the compound was identified as 3β-methoxy-urs-12-en-28-oic acid (Figure 7). Its molecular formula is $C_{31}H_{50}O_3$. Its molecular weight from HREIMS analysis was found to be 471.0338.

Antioxidant activity of the compounds: The isolated triterpenoids from CC2 to CC6 were tested for their antioxidant activities and the results are shown in table 2.

Antioxidant activity of the isolated compounds: Among the five compounds tested, the compound CC6 showed better DPPH activity ranging from 46.05 to 77.12µg/mL, FRAP activity ranging from 39.20 to 53.36 µg/mL and reducing power activity ranging from 71.66 to 91.00 µg/mL followed by CC5, CC4, CC3 and CC2 respectively.

***In vitro* anti-inflammatory activity of CC6**

Protein denaturation: The inhibitory effect of compound 6 (CC6) on protein denaturation was studied using diclofenac sodium as a positive control. The compound 6 showed protein inhibition in a concentration-dependent manner. At a concentration of 0.5mg, the percentage of inhibition was

found to be 41.05 units and is almost similar to the effect of the positive control (Table 3).

***In vitro* cytotoxicity activity of CC6:** MTT assay was used to determine the cytotoxicity of compound 6 using A549 cells. The results showed that increasing concentrations of the compound decrease cell viability. At a concentration of 200µg/ml, the % of cell viability decreased up to 15.02 and the IC50 value was found to be 83.24µg/ml. (Fig. 8 and 9).

DNA fragmentation assay: DNA isolated from A549 cells was treated with 100µg/ml of compound 6 for 48 h and the DNA was analyzed by agarose gel electrophoresis. The results are shown in fig. 10.

Table 2
DPPH, FRAP and Reducing power of isolated compounds from the leaf of *C. carandas*

	DPPH					FRAP					Reducing Power			
Concentration of the compounds (µg/mL)	**CC2**	**CC3**	**CC4**	**CC5**	**CC6**	**CC2**	**CC3**	**CC4**	**CC5**	**CC6**	**CC2**	**CC3**	**CC4**	**CC5**	**CC6**
50	40.33± 0.57	30.58± 0.52	39.08 ±1.00	45.87± 2.90	46.05± 1.93	30.00 ±1.00	33.50 ±1.00	36.66 ±1.00	37.40 ±1.00	39.20 ±1.00	53.00 ±1.00	14.00 ±1.00	44.00 ±1.00	71.83 ±0.76	71.66 ±1.52
100	41.66± 1.52	45.58± 4.69	47.04 ±0.93	52.24± 0.98	57.48± 0.90	36.2 ±1.00	34.00 ±1.00	41.33 ±1.00	39.7 ±1.00	45.9 ±1.00	70.00 ±1.00	15.50 ±0.50	49.50 ±0.50	81.00 ±2.64	77.33 ±1.52
150	51.66± 1.52	56.10± 1.01	55.05 ±0.92	60.27± 0.33	65.22± 1.00	37.8 ±1.00	37.00 ±1.00	47.80 ±1.00	48.2 ±1.00	50.40 ±1.00	74.00 ±1.00	24.00 ±4.00	56.16 ±1.25	88.00 ±1.00	83.33 ±1.52
200	52.50± 1.50	59.78± 0.69	60.17 ±0.75	74.93± 1.51	77.12± 1.75	38.00 ±1.00	49.50 ±1.00	51.2 ±1.00	52.10 ±1.00	53.36 ±1.00	80.00 ±1.00	33.83 ±0.76	64.00 ±1.00	90.33 ±0.57	91.00 ±1.00
BHT (10µg/ml)	82.19± 1.08	82.19± 1.08	82.19 ±1.08	82.19± 1.08	82.19± 1.08	52.33 ±1.00	52.33 ±1.00	52.33 ±1.00	52.33 ±1.00	52.33 ±1.00	88.00 ±1.00	88.00 ±1.00	88.00 ±1.00	88.00 ±1.00	88.00 ±1.00
	F=52.59*	F=155.02*	F=272.07*	F=268.06*	F=397.69*	F=14.73*	F=51.00*	F=72.33*	F=54.69*	F=59.13*	F=513.00*	F=206.53*	F=173.65*	F=120.43*	F=159.94*

Each value represents Mean±SD of three independent experiments. The values was significant at P<0.05.

Table 3
Inhibitory effect of compound 6 (CC 6) on protein denaturation

S.N.	Concentration (mg/ml)	Units (%inhibition)
1	0	0.00
2	0.1	21.25 ± 1.02
3	0.2	27.37 ± 0.52
4	0.3	33.19 ± 1.05
5	0.4	38.72 ± 1.07
6	0.5	41.05 ± 1.08
7	Diclofenac sodium (0.5 mg/mL)	41 ± 1

Each value represents mean ± SD of three replicates.

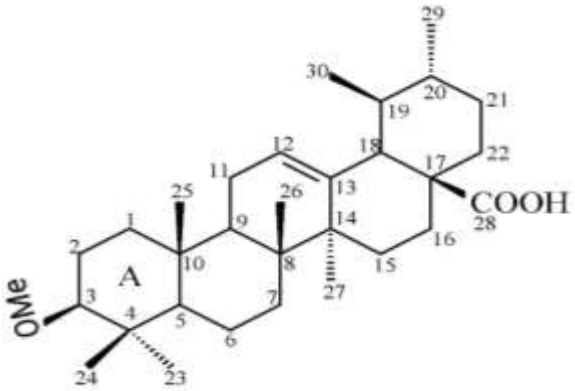


Figure 7: Structure of CC6; 3β-methoxy-urs-12-en-28-oic acid

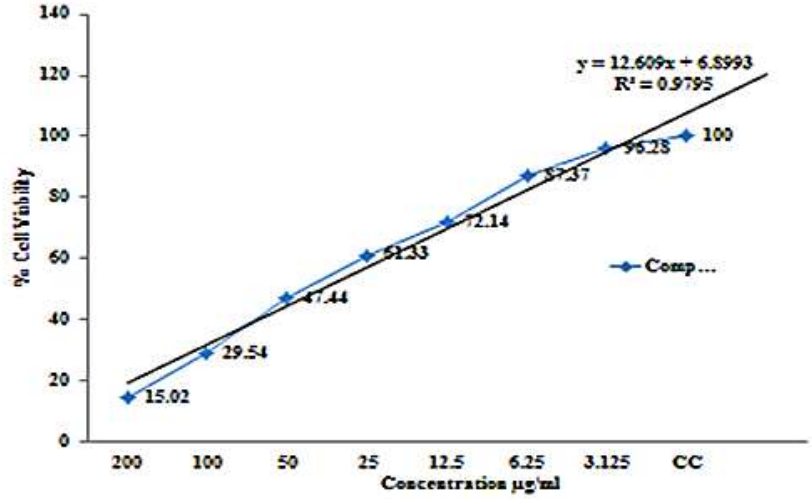


Figure 8: Anticancer activity of pure compound against the A549 Cell lines

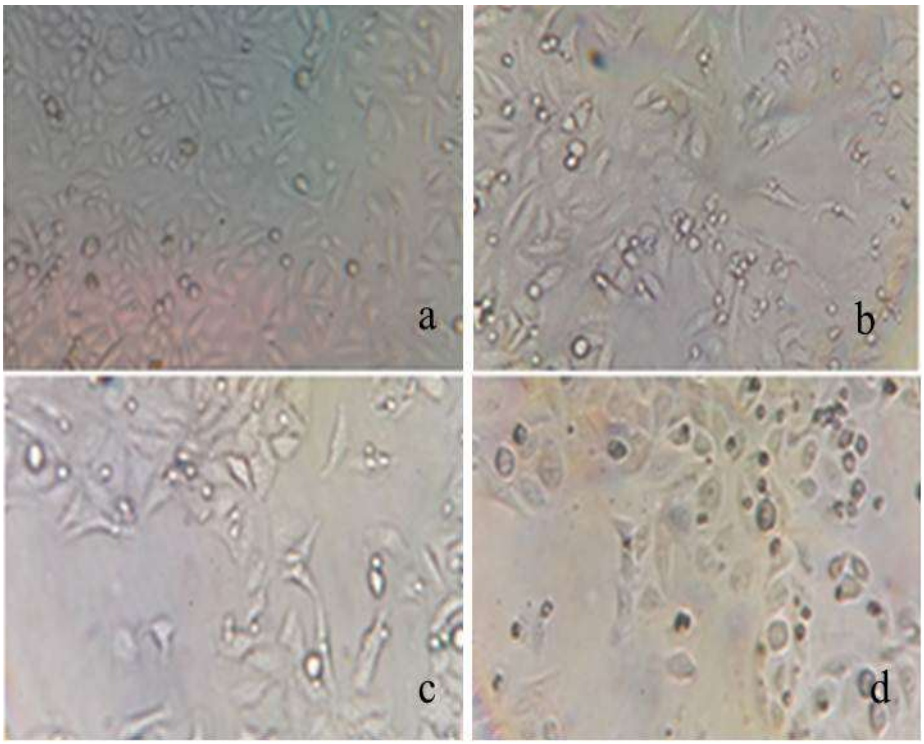


Figure 9: Anticancer activity of different concentrations of CC6 against the A549 Cell lines a=control; b= 50 µg/mL; c= 100 µg/mL; d= 200 µg/mL.

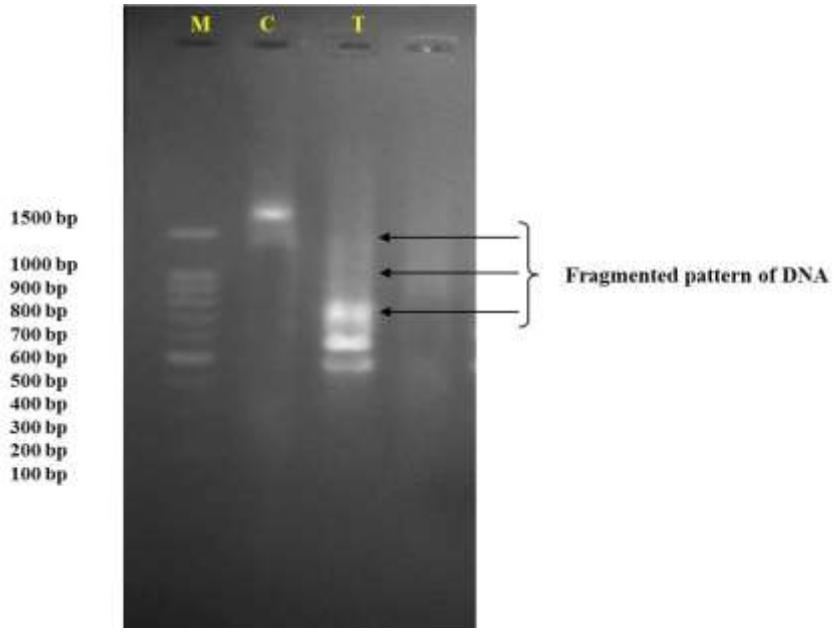


Figure 10: Well M: 100 bp DNA ladder, C: Control Cell DNA; T: 100 µg/mL (Showing the fragmented DNA pattern)

The presence of different bands in the isolated DNA indicates that the DNA was cleaved to fragments of different size by CC6.

Discussion

In the present study, four different types of solvents with variable polarity were used to extract the bioactive compounds from *C. carandas* leaves. The sequential extractions using non-polar hexane to polar methanol resulted in the extraction of different bioactive compounds[6,12,21]. Initially, all the four extracts were tested for their antioxidant potential. The results obtained from the present study indicate that methanol extract showed the strongest antioxidant activity, followed by ethyl acetate, hexane and chloroform extracts. Similar studies by Saika et al[18] and Anupama and Madhumitha[1] showed antioxidant activity of fruit extract of *C. carandas* using methanol as solvent. The results showed the presence of antioxidant compounds in the leaf extract of *C. carandas*. Methanol is one of the very good solvents not only for the extraction of phenolic compounds but is also suitable for extraction of triterpenoids. Hence, further studies were continued with methanol extract. Six compounds were isolated and identified. Compound 1 (CC1) was identified as β-sitosterol and stigmasterol, compound 2 (CC2) as 3 β -Lup-20(29)-en-3-ol (lupeol), compound 3 (CC3) as 3β- hydroxy-lup-20(29)-en-28-ol (betulin), compound 4 (CC4) as 3β-Hydroxy-Lup-20(29)-en-28-oic acid (betulinic acid), compound 5 (CC5) as 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid) and compound 6 (CC6) as 3β-methoxy-urs-12-en-28-oic acid (methoxy urosolic acid). All the isolated compounds were identified as pentacyclic triterpenoids.

Similarly, studies were conducted by Ganapathy et al.[9] where they isolated carissone, carindone, lupeol etc. from methanolic root extracts of *C. conjesta*. Sunita et al[23] reported three triterpenoids lupeol, stigmasterol and β -sitosterol from *C. carandas*. Wangteeraprasert et al[26] isolated sesquiterpene glucoside, carandoside, lignin glycoside, lignans, sesquiterpene glucoside lyoniresinol etc. from *C. carandas* stem. Siddiqui et al[20] reported four pentacyclic triterpenoids characterized as carissin, 3- β -hydroxy-27-p-E-coumaroyloxy urs-12-en-28-oic acid, oleanolicacid and ursolic acid.

In the present study, six potentially bioactive triterpenoids were isolated using different concentrations of ethyl acetate and hexane. Among 6 compounds isolated and identified from *C. carandas* leaves, compound 3, 3β- hydroxy-lup-20(29)-en-28-ol, compound 4, 3β- hydroxy-lup-20(29)-en-28-oic acid and compound 6 3β-methoxy-urs-12-en-28-oic acid were not reported earlier from the genus *Carissa.* Due to the wide applications and therapeutic value of *C.carandas,* several studies were conducted on fruits and roots. But studies on leaves and their biological properties were meager. As there is a need to understand the biological effects of compounds isolated from *C.carandas* leaf, in the present study the isolated compounds were tested for their antioxidant, anti-inflammatory and anticancer activities in *C. carandas.*

The antioxidant activities of all the five purified compounds (CC2-CC6) were significantly higher than the crude methanol extract. It was observed that among five isolated compounds, compound CC6 isolated from *C. carandas* leaf showed high antioxidant activity. Similar studies regarding triterpenes having high antioxidant activity were reported by Nzogong et al[17] in *Dissotis senegambiensis,* by Santiago and Mayor[19] in *Ficus pseudopalma Blanco* (Moraceae) and Begum et al[3] in *C. carandas*.

As compound CC6 showed the highest antioxidant activity, it was tested for anti-inflammatory and cytotoxic potential. Significant *in vitro* anti-inflammatory activity was observed

with compound 6. It showed maximum inhibition of 41.05% at a concentration of 0.5mg. Similar studies regarding triterpenes acting as anti-inflammatory compounds were reported by De Almeida et al[5] in *Protium paniculatum* oil-resins and by Wu et al[27] in *Ligustrum.* The apoptotic potential of compound 6 was estimated by a DNA fragmentation assay where DNA was isolated from A549 cells when treated with compound 6, a clear fragmentation was observed, whereas the control DNA showed a single band in the agarose gel. It indicates compound 6 promotes apoptosis of cancer cells.

The cytotoxic activity of compound 6 was assessed using an MTT assay. MTT is the commonly applied method for evaluation of cell viability and cytotoxicity for screening the drugs, compounds etc. It is based on the reduction of MTT (yellow colored) and other tetrazolium dyes and depends upon cellular metabolic activities due to NAD(P)H-dependent cellular oxidoreductase enzymes[5]. Compound 6 was found to decrease cell viability in a concentration-dependent manner. It was found to be more effective on A549 lung cancer cells. Pentacyclic triterpenoids are found to exhibit cytotoxicity by several mechanisms like proliferation inhibition, enhancing apoptosis, growth receptor modulation, reducing cellular oxygen consumption and increasing cellular level.

From the present study, compound 6 was found to be a derivative of ursolic acid which exhibits similar functions like urosolic acid and other triterpenes. Begum et al[4] reported isohopane triterpene carandinol from the leaves of *Carissa carandas* L. and tested its cytotoxicity against cancer cell lines.

Kurimoto et al[13] reported triterpenes and a triterpene glucoside from *Dysoxylum cumingianum*. They showed cytotoxicity against KB (epidermal nasopharyngeal cancer), MCF-7 (breast cancer), as well as multi drug resistant cellular line KB-C2.

Conclusion

From the present study, it was concluded that a mixture of ethyl acetate and methanol in different ratios helped in the isolation of six potent pentacyclic triterpenes from the leaves of *C. carandas* and all of them are having a wide spectrum of biological activities like antioxidant, anti-inflammatory, cytotoxic and apoptotic potential against the lung cancer cell line A549.

Future studies will be continued on *in vivo* models in order to establish pentacyclic triterpenes as therapeutic agents in the treatment of infectious diseases, antibiotic resistance and human disorders like cancer, inflammation etc.

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