Journal of Advanced Biomedical and Pharmaceutical Sciences

Journal Homepage: http://jabps.journals.ekb.eg

Phytochemical and Biological Studies of *Carissa macrocarpa*, F. Apocyanaceae

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Received: September 14, 2020; revised: October 18, 2020; accepted: October 25, 2020

Abstract

The present work comprises the isolation, purification and identification of eight known compounds obtained from the powdered leaves & stems of *Carissa macrocarpa* (F. Apocyanaceae) named; *α*-amyrin acetate (**1**), *α*-amyrin (**2**), lupeol acetate (**3**), stearic acid (**4**), ursolic acid (**5**), *β*-sitosterol-3-*O*-*β*-D-glucopyranoside (**7**), quercetin (**7**) and kaempferol-3-*O*-rutinoside (**8**). The structural elucidations of the isolated compounds were confirmed on the basis of their physical, chemical, chromatographic and spectroscopic methods. In addition; the study of some biological activities of *Carissa macrocarpa* 70% methanolic extract (CMME) and its fractions (*n*-hxane, dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol). The biological evaluations included cardiovascular activity, together with anti-inflammatory & antipyretic and antimicrobial activities. CMME and its fractions showed promising increase in the force of contractility (positive inotropic effect) and decrease in the heart rates (negative chronotropic effect) of the isolated rabbit's heart. They showed hypotensive effect on the rabbits mean arterial blood pressure (MABP). The EtOAc and DCM fractions showed the maximum anti-inflammatory activity after 5 h. (100 mg/kg) with percentage of inhibition 43% and 41%, respectively, in comparison with indomethacin reference drug (47%, 10 mg/kg). Yeast-induced hyperpyrexia method was used for antipyretic activity. The CMME and *n*-butanol fraction showed the highest antipyretic activity after 5 h. The CMME and fractions provided remarkable antibacterial activity. The EtOAc fraction showed the most potent effect against the tested microorganisms *Bacillus subtilis & Staphylococcus aureus* (Gram +ve bacteria) and *Escherichia coli & Pseudomonas aeruginosa* (Gram -ve bacteria).

Key words
Carissa macrocarpa, cardiovascular, anti-inflammatory, antipyretic, antimicrobial.

1. Introduction

Family Apocynaceae considered as one of the largest families comprising about 366 genera & 5000 species distributed all over many countries [1, 2], most of them are grow in shrubs about 3-5 m height. The geographical source of the genus Carissa mainly the tropical and subtropical areas of different countries as Africa, Australia and Asia. It was introduced into the United States in 1886 by the horticulturist [3]. *Carissa macrocarpa* commonly known as Natal plum, native to the coastal region of Natal, South Africa and consumed as edible fruits [4]. The plant also cultivated in Egypt El-Orman garden and grows as a shrub (3-4 m height). It is a spiny, evergreen latex containing shrub with yellowish white flowers and delicious red fruits [5, 6]. In traditional medicine Carissa species used in the treatment of diarrhea and venereal diseases [5]. A large number of diverse chemical constituents having important economic and biological values were detected in Carissa species such as; sterols & triterpenes [7, 8], anthraquinones, saponins, tannins, [9, 10], cardiac glycosides [11, 12] alkaloids [13]. Carissa species considered as a rich source of polyphenols, they are rich of flavonoids, coumarins, chalcones, stilbenes, lignans and xanthones 14]. Accordingly, the plant genus comprising several medicinal activities such as cardiovascular [15, 16], anti-inflammatory [17], anti-microbial [18], cytotoxic [19] in addition to analgesic, antioxidant, hepatoprotective wound healing, antiepileptic, antidiabetic, diuretic and hypolipidemic activities [20]. This provoked us to carry out the isolation, purification of the active constituents from the powdered leaves & stems of *Carissa macrocarp* together with the study of some biological activities of 70% methanolic extract and its fractions; *n*-hxane, dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol. Our biological evaluations were focused on the pharmacological action of the plant under investigation on cardiovascular activity, together with anti-inflammatory & antipyretic and antimicrobial activities.

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2. Experimental

2.1. Plant materials

The aerial parts of the plant (*Carissa macrocarpa)* were collected from El-Orman Botanical Garden (Giza, Egypt) and authenticated as soon as possible by the help of Agr. Eng./ Tereza Labib, a taxonomist at the Egyptian Ministry of Agriculture. Part of the collected plant was maintained as an herbarium sample and deposited in the herbarium Lab., Department of Pharmacognosy, Faculty of Pharmacy, Minia University (Regs. No. is Mn-Ph-Cog-020).

2.2. Materials used for biological study

2.2.1. Microbial strains

The tested microorganisms *Bacillus subtilis, Staphylococcus aureus Pseudomonas aeruginosa, Escherichia coli, Candida albicans* and *Aspergillus flavus* were obtained from Micro Analytical Center, Faculty of Science, Cairo University.

2.2.2. Animals

2.2.2.1. Rats

Adult male albino rats (each 120-200 g) were used. The animals were bred and housed under standardized environmental conditions in the pre-clinical animal house, Pharmacology Department, Faculty of Medicine, Assiut University. They were fed with standard diet and allowed free access to drinking water.

2.2.2.2. Rabbits

Adult male-Neizuland rabbits (each weighing 1.2-2 kg) were used. The animals were bred and housed under standardized environmental conditions in the pre-clinical animal house, Pharmacology Department, Faculty of Medicine, Assiut University. They were fed with standard diet and allowed free access to drinking water.

All animals' procedures were done based on care and use of animals for scientific purposes according to the code of practice for the Animals ethics committee of Al-Azhar University.

2.3. Equipment and Chemicals

Electrothermal 9100 Digital Ins. (England) for measurement of Melting points. ^{1}H, ^{13}C-NMR spectral analysis was done by a Brucker 400 MHz spectrometer (Germany). EI-MS data were estimated on JEOL JMS-600 (mass spectrometer, Japan). The chromatographic separation was done by using column chromatography packed with silica gel with 70-230, mesh diameter (E-Merck) and Sephadex LH-20 with 20-100 μm id. (Sigma-Aldrich chemicals). Monitoring by TLC was done by using precoated Aluminum silica gel plates (G60 F254) and RP-18 F254 Merck, Germany). Indomethacin was obtained from El-Nile Company, Egypt. Acetyl salicylic acid (ASA) was obtained from America pharmaceutical Industry, Egypt. Ringer-Locke solution was prepared at pharmacology department, faculty of medicine, Al-Azhar University, Assuit. Mueller-Hinton agar obtained from Himedia Laboratories, India. Perfusion apparatus & Force transducer inst. (Australia) were used for determination of cardiovascular activities. Urethane solution purchased from Sigma-Aldrich, Inc. U.S.A. Heparin (Cal-Heparin®, 12000 I.U.) purchased from Amoun company. Blood pressure transducer (Research grade, EM 60-3003; Harvard) was used for measurement of mean arterial blood pressure (MABP). Ampicillin, Amphotericin-B were obtained from Sigma-Aldrich Chemicals Co, Germany.

2.4. Extraction and isolation

About 2 kg of the air-dried powdered leaves & stems of *C. macrocarpa* was extracted by maceration and percolation at room temperature with 70% methanol till complete exhaustion. The alcoholic extract was collected and concentrated in rotary evaporator under reduced pressure to give syrupy residue (350 g, CMME). The CMME (350 g) was successively partitioned using *n*-hexane (150 g), DCM (10 g), EtOAc (10 g) and finally with *n*-butanol (80 g). Certain weight of *n*-hexane soluble fraction (20 g) was portioned over silica gel column with *n*-hexane-EtOAc gradient elution system, six sub-fractions were obtained and named CHI to CHVI. A part of sub-fraction CH-I (100 mg) was packed on silica gel CC, using *n*-hexane-EtOAc solvent mixture. The fractions eluted with mixture of 97% *n*-hexane-EtOAc, compiled together to afford compound **1** (25 mg). Sub-fraction CH-II (500 mg) was packed on silica gel CC, eluted with *n*-hexane-EtOAc solvent mixture. The fraction eluted with mixture of 95% *n*-hexane-EtOAc yielded compound **2** (40 mg), while the fraction eluted with 93% *n*-hexane-EtOAc, resulted in separation of pure compound **3** (15 mg) and the fraction eluted with 90% *n*-hexane-EtOAc resulted in separation of pure compound **4** (20 mg). DCM fraction was packed on silica gel column, followed by elution with DCM-MeOH gradients. Five sub-fractions were obtained and labeled CD-I to CD-V. A part of sub-fraction CD-I (110 mg) was chromatographed using silica gel CC, eluted with DCM-MeOH gradients. The fraction eluted with DCM-MeOH (95:5) afforded the pure compound **5** (20 mg). Sub-fraction CD-II (150 mg) was packed on silica gel CC, using gradient mixture of DCM-MeOH for elution. The fraction, which was eluted with 90% DCM-MeOH solvent mixture resulted in separation of compound **6** (35 mg).

The EtOAc soluble fraction (10 g) was portioned over silica gel CC, eluted gradiently with DCM-MeOH solvent mixture. Five sub-fractions were obtained and labeled CE-I to CE-V. A part of sub-fraction CE-I (130 mg) packed over silica gel CC, eluted with 90% DCM-MeOH mixture followed by packing on sephadex LH-20 (eluted by 50% DCM-MeOH) for further purification, afforded pure compound **7** (18 mg). A part of sub-fraction CE-III (120 mg) was applied on silica gel CC, using solvent mixture of 80% DCM-MeOH elution system. The fractions from 25-36 compiled together and finally purified by the aid of sephadex LH-20 resulted in separation of pure compound **8** (15 mg).

Compound 1 (*α*-amyrin acetate) was separated as a white amorphous powder. The EI-MS spectrum showed fragment at *m/z* 468 [M^+] calculated to the molecular formula $C_{32}H_{52}O_2$. The ^{1}H-NMR spectral data (400 MHz, $CDCl_3$) displayed signals at δ_H 5.14 (1H, t, *J*=3.4, CH-12), 4.52 (1H, m, CH-3), 2.06 (3H, s, CH_3-2'), 1.08 (3H, s, CH_3-23), 1.03 (3H, s, CH_3-27), 0.99 (3H, s, CH_3-24), 0.98 (3H, d , *J*=1.8, CH_3-29), 0.93 (3H, s, CH_3-26), 0.89 (3H, d, *J*=3.2, CH_3-30), 0.85 (3H, s, CH_3-25) and 0.81 (3H, s, CH_3-28). DEPT-Q NMR experiment (100 MHz, $CDCl_3$) showed signals at δ_C 38.47 (C1), 28.04 (C2), 80.95 (C3), 37.71 (C4), 55.26 (C5), 18.25 (C6), 32.87 (C7), 40.03 (C8), 47.64 (C9), 36.80 (C10), 23.38 (C11), 124.32 (C12), 139.63 (C13), 42.07 (C14), 26.60 (C15), 28.10 (C16), 33.75 (C17), 59.06 (C18), 39.65 (C19), 39.61 (C20), 31.25 (C21), 41.54 (C22), 28.76 (C23), 16.87 (C24), 15.75 (C25), 16.75 (C26), 23.70 (C27), 28.76 (C28), 17.52 (C29), 21.41 (C30), 171.01 (C1') and 21.33 (C2').

Compound 2 (*α*-amyrin) was isolated as a white amorphous powder. EIMS showed fragment at *m/z* 426 [M^+] calculated to the molecular formula $C_{30}H_{50}O$. The ^{1}H-NMR spectral data (400 MHz, $CDCl_3$) exhibited signals at δ_H 5.15 (1H, t, *J*=3.62, CH-12), 3.24 (1H, dd, *J*=5.12, 10.6, CH-3), 1.09 (3H, s, CH_3-23), 1.03 (3H, s, CH_3-27), 1.02 (3H, s, CH_3-24), 0.97 (3H, brs, CH_3-29), 0.93 (3H, s, CH_3-26), 0.89 (3H, brs, CH_3-30), 0.85 (3H, s, CH_3-25) and 0.81 (3H, s, CH_3-28). ^{13}C-NMR (100 MHz, $CDCl_3$) data revealed signals at δ_C 38.78 (C1), 27.28 (C2), 79.05 (C3), 38.81 (C4), 55.20 (C5), 18.37 (C6), 32.95 (C7), 40.02 (C8), 47.73 (C9), 36.91 (C10), 23.38 (C11), 124.43 (C12), 139.59 (C13), 42.09 (C14), 28.76 (C15), 26.63 (C16), 33.76 (C17), 59.08 (C18), 39.62 (C19), 39.67 (C20), 31.27 (C21), 41.55 (C22), 28.15 (C23), 15.64 (C24), 15.69 (C25), 16.88 (C26), 23.28 (C27), 28.12 (C28), 17.49 (C29) and 21.41 (C30).

Compound 3 (lupeol acetate) was separated as a white crystalline needle with MP = 216°C. The EI-MS showed fragment at *m/z* 468 [M^+] calculated to the molecular formula $C_{32}H_{52}O_2$. The ^{1}H-NMR spectral analysis (400 MHz, $CDCl_3$) showed proton signals at δ_H 4.70 (1H, brs, CH-29 a), 4.59 (1H, brs, CH-29 b), 4.50 (1H, dd, *J*=6, 10.8, CH-3), 2.38 (1H, m, CH-19), 2.06 (3H, s, CH_3-2'), 1.70 (3H, s, CH_3-30), 1.05 (3H, s, CH_3-25), 0.96 (3H, s, CH_3-28), 0.87 (3H, s, CH_3-23), 0.86 (3H, s, CH_3-24), 0.85 (3H, s, CH_3-26) and 0.80 (3H, s, CH_3-27). DEPT-Q NMR experiment (100 MHz, $CDCl_3$) showed carbon signals at δ_C 38.39 (C1), 20.95 (C2), 80.89 (C3), 37.80 (C4), 55.39 (C5), 18.21 (C6), 34.21 (C7), 40.85 (C8), 50.35 (C9), 37.09 (C10), 20.95 (C11), 23.72 (C12), 38.04 (C13), 42.83 (C14), 25.10 (C15), 35.57 (C16), 43.0 (C17), 48.29 (C18), 48.01 (C19), 150.86 (C20), 29.84 (C21), 40.0 (C22), 27.44 (C23), 16.50 (C24), 16.19 (C25), 15.98 (C26), 14.51 (C27), 18.01 (C28), 109.43 (C29), 19.29 (C30), 171.20 (C1') and 21.34 (C2').

Compound 4 (stearic acid) was obtained as a white waxy residue. EI-MS showed fragment at *m/z* 284 [M^+] established to the molecular formula $C_{18}H_{36}O_2$. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) showed proton signals at δ_H 2.36 (2H, t, *J*=7.5, CH_2-2), 1.65 (2H, m, CH_2-3), 1.27 (28H, brs, CH_2 4-17) and 0.90 (3H, t, *J*=7, CH_3-18). The ^{13}C-NMR spectral analysis (100 MHz, $CDCl_3$) revealed carbon signals at δ_C 180.54 (C1), 34.12 (C2), 31.94 (C3), 29.71–29.07 (C4–15), 24.67 (C16), 22.70 (C17) and 14.10 (C18).

Compound 5 (**ursolic acid**) was isolated as a white needle crystal with MP = 289-292 °C. The EI-MS showed base peak [M^+] at *m/z* 456, calculated to molecular formula $C_{30}H_{48}O_3$, While the ^{1}H-NMR spectrum (DMSO-d_6, 400 MHz) demonstrated proton signals at δ_H 5.13 (1H, t, *J*=2.92, CH-12), 3.00 (1H, m, CH-3), 2.12 (1H, d, *J*=11.4, CH-18), 1.04 (3H, s, CH_3-27), 0.91 (3H, s, CH_3-23), 0.89 (3H, s, CH_3-25), 0.87 (3H, br s., CH_3-30), 0.82 (3H, d, *J*=6.3, CH_3-29), 0.75 (3H, s, CH_3-26) and 0.68 (3H, s, CH_3-24). The DEPT-Q NMR experiment (DMSO-d_6,100 MHz) revealed carbon signals at δ_C 38.83 (C1), 27.39 (C2), 77.42 (C3), 38.69 (C4), 55.23 (C5), 18.44 (C6), 33.15 (C7), 39.20 (C8), 47.47 (C9), 36.97 (C10), 23.30 (C11), 124.94 (C12), 138.55 (C13), 42.09 (C14), 28.06 (C15), 24.25 (C16), 47.30 (C17), 52.84 (C18), 38.96 (C19), 38.89 (C20), 30.62 (C21), 36.78 (C22), 28.70 (C23), 16.52 (C24), 15.67 (C25), 17.38 (C26), 23.71 (C27), 178.77 (C28), 17.47 (C29) and 21.53 (C30).

Compound 6 (*β*-sitosterol-3-*O*-*β*-D-glucopyranoside) was obtained as a white amorphous powder. EI-MS showed base peak [M^+] at m/z 576, corresponding to molecular formula $C_{35}H_{60}O_6$. The ^{1}H-NMR spectral analysis (DMSO-d_6, 400 MHz) showed proton signals at δ_H 5.35 (1H, m, CH-6), 4.25 (1H, d, *J*=7.7, CH-1'), 3.65-4.89 (6H, m, Sugar protons), 3.47 (1H, m, CH-3), 0.98 (3H, s, CH_3-19), 0.93 (3H, d, *J*=6.2, CH_3-21), 0.84 (3H, s, CH_3-26), 0.82 (3H, s, CH_3-27), 0.81 (3H, m, CH_3-29) and 0.67 (3H, s, CH_3-18). ^{13}C-NMR spectrum (DMSO-d_6,100 MHz) revealed carbon signals at δ_C 36.67 (C1), 29.17 (C2), 77.43 (C3), 37.29 (C4), 140.91 (C5), 121.66 (C6), 31.88 (C7), 33.81 (C8), 50.07 (C9), 35.95 (C10), 21.06 (C11), 40.52 (C12), 42.31 (C13), 56.65 (C14), 24.32 (C15), 28.26 (C16), 55.90 (C17), 12.12 (C18), 19.08 (C19), 35.95 (C20), 19.56 (C21), 33.81 (C22), 24.32 (C23), 45.61 (C24), 29.72 (C25), 19.39 (C26), 20.17 (C27), 23.07 (C28), 12.24 (C29), 101.25 (C1'), 73.92 (C2'), 77.43 (C3'), 70.54 (C4'), 77.20 (C5') and 61.54 (C6').

Compound 7 (quercetin) was isolated as a yellow needle crystal with MP = 314-316°C. EI-MS showed [M^+] at *m/z* 302, corresponding to molecular formula $C_{15}H_{10}O_7$. The ^{1}H-NMR (DMSO-d_6, 400 MHz) revealed proton signals at δ_H 12.49 (1H, s, 5-(OH)), 7.69 (1H, d, *J*=1.8, CH-2'), 7.56 (1H dd, *J*=1.8, 8.4, CH-6'), 6.90 (1H d, *J*=8.4, CH-5'), 6.41 (1H d, *J*=1.8, CH-8) and 6.19 (1H d, *J*=1.8, CH-6). The spectral data of ^{13}C-NMR (DMSO-d_6, 100 MHz) showed carbon signals at δ_C 147.24 (C2), 136.18 (C3), 176.28 (C4), 161.17 (C-5), 98.67 (C-6), 164.38 (C-7), 93.83 (C-8), 156.60 (C-9), 103.46 (C-10), 122.44 (C-1'), 115.53 (C-2'), 145.5 (C-3'), 148.14 (C-4'), 116.07 (C-5') and 120.46 (C-6').

Compound 8 (kaempferol-3-*O*-rutinoside) was separated as a yellow amorphous powder. EI-MS showed [M^+] at *m/z* 594, corresponding to molecular formula $C_{27}H_{30}O_{15}$. ^{1}H-NMR analysis (DMSO-d_6, 400 MHz) represented the proton signals at δ 12.53 (1H, s, 5-(OH)), 7.99 (2H, d, *J*=8.8, CH-2' & CH-6'), 6.90 (2H, d, *J*=8.8, CH-3' & CH-5'), 6.42 (1H, d, *J*=1.7, CH-8), 6.20 (1H, d, *J*=1.7, CH-6), 5.31 (1H, d, *J*=7.4, anomeric proton of Glc.) 4.38 (1H, brs, anomeric proton of Rha.) 3.05-3.77 (m, Other sugar protons) and 0.99 (3H, d, *J*=6.1, CH_3 of Rha.). The DEPT-Q NMR spectral analysis (DMSO-d_6,100 MHz) showed carbon signals at δ_C 156.98 (C2), 133.63 (C3), 177.75 (C4), 161.60 (C5), 99.34 (C6), 165.12 (C7), 94.29 (C8), 157.25 (C9), 104.20 (C10), 121.30 (C1'), 131.31 (C2'), 115.57 (C3'), 160.41 (C4'), 115.57 (C5'), 131.31 (C6'), 101.84 (C1"), 74.62 (C2"), 76.82 (C3"), 71.05 (C4"), 76.16 (C5"), 67.35 (C6"), 101.23(C1'"), 70.36 (C2'"), 70.78 (C3'"), 72.27 (C4'"), 68.70 (C5'") and 18.13 (C6'").

2.5. Methods for biological study

2.5.1. Evaluation of the activity on the cardiac parameters *(Rabbit Isolated Heart Method)* Certain weighs of CMME and its fractions (*n*-hexane, DCM, EtOAc and *n*-butanol) were suspended in Ringer-Locke solution to prepare the concentration of 50 mg/mL. Six groups each of six male-Neizuland rabbits (1.75±0.25 kg) were used. The experiment was carried out according to Langendorff procedure [21, 22]**.** At the beginning, the selected rabbits were injected with heparin (1000 IU) through the marginal ear vein. Few minutes later after heparin administration, we made a blow on the neck each tested rabbit till it became unconscious completely. After that, the rabbit' heart was picked up with 1 cm of aorta attached to them and rapidly washed by immersing them in oxygenated Ringer-Locke solution. The aorta was cannulated with a stainless-steel cannula in the perfusion apparatus. Ringer-Locke solution was mixed with 5% carbon dioxide & 95% oxygen and introduced fixed pressure (70 mm Hg). The temperature was maintained between 36-37 °C and monitored continually by the thermo-probe. The hearts were acclimatized for 30 min before any drug administration. For recording the sensitivity of the heart, 1 mL of 0.05 mM adrenaline solution was injected to it. After adrenaline administration, 1 mL of the total plant extract and fraction in a dose of 50 mg/mL was injected over 30 sec, then the change in the cardiac parameters were monitored and recorded. The spontaneous contractions of heart were monitored by attaching the heart apex with a thread end and the other end hanged directly to a force transducer connected by Oscillograph (Harvard) for recording. The Parameters measured were the Heart rate (beats/min) and Mean of contractility (mean cycle height in g). The obtained results of cardiac parameters (contractility and heart rate) were compiled in (**Table 1** and **2**) and illustrated in (**Figure 3**).

2.5.2. Evaluation of hypotensive activity

The plant samples (CMME, *n*-hexane, DCM, EtOAc and *n*-butanol fractions) were solubilized in normal saline with the aid of tween 80 (2%). The extracts (50 mg/kg per body weight) then injected to the rabbits at the right jugular vein. Six groups each of six male-Neizuland rabbits were used, each weighing 1.1±0.1 kg. The selected rabbits were acclimatized for 30 min. before commencing the experiment. The animals were anesthetized with the injection urethane solution (concentration 100 mg/mL) in a dose of 6.4 mL/kg. The anaesthetized rabbits were stablished on a dissecting disc. Spontaneous respiration was maintained by inserting tracheal cannula after tracheotomy. Overhead lamp was used to maintain the temperature at about 37° C. The left carotid artery and right jugular vein were exposed by making longitudinal incision at the middle of the trachea and the free airway was maintained by introducing polyethylene tube 92.75 mm to the trachea [23] . The drugs and isotonic saline solution were administered by polyethylene (1 mm id.) tube introduced to the right jugular vein. Another tube of polyethylene introduced to the carotid artery but filled with heparin sodium dissolved in saline solution [24]. The arterial BP was monitored from the left carotid arterial cannula by blood pressure transducer connected with Oscillograph to record the results [25] . The MABP was calculated as follow:

MABP = DP + 1/3 (SP-DP) [26] , Where, DP= diastolic pressure & SP= systolic pressure.

The BP measured immediately before injection and the change percent from the control considered was calculated as change results [27] . The obtained results were compiled in (**Table 3**) and illustrated in (**Figure 3**).

2.5.3. Evaluation of anti-Inflammatory activity

The pedal inflammation in rat paws was made by injection of 0.1 mL carrageenan suspension (1% w/v in normal saline solution) to the right hind paw of rats. The male albino rats weighting 150±30 g were selected and grouped to seven groups each of five rats. The 1st group was injected with tween 80 (2%) in normal saline in the intraperitoneal (negative control). The 2nd group was injected by indomethacin (10 mg/kg) [28] and kept as a reference group. The other tested groups were administered with the total extract & different fractions in a dose of 100 mg/kg body weight. The paws thickness then measured in mm immediately (0 h) using Vernier Caliber and after 30 min, as well as at intervals after 1, 2, 3, 4 and 5 h. from administration of the extracts. The percentage of inhibition [29] was calculated by the following equation:

$$\% \text{ Variation (edema)} = \frac{(\text{Right paw thickness - Left paw thickness}) \times 100}{\textit{Right} \text{ paw thickness}}$$

,

$$\% \text{ Inhibition} = \frac{(V_o - V_t) x 100}{V_o}$$

Where V_o is the paw thickness of control group, V_t: paw thickness of the treated groups. The obtained results were collected in (Table **4** and **5)**.

2.5.4. Antipyretic Activity

It was done by yeast-induced pyrexia method [29]. The pyrexia was made by subcutaneous injection of 10 mL/kg body weight of 20% aqueous suspension of yeast to the selected male albino rats weighing about 165±15 g. The rectal temperature was measured for each animal before and after 19 h of the yeast injection by inserting a digital thermometer to the rectum in a depth of 2 cm. The animals that showed an increase of elevated rectal temperature (not less than 0.5 °C) were selected for the for operation of the experiment [30]. The animals were divided into seven groups, five animals each. The 1st group was injected by 2% tween 80 in normal saline (negative control group). The 2nd group administered ASA at a dose of 100 mg/kg (reference group) [31] . The other groups were given the total plant extract and fractions in a dose of 100 mg/kg body weight (tested groups). The rectal temperature was monitored at intervals after 30 min, 1, 2, 3, 4 and 5 h. following administration of the drugs.

2.5.5. Evaluation of antimicrobial activity

For determination of antimicrobial, the zones of inhibition obtained from the effect of the tested samples were measured by agar cup diffusion method [32]. The bacterial cultures were done on nutrient agar media poured in petri dishes with about 15 cm diameter for each. The bacterial inoculate were prepared according to 0.5 McFarland turbidity standards. The *Candida albicans* was chosen for determination of antimycotic activity and prepared in concentration of 10^6 cfu/mL. Under aseptic conditions by the aid of a sterile corkborer, cups were made in the medium. Fixed volumes from the different concentrations of the tested fractions were dispensed to fill the cups, using sterile micropipette. Plates were placed carefully in the incubators and the temperature was adjusted at 37 °C for 24 h. for bacteria, while the fungal growth was done at 28 °C for 48 h. After incubation, the diameter of clear zones around the samples were measured carefully and compared to the standard. The zones of inhibition considered as the inhibitory effect of the sample against selected microorganisms. The results were listed in (**Table 7**).

2.5.6. Statistical analysis

The obtained results were calculated using Statistical Package for the Social Sciences version 16 (SPSS-16) program. The results are expressed in term of mean ± S.E.M. The significant difference when compared to the control was determined using one-way analysis of variance (ANOVA), followed by Dunnett's t test for comparison between different groups. [$p < 0.05$ was considered as significant, $p < 0.01$ was considered as highly significant].

3. Results and discussion

The present study of the plant under investigation resulted in separation and identification of eight compounds. The identification based on their physical, chemical, co-chromatography in addition to comparison of the obtained spectral information with literature. The isolated compounds were identified as *α*-amyrin acetate [33, 34] , *α*-amyrin, lupeol acetate [35, 36] , stearic acid [16], ursolic acid [35] , quercetin [37] , *β*-sitosterol-3-*O*-*β*-glucopyranoside [38] and kaempferol-3-*O*-rutinoside (nicotiflorin) [39, 40], all of these compounds are firstly isolated from *Carissa macrocapa.*

The cardiovascular diseases (Heart attacks, coronary artery disease, heart failure, stroke in addition to high blood pressure) have threatened effects on people's life. Approximately 30,000 people cold attacked daily according to the reports of World Health Organization [41] . Natural products offer unique source and play an important role as therapeutic agents for cardiovascular diseases. From the obtained literature on Carissa species, we focused our biological work on the effect of plant under investigation on cardiovascular activities. Our results revealed that; the total extract and its fractions (DCM, EtOAc and *n*-butanol) of *C. macrocarpa* showed remarkable increase in the force of contractility and lowering the heart rate of the tested rabbit's heart. The promising effects was done by DCM and EtOAc fractions, which they showed significant deference from control at $P<0.01$ (**Figure 2**), while the *n*-hexane fraction did not show any effect. The remarkable contractility may be due to the presence of cardiotonic active principles in the plant extract, which are responsible for these activities. The total extract & different fractions of the studying plant also showed obvious hypotensive effect on the rabbits MABP. The extracts that showed the most potent hypotensive effect were DCM and ethyl acetate fractions (**Figure 3**); which may be attributed to the presence of cardiac glycosides in these fractions, while the *n*-Hexane fraction did not show any hypotensive activity. In this study, the suggested mechanism of action may attribute to the enhanced contractility due to enhanced Ca^{2+} membrane influx and inhibition of myocardial Na+/K+ ATPase. which resulted in increase in contractility [42]. Preliminary anti-inflammatory study was done on male albino rats. The obtained results motivated us to conclude that; the DCM and EtOAc fractions showed pronounced activity at doses of 100 mg/kg in comparison to reference drug (indomethacin 10 mg/kg), the percentage of inhibition is 43% and 41% respectively, in comparison with indomethacin 47%. The other samples showed mild activity with percentage of inhibition of 28% (CMME), 30% (*n*-hexane) and 28% (*n*-butanol) after 4-5 h. (**Table 5**). The observed anti-inflammatory effect due to the presence of active principles such as triterpenes & sterols [43] and flavonoids [44, 45]. The CMME and *n*-butanol fraction showed promising decrease in body temperature at doses of 100 mg/kg after two hours in comparison with ASA reference drug (100 mg/kg body weight) (**Table 6**). The other fractions (*n*-hexane, DCM and EtOAc) fractions showed mild activity within 4-5 hours. The obtained antipyretic activity related to the presence of triterpenes & sterols and tannins [46]. The CMME and its fractions (*n*-hexane, DCM and EtOAc) showed remarkable antibacterial activity against Gram +ve (*B. subtilis* & *S. aureus*) and Gram -ve bacteria (*E. coli* & *P. aeruginosa*) (**Table 7**).

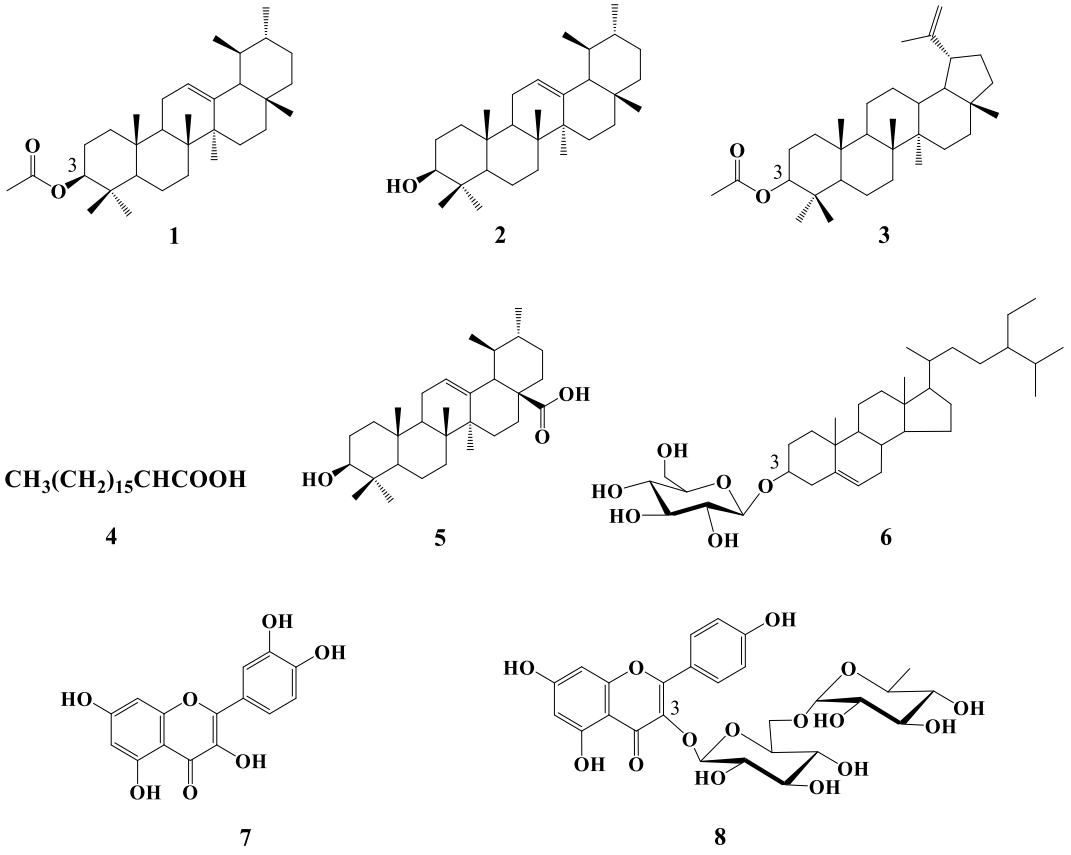

Figure 1: The chemical structures of the isolated compounds 1-8.

3.1. Cardiovascular activity

Table 1: The mean of contractility of isolated rabbit's heart after administration of the CMME and its fractions.

Groups	Sample					
	Negative Control	CMME	*n*-Hexane	DCM	EtOAc	*n*-Butanol
Untreated rabbits	2±0.02	2±0.03	2±0.03	1.9±0.03	2± 0.02	1.9± 0.03
Treated rabbits	1.9±0.05	2.4±0.08**	1.9±0.05	2.4±0.05**	2.5±0.04**	2.1±0.03

The data expressed in Mean ± S.E, (*) Significance from control at $p<0.05$ and Significance (**) at $p<0.01$.

Table 2: The mean of heart rate of isolated rabbit's heart after administration of the CMME and its fractions.

Groups	Sample					
	Negative Control	CMME	*n*-Hexane	DCM	EtOAc	*n*-Butanol
Untreated rabbits	71±0.44	70.0±0.25	71.5±0.34	71.5±0.34	71.0±0.36	71.3±0.33
Treated rabbits	70±0.55	59.1±1.0**	70.4±0.22	57.2±0.74*	56.5±1.1**	60.8±1.1**

The data expressed in Mean ± S.E, (*) Significance from control at $p<0.05$ and Significance (**) at $p<0.01$.

Table 3: The MABP of isolated rabbit's heart after administration of the CMME and its fractions.

Groups	Sample					
	Negative Control	CMME	*n*-Hexane	DCM	EtOAc	*n*-Butanol
Untreated rabbits	95.1±0.94	96.6±0.65	94.5±0.69	94.3±0.67	95.2±0.94	96.5±0.65
Treated rabbits	94.5±0.71	82.0±0.50*	94.0±0.56	75.0±0.96**	76.8±0.97**	82.7±0.49**

The data expressed in Mean ± S.E, (*) Significance from control at $p<0.05$ and Significance (**) at $p<0.01$.

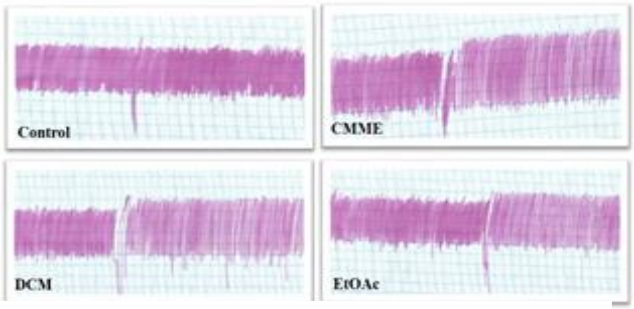

Figure 2: Tracing showing the effect of CMME and DCM & EtOAc fractions on heart rate and contractility of tested rabbit's hear.

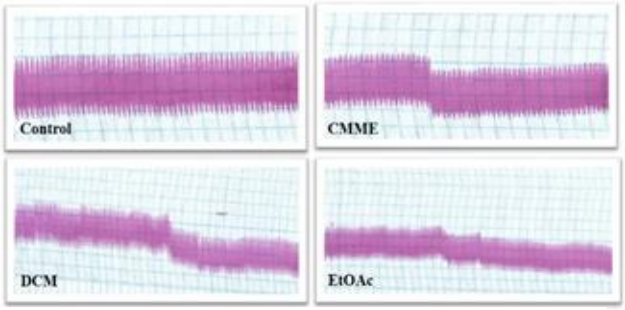

Figure 3: Tracing showing the effect of CMME and DCM & EtOAc fractions on MABP.

3.2. Anti-inflammatory & antipyretic activities

Table 4: Results of anti-inflammatory activity of the CMME and its fractions.

Group	Paw Thickness (mm), Time/h						
	0	**0.5**	**1**	**2**	**3**	**4**	**5**
Negative control	3.0±0.02	7.2±0.14	7.2±0.04	7.5±0.20	7.6±0.08	7.5±0.10	7.3±0.10
Indomethacin (Positive control)	3.0±0.05	6.7±0.14	5.7±0.08**	5.0±0.20**	4.6±0.12**	3.8±0.08**	3.8±0.04**
CMME	3.0±0.05	7.2±0.04	7.1±0.06	6.7±0.08**	6.3±0.12**	6.0±0.10**	5.3±0.10**
***n*-Hexane**	3.0±0.05	7.2±0.05	7.0±0.10	6.3±0.12**	6.2±0.12**	5.6±0.04**	5.1±0.04**
DCM	3.0±0.07	7±0.06	6.6±0.04**	5.8±0.05**	5.3±0.10**	4.7±0.08**	4.2±0.10**
EtOAc	3.0±0.05	7±0.20	6.5±0.10**	5.8±0.05**	5.3±0.12**	4.8±0.06**	4.3±0.04**
***n*-Butanol**	3.0±0.02	7±0.06	6.8±0.04*	6.3±0.12**	6.2±0.08**	5.7±0.04**	5.1±0.14**

The data expressed in Mean ± S.E, (*) Significance from control at $p<0.05$ and Significance (**) at $p<0.01$.

Table 5: Inhibition % of the anti-inflammatory activity of the CMME and its fractions.

Groups	Percentage of inhibition/h					
	0.5	**1**	**2**	**3**	**4**	**5**
Negative control	-	-	-	-	-	-
Indomethacin (positive control)	7	20	33	39	49	47
CMME	0	2	11	17	20	28
***n*-Hexane**	0	3	16	18	25	30
DCM	2.8	8	23	30	38	43
EtOAc	2.8	10	23	30	36	41
***n*-Butanol**	2.8	6	16	16	24	28

Table 6: Results of the antipyretic activity of the CMME and its fractions.

Group	Rectal temperature (°C)/ h						
	0	**0.5**	**1**	**2**	**3**	**4**	**5**
Negative control	36.9±0.04	39.6±0.14	39.6±0.12	39.7±0.09	39.4±0.06	39.7±0.08	39.3±0.08
ASA (positive control)	36.9±0.04	38.6±0.20**	37.4±0.15**	37.5±0.11**	37.3±0.11**	37.1±0.05**	37.1±0.06**
Total	37±0.15	39.6±0.12	38.9±0.22	38.6±0.08**	38.5±0.12**	38.5±0.18**	37.7±0.13**
***n*-Hexane**	37.1±0.13	39.5±0.08	39.5±0.08	39.5±0.08	39.6±0.04	39.4±0.05	38.7±0.13**
DCM	36.9±0.02	39.6±0.04	39.4±0.06	39.4±0.07	39.1±0.22	38.9±0.34*	38.7±0.22**
EtOAc	37.1±0.14	39.5±0.04	39.5±0.06	39.4±0.08	39.5±0.04	39.4±0.06	38.7±0.15**
***n*-Butanol**	37±0.23	39.6±0.07	38.9±0.20	38.5±0.17**	38.1±0.13**	37.8±0.14**	37.8±0.17**

The data expressed in Mean ± S.E, (*) Significance from control at $p<0.05$ and Significance (**) at $p<0.01$. ASA: Acetyl salicylic acid

. 3.3. Antimicrobial activity

Table 7: The antimicrobial effects of CMME and its fractions.

Sample	Diameters of Inhibition zones (mm)					
	S. aureus	***B. subtilis***	***P. aeruginosa***	***E. coli***	***A. flavus***	***C. albicans***
Control	0.0	0.0	0.0	0.0	0.0	0.0
Ampicillin	18	21	23	21	--	--
Amphotericin B	--	--	--	--	17	23
CMME	9	10	10	11	0.0	0.0
***n*-Hexane**	9	10	9	10	0.0	0.0
DCM	11	10	10	11	0.0	0.0
EtOAc	13	13	14	13	0.0	0.0
***n*-Butanol**	0.0	0.0	0.0	0.0	0.0	0.0

The EtOAc fraction exert the highest antibacterial activity, while the *n*-butanol did not show any activity. The antibacterial activity may be attributed to the present active secondary metabolites such as triterpenes & sterols and phenolic constituents [14, 47]. The total alcoholic extract and all tested fractions lack the antifungal activity.

4. Conclusion

Carissa macrocarpa methanolic extract and its fractions showed promising increase in contractile function accompanied with the decrease on heart rates of isolated rabbit's heart suggested the probability of future usage of *Carissa macrocarpa* in treatment of congestive heart failure (CHF) after extensive studies. Our hope future study is the isolation and identification of pure compounds responsible for the cardiovascular activities. It may be considered in the future as an additional plant in treatment of CHF.

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