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Phytotherapeutic Studies On *Calothamnus Quadrifidus* R. Br (Myrtaceae)

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ABSTRACT

The aqueous ethanolic extract of the aerial parts of *Calothamnus quadrifidus,* R. Br (Myrtaceae) showed significant biological activities including analgesic, anti-inflammatory, hypoglycaemic and antioxidant activities. Fifteen compounds were isolated and characterized from the same extract after chromatographic column fractionation. Compounds **(1-3)** are phenolic acids. The remaining compounds **(4-15)** belong to the group of plant natural products flavonoid. This is the second report regarding the natural occurrence of compound **(8)**, quercetin 3-*O*-β-$^4C_1$-D-glucuronide-3′-sulphate.

KEYWORDS

Calothamnus quadrifidus;
Myrtaceae;
Analgesic activity;
Anti-inflammatory activity;
Hypoglycaemic activity;
Antioxidant activity;
Phenolics;
Flavonoids.

INTRODUCTION

Calothamnus (Myrtaceae) contains about 40 species, all of which are native to west Australia and are described as erect shrubs that reach 8ft. They have red or reddish flowers; some also have yellow or brown flowers, most have linear to needle shaped leaves, but a few have slightly broader leaves. Several members of the genus are called 'Claw-flowers' because of the claw-like shape of the stamen bundles. The fruit is a capsule enclosed in the hardened and enlarged calyx-tube. The species *C.quadrifidus*, R. Br is known as 'one-sided-bottlebrush' because the red, brush-like flowers are on one side of the stem[1].

Myrtaceae plants are well known as rich sources of valuable essential oils as well as other medicinally significant phytoconstituents including various types of phenolic compounds[2,3]. As a part of an ongoing study on medicinal plants belonging to family Myrtaceae, *C.quadrifidus* R. Br cultivated in Egypt was chosen to be the subject of a detailed biological and phytochemical investigation for its aqueous ethanolic extract. No reports concerning the phytoconstituents of this genus were traced in literature;

hence, to the best of our knowledge this is the first report of its kind on this species.

EXPERIMENTAL

Plant material

The aerial parts of *C.quadrifidus* were collected from private gardens in Cairo in July, 2003 and authenticated by Prof. Dr. Abdel Salam El-Nowiahi, Professor of Taxonomy, Faculty of Science, Ain-Shams University, Egypt. Voucher specimens were deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt.

Plant extract

An aqueous ethanolic (70% ethanol) extract was prepared from 5 Kg of the plant material completely dried to give 300g of powdered extract.

BIOLOGICAL ASSAYS

1.Experimental animals

Experimental animals used consisted of albino mice of 25-30g body weight and adult male albino rats of sprauge dawely strain of 130-150g body weight. Doses of the drugs used were calculated according to Paget and Barne's[4] and were administered orally by gastric tubes.

2. Determination of the median lethal dose (LD_{50})

Determination of the LD_{50} of the aqueous ethanolic extract of *C.quadrifidus* was estimated according to the Karber procedure[5]. Preliminary experiments were done to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses, each dose was injected in a group of 6 animals by subcutaneous injection. The mice were then observed for 24 hours and symptoms of toxicity and mortality rates in each group were recorded and the LD_{50} was calculated.

3. Acute anti-inflammatory activity

This effect was determined according to the method described by Winter et al.[6]. Twenty-four male albino rats, weighing 130-150g were divided into four groups, each of six animals:

1. First group: Rats that received 1ml of saline serving as control.
2. Second group: Rats that received 50mg/kg of the plant extract.
3. Third group: Rats that received 100mg/kg of the plant extract.
4. Fourth group: Rats that received 20mg/kg of the reference drug, indomethacin.

One hour later, all the animals received a sub plantar injection of 0.1 ml of 1% carrageenan solution in saline, in the right hind paw and 0.1 ml saline in the left hind paw. Four hours after drug administration, the rats were sacrificed; both hind paws excised and weighed separately.

$$\% \text{ Edema} = \frac{\text{weight of right paw - weight of left paw}}{\text{weight of left paw}} \times 100$$

4. Analgesic activity

This activity was evaluated according to the method of Charlier et al.[7]. by using an electric current as anoxious stimulus where electrical stimulation was applied to the rat's tail by means of 515 Master Shocker (Lafayette Inst. Co.) using alternative current of 50 cycles /sec. for 0.2 sec. Eighteen male albino rats, weighing 130-150g were divided into three groups, each of six animals:

1. First group: Rats that received 1ml of saline serving as control.
2. Second group: Rats that received 100mg/kg of the plant extract.
3. Third group: Rats that received 50mg/kg of the reference drug, novalgin.

The minimum voltage required for the animal to emit a cry was recorded for the three groups after one and two hour's intervals.

5. Hypoglycaemic activity

Male albino rats of the sprague dawely strain (130-140g) were injected intra-peritoneal with alloxan (150mg/kg body weight) to induce diabetes mellitus[8]. Hyperglycaemia was assessed after 72 hours by measuring blood glucose[9] and after 1 and 2 months intervals. Animals were divided into 3 groups:

1. First group: Diabetic rats that served as positive control.
2. Second group: Diabetic rats that received 100

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mg/kg b.wt. of plant extract.

3. Third group: Diabetic rats that received 150mg/kg b.wt. of cidophage drug as reference drug.

At the end of each study period, blood samples were collected from the retro orbital venous plexus through the eye canthus of aneasthetized rats after an overnight fast. Serum was isolated by centrifugation and the blood glucose level was measured.

6. Antioxidant activity

This method depends on measuring the blood glutathione level and was determined according to the method of Beutler et al.[10]. The method depends on the fact that both protein and non protein SH-groups react with Ellman's reagent [5,5-dithiobis- (2-nitrobenzoic acid)] to form a stable yellow colour of 5-thio-2-nitrobenzoic acid, which can be measured at 412nm. In order to determine the glutathione level in the blood, precipitation of protein SH-groups was necessary before the addition of Ellman's reagent. Blood sample (0.1ml) was haemolysed by the addition of 0.9ml bidistilled water. To the haemolysate, 1.5 ml of the precipitating solution was added, mixed and allowed to stand for 5 min. Centrifugtion at 3000 rpm was carried out for 15 min. To 1 ml of the resulting supernantant, 4 ml of phosphate solution was added followed by 0.5ml of Ellman's reagent. The optical density was measured within 5 min. at 412 nm using the Shimadzu double beam spectrophotometer (UV-150-02). The blank solution for the samples was prepared with 4 ml phosphate solution, 1 ml dilute precipitating solution (3:2) and 0.5ml Ellman's reagent. To 1 ml standard glutathione solution, 4ml phosphate solution and 0.5ml Ellman's reagent were added and the optical density was measured at 412nm against blank containing 1ml bidistilled water instead of the standard solution using the following equation:

$$\text{GSH (mg\%)} = \frac{\text{absorbance of sample}}{\text{absorbance of the standard}} \times \frac{37.5}{1000} \times \frac{2.5}{0.1} \times 100$$

All statistical analyses were performed using the Student 't' test as described by Spedecor and Cochran[11].

Phytochemical isolation

The prepared extract (200g) was fractionated on a sephadex LH-20 column, (100cm×5cm) eluted with distilled water, followed by mixtures of water/methanol of decreasing polarities and finally by acetone. Fractions of 1-2L. were collected and monitored by 2D paper chromatography using solvent systems; BAW/6% acetic acid. Similar fractions were pooled together to give 8 main fractions (I-VIII). 2D PC, sprayed with specific chromogenic spray reagents showed that, fraction I consisted mainly of carbohydrates, fractions II-VII contain mainly phenolic constituents and fraction VIII contained condensed tannins. A combination of column fractionation and Prep. PC led to isolation of fifteen pure phenolics from fractions II- VII.

3,4-dihydroxy benzoic acid, Protocatechuic acid (1) was obtained as an off white amorphous powder (10mg); R_f values:80(HOAc-15%) and 75 (BAW); UV λ_{max} : MeOH (293.40 and 259.40). ^{1}HNMR data: δ ppm 7.32(H-2, d, J=2.5 Hz), 6.75(H-5, d, J=7 Hz) and 7.25(H-6, dd, J=7 Hz and 2.5 Hz). ^{13}C-NMR data: δ ppm 123.52(C-1), 117.19(C-2), 145.23(C-3), 150.09(C-4), 115.50(C-5), 122.00(C-6), 167.96 (COOH). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[12].

3,4,5-trihydroxy benzoic acid, Gallic acid (2) was obtained as an off white amorphous powder (20 mg); R_f=56(HOAc-15%) and 78 (BAW); UV λ_{max} : MeOH (272nm). ^{1}H-NMR data: δ ppm 6.91 (s, H-2 and H-6). ^{13}C-NMR data: δppm 109.2(C-2 and C-6), 122.93(C-1), 137.92(C-4), 145.83(C-3 & C-5), 169.24(C-7).^{1}H- and ^{13}C-NMR data were identical to those reported in literature[13].

3-methoxy, 4-hydroxy cinnamic acid, Ferulic acid (3) was obtained as as colorless crystals (10mg); R_f values: 55 (HOAc-15%) and 90 (BAW); UV λ_{max} : MeOH (235 and 324). ^{1}H-NMR data: δ ppm 7.50 (H-α, d, J=16 Hz), 7.26 (H-2, d, J=1.9 Hz), 7.07 (H-6, dd, J=1.9 Hz and 8.1 Hz), 6.79 (H-5, d, J=8.1 Hz), 6.35 (H-β, d, J=16 Hz), 3.81 (OCH_3). ^{13}C-NMR data: δ ppm 55.75 (OCH_3), 111.21 (C-1), 115.61 (C-2), 115.71 (C-β), 122.91 (C-5), 125.87 (C-6), 144.62 (C-α), 147.99 (C-3), 149.16 (C-4), 168.12 (COOH). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[14].

Quercetin 3-O-$^4C_1$-β-D-galactopyranoside (4) was obtained as pale yellow amorphous powder (15 mg); R_f values: 58 (HOAc-15%) and 36 (BAW); UV

λ_{max} : MeOH (257, 269sh, 299sh, 362), MeOH/ NaOAc (274, 324, 380), MeOH/ NaOAc/ H_3BO_3 (262, 298sh, 377), MeOH/ $AlCl_3$ (275, 305sh, 331sh, 438), MeOH/ NaOMe (272, 327, 409). ^{1}H-NMR data: δ ppm 5.36 (d, J=7.0 H-1″), 6.19 (d, J=2.0 Hz, H-6), 6.40 (d, J=2.0 Hz, H-8), 6.82(d, J=8.5 Hz, H-5′), 7.54(d, J=2.0 Hz, H-2′), 7.65(dd, J=2.0 Hz and J=8.5 Hz, H-6′). ^{13}C-NMR data: δ ppm 60.45(C-6″), 67.99(C-4″), 71.26(C-2″), 73.22(C-3″), 75.91(C-5″), 93.59(C-8), 98.75(C-6), 101.80(C-1″), 104.0(C-10), 115.25(C-2′), 116.0(C-5′), 121.15(C-1′), 122.20(C-6′), 133.52(C-3), 144.91(C-3′), 148.53(C-4′), 156.30(C-9), 156.37(C-2), 161.30(C-5), 164.19(C-7), 177.57(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[15].

Quercetin 3-O-$^4C_1$-β-D-glucopyranoside (5) was obtained as dark brown amorphous powder (12 mg); R_f values: 60 (HOAc-15%) and 30 (BAW); UV λ_{max} : MeOH (258, 267sh, 356), MeOH/ NaOAc (256, 362, 374), MeOH/ NaOAc/ H_3BO_3 (265, 272, 380, 420sh), MeOH/ $AlCl_3$ (263, 430), MeOH/ NaOMe (275, 470). ^{1}H-NMR data: δ ppm 5.10(d, J=7.5 Hz, H-1″), 6.23(d, J=2.2 Hz, H-6), 6.60(d, J=2.2 Hz, H-8), 7.03(d, J=8.2 Hz, H-5′), 7.19(dd, J=2.0 Hz and J=8.2 Hz, H-6′), 7.21(d, J=2.0 Hz, H-2′). ^{13}C-NMR data: δ ppm 60.95(C-6″), 69.92(C-4″), 73.42(C-2″), 76.77(C-3″), 77.50(C-5″), 94.94(C-8), 99.71(C-6), 100.23(C-1″), 102.03(C-10), 115.30(C-2′), 116.32(C-5′), 120.03(C-1′), 122.20(C-6′), 133.0(C-3), 142.0(C-3′), 149.0(C-4′), 156.30(C-9), 157.24(C-2), 161.30(C-5), 163.08(C-7), 177.40(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[16].

Kaempferol 3-O-$^4C_1$-β-D-glucuronide (6) was obtained as buff amorphous powder (15 mg); R_f values: 22 (HOAc-15%) and 68 (BAW); UV λ_{max} : MeOH (267, 353), MeOH/ NaOAc (268, 355), MeOH/ NaOAc/ H_3BO_3 (271, 355), MeOH/ $AlCl_3$ (272, 408), MeOH/ NaOMe (275, 310, 402). ESI-MS: $[M-H]^-$ at m/z = 461.23 (molecular mass = 462 indicating molecular formula of $C_{21}H_{18}O_{12}$). ^{1}H-NMR data: δ ppm 5.20(d, J=7.5 Hz, H-1″), 6.16(d, J=2.2 Hz, H-6), 6.37(d, J=2.2 Hz, H-8), 6.86(d, J=8.5 Hz, H-3′and H-5′), 8.02(d, J=8.5 Hz, H-2′and H-6′). ^{13}C-NMR data: δ ppm 71.88(C-4″), 73.78(C-2″), 74.71(C-3″), 76.31(C-5″), 93.87(C-8), 98.96(C-6), 101.79(C-1″), 103.68(C-10), 115.23(C-3′), 115.23(C-5′), 120.52(C-1′), 131.10(C-2′), 131.10(C-6′), 133.65(C-3), 156.41(C-9), 156.58(C-2), 160.29(C-4′), 160.98(C-5), 164.95(C-7), 172.75(C-6″), 177.64(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[17].

Quercetin 3-O-$^4C_1$-β-D-glucuronide (7) was obtained as buff amorphous powder (12 mg); R_f values: 41 (HOAc-15%) and 38 (BAW); UV λ_{max} : MeOH (255, 267sh, 357), MeOH/ NaOAc (256, 360, 376), MeOH/ NaOAc/ H_3BO_3 (272, 363), MeOH/ $AlCl_3$ (271, 394), MeOH/ NaOMe (272, 406). ESI-MS: $[M-H]^-$ at m/z=477.24 (molecular mass=478 indicating molecular formula of $C_{21}H_{18}O_{13}$). ^{1}H-NMR data: δ ppm 5.40(d, J=7.5 Hz, H-1″), 6.20(d, J=2.2 Hz, H-6), 6.42(d, J=2.2 Hz, H-8), 6.84(d, J=8.2 Hz, H-5′), 7.55(dd, J=2.2 Hz and J=8.2 Hz, H-6′), 7.75(d, J=2.2 Hz, H-2′). ^{13}C-NMR data: δ ppm 71.73(C-4″), 73.81(C-2″), 74.47(C-3″), 76.46(C-5″), 93.85(C-8), 99.12(C-6), 101.27(C-1″), 103.59(C-10), 115.53(C-5′), 117.17(C-2′), 120.49(C-6′), 121.32(C-1′), 134.15(C-3), 144.93(C-3′), 148.71(C-4′), 156.46(C-2), 157.14(C-9), 160.93(C-5), 165.17(C-7), 172.74(C-6″), 177.67(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[18].

Quercetin 3-O-$^4C_1$-β-D-glucuronide-3′-O-sulphate (8) was obtained as yellow amorphous powder (15 mg); R_f values: 76 (HOAc-15%) and 27 (BAW); UV λ_{max} : MeOH (268, 283sh, 350), MeOH/ NaOAc (275, 310, 390), MeOH/ NaOAc/ H_3BO_3 (268, 295, 352), MeOH/ $AlCl_3$ (267, 295sh, 350, 400), MeOH/ $AlCl_3$ / HCl (276, 295sh, 350, 400), MeOH/ NaOMe (275, 325, 400). ^{1}H-NMR data: δ ppm 5.10(d, J=7.5 Hz, H-1″), 6.21(d, J=2.2 Hz, H-6), 6.42(d, J=2.2 Hz, H-8), 6.84(d, J=8.2 Hz, H-5′), 7.44(dd, J=2.2 Hz and J=8.2 Hz, H-6′), 7.94(d, J=2.2 Hz, H-2′). ^{1}H- NMR data were identical to those reported in literature[19]. Partial acid hydrolysis of compound **(8)** using 0.05M HCL in 50% MeOH yielded an intermediate (8a), that was isolated by preparative PC identified as Quercetin 3-O-glucuronide by CoPC with compound **(7)** as well as typical UV shifts and ^{1}H-NMR spectral analysis. An electrophoresis experiment of compound **(8)** on Whatman paper No. 3MM using acetic acid-formic acid buffer, pH 2.2 for 1.5 hrs showed a movement

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of the spot equivalent to 1 cm. from the basal line towards the anode.

Kaempferol 3-O-$^1C_4$-α-L-rhamnopyranoside (9) was obtained as pale yellow amorphous powder (20 mg); R_f values: 68 (HOAc-15%) and 42 (BAW); UV λ_{max} : MeOH (267, 353), MeOH/ NaOAc (268, 355), MeOH/ NaOAc/ H_3BO_3 (271, 355), MeOH/ $AlCl_3$ (272, 408), MeOH/ NaOMe (275, 310, 402). ^{1}H-NMR data: δ ppm 0.9 (d, J=6 Hz., CH_3), 5.29 (1 H, d, J=1.5 Hz H-1″), 6.21 (1H, d, J=2.5 Hz, H-6), 6.42 (1H, d, J=2.5 Hz, H-8), 6.91 (2H, d, J=8.4 Hz, H-3′ and H-5′), 7.74 (2H, d, J=8.4 Hz, H-2′ and H-6′). ^{13}C-NMR data: δ ppm 17.53(C-6″), 70.11(C-5″), 70.35(C-2″), 70.69(C-3″), 71.13(C-4″), 93.88(C-8), 98.92(C-6), 101.77(C-1″), 103.98(C-10), 115.48(C-3′) and (C-5′), 120.49(C-1′), 130.64(C-2′) and (C-6′), 134.15(C-3), 156.56(C-9), 157.24(C-2), 160.14(C-4′), 161.28(C-5), 164.80(C-7), 177.70(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[15].

Quercetin 3-O-$^1C_4$-α-L-rhamnopyranoside (10) was obtained as pale yellow amorphous powder (20 mg); R_f values: 70 (HOAc-15%) and 52 (BAW); UV λ_{max} : MeOH (259, 297sh, 348), MeOH/ NaOAc (276, 372), MeOH/ NaOAc/ H_3BO_3 (272, 383), MeOH/ $AlCl_3$ (268, 352, 408), MeOH/ NaOMe (270, 355, 402). ^{1}H-NMR data: δ ppm 0.9 (d, J=6 Hz, CH_3), 5.2(d, J=1.5 Hz, H-1″), 6.15(d, J=2.2 Hz, H-6), 6.36(d, J=2.2 Hz, H-8), 6.95(d, J=8.2 Hz, H-5′), 7.25(dd, J=2.2 Hz and J=8.2 Hz, H-6′), 7.35(d, J=2.2 Hz, H-2′). ^{13}C-NMR data: δ ppm 17.67(C-6″), 70.21(C-5″), 70.73(C-3″), 70.50(C-2″), 71.35(C-4″), 93.97(C-8), 99.20(C-6), 101.91(C-1″), 103.79(C-10), 115.68(C-2′), 115.78(C-5′), 120.79(C-1′), 121.21(C-6′), 134.24(C-3), 145.44(C-3′), 148.78(C-4′), 156.66(C-2), 157.25(C-9), 161.35(C-5), 165.59(C-7), 177.72(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[20].

Luteolin 7-O-$^4C_1$-β-D-glucopyranoside (11) was obtained as faint yellow crystals (20 mg); R_f values: 54 (HOAc-15%) and 18 (BAW); UV λ_{max} : MeOH (255, 267, 346), MeOH/ NaOAc (259, 265, 360, 398), MeOH/ NaOAc/ H_3BO_3 (260, 370), MeOH/ $AlCl_3$ (272, 300, 330, 430), MeOH/ NaOMe (264, 300, 398). ^{1}H-NMR data: δ ppm 3.30-3.80 (m, H-2″-H-6″), 5.10 (d, J=7.50 Hz, H-1″), 6.66 (s, H-3), 6.41 (d, J=2.5 Hz, H-6), 6.78(d, J=2.5 Hz, H-8), 6.94 (d, J=8.0 Hz, H-5′), 7.39 (dd, J=2.5 Hz and J=8.0 Hz, H-6′), 7.48 (d, J=2.5 Hz, H-2′). ^{13}C-NMR data: δ ppm 60.67(C-6″), 69.64(C-4″), 73.19(C-2″), 76.47(C-3″), 77.22(C-5″), 94.63(C-8), 99.45(C-6), 99.93(C-1″), 101.67(C-3), 105.30(C-10), 112.21(C-2′), 116.16(C-5′), 117.83(C-6′), 119.79(C-1′), 147.14(C-3′), 149.6(C-4′), 156.99(C-9), 161.24(C-5), 162.79(C-7), 165.23(C-2), 175.0(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[21].

Naringenin (12) was obtained as faint yellow amorphous powder (12 mg); R_f values: 15 (HOAc-15%) and 85 (BAW); UV λ_{max} : MeOH (289, 326sh), MeOH/ NaOAc (284sh, 323), MeOH/ NaOAc/ H_3BO_3 (290, 322sh), MeOH/ $AlCl_3$ (312, 375), MeOH/ NaOMe (245, 323). ^{1}H-NMR data: δ ppm 2.68 (1H, dd, J=3, 17 Hz, H-3), 3.22 (1H, dd, J=13, 17 Hz, H-3), 5.41 (1H, dd, J=3, 13Hz, H-2), 5.89(2H, brs, H-6 & H-8), 6.80 (2H, d, J=8.5 Hz, H-3′, H-5′), 7.30 (2H, d, J=8.5 Hz, H-2′, H-6′). ^{13}C-NMR data: δ ppm 42.08(C-3), 78.54(C-2), 95.10(C-8), 96.0(C-6), 101.87(C-10), 115.29(C-3′) and (C-5′), 128.41 (C-6′) and (C-2′), 128.95(C-1′), 157.83(C-4′), 163.02(C-9), 163.62(C-5), 166.74(C-7), 196.39(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[17].

Quercetin (13) was obtained as yellow amorphous powder (18 mg); R_f values: 58 (HOAc-15%) and 63 (BAW); UV λ_{max} : MeOH (256, 263sh, 350), MeOH/ NaOAc (273, 323sh, 373), MeOH/ NaOAc/ H_3BO_3 (260, 302sh, 309), MeOH/ $AlCl_3$ (276, 305sh, 333, 430), MeOH/ NaOMe (270, 326, 393). ^{1}H-NMR data: δ ppm 6.18 (1H, d, J=2.1, H-6), 6.41 (1H, d, J=2.1, H-8), 7.66 (1H, d, J=2.1, H-2′), 6.90 (1H, d, J=8.5, H-5′), 7.53 (1H, dd, J=2.1 and 8.5, H-6′). ^{13}C-NMR data: δ ppm 93.73(C-8), 98.57(C-6), 103.33(C-10), 115.40(C-2′), 115.98(C-5′), 120.35(C-6′), 122.30(C-1′), 136.06(C-3), 145.40(C-3′), 147.14(C-2), 148.05(C-4′), 156.47(C-9), 161.03(C-5), 164.30(C-7), 176.17(C-4). ^{1}H- and ^{13}C-NMR data were identical to those reported in literature[22].

Kaempferol (14) was obtained as yellow amorphous powder (20 mg); R_f values: 40 (HOAc-15%) and 79 (BAW); UV λ_{max} : MeOH (253sh, 266, 322sh, 367), MeOH/ NaOAc (272, 387), MeOH/ NaOAc/ H_3BO_3 (276, 297sh, 320sh, 372), MeOH/ $AlCl_3$

(260, 269, 350, 424), MeOH/ NaOMe (278, 416). ^{1}H-NMR data: δ ppm 6.19 (1H, d, J=2.5, H-6), 6.43 (1H, d, J=2.5, H-8), 8.03 (1H, d, J=2.1, H-2′), 8.03 (1H, dd, J=2.1 and 8.5, H-6′), 6.92 (1H, d, J=8.0, H-3′), 6.92 (1H, dd, J= 8.0, H-6′). ^{1}H- NMR data was identical to that reported in literature[22].

Luteolin (15) was obtained as yellow amorphous powder (15 mg); R_f values: 66 (HOAc-15%) and 78 (BAW); UV λ_{max} : MeOH (253, 267, 350), MeOH/ NaOAc (268, 384), MeOH/ NaOAc/ H_3BO_3 (262, 370, 426), MeOH/ $AlCl_3$ (272, 300, 330, 422), MeOH/ NaOMe (266, 330sh, 400). ^{1}H-NMR data: δ ppm 6.20 (d, J=2.5 Hz, H-6), 6.45 (d, J=2.5 Hz, H-8), 6.65 (s, H-3), 6.92 (d, J=8.0 Hz, H-5′), 7.45 (dd, J=2.5 Hz and J=8.0 Hz, H-6′), 7.50 (d, J=2.5 Hz, H-2′). ^{1}H- NMR data was identical to that reported in literature[22].

RESULTS AND DISCUSSION

The aqueous ethanolic extract of the aerial parts of *C.quadrifidus* was shown to possess significant biological activities represented in graphs compared to reference drugs (figures 1-6). The median lethal dose (LD_{50}) of the extract was revealed to be 7.3g/kg b.wt.

The anti-inflammatory activity for the aqueous ethanolic extract of *C.quadrifidus* (figure 1) was investigated in two dose levels (50 and 100mg/kg b.wt). These results nearly matched that of the reference drug, indomethacin, proving the extract possesses a reasonable anti-inflammatory activity. Concerning the analgesic activity (figure 2), the potency of the extract almost resembles that of the reference drug, novalgin, indicating reasonable analgesic effect of the extract. In case of the hypoglycemic activity of the extract showed remarkable results (figure 3). The percent change produced by the extract after 8 weeks exceeded that of the reference drug, cidophage, after 4 weeks indicating the extract's significantly high hypoglycemic effect. Finally, the aqueous ethanolic extract of *C.quadrifidus* was shown to possess significantly high antioxidant activity (figure 4) revealed in its remarkable reduction of blood glutathione level which is nearly similar to that of the reference drug, vitamin E.

These potent biological activities of the extract including the analgesic, anti-inflammatory and hypoglycaemic activities are probably attributed to the presence of phenolic compounds as was revealed by the phytochemical investigation. A total of fifteen compounds were identified from four fractions of the extract including three known phenolic acids **(1-3)**, eight flavonoid glycosides **(4-11)** and four flavonoid aglycones **(12-15)**, all of which are known and have been previously isolated from family Myrtaceae except the sulphated flavonoid (8).

Compound **(8)** was separated as yellow amorphous powder (15 mg), and appeared as a dark purple spot on PC under UV light that changed to yellow on exposure to ammonia vapors. The chromatographic properties and UV spectral data were found in consistence with the only report of quercetin 3-O-β-$^4C_1$-D-glucuronide-3′-sulphate[21]. ^{1}H-NMR spectral

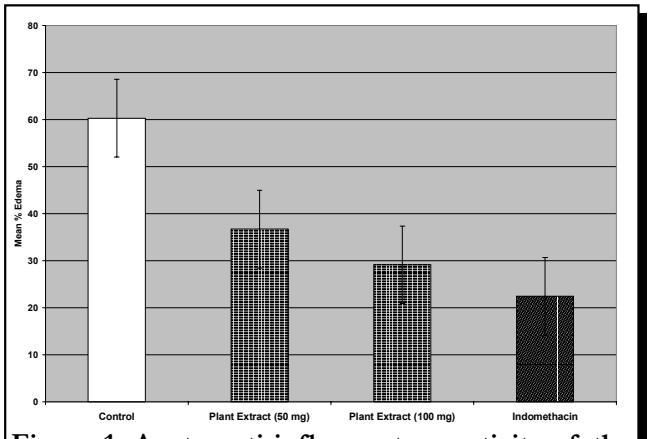

Figure 1: Acute anti-inflammatory activity of the aqueous ethanolic extract of *C.quadrifidus* and reference drug (Indomethacin) in male albino rats (n=6)

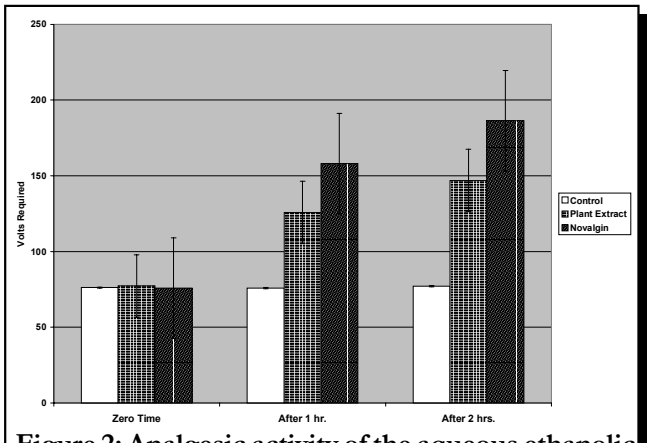

Figure 2: Analgesic activity of the aqueous ethanolic extract of *C.quadrifidus* and reference drug (Novalgin) in male albino rats (n=6)

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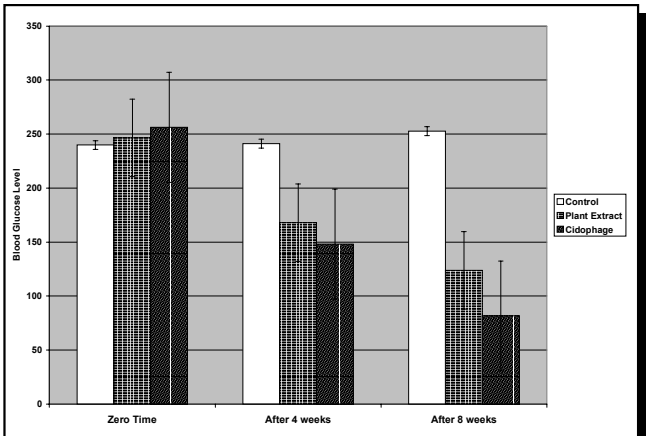


Figure 3: Effect of the aqueous ethanolic extract of *C.quadrifidus* and reference drug (Cidophage) on serum glucose level in diabeticrats (n=10)

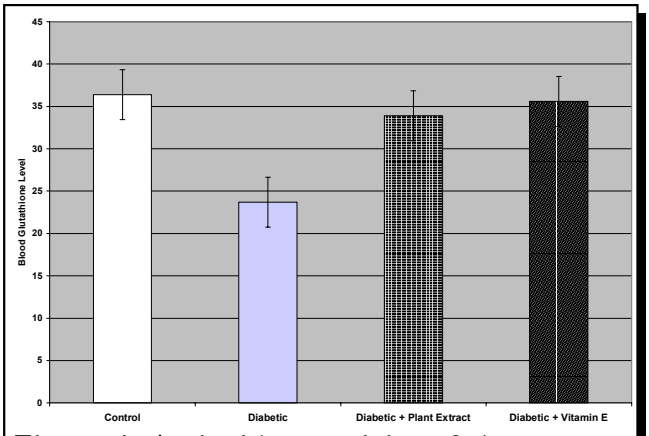
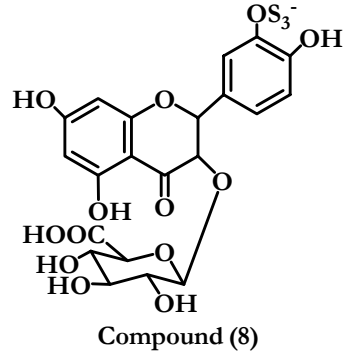


Figure 4: Antioxidant activity of the aqueous ethanolic extract of *C.quadrifidus* and reference drug (vitamin E) in male albino rats (n=6)

analysis revealed the characteristic pattern of quercetin proton resonances in the aromatic region [δ ppm: 7.44(dd, J=2.2 Hz and J=8.5 Hz, H-6′), 6.84(d, J=8.5 Hz, H-5′), 6.42(d, J=1.6 Hz, H-8), and 6.21(d, J=1.6 Hz, H-6)] with a deviation of H-2′ resonance showing a downfield chemical shift of δ ppm: 7.94(d, J=2.2 Hz.). This proton resonance was proved to be genuine to compound **(8)** by the proton-proton COSY spectrum that showed two cross peaks correlating the H-2′ to H-5′ as well as H-6′. The presence of a sulphate substituent at position 3′ was concluded from the downfield shift of H-2′[23]. This conclusion was also supported by partial acid hydrolysis of compound **(8)** to yield an intermediate (8a), that was isolated by preparative PC identified as quercetin 3-O-glucuronide by CoPC with compound **(7)** as well as typical UV shifts and ^{1}H-NMR spectral analysis. Finally, compound **(8)** was confirmed to be quercetin 3-O-$^4C_1$-β-D-glucuronide-3′-sulphate by electrophoresis on Whatman paper No. 3MM using acetic acid-formic acid buffer, pH 2.2 for 1.5 hrs. where the spot moved 1 cm. from the basal line towards the anode.



Compound (8)
Quercetin 3-*O*-$^4C_1$- β-D-glucuronide-3′-*O*-sulphate

Among the isolated phenolic compounds: protocatechuic acid **(1)**, gallic acid **(2)** ferulic acid **(3)**, quercetin 3-O-$^4C_1$-β-D-galactoside **(4)**, quercetin 3-O-$^4C_1$-β-D-glucoside **(5)**, kaempferol 3-O-$^4C_1$-β-D-glucuronide **(6)**, quercetin 3-O-$^4C_1$-β-D-glucuronide (7), kaempferol 3-O-$^1C_4$-α-L-rhamnoside **(9)**, luteolin 7-O-$^4C_1$-β-D-glucoside **(10)** and quercetin 3-O-$^1C_4$-α-L-rhamnoside **(11)**, naringenin **(12)**, quercetin **(13)**, kaempferol **(14)** and luteolin **(15)**.

In addition, fraction I (40g) gave a positive test for carbohydrates (Molisch test). The concentrated aqueous extract of the fraction was precipitated with acetone to yield a white crystalline precipitate, which was confirmed to be complicated polysaccharide through NMR analysis. ^{1}H-NMR revealed (broad multiplet between 3-5 δppm and anomeric proton resonance signals at 4.9 and 5.2 ppm). ^{13}C-NMR confirmed this result showing carbon resonance (61-83 δ ppm) corresponding to sugar carbons, and carbon resonance (92-104 δ ppm) corresponding to anomeric carbons. MALDI mass spectrometric analysis indicated a mixture of 2 major oligosaccharides.

Therefore, an in-depth study is recommended to resolve this mixture.

Finally, the highly effective antioxidant activity of the extract is obviously related to the richness in phenolic compounds (fractions II-VII) as well as condensed tannins that constituted most of the last fraction collected (Fraction VIII). This fraction was concentrated into a dark precipitate (2.3g) and showed

typical chromatographic properties of condensed tannins on PC. This included dark purple elongated streaks that were observed under UV light, which remained at the basal line when using both polar (15% AcOH) and nonpolar (BAW) solvent systems. The observed tailing gave a green colour when sprayed with $FeCl_3$ and a pink colour when sprayed with vanillin HCl[24] confirming fraction VIII to mainly constitute condensed tannins.

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