Antimicrobial and Immunomodulatory Activities of Flavonol Glycosides Isolated From *Atriplex halimus* L. Herb.

Mona El-Aasr,[1,2,*] Amal Kabbash,[1] Kamilia A. Abo El-Seoud,[1]Lamiaa A. Al-Madboly,[3] and Tsuyoshi Ikeda[2]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta (31527), Egypt.*

[2]*Natural Medicines Laboratory, Faculty of Pharmaceutical Sciences, Sojo University,4-22-1, Ikeda, Nishi-ku, Kumamoto 860-0082, Japan.*

[3]*Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University, Tanta (31527), Egypt.*

Correspondence: Mona El-Aasr,Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta (31527), Egypt.

Abstract

Phytochemical investigation of *Atriplex halimus* L. aerial parts resulted in isolation of four flavonol glycosides, syringetin 3-*O*-*β*-D-rutinoside **(1)**, syringetin 3-*O*-*β*-D-glucopyranoside **(2)**, isorhamnetin 3-*O*-*β*-D-rutinoside (narcissin) **(3)** and artiplexoside A **(4)**. Compound **(1)**, **(2)** and **(3)** were isolated for the first time from this species. The chemical structure of the compounds were determined using ¹H-NMR, ¹³C-NMR and 2D-NMR. The antimicrobial activity of the isolated compounds was investigated and the results showed that all of the tested compounds displayed broad spectrum antibacterial activity. Isorhamnetin 3-*O*-*β*-D-rutinoside (narcissin) **(3)** was effective against Gram–negative isolates such as *Escherichia coli* and *Acinetobacter baumanii*. Artiplexoside A **(4)** was the most active against Gram-positive bacteria including; *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus feacalis*. In addition, artiplexoside A **(4)** was the most effective anticandida compound. Moreover, the immunomodulatory role of these flavonol glycosides on human macrophages was investigated *in vitro*. Isorhamntin 3-*O*-*β*-D-rutinoside (narcissin) **(3)** and artiplexoside A **(4)** reduced the level of the induced IL-6 from 255.13 pg/mL to 77.34 and 32.106 pg/mL, respectively. Also, the level of IL-1β decreased by compound **(3)** and **(4)** from 287.22 to 82.11 and 45.12 pg/mL, respectively. Furthermore, the level of TNF-α and COX-2 was reduced by artiplexoside A **(4)** to approximately the normal level in LPS-inflammation model. On the other hand, syringetin 3-*O*-*β*-D-rutinoside **(1)** and syringetin 3-*O*-*β*-D- glucopyranoside **(2)** markedly increased all the above cytokines and pro-inflammatory mediators, which suggest that the isolated compounds act as immunomodulators.

Keywords: *Atriplex halimus* L., syringetin 3-*O*-*β*-D-rutinoside, syringetin 3-*O*-*β*-D- glucopyranoside, isorhamntin 3-*O*-*β*-D-rutinoside (narcissin), artiplexoside A, antimicrobial and immunomodulatory.

1. INTRODUCTION

Limited water resources and salty water are major challenges for world food security, especially in many developing countries. The xero-halophyte saltbush (Atriplex) is well adapted to saline clay soils [1]. Over 400 species of Atriplex have been identified in all continents. The Mediterranean Basin has 40–50 Atriplex species [2]. *Atriplex halimus* L. is a halophytic shrub of semi-arid and arid zones. It is used for livestock feed and soil protection and in traditional medicine. The possible new uses of *A. halimus* L. such as phytoremediation and biomass energy provision may ensure that *A. halimus* L. will remain a vital plant species in low-rainfall regions [3].

A. halimus L. produces polyphenols and other bioactive substances potentially useful for medicinal properties and as natural food preservation [4]. It contains tannins, flavonoids, saponins, alkaloids and resins [5]. These molecules were known to show medicinal activity as well as exhibiting physiological activity. The flavonoids of *A. halimus* L. leaves have more hydrogen donating ability to reducing iron and the higher DPPH radical scavenging activity [4]. The aqueous leaf extract of *A. halimus* L. has beneficial effect in reducing the elevated blood glucose level and hepatic levels in streptozotocin-induced diabetic rats [5]. Chromatographic investigation of ethyl acetate extract indicated that the plant contained flavonol, flavanone and flavone glycosides [6]. Flavonoids are antioxidants, metal chelators and possess anti-inflammatory, antiallergic, antiviral, anticarcinogenic and antithrombotic activities. In our previous study, two new flavonol glycosides, designated as atriplexoside A and atriplexoside B, together with phenolic glucosides, ecdysteroid, megastigmane, and methoxylated flavonoid glycosides were reported from this plant. In addition, the antioxidant, antileishmanial and anti-multidrug resistance activities were evaluated [7]. Furthermore, the total extract and different fractions of *A. halimus* L. showed cytotoxic activity with specific selectivity against breast (MCF-7) and prostate (PC3) carcinoma cells [8]. However, There are few studies related to phytochemical investigation of *A. halimus* L.

Modulation of the immune system involves amplification, expression, induction, or inhibition of any phase of the immune response. The immune system could be modulated through various mechanisms such as activation or inhibition of the complement, proliferation of lymphocytes, macrophages activation / inhibition or affecting the cytokine production by various immune cells [9]. It is important to modulate the immune response to cure many diseases particularly if plants are used as alternative to

traditional drugs. Studies done in recent years have shown that many plants have been used as immunomodulators and were shown to directly stimulate the immune system, while others were shown to have immunosuppressive actions [10–12].

The probable uses of immunomodulators in medicine involve stimulating the immune response as in case of AIDS patients as well as suppressing the excessive undesired immune function as in case of autoimmune diseases or graft rejection. Moreover, immunomodulators could also be utilized in conjunction with antigens to boost the immune response to the constituents of vaccines [13]. During the inflammatory process, huge amounts of pro-inflammatory mediators such as tumor necrotic factor alpha (TNF-α), prostaglandin E2 (PGE2), nitric oxide (NO), and cyclooxygenase-2 (COX-2) are generated [14]. Therefore, using plants as immunomodulators is advantageous due to being accepted by patients and physicians alike [15].

Based on the aforementioned information, this study was designated to investigate this valuable plant for further isolation and characterization of the biologically active secondary metabolites. The isolated compounds were evaluated for the antibacterial and antifungal effects as well as immunomodulatory role on human macrophages *in vitro*.

2. MATERIALS AND METHODS

2.1. General experimental procedure

^{1}H- and ^{13}C-NMR spectra were measured in pyridine-d_5 and in DMSO-d_6 with a JEOL ECA 500 NMR (JEOL Ltd., Tokyo, Japan) spectrometer at 500 and 125 MHz, respectively. The chemical shift (δ) was reported in parts per million (ppm). The *J* value was reported in Hz. Preparative HPLC was performed on a Shimadzu HPLC system equipped with an LC-20AT pump (Shimadzu Co. Ltd., Kyoto, Japan), JASCO 830-RI detector (JASCO Co. Ltd., Tokyo, Japan), Sugai U-620 column heater (Sugai Chemie Inc.,Wakayama, Japan) and COSMOSIL 5C$_{18}$ AR-II (5 μm, ϕ 10.0 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) and Atlantis T$_3$ (5 μm, ϕ 10 × 250 mm, Waters Co., MA, USA) HPLC columns at a flow rate of 2.0 mL/min and a column temperature of 40 °C. Neubaurhaemocytometer (Weber, Teddington, UK, (CO$_2$ incubator (Heal Force, Shanghai), an inverted microscope (Olympus, USA), and 96-well TC plates (Griener, Germany) were used for biological study.

TLC was performed on pre-coated silica gel 60 F$_{254}$ (Merck Ltd., Frankfurt, Germany). Detection was achieved by spraying the plates with 10 % H$_2$SO$_4$ followed by heating. Column chromatography was carried out on MCI-gel CHP20P (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 (GE Healthcare Bioscience Co., Uppsala, Sweden), μ-Bonda Pak C$_{18}$ (Waters Co., MA, USA) and silica gel 60 columns (230–400 mesh, Merck Ltd., Frankfurt, Germany). The antimicrobial activity was carried out using Muller Hinton agar for bacterial strains and Sabrouid agar for fungi (Oxoid, England). Immunomodulatory effect was tested using lipopolysaccharide (LPS) from *Escherichia coli* (serotype O55:B5, Sigma–Aldrich), recombinant human GM-CSF (Novartis Pharma, Arnhem,The Netherlands), antihuman

CD80 FITC-conjugated and antihuman CD14 PE-conjugated (ImmunoTools, Germany), a FACS Calibur by CELLQUEST software (BD Biosciences, Singapore), trypsin-EDTA in Hanks' balanced salt solution without Ca/Mg (Sigma-Aldrich, USA), IL-1β, IL-6, TNF-α and cyclooxygenase 2 (COX-2) were quantitatively measured using Human IL-1β, IL-6, TNF-α and COX-2 ELISA Kits (Thermo Scientific®, USA).

2.2 Plant materials, Bacterial and fungal isolates
Plant materials:
The aerial parts of *Atriplex halimus* L. (Amaranthaceae) was collected from International road (Balteem area) in February, 2014 and identified by Prof. Kamal Shaltoot, Professor of taxonomy, Department of Botany, Faculty of Sciences, Tanta University-Egypt. A voucher specimen was deposited at the international herbarium of Faculty of Sciences, Tanta University, Egypt.

Bacterial and fungal isolates:
In vitro antimicrobial activity was carried out using Gram-positive bacteria including; *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 12204) and *Enterococcus faecalis* (ATTC 29212). In addition, Gram-negative bacterial strains were tested including; *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (MTCC109), *Enterobacter aerogenes* (MTCC111), *Proteus mirabilis* (MTCC425), *Pseudomonas aeruginosa* (ATCC 27950), *Shigella flexneri*, and *Salmonella typhimurium* (MTCC98). Moreover, fungi like *Candida albicans* (ATCC 90028) were also used. All the above reference strains were obtained from Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University.

2.3. Extraction and isolation:
Plant powdered material (1906.1 g) was extracted trice with MeOH by sonication for 6 h (30 min × 12) at room temperature. The extract was concentrated under reduced pressure to afford a residue (309.5 g). The residue was partitioned between *n*-hexane and 80 % MeOH, after which the 80 % MeOH layer was concentrated to yield a residue [169.5 g], which was loaded onto an MCI-gel CHP20P column (ϕ 50 × 300 mm) and eluted sequentially with (H$_2$O, H$_2$O-MeOH = 80:20, H$_2$O-MeOH = 60:40, H$_2$O-MeOH = 40:60, H$_2$O-MeOH = 20:80, 100% MeOH and acetone; 1.5 L of each solution) to afford seven fractions (fr.1–7). Fr.1 (147.45 g, H$_2$O eluate), Fr.2 (1.925 g, H$_2$O-MeOH; 80:20), Fr.3 (2.671 g, H$_2$O-MeOH; 60:40), Fr.4 (3.940 g, H$_2$O-MeOH; 40:60), Fr.5 (3.428 g, H$_2$O-MeOH; 20:80), Fr.6 (5.936 g; 100% MeOH), Fr.7 (3.142 g, acetone). Fraction 4 (3.940 g) eluted with 60% methanol in water from MCI gel were further subjected to column chromatography using Sephadex LH-20 column (ϕ 20 × 1000 mm, eluted with 100% MeOH) to give 6 fractions (fr 4-1 to fr 4-6). Fr 4-1 (0.708 g), fr 4-2 (1.0 g), fr 4-3 (1.113 g), fr 4-4 (0.658 g), fr 4-5 (0.275 g) and fr 4-6 (79.0 mg). Fr 4-4 (0.658 g) was subjected to μ-Bonda Pak C$_{18}$ HPLC column chromatography (ϕ 25 × 200 mm) and eluted with H$_2$O-MeOH gradient (40, 50, 60, 70, and 80% MeOH; 135 mL of each gradient solution) to afford 10 subfractions (fr

4-4-1 to fr-4-4-10). Fr 4-4-8 (62.7 mg, eluted with 60% MeOH in water) was subjected to silica gel column chromatography (ϕ 10 × 100 mm), eluted with $CHCl_3$:MeOH:H_2O = 8:2:0.2 (v/v) to obtain 2 fractions, fraction 1 (47.0 mg) and fraction 2 (4.4 mg). Fraction 1 (47.0 mg) was applied to a COSMOSIL $5C_{18}$ AR-II and eluted with 40% MeOH to give compound 1 (22.9 mg). Fr 4-4-9 (60.4 mg, eluted with 60% MeOH in water) was subjected to silica gel column chromatography (ϕ 10 × 100 mm), eluted with [$CHCl_3$:MeOH:H_2O = 8:2:0.2 (v/v)] to obtain 3 fractions, fraction 1 (4.9 mg) and fraction 2 (43.3 mg) and fraction 3 (2.1 mg). Fraction 1 (4.9 mg) were applied to a COSMOSIL $5C_{18}$ AR-II and eluted with 40% MeOH to give compound 2 (3.1 mg). Fraction 2 (43.3 mg) was applied to a COSMOSIL $5C_{18}$ AR-II HPLC column and eluted with 40% MeOH to give compound 3 (7.0 mg). Fr.2 (1.925 g, H_2O-MeOH; 80:20, from MCI gel) was further subjected to column chromatography using Sephadex LH-20 column (ϕ 20 × 1000 mm, eluted with 90% MeOH in water) to give 5 fractions (fr 2-1 to fr 2-5). Fr 2-1 (69.1 mg), fr 2-2 (0.294 g), fr 2-3 (0.405 g), fr 2-4 (0.714 g) and fr 2-5 (0.390 g). Fr 2-4 (0.714 g) was subjected to μ-Bonda Pak C_{18} HPLC column (ϕ 25 × 200 mm) and eluted with H_2O-MeOH gradient (10, 20, 30, 40, 50 and 60% MeOH; 135 mL of each gradient solution) to afford 6 subfractions (fr 2-4-1 to fr-2-4-6). Fr 2-4-3 (0.206 g, eluted with 30% MeOH in water) was subjected to silica gel column chromatography (ϕ 10 × 100 mm), eluted with [$CHCl_3$:MeOH:H_2O = 7:3:0.5→6:4:1 and→5:1:1 (v/v)] to obtain 12 fractions. Fraction 8 (37.4 mg, eluted with $CHCl_3$:MeOH:H_2O = 6:4:1) was applied to a Atlantis T_3 HPLC column and eluted with [30% MeOH in 0.1 % Trifluoroacetic acid (TFA)] to give compound 4 (4.0 mg).

Compound 1:
Yellow amorphous powder, ^{1}H-NMR (in DMSO-d_6, 500 MHz) δ 7.81 (1H, d, J = 1.75 Hz, H-2`), 7.52 (1H, d, $J$ = 1.75 Hz, H-6`), 6.42 (1H, d, J = 1.70 Hz, H-8), 6.20 (1H, d, J = 1.70 Hz, H-6), 5.43 (1H, d, J = 7.45 Hz, Glc H-1), 4.43 (1H, br s, Rha H-1), 3.85 (6H, s, OCH$_3$-3`, OCH$_3$-5`) and 0.97 (3H, d, J = 6.3 Hz, Rha C-5-Me). ^{13}C-NMR (in DMSO-d_6, 125 MHz) δ: 177.8 (C-4), 164.7 (C-7), 161.7 (C-5), 157.0 (C-2 or C-9), 156.9 (C-2 or C-9), 147.9 (C-3` or C-5`), 147.4 (C-3` or C-5`), 139.1 (C-4`), 133.5 (C-3), 121.6 (C-1`), 107.4 (C-2` and C-6`), 104.5 (C-10), 99.2 (C-6), 94.5 (C-8), 56.7 (OCH$_3$-3` or OCH$_3$-5`), 56.2 (OCH$_3$-3` or OCH$_3$-5`). Glucose moiety: 101.6 (C-1``), 76.4 (C-2``), 76.9 (C-3``), 72.3 (C-4``), 74.8 (C-5``) and 67.5 (C-6``). Rhamnose moiety: 101.2 (C-1```), 70.6 (C-2```), 71.1 (C-3```), 71.3 (C-4```), 68.8 (C-5```) and 18.7 (C-6```).

Compound 2:
Yellow amorphous powder. ^{1}H-NMR (in pyridine-d_5, 500 MHz) δ: 7.87 (2H, s, H-2`, H-6`), 6.78 (1H, d, J = 1.75, H-6), 6.73 (1H, d, J = 1.75, H-8), 6.44 (1H, d, J = 8.0 Hz, Glc H-1) and 3.93 (6H, s, OCH$_3$-3` and OCH$_3$-5`). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 178.1 (C-4), 165.6 (C-7), 161.9 (C-5), 157.0 (C-2 or C-9), 156.7 (C-2 or C-9), 148.1 (C-3` and C-5`), 140.0 (C-4`), 134.3 (C-3), 120.3 (C-1`), 107.6 (C-2` and C-6`), 104.6 (C-10), 99.4 (C-6), 94.3 (C-8)

and 56.2 (OCH$_3$-3` and OCH$_3$-5`). Glucose moiety: 102.5 (C-1``), 75.5 (C-2``), 78.4 (C-3```). 70.7 (C-4``), 77.7 (C-5``) and 61.4 (C-6``).

Compound 3
Yellow amorphous powder. ^{1}H-NMR (in pyridine-d_5, 500 MHz) δ: 8.51 (1H, d, J = 1.70 Hz, H-2`), 7.74 (1H, dd, $J$ = 8.0, 1.70 Hz, H-6`), 7.26 (1H, d, J = 8.0 Hz, H-5`), 6.76 (1H, s, H-8), 6.71 (1H, s, H-6), 6.16 (1H, d, $J$ = 7.45 Hz, Glc H-1), 5.14 (1H, br s, Rha-H-1). 4.03 (3H, s, OCH$_3$-3`) and 1.45 (3H, d, J = 6.3 Hz, Rha C-5-Me). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 178.1 (C-4), 165.3 (C-7), 161.9 (C-5), 157.1 (C-2 or C-9), 157.0 (C-2 or C-9), 150.6 (C-4`), 147.6 (C-3`), 134.2 (C-3), 122.5 (C-6`), 121.6 (C-1`), 115.6 (C-5`), 114.0 (C-2`), 104.7 (C-10), 99.3 (C-6), 94.2 (C-8) and 56.1 (OCH$_3$-C-3`). Glucose moiety: 103.5 (C-1``), 74.3 (C-2``), 74.5 (C-3``), 71.3 (C-4``), 73.1 (C-5``), 65.9 (C-6``). Rhamnose moiety: 101.2 (C-1```), 69.1 (C-2```), 71.9 (C-3```), 72.5 (C-4```), 68.7 (C-5```) and 17.9 (C-6```).

Compound 4
Yellow amorphous powder. ^{1}H-NMR (in pyridine-d_5, 500 MHz) δ: 8.10 (2H, d, J = 1.70 Hz, H-6, H-8), 7.88 (1H, dd, J = 1.75, 9.10 Hz, H-6`), 7.33 (1H, d, $J$ = 8.55 Hz, H-5`), 7.31 (1H, s, H-2`), 5.86 (1H, d, $J$ = 7.45 Hz, Glc H-1), 5.43 (1H, d, $J$ = 3.45 Hz, Api H-1), 4.98 (1H, d, $J$ = 1.35 Hz, Rha H-1), 4.46 (1H, d, $J$ = 8.6 Hz, Glc H-5), 4.26 (1H, m, Api HA-5), 4.13–4.06 (overlapped, m, sugar protons), 4.05 (3H, s, OCH$_3$-3`), 3.90 (1H, d, J = 9.2 Hz, Api HA-4), 3.88 (1H, d, J = 9.2 Hz, Api HB-4), 3.83 (1H, m, Api H-2), 3.77 (1H, d, J = 6.9 Hz, Api HB-5), 3.66 (1H, m, Rha H-5), and 1.31 (3H, d, J = 6.3 Hz, Rha C-5-Me). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 177.7 (C-4), 165.0 (C-7), 161.2 (C-5), 158.0 (C-2 or C-9), 156.9 (C-2 or C-9), 150.1 (C-4`), 147.3 (C-3`), 133.4 (C-3), 121.6 (C-6`and C-1`), 115.6 (C-5` and C-2`), 104.4 (C-10), 99.4 (C-6), 98.3 (C-8) and 56.0 (OCH$_3$-C-3`). Glucose moiety: 101.1 (C-1``), 76.5 (C-2``), 76.3 (C-3``), 72.5 (C-4``), 78.0 (C-5``) and 67.3 (C-6``). Rhamnose moiety: 101.3 (C-1```), 70.4 (C-2```), 70.8 (C-3```), 71.3 (C-4```), 68.2 (C-5```) and 17.3 (C-6```). Apiose moiety: 111.6 (C-1```), 76.0 (C-2````), 79.8 (C-3````), 68.6 (C-4````) and 73.9 (C-5````). The data was in accordance with the data reported in the literature for atriplexoside A (3'-O-methylquercetin-4'-O-β-D-apiofuranoside-3-O-(6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside) [7].

Acid hydrolysis:
Each compound (2 mg) was dissolved in (5 mL) MeOH and refluxed with (2 mL) 8% HCl for 2 h. The reaction mixture was evaporated to dryness, dissolved in (2 mL) H_2O and neutralized with NaOH. The neutralized product was analyzed by TLC [silica gel, n-PrOH-EtOAc-H_2O = 7:2:1] in the presence of authentic samples [16].

2.4. Biological activities
2.4.1. Antimicrobial activity
The test was performed on Muller Hinton agar for bacterial strains and Sabrouid agar for *C. albicans* using well diffusion method. The clinical laboratory standards institute (CLSI, 2014) methods were followed [17]. The bacterial

suspension prepared from an overnight culture was adjusted to 1×10^7 cfu/mL and the dried surface of the Muller Hinton or Sabrouid agar plates was streaked. Sterile corkporer (6 mm) was utilized to cut wells, which were filled with 100 µl of each sample at a concentration of 1 mg/mL dissolved in DMSO. Also, the negative control (DMSO) was included. The plates were then incubated at 37 °C for 18–24 h. Microbial growth was indicated by measuring the diameter of the inhibition zone [18, 19].

2.4.2. Immunomodulatory effects
2.4.2.1 Collection, separation and differentiation of monocytes into macrophage (M1Mφ):
Normal human peripheral blood mononuclear cells (PBMC) cultured in RBMI media were used. They were isolated from 10 mL whole blood drawn from a healthy donor by gradient centrifugation using equal volume of Histopaque [20]. The mixed solution was centrifuged at 1,000 rpm for 30 min. The mononuclear layer was transferred out and washed, then preciptated with 30 mL PBS (phosphate buffer saline) and centrifuged at 1,000 rpm for 10 min for thrice then re-suspended in RPMI media. Cell counting was done using Neubaurhaemocytometer to find out the number of PBMC with equal volume of trypan blue. Appropriate number of cells were left to adhere in TC flask. Following 2 hrs of incubation in the presence of 5% CO_2 at 37 °C in a CO_2 incubator, the medium was replaced with new RPMI supplemented with 2 mM glutamine, 10% human serum, 1% sodium pyruvate, 1% pen/strep, 20 ng/mL lipopolysaccharide (LPS) extracted from *E. coli*, 50 units/mL recombinant human GM-CSF then incubated for 5 days [21]. Monocytes (negative control) and macrophages were photographed using an inverted microscope then collected and subjected to flowcytometry.

2.4.2.2 Cell cytometry:
To analyze cell surface marker expression, aliquots of 10^5 M1Mφ as well as the monocytes were stained for 30 min at 4 °C by using antihuman CD14 PE-conjugated and antihuman CD80 FITC-conjugated. Samples were analyzed on a FACS Calibur using certain software (CELLQUEST).

2.4.2.3. Cytotoxicity assay:
The safety patterns of the tested drugs were checked on PBMCs using neutral red assay. An aliquot of 100 *µ*l of each compound (1mg/ml) was serially diluted and incubated with pre-cultured (6×10^4 cell/mL) cell on 96-well plates. After 48 hours, the cellular cytotoxic effects were quantified using neutral red test as described by Borenfreund and Puerner [22].

2.4.2.4. Immunomodulatory activities of the isolated compounds:
Polarized M1Mφ were harvested using trypsin-EDTA in Hanks' balanced salt solution without Ca/Mg. Macrophages were washed, counted, seeded in RPMI-phenol red free in triplicate at 2×10^5 cells per 200 µl in 96-well flat-bottom culture plates then incubated for 24 hrs. At the end of incubation, the inflammatory model was

induced by stimulating M1Mφ with 20 ng/mL LPS for 24 hrs in the presence or absence of the isolated tested compounds (125 µg/mL). At the end of the incubation period, the levels of IL-1β, IL-6, TNF-α and cyclooxygenase 2 (COX-2) were quantitatively measured using Thermo Scientific® Human IL-1β, IL-6, TNF-α and COX-2 ELISA Kit according to the manufacturer's instructions.

2.4.3. Statistical Analysis:
Values were expressed as the means (triplicates) ± standard error (SE). The differences between groups were determined by one-way analysis of variance (ANOVA) considering a value of $P < 0.01$. Data were analyzed using SPSS (version 17).

3. RESULTS AND DISCUSSION
3.1. Phytochemical investigation
The aerial parts of *Atriplex halimus* L. (1906.1) were extracted with methanol. The extract was partitioned between n-hexane and 80% methanol, after which the 80% methanol layer was concentrated under reduced pressure to obtain a residue (169.5 g) which was subjected to column chromatography on an MCI-gel CH_2-20P column and eluted with water; water-methanol 80:20; water-methanol 60:40; water-methanol 40:60; water-methanol 20:80; 100% methanol; and acetone to obtain seven fractions. Fraction 2 eluted with 20% methanol in water and fraction 4 eluted with 60% methanol in water were further subjected to column chromatography using Sephadex LH-20 and silica gel chromatography, followed by preparative high-performance liquid chromatography (HPLC) purification to afford compound **1** (22.9 mg), compound **2** (3.1 mg), compound **3** (7.0 mg) and compound **4** (4.0 mg). Compounds **1**, **2** and **3** were isolated from fraction 4 (60% methanol) and were identified as syringetin 3-*O*-β-D-rutionside, syringetin 3 *O*-β-D-glucopyranoside and isorhamnetin 3-*O*-β-D-rutinoside (narcissin), respectively. Compound **4** isolated from fraction 2 (20% methanol) and was identified as atriplexoside A (3'-*O*-methylquercetin-4'-*O*-β-D-apiofuranoside-3-*O*-(6"-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside). The structure of the isolated compounds (Figure 1) were elucidated using 1D and 2D-NMR and by comparison their physical and spectroscopic data with those reported in literatures [23–26, 7].

Compound 1 was obtained as a yellow amorphous powder. The ^{1}H-NMR spectrum of **1** indicated the presence of four aromatic protons at δ 6.20 (1H, d, *J* = 1.70 Hz, H-6), 6.42 (1H, d, *J* = 1.70 Hz, H-8), 7.52 (1H, d, *J* = 1.75 Hz, H-6`), 7.81 (1H, d, *J* = 1.75 Hz, H-2`) together with 2 anomeric protons at δ 5.43 (1H, d, *J* = 7.45 Hz, Glc H-1), 4.43 (1H, br s, Rha H-1) and doublet methyl protons at 0.97 (3H, d, *J* = 6.30, Rha C-5-Me). In addition two methoxy groups at δ 3.85 (6H, s, 2 × OCH_3). ^{13}C-NMR spectrum indicated 29 signals, 15 were assigned to myricetin, 2 methoxy groups at δ 56.2 and 56.7, and 12 signals for the sugar moieties. The anomeric carbons of the sugar moieties at δ 101.2 (Rha C-1) and 101.6 (Glc C-1). The sugar were identified as D-glucose and L-rhamnose

after acid hydrolysis and confirmed by Co-TLC with authentic samples. The chemical shifts were assigned by 2D-NMR experiments (COSY, HMQC, HMBC). The HMBC revealed the inter glycosidic linkage of Glc H-1 at δ 5.43 to C-3 at δ 133.5 and Rha H-1 at δ 4.43 to Glc C-6 at δ 67.5. The sites of attachment of the two methoxy group at ring B were determined from HMBC correlations between methyl protons at δ 3.85 to C-3` and C-5` at δ 147.9 and 147.3. The chemical structure of compound **1** was identified as syringetin 3-*O*-β-(6``-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) or syringetin 3-*O*-β-D-rutionside [23].

Compound 2 was obtained as a yellow amorphous powder. The ^{1}H-NMR spectrum of **2** indicated the presence of 4 aromatic protons at δ 6.73 (1H, d, *J* = 1.75 Hz, H-8), 6.78 (1H, d, *J* = 1.75 Hz, H-6) and 7.87 (2H, s, H-2`, H-6`). In addition to one anomeric proton at δ 6.44 (1H, d, *J* = 8.0 Hz, Glc H-1), and 2 methoxy groups at 3.93 (6H, s, 2 × OCH$_3$). ^{13}C-NMR spectrum indicated 23 signals, 15 were assigned to myricetin, 2 methoxy groups at δ 56.2 and 6 signals for glucose. The anomeric carbon of glucose at δ 102.5. The sugar moiety was confirmed after acid hydrolysis by Co-TLC using authentic samples. All the chemical shift were assigned by 2D-NMR experiments (COSY, HMQC, HMBC). The HMBC correlations revealed the attachment of Glc H-1 at δ 6.44 to C-3 at δ 134.3. The sites of attachment of the two methoxy groups at ring B was determined from HMBC correlation between methyl protons at δ 3.93 to C-3` and C-5` at δ 148.1. The chemical structure of compound **2** was identified as syringetin 3-*O*-β-D-glucopyranoside [24–26].

Compound 3 was obtained as a yellow amorphous powder. The ^{1}H-NMR spectrum of **3** indicated the presence of 5 aromatic protons at δ 6.71 (1H, s, H-6), 6.76 (1H, s, H-8), 7.26 (1H, d, *J* = 8.0 Hz, H-5`), 7.74 (1H, dd, *J* = 8.0, 1.70 Hz, H-6`) and 8.51 (1H, d, *J* = 1.70 Hz, H-2`). Two anomeric protons at δ 6.16 (1H, d, *J* = 7.45 Hz, Glc H-1) and 5.14 (1H, br s, Rha H-1). In addition to doublet methyl protons at δ 1.45 (3H, d, *J* = 6.30 Hz, Rha C-5-Me) and one methoxy group at δ 4.03 (3H, s, OCH$_3$). ^{13}C-NMR spectrum indicated 28 signals, 15 were assigned to quercetin, one methoxy at δ 56.1 and 12 signals for the sugar moieties. The anomeric carbons of the sugar moieties at δ 101.2 (Rha C-1) and 103.5 (Glc C-1). As in compound **1** and **2**, the sugar moieties of compound **3** were identified as D-glucose and L-rhamnose after acid hydrolysis and confirmed by CO-TLC with authentic samples. The chemical shifts were assigned by 2D-NMR experiment. The HMBC experiment revealed the inter glycosidic linkage of Glc H-1 at δ 6.16 to C-3 at δ 134.2 and Rha H-1 at δ 5.14 to Glc C-6 at δ 65.9. The site of attachment of the methoxy group at ring B was determined from HMBC correlation between methyl protons at δ 4.03 to C-3` at δ 147.6. The chemical structure of compound **3** was identified as isorhamnetin 3-*O*-β-D-rutinoside (narcissin) [26, 27]. This compound is isolated for the first time from *Atriplex halimus* L. and was previously only identified in the same species [28].
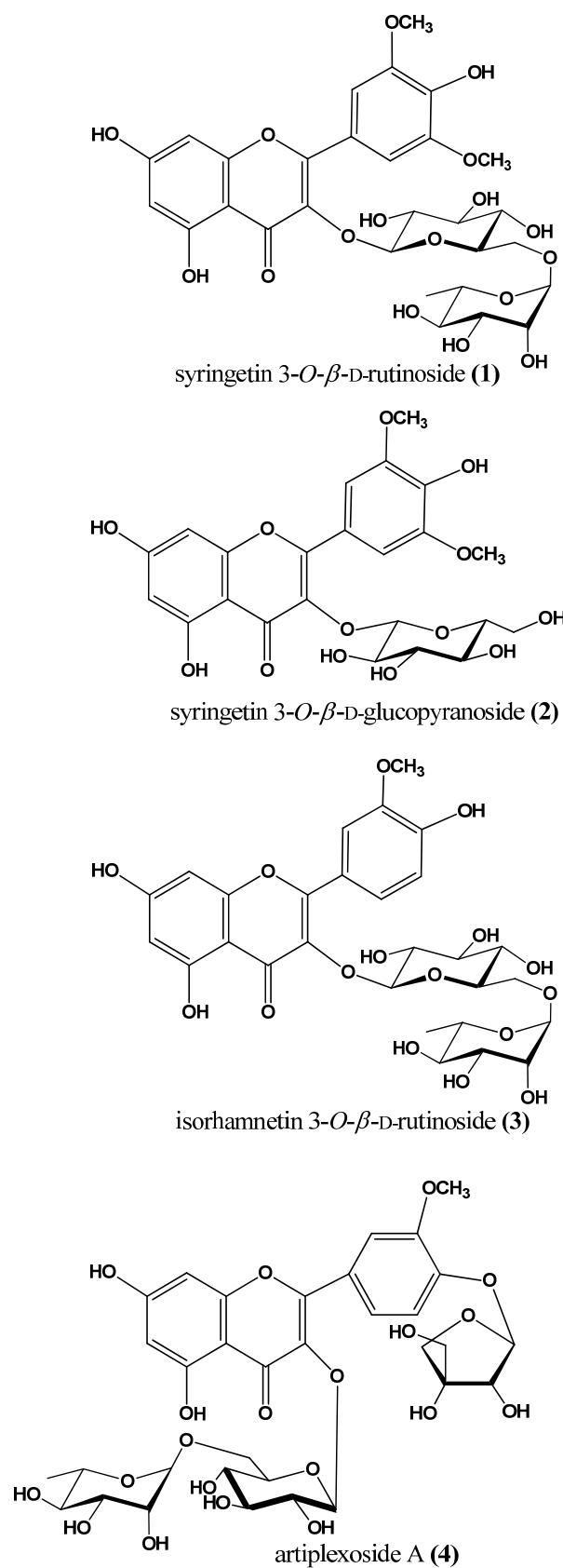

syringetin 3-*O*-β-D-rutinoside **(1)**

syringetin 3-*O*-β-D-glucopyranoside **(2)**

isorhamnetin 3-*O*-β-D-rutinoside **(3)**

artiplexoside A **(4)**

Figure (1): Chemical structure of isolated compounds

3.2. Biological activities

The increase in the rates of bacterial resistance among common pathogens is threatening the effectiveness of even the most potent antibiotics creating a major public health problem. The spread of multi-drug resistant Gram-positive organisms, such as methicillin-resistant *S. aureus*, as well as Gram-negative pathogens such as *Ps. aeruginosa* and *E. coli* were associated with serious public health concerns [29]. Hence, there was a need for novel antimicrobial agents.

Flavonoids had been reported to have a major antimicrobial activity because they can interact with the bacterial cell wall. Moreover, lipophilic flavonoids could disrupt the microbial cell membranes. Choudhury *et al.* evaluated the antibacterial activity of acetone and methanol extracts of *M. malabathricum* against *S. aureus*, *Streptococcus* sp. and *E. coli* using disc diffusion method. They found significant inhibition zones against all the indicator strains [30]. Sunilson *et al.* and Wang *et al.* presented similar results [31, 32]. These findings were in consistent with the present study because all of the tested compounds displayed broad spectrum antibacterial activity. However, their effect against Gram-positive bacteria was more noticeable than Gram-negative ones except for compound **3** as shown in Table (1) and figure 2. Compound **3** was also effective against Gram-negative isolates such as *Escherichia coli* and *Acinetobacter baumanii* (inhibition zone = 16 mm) that showed resistance to the carbapenem antibiotic (ertapenem). On the other hand, Compound **4** was the most effective one against Gram-positive bacteria including; *S. aureus*, *S. pyogenes* and *E. feacalis* showing inhibition zones of 20, 23, and 20 mm respectively. Inhibition of *C. albicans* was noticed for all compounds with more emphasis on compound **4** which showed the highest activity (inhibition zone = 24 mm) as presented in Table (1). Also, it was found that dimethyl sulfoxide (DMSO) did not show any antimicrobial activity (Figure 2). In general, this study showed that Gram-positive bacteria were more susceptible to the antimicrobial agents in medicinal plants than Gram-negative strains. Susceptibility differences could be explained by the presence of an outer-membrane permeability barrier in Gram-negative bacteria that acts as a barrier outside the cytoplasmic membrane limiting the access of such antimicrobial agents to their targets within the bacterial cells [33, 34].

Table 1: Evaluation of antibacterial activity of the isolated flavonol glycosides using well-diffusion method.

Bacterial pathogens	Inhibition zone diameter (mm)* by the isolated compounds			
	1	2	3	4
S. aureus	10±0.4	17±0.06	14±0.31	20±0.45
S. pyogenes	11±0.09	19±0.2	17±0.09	23±0.29
E. faecalis	11±0.12	17±0.33	16±0.7	20±0.78
E. coli	0±0	11±0.25	16±0.09	14±0.03
Ps. aeruginosa	0±0	12±0.07	12±0.11	12±0.17
Ac. baumanii	0±0	10±0.08	16±0.13	12±0.09
Sh. flexneri	0±0	11±0.08	14±0.11	13±0.11
Pr. mirabilis	0±0	11±0.011	13±0.11	11±0.74
S. typhimurium	0±0	12±0.5	14±0.33	14±0.05
E. aerogenes	0±0	11±0.33	13±0.09	13±0.8
C. albicans	15±0.04	16±0.01	15±0.56	24±0.05

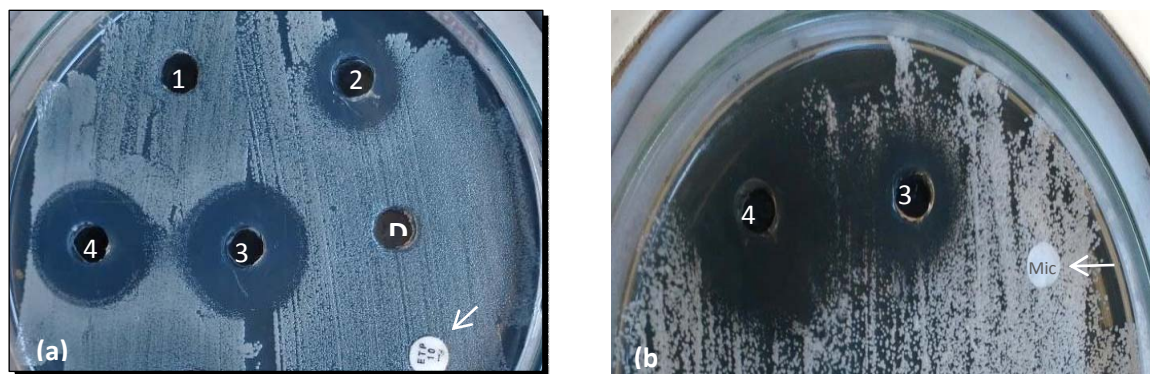*Values were presented as mean inhibition zone (mm) ± SEM of triplicates.

Figure (2): Effect of the isolated compounds on *E. coli* (a), and *C. albicans* (b), showing different inhibition zone diameters. D, refers to DMSO control. The arrow in left photo points to an ertapenem disc, while that to the right refers to miconazole disc, showing resistance of the indicator pathogens to such antimicrobials.

In the present study, we evaluated the cytotoxicity of the tested compounds on PMNCs using neutral red assay. It revealed IC_{50} values ranged from (125-450) µg/mL as shown in (Figure 3). Our data demonstrated that the IC_{50} of compounds **3** recorded at approximately 450 µg/mL suggesting less cytotoxic effect against PMNCs. Matsuo *et al.* reported that some of the flavonoids presented cytotoxicity at high concentrations and in a dose-dependent manner [35].

Macrophages were the major population of tissue-resident mononuclear phagocytes and the predominant targets for infection by pathogens. They considered to be the first line of defense in innate immunity as they could engulf and kill microorganisms then present antigens for triggering adaptive immune responses. There are two subsets of polarized phenotypes of macrophages; M1 with pro-inflammatory and M2 with anti-inflammatory functions [36]. In the present study, human monocytes were differentiated into macrophage (M1) using LPS and GM-CSF. Following 5 days of incubation, both control and treated cells were investigated under the phase-contrast inverted microscope that showed rounded cells with irregular edges suggesting the presence of macrophages compared to the control monocytes that were oval cells with central nucleus as presented in (Figure 4). To confirm the presence of M1Mφ, they were tested for the presence of CD14 and CD80 using flow cytometry. It was noticed that M1Mφ overexpressed CD80 but both of them showed no or little difference in the expression of CD14 as shown in (Figure 5). Interestingly, Takashiba *et al.* reported similar results [37].

The use of immunomodulators could improve the innate immunity and thus could enhance host resistance to pathogens. Microorganisms, fungi and plants represented different sources from which various immunomodulators had been isolated and identified [38]. Such compounds could be used for inhibition of the pro-inflammatory mediators and cytokines that play a major role in inflammatory diseases suggesting novel therapy for inflammation [39]. In the present study, the effect of the test drugs on the M1Mφ function was investigated. It revealed that compounds **3** and **4** reduced the level of the induced IL-6 from 255.13 pg/mL to 77.34 & 32.106 pg/ml, respectively. Also, the level of IL-1β decreased from 287.22 to 82.11 & 45.12 pg/mL as shown in (Figure 8). Moreover, the level of TNF-α and COX-2 was reduced by compound **4** to approximately the normal level in LPS-inflammation model. On the other hand, compounds **1** and **2** markedly increased all the above cytokines and pro-inflammatory mediators (Figures 6–8). Interestingly, these findings were consistent with the results obtained by Geng *et al.* who reported that flavonoid genistein was found to prevent IL-6, IL-1 β and TNFα formation in LPS-induced macrophages of human origin [40]. In addition, it was found that both quercetin and luteolin were capable of inhibiting TNF-α production by nearly 80% [41]. Moreover, genistein, apigenin, kaempferol, catechin, myricetin were found to inhibit COX-2 in LPS induced macrophages [42].
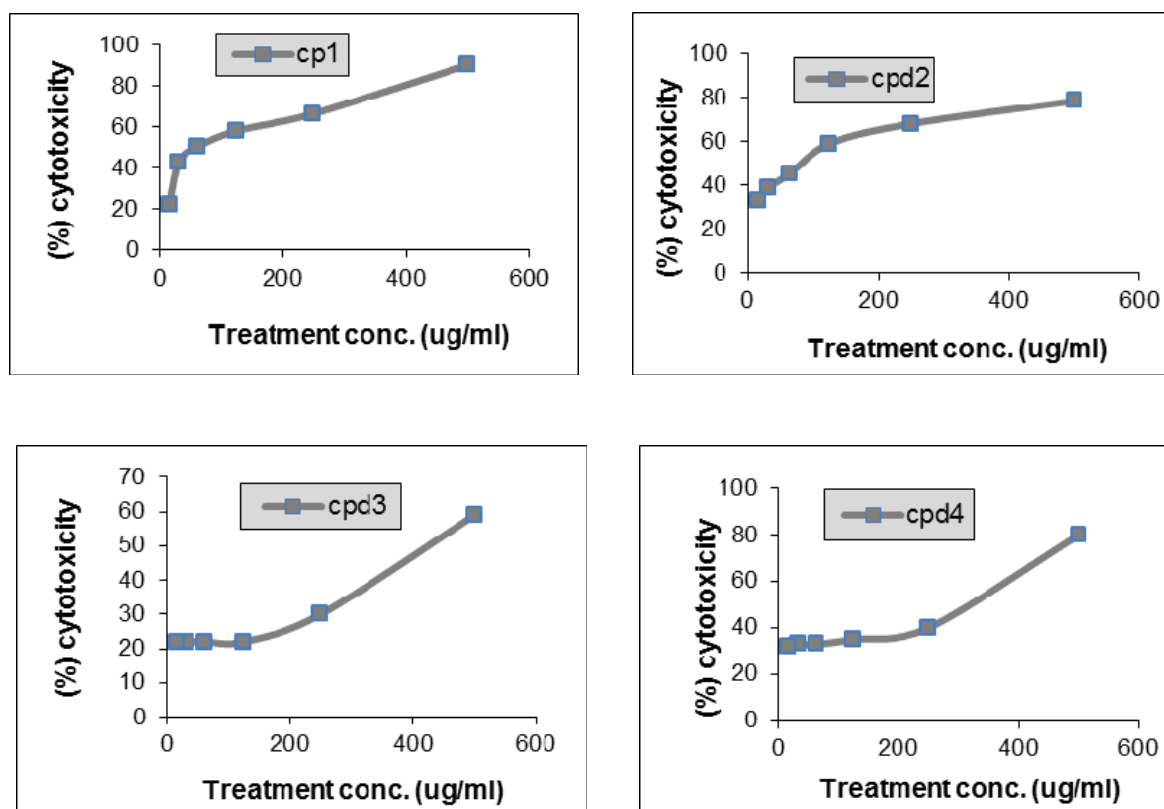

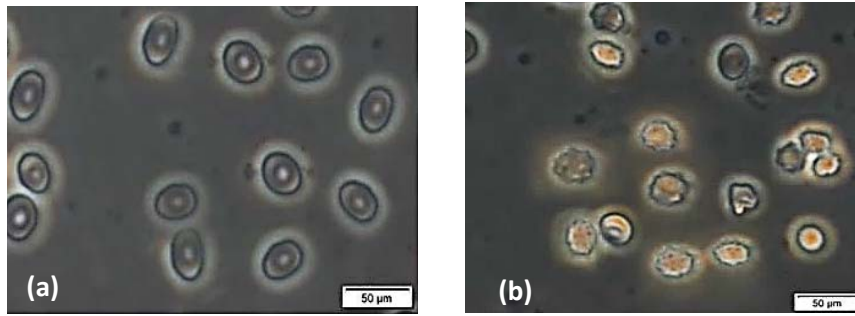Figure (3): Cytotoxicity assay of the isolated compounds on human PMNCs.

Figure (4): Induction of monocyte differentiation into macrophage. Monocytes were incubated for 5 days without (a; monocyte) or with (b; Mφ) LPS (20ng/mL). The cells were photographed at 2500 magnification with a phase-contrast inverted microscope.
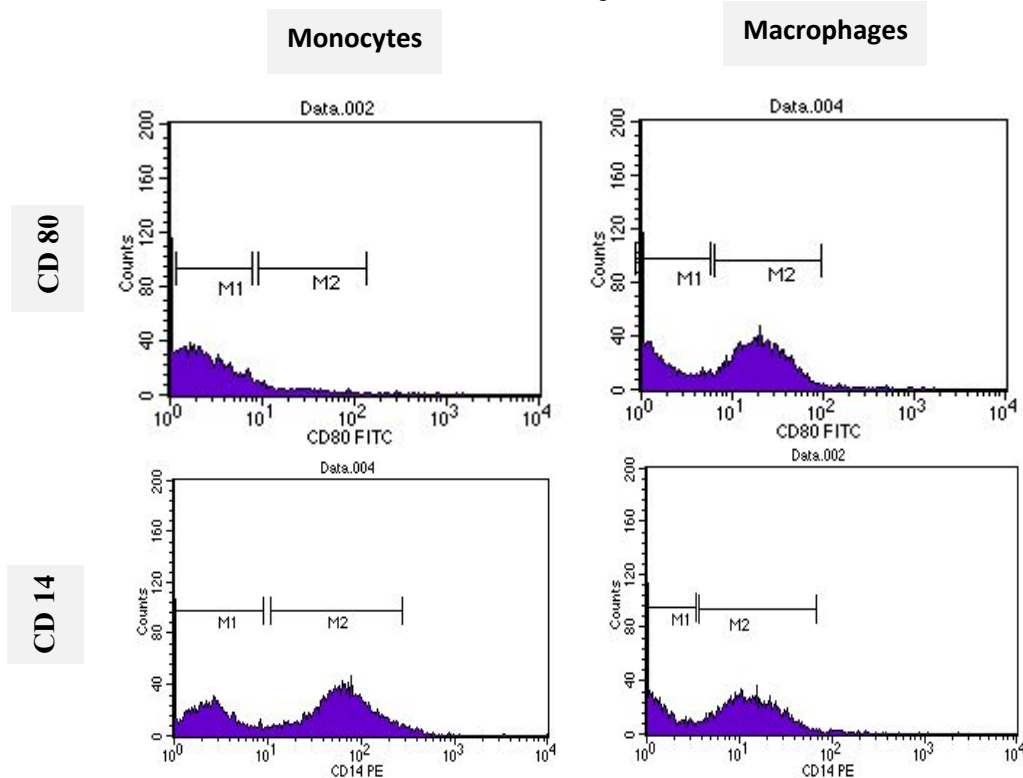

Figure (5): Differentiation of human blood monocytes into Mφ-1 after activation by LPS as determined by flow cytometry. In contrast to monocytes, Mφ-1highlyexpressed CD80 but showed little difference in the expression of CD14.
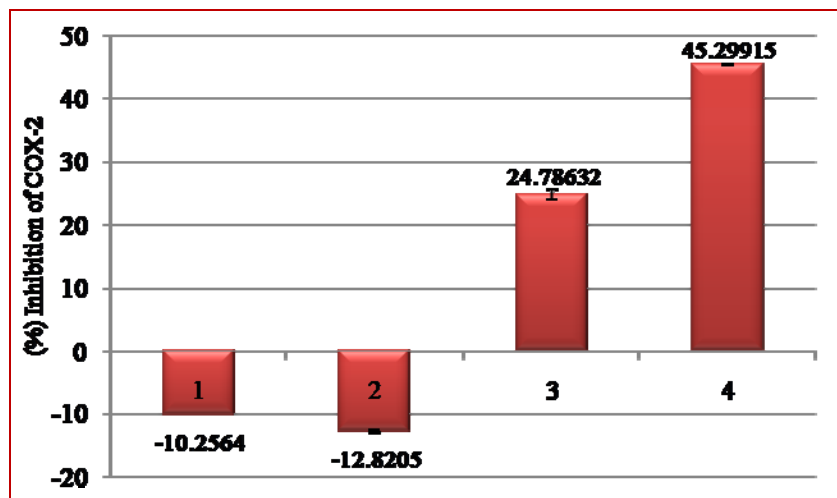

Figure (6): Effect of the isolated flavonol glycosides on COX-2 production by macrophage cells. Showing inhibition of LPS-stimulated COX-2 production from Mφ by compound **(3)** while compounds **(1)** and **(2)** stimulated its production.
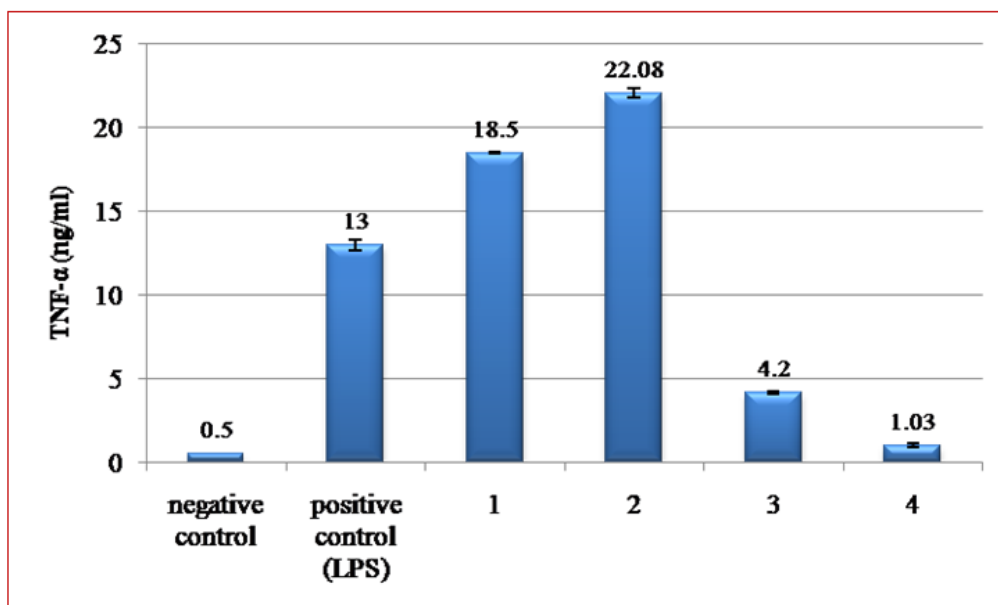
Figure (7): Effect of the isolated flavonol glycosides on TNF-α production from LPS-stimulated macrophages. Data were presented as mean ± SE

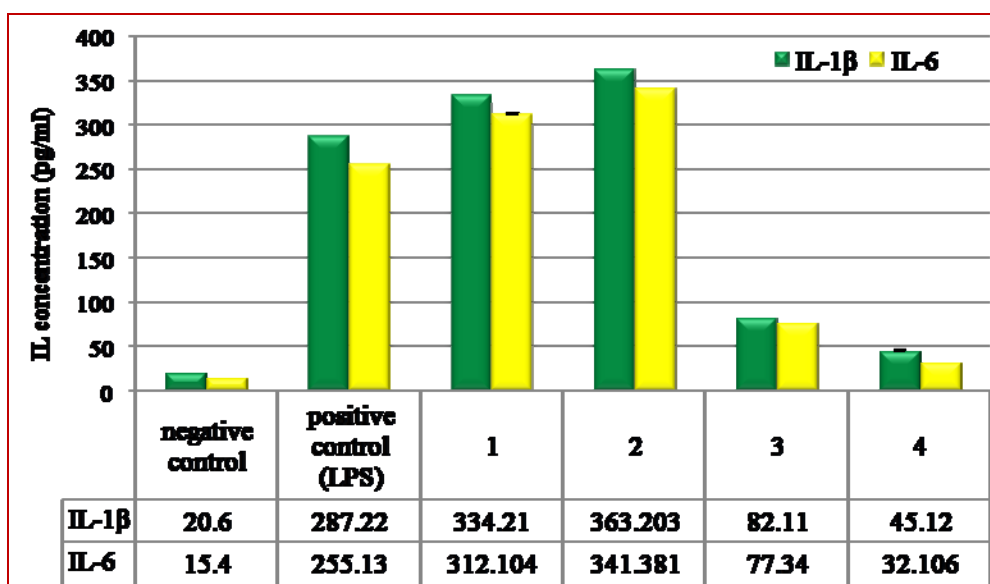|	negative control	positive control (LPS)	1	2	3	4
IL-1β	20.6	287.22	334.21	363.203	82.11	45.12
IL-6	15.4	255.13	312.104	341.381	77.34	32.106

Figure (8): Effect of the isolated flavonol glycosides on the production of IL-1β and 6 from LPS-stimulated macrophages. Data were presented as mean ± SE

4. CONCLUSION

From the aerial parts of *Atriplex halimus* L., four flavonol glycosides were isolated: syringetin 3-*O*-β-D-rutionside **(1)**, syringetin 3-*O*-β-D-glucopyranoside **(2)** and isorhamnetin 3-*O*-β-D-rutinoside (narcissin) **(3)** for the first time and atriplexoside A **(4)**. The isolated compounds exhibited antibacterial and anticandida activity with variable degrees. Compounds **1** and **2** could stimulate the immune response and could be a candidate agent for treatment of infections, on the other hand compounds **3** and **4** could suppress the immune response indicating its suggested role in case of organs transplantation.

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