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Immunomodulatory and anti-inflammatory activities of the defatted alcoholic extract and mucilage of *Hibiscus sabdariffa* L. leaves, and their chemical characterization

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Abstract
In this study, the immunomodulatory and anti-inflammatory activities of chemically characterized defatted ethanolic extract (DEE) and mucilage (ML) of *Hibiscus sabdariffa* L. (roselle) leaves, were evaluated. Seven compounds were isolated and identified, as daucosterol (1), rutin (2), isoquercetrin (3), kaempferol (4), quercetin (5), caffeic (6) and neochlorogenic (7) acids, based on their spectral data. This is the first report on the isolation of these compounds from *Hibiscus sabdariffa* L. leaves, except for rutin. Total phenolic and flavonoid contents were estimated as 120.13 ± 0.27 µg GAE and 72.33 ± 0.061µg QE /mg DEE, respectively using spectrophotometric methods. HPLC fingerprint analysis of DEE and its ethyl acetate fraction was carried out. ML hydrolysate was analyzed using HPLC. DEE (50 µg/ml) possessed significant stimulatory effect on lymphocytes using MTT assay, while ML revealed inhibitory proliferative effects at different concentrations (10, 20, 50 µg/ml). Both possessed anti-inflammatory activities *via* inhibition of COX-2 and 5-LOX enzymes, compared to celecoxib and meclofenamate sodium, respectively. However, DEE exhibited more selective inhibition of COX-2 as well as more potent 5-LOX inhibitory activities, compared to ML. DEE (200, 400 mg/kg) dose-dependently inhibited carrageenan-induced paw edema, revealing maximum inhibition (65.71%) at 400 mg/kg, after 6 hr, compared to indomethacin (78.57%). These findings highlight the beneficial effect of *Hibiscus sabdariffa* L. leaves on immune response and inflammation, encouraging further investigation of this underutilized part.

Keywords: Anti-inflammatory, COX, *Hibiscus sabdariffa* L., HPLC, immunomodulatory, LOX

1. Introduction
Inflammation has significant implications in the pathogenesis of different multifactorial diseases encompassing rheumatoid arthritis, type 2 diabetes, asthma, cancer, ageing as well as cardiovascular and neurodegenerative diseases [1]. The concepts of immunomodulatory and anti-inflammatory are often strongly related [2]. Regulation of the immune system is a vital factor in maintaining a healthy equilibrium of the body [2]. Therefore, the world attention has been attracted to the discovery of medicinal plants with immunomodulatory and anti-inflammatory properties in order to control several diseases.

Although non-steroidal anti-inflammatory drugs (NSAIDs) have been used for several years, the long-term use of these drugs is associated with various side effects such as gastrointestinal bleeding, platelet dysfunction, kidney disorders, cardiovascular and cerebrovascular complications [3]. These side effects were attributed to the inhibition of COX-1, which is a cytoprotective constitutive enzyme, involved in several homeostatic processes. Thus, selective COX-2 inhibitors (COXIBs) were developed to overcome these side effects. However, inhibition of COX-2 shifts arachidonic acid metabolism from prostaglandins synthesis to leukotriene synthesis *via* upregulation of 5-LOX, resulting in increased levels of leukotrienes [4, 5]. Therefore, dual COX-2/5-LOX inhibitors constitute an emerging therapy for inflammation.

Hibiscus sabdariffa L. (known as roselle), belonging to the Malvaceae family, is widely grown in many countries including Egypt. It has been traditionally used for treatment of various diseases including hypertension, inflammation and liver disorders [6, 7]. Roselle is mainly grown for its calyces, representing the commercial important part of the plant, which is extensively used in preparation of herbal drinks, hot and cold beverages, jams and jellies [8].

The world demand for roselle calyces has steadily increased over the past decades [9], yielding huge amounts of underutilized leaves which are usually discarded in some countries, including Egypt. However, in Sudan, roselle leaves are eaten raw in salad or cooked [7]. In Mexico, India and Africa, leaves infusion is traditionally used as diuretic, choleretic, febrifugal, hypotensive and to stimulate intestinal peristalsis [8]. In African folk medicine, the leaves are also used as antimicrobial, emollient and sedative [6].

Researchers have recently focused on *H. sabdariffa* L. leaves, demonstrating a good source of several nutrients like protein, carbohydrates, iron, calcium, ascorbic acid, riboflavin and β-carotene [10]. Besides, they contain high levels of phenolic compounds including different phenolic acids and flavonoids that contribute to their various activities [11-13]. Several biological activities have been reported for the leaves as hypolipidemic, hepatoprotective, nephroprotective, anticancer and antioxidant [8]. In addition, the immunomodulatory activity of the total crude extract of *H. sabdariffa* L. leaves from Uganda was previously demonstrated in rats [14]. The methanolic extract of the plant leaves from Nigeria was previously evaluated for its anti-inflammatory activity using carrageenan induced inflammation model, revealing significant effect only at high dose levels (500 mg/kg) [15]. In contrast, Patil and Munoli, 2017 [16], reported the lack of any anti-inflammatory effect for the aqueous extract of the leaves in acute and chronic models of induced inflammation. Yet, these reports lack chemical characterization of the investigated extracts.

Chemical variation in fingerprints and flavonoid contents have been demonstrated in *H. sabdariffa* L. leaves from different countries and accessions [11], which can explain discrepancies between different reports regarding the biological activities. Therefore, chemical characterization of *H. sabdariffa* L. leaves extracts is an essential prerequisite step prior to its biological evaluation. Estimation of total phenolic and flavonoid contents as well as fingerprint analysis have been accepted as means for chemical characterization of medicinal plants. Phenolic compounds possess a wide range of pharmacological effects. They exert beneficial anti-inflammatory and immunomodulatory activities by interfering with immune cell regulation, synthesis of proinflammatory cytokines and gene expressions [2, 17]. However, no reports were traced on the immunomodulatory and anti-inflammatory activities of chemically characterized defatted ethanolic extract of *H. sabdariffa* L. leaves.

Mucilages are complex polysaccharides consisting of monosaccharides and uronic acids. They have been recently used in pharmaceutical preparations as binders, emulsifying and suspending agents [18]. In addition, their therapeutic values have been investigated in many diseases as antidiabetic, immunostimulatory, anticancer, anti-inflammatory, wound healing and antioxidant [18]. The evaluation of novel and safe mucilage from plants has become a popular research topic to detect an alternative for the treatment of many diseases. *H. sabdariffa* L. is generally characterized by the presence of mucilage [8]. However, no data was traced concerning the mucilage composition of its leaves. Though, its pharmaceutical use as suspending agent was previously evaluated [19].

Our research team has recently reported the hepatoprotective potential of *H. sabdariffa* L. seed oil and its nano-formulation[20]. In the current study, we aimed to assess the immunomodulatory and anti-inflammatory activities of the defatted ethanolic extract of *H. sabdariffa* L. leaves (DEE) and its mucilage, and to investigate their chemical composition**.**

2. Material and Methods

2.1 Plant material and chemicals

The leaves of *Hibiscus sabdariffa* L. were collected, in the mature fruiting stage, from Medicinal, Aromatic and Poisonous Plants Experimental Station, Faculty of Pharmacy, Cairo University, Giza, Egypt, in December 2015. The plant was identified by Dr. Mohammed El-Gebaly (Senior botanist and consultant at Orman Botanical Garden, Dokki, Egypt), and a voucher specimen (No. 6-3-2016) was deposited at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Reference sugars, gallic acid, quercetin and carrageenan were obtained from Sigma-Aldrich, St. Louis, MO, USA. Celecoxib (Celebrex®) was purchased from Pfizer, Pharmaceutical Industries Company. Meclofenamate sodium was procured from Cayman Chemicals, USA. Indomethacin (Indomethacin®) was obtained from Epico, Egyptian Int. Pharmaceutical Industries Company. Solvents used in HPLC analysis were of HPLC grade, whereas other chemicals and reagents were of analytical grade.

2.2 Extraction and isolation

Defatted air dried powdered leaves (1.5 kg) were extracted with 70% ethanol (15 x 2 L), yielding 150 g of *H. sabdariffa* L. leaves defatted ethanolic extract (DEE). DEE (100 g) was suspended in water and partitioned successively with methylene chloride, ethyl acetate and *n*-butanol saturated with water (5 x 350 ml, each) to give respectively 20, 12 and 5 g of each fraction. Aliquot of the methylene chloride fraction (15 g) was submitted to vacuum liquid chromatography column (VLC) (15 D x 6 L cm, 300 g silica gel H). Gradient elution was performed using methylene chloride increasing the polarity with ethyl acetate, then with ethyl acetate increasing the polarity with methanol. Fractions (500 ml, each) were collected and monitored by TLC. Fractions with similar chromatographic pattern were pooled together and sub-fraction eluted with 10%-30% methanol in ethyl acetate was repeatedly chromatographed on silica gel 60 column for purification to afford 1 compound (50 mg). Aliquot of the ethyl acetate fraction (8 g) was subjected to polyamide column chromatography (5 D x 25 L cm, 750 g), eluted with water/methanol (decreasing polarity) in fractions (250 ml, each) and similar ones were pooled together. Selected subfractions were further purified using sephadex LH-20, silica gel 60 and Rp-18 columns, affording 6 compounds (Fig. 1).

2.3 Characterization of isolated compounds

The isolated compounds were identified based on their spectral data (UV, ^{1}H NMR, ^{1}H ^{1}H COSY, ^{13}C NMR and MS), compared with reported ones. A Schimadzu double beam spectrophotometer (UV-1650PC, USA) for running UV spectra, a Bruker NMR-spectrometer (Japan) for running ^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz spectra, and a Mass spectrometer (Thermo scientific ISQLT single quadrupole, USA) for recording mass spectra, were used.

Compound 1: 50 mg, white powder. ^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm, J/Hz): 0.69 (3H, *d*, *J* = 7.4, CH_3-21), 0.82 (3H, *t*, *J* = 7.7, CH_3-29), 0.83 (3H, *d*, *J* = 7, CH_3-26), 0.86 (3H, *s*, CH_3-18), 0.92 (3H, *d*, *J* = 6.5, CH_3-27), 1.01 (3H, *s*, CH_3-19), 3.58 (1H, *m*, H-3), 5.35 (1H, *d*, *J* = 5, H-6), 4.38 (1H, *d*,

J=7.8, anomeric H-1'), 3.18-3.85 (sugar protons). EI/MS m/z (70 ev): 577 $[M+H]^+$, 414 [aglycone], 396 (100%) [M – Glucose]$^+$.

Compound 2: 20 mg, yellow powder. UV λmax (MeOH): 258, 300 sh, 358. ^{1}H-NMR (400 MHz, DMSO, δ ppm, J/Hz): 6.12 (1H, *br s*, H-6), 6.32 (1H, *br s*, H-8), 6.83 (1H, *d*, *J* = 8.4 Hz, H-5'), 7.55 (1H, *dd*, *J* = 1.8, 8.5 Hz, H-6'), 7.52 (1H, *br s*, H-2'), 5.31 (1H, *d*, *J* = 6.9 Hz, H-1"), 4.40 (1H, *br s*, H-1'"), 1.00 (3H, *d*, *J* = 6.1 Hz, Me-6'"). 3.00 - 3.72 (sugar protons). ^{13}C-NMR (100 MHz, DMSO, δ ppm): 156.5 (C-2), 133.5 (C-3), 177.4 (C-4), 161.1 (C-5), 99.9 (C-6), 164.2 (C-7), 93.9 (C-8), 156.8 (C-9), 105.3 (C-10), 121.3 (C-1'), 115.8 (C-2'), 145.5 (C-3'), 148.9 (C-4'), 116.4 (C-5'), 122.1 (C-6'), 103.7 (C-1"), 74.5 (C-2"), 76.9 (C-3"), 71.0 (C-4"), 75.7 (C-5"), 67.5 (C-6"), 101.9 (C-1'"), 70.9 (C-2'"), 70.3 (C-3'"), 72.4 (C-4'"), 68.8 (C-5'"), 18.2 (C-6'").

Compound 3: 15 mg, yellow powder. UV λmax (MeOH): 256, 265 sh, 301 sh, 359. ^{1}H-NMR (400 MHz, DMSO, δ ppm, J/Hz): 6.21 (1H, *br s*, H-6), 6.42 (1H, *br s*, H-8), 6.86 (1H, *d*, *J* = 9 Hz, H-5'), 7.59 (2H, *br d*, *J* = 5.6 Hz, H-2', H-6'), 5.47 (1H, *d*, *J* = 6.8 Hz, H-1"), 3.09 - 3.60 (sugar protons). ^{13}C-NMR (100 MHz, DMSO, δ ppm): 156.6 (C-2), 133.8 (C-3), 177.9 (C-4), 161.7 (C-5), 99.2 (C-6), 164.7 (C-7), 94.0 (C-8), 156.8 (C-9), 104.4 (C-10), 121.6 (C-1'), 115.7 (C-2'), 145.3 (C-3'), 148.9 (C-4'), 116.7 (C-5'), 122.1 (C-6'), 101.3 (C-1"), 74.6 (C-2"), 77.0 (C-3"), 70.4 (C-4"), 78.0 (C-5"), 61.4 (C-6").

Compound 4: 29 mg, yellow powder. UV λmax (MeOH): 265, 292 sh, 329 sh, 366. ^{1}H-NMR (400 MHz, CD_3OD, δ ppm, J/Hz): 6.19 (1H, *d*, *J*= 2Hz, H-6), 6.40 (1H, *d*, *J*= 2Hz, H-8), 6.91 (2H, *d*, *J*=8.9 Hz, H-3', H-5'), 8.09 (2H, *d*, *J*=8.9 Hz, H-2', H-6'). ^{13}C-NMR (100 MHz, CD_3OD, δ ppm): 146.6 (C-2), 135.7 (C-3), 175.9 (C-4), 161.1 (C-5), 97.9 (C-6), 164.2 (C-7), 93.1 (C-8), 156.8 (C-9), 103.1 (C-10), 122.3 (C-1'), 129.3 (C-2',6'), 114.9 (C-3',5'), 159.1 (C-4').

Compound 5: 91 mg, yellow powder. UV λmax (MeOH): 256, 301 sh, 372. ^{1}H-NMR (400 MHz, CD_3OD, δ ppm, J/Hz): 6.20 (1H, *d*, *J*=1.9 Hz, H-6), 6.42 (1H, *d*, *J*= 1.9 Hz, H-8), 6.90 (1H, *d*, *J*=8.5 Hz, H-5'), 7.55 (1H, *dd*, *J*=2, 8.5 Hz, H-6'), 7.69 (1H, *d*, *J*= 1.9 Hz, H-2'). ^{13}C-NMR (100 MHz, CD_3OD, δ ppm): 148.1 (C-2), 136.2 (C-3), 176.3 (C-4), 161.2 (C-5), 98.6 (C-6), 164.3 (C-7), 93.8 (C-8), 156.6 (C-9), 103.5 (C-10), 122.4 (C-1'), 115.5 (C-2'), 145.5 (C-3'), 147.2 (C-4'), 116.1 (C-5'), 120.5 (C-6').

Compound 6: 20 mg, white powder. UV λmax (MeOH): 245, 290 sh, 330. ^{1}H-NMR (400 MHz, CD_3OD, δ ppm, J/Hz): 6.24 (1H, *d*, *J*=15.9 Hz, H-8), 6.80 (1H, *d*, *J*= 8.1 Hz, H-5), 6.95 (1H, *dd*, *J*=1.6, 8.1 Hz, H-6), 7.06 (1H, *d*, *J*= 1.6 Hz, H-2), 7.56 (1H, *d*, *J*=15.9 Hz, H-7).

Compound 7: 15 mg, white powder. UV λmax (MeOH): 241, 290 sh, 326. ^{1}H-NMR (400 MHz, CD_3OD, δ ppm, J/Hz): 2.08 (1H, *br d*, *J*=13.2 Hz, H-6 ax), 2.19 (2H, m, H-2eq and H-6 eq), 2.28 (1H, *dd*, *J*=13.1, 2.2 Hz, H-2ax), 3.77 (1H, *dd*, *J*=3, 8.6 Hz, H-4), 4.21 (1H, *m*, H-5), 5.38 (1H, *m*, H-3), 6.28 (1H, *d*, *J*=15.9 Hz, H-8'), 6.81 (1H, *d*, *J*=8.2 Hz, H-5'), 6.96 (1H, *dd*, *J*=1.7, 8.2 Hz, H-6'), 7.08 (1H, *d*, *J*=1.7 Hz, H-2'), 7.57 (1H, *d*, *J*=15.9 Hz, H-7').^{13}C-NMR (100 MHz, CD_3OD, δ ppm): 74.9 (C-1), 36.8 (C-2), 70.6 (C-3), 72.2 (C-4), 70.1 (C-5), 37.5 (C-6), 175.8 (C-7), 126.5 (C-1'), 114.0 (C-2'), 145.3 (C-3'), 148.1 (C-4'), 115.2 (C-5'), 121.8 (C-6'), 145.8 (C-7'),114.9 (C-8'), 167.5 (C-9').

2.4 Determination of total phenolic and flavonoid contents

Total phenolic (TPC) and total flavonoid (TFC) contents of DEE were determined spectrophotometrically using Folin-Ciocalteu and aluminum chloride methods, respectively [21]. Total phenolic content was expressed as µg gallic acid equivalents (GAE) per mg of DEE, based on established calibration curve (Y=0.0052 X - 0.0097, R^2 =0.99) using serial dilutions of gallic acid (20 - 280 µg/ml). Total flavonoid content was expressed as µg quercetin equivalents (QE) per mg of DEE, based on established calibration curve (Y=0.0094 X + 0.0181, R^2=0.99) using serial dilutions of quercetin (5-100 µg/ml).

2.5 HPLC fingerprint analysis

2.5.1 Preparation of samples

An analytical HPLC technique was developed for analysis of DEE and its ethyl acetate fraction, where DEE (4 mg) and its ethyl acetate fraction (2 mg) were dissolved separately in 10 ml methanol in a measuring flask 10 ml capacity. Mixed solutions of isolated compounds were prepared at final concentrations of 0.1 mg/ml methanol for each. The developed solutions were filtered through 0.2 µm membrane filter and an aliquot (20 µl) of each filtrate was subjected to HPLC analysis.

2.5.2 Chromatographic conditions

HPLC analysis was performed using an Agilent HPLC 1100 series, equipped with a quaternary pump, degasser G1322A and a UV detector. The column employed was a LiChrospher 100 RP-C18 (250 × 4.6 mm, 5µm), preceded by RP-C18 guard column (10 × 4 mm, 5 µm). The mobile phase consisted of acetonitrile (A) and acidulated water (0.3% phosphoric acid) (B). The stepwise gradient elution program adopted was as follows: 0-13 min (0-14% A), 13-20 min (14-23% A), and 20-28 min (23-40% A), then up to 100% A within 2 min. Injection volume was 20 µL, the flow rate was adjusted at 1 ml/min and the UV detector was set at 325 nm. Agilent Chem Station software was used for data acquisition and processing.

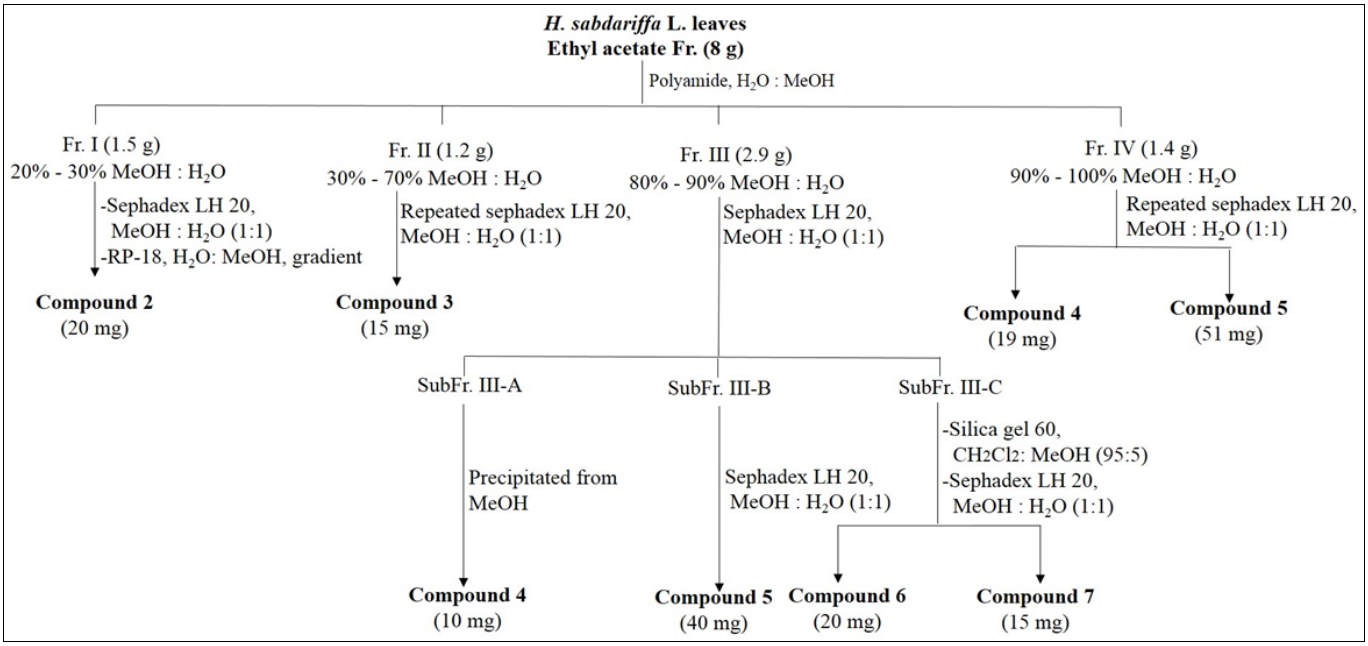

Fig 1: Scheme showing the isolation of compounds 2-7 from the ethyl acetate fraction of defatted ethanolic extract of *H. sabdariffa* L. leaves (DEE)

2.6 HPLC analysis of the mucilage hydrolysate

2.6.1 Isolation of mucilage

The mucilage (ML) was isolated from air-dried leaves (200 g) according to previously described method [22]. The precipitated mucilage was dried in a desiccator till constant weight, yielding dried isolated mucilage (6.5 g, 3.25%) of leaves.

2.6.2 Hydrolysis of the isolated mucilage (ML)

Hydrolysis of the purified isolated mucilage (13 mg) was carried out according to previously described method [23]. The resulting hydrolysate was evaporated under vacuum and the obtained dried residue was extracted with hot, distilled pyridine. The pyridine solutions were evaporated to dryness and the residue was reserved for chromatographic study.

2.6.3 Chromatographic conditions

The dried hydrolysate (3 mg), as well as the individual reference sugars were separately solubilized in DMSO, then purified through C18 Sep-Pak cartridge, followed by filtration through a 0.45 µm membrane filter. Then, they were subjected to HPLC analysis, using Shimadzu Class-VP 5.03 (Kyoto, Japan), equipped with refractive index RID-10A Shimadzu detector, an LC-16 ADVP binary pump and a DCou-14 degasser. Shodex PL Hi-Plex Pb column (Sc 1011 No. H706081) and guard column Sc-Lc Shodex were used. Acetonitrile/water (80:20 v/v) was used as mobile phase at flow rate, 1 ml/min. The chamber temperature was set at 65°C.

2.7 *In vitro* study

2.7.1 *In vitro* immunomodulatory activity

This study was performed according to previously reported method [24]. Briefly, blood was collected in heparinized tubes from seven rats. Peripheral blood lymphocytes (PBL) were isolated from heparinized blood and diluted 1:1 using hank's balanced salt solution. Diluted blood was layered above ficoll-hypaque in 2:1v/v ratio and centrifuged at 400 x g for 30 minute at 4 ºC. The lymphocyte cells were collected from interface layer carefully and washed twice using phosphate buffered saline (PBS, pH = 7.4) and resuspended in Roswell Park Memorial Institute (RPMI) RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin–streptomycin. 0.1 ml of cell suspension from RPMI-1640 medium with 10% fetal calf serum was added to the wells of flat-bottomed 96-well microplates. Wells also received 0.1 ml of tested samples, each at four different concentrations (5, 10, 20, 50 µg/ml). The control wells received medium free of tested samples. The plates were incubated for 72 hr at 37 °C with 5% CO_2 in a humidified atmosphere. After incubation, 20 µL MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, 5 mg/ml] was added to each well and incubation was continued for an additional 4 hrs. Thereafter, 200 µl DMSO was added to each well to dissolve the formazan particles and the optical density (OD) was measured at 570 nm using ELISA microplate reader (Biotek – 8000, USA). Proliferative percentage of different tested samples was calculated according to the following equation:

Proliferative percentage =
(OD of test - OD of control)/ OD of control ×100

2.7.2 *In vitro* COX-1/ COX-2 inhibitory assay

The assay investigated the ability of tested samples to inhibit ovine COX-1 and human recombinant COX-2 using cyclooxygenase (COX) inhibitor screening assay kit assay (Cayman Chemicals, USA), according to the manufacturer's instructions and as previously reported [25]. The concentration of the tested samples causing 50% enzyme inhibition (IC_{50}) was calculated and compared to standard celecoxib.

2.7.3 *In vitro* 5-LOX inhibitory assay

The assay investigated the ability of tested samples to inhibit 5-Lipoxygenase enzyme using Abnova 5-lipoxygenase inhibitor screening assay kit (Cayman Chemicals, USA), according to manufacturer's instructions and as previously reported [26]. The concentration of tested samples causing 50% enzyme inhibition (IC_{50}) was calculated and compared to standard meclofenamate sodium.

2.8 *In vivo* anti-inflammatory study

2.8.1 Experimental animals

Adult male Wistar albino rats (180-220 g) were purchased from Helwan farm, Cairo, Egypt. They were kept in polypropylene cages in the laboratory animal house of Faculty of Veterinary Medicine, Cairo University, Egypt. Animals were acclimatized for 7 days under laboratory conditions (24 ± 2 ºC and 12 h light/12 h dark cycles) with free access to commercial diet and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (Approval number of ethics committee, CU/III/F/17/18).

2.8.2 Experimental design

The anti-inflammatory activity was assessed using carrageenan-induced paw edema model [27]. After acclimatization, rats were fasted 12 hr prior to experiment with free water access, then randomly allocated into 4 groups (7 rats /group) as follows; (group 1): rats received only saline, (group 2): rats received indomethacin as standard drug (10 mg/kg), (group 3): rats received DEE (200 mg/kg), (group 4): rats received DEE (400 mg/kg). A small volume (0.05 ml) of 1% carrageenan was subcutaneously injected into the plantar surface of rat right hind footpad, 1 hr after oral administration of tested samples or vehicle. The paw oedema of each rat was measured at different time intervals (2, 4, and 6 hr) after carrageenan injection by means of a digital plethysmometer. The edema was assessed by the difference between the paw volume before and after injection of carrageenan. The percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 100\ (1\text{-}Vt/Vc)$$

Where
Vc is the mean edema volume observed in the control group
Vt is the mean edema volume measured in the treated groups

2.8.3 Statistical analysis

The results were expressed as mean ± S.D. and statistical comparisons were carried out using one way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. The minimal level of significance was identified at $p<0.05$.

3. Results and Discussion

Seven compounds were successfully isolated from DEE. Phytochemical investigation of the methylene chloride fraction led to the isolation of a sterol glycoside (compound 1), while that of the ethyl acetate fraction yielded four flavonoid compounds and two phenolic acids, designated as compounds 2-7 (Fig. 2). Compound 1 was identified as daucosterol *via* its spectral data (^{1}H NMR and MS) and its comparison with published ones [28, 29]. The identities of compounds 2, 3, 4 and 5 were deduced as rutin, isoquercetrin, kaempferol and quercetin, respectively based on their UV, ^{1}H NMR and ^{13}C NMR data and its comparison with published data [30-33]. The spectral data of compounds 6 (UV and ^{1}H NMR) and 7 (UV, ^{1}H NMR, ^{1}H ^{1}H COSY and ^{13}C NMR) complied with published data of caffeic [34] and neochlorogenic [35] acids, respectively.

The *n*-butanol and ethyl acetate fractions had the same chromatographic pattern using TLC but with different intensities of spots. Thus, we investigated the more enriched fraction; the ethyl acetate fraction revealing higher compounds intensities. All these compounds, reported herein are isolated from the leaves of *H. sabdariffa* L. for the first time except rutin [30]. However, they have been previously identified in *H. sabdariffa* L. leaves growing worldwide [11, 12, 36]. The total phenolic and flavonoid contents of *H. sabdariffa* L. were estimated as 120.13 ± 0.27 µg GAE and 72.33 ± 0.061µg QE /mg DEE, respectively. Based on the previous results, DEE is shown to be rich in phenolic compounds, which may be responsible for its therapeutic efficacy. Therefore, an HPLC method was developed to establish a fingerprint chromatographic profile of DEE and its ethyl acetate fraction (Fig. 3). The HPLC fingerprints of DEE (Fig. 3A) and its ethyl acetate fraction (Fig. 3B) showed almost identical chromatographic profiles regarding peak numbers and their corresponding retention times, but at varying intensities. Two major peaks were monitored in DEE at R_t of 5.8 and 8.3 min, respectively versus six major peaks in the ethyl acetate fraction at R_t of 5.8 and 8.3, 12.5, 13.3, 21.9 and 24.5 min, respectively. These peaks were identified as neochlorogenic acid, caffeic acid, rutin, isoquercetrin, quercetin and kaempferol, respectively by comparing their retention times to those of isolated compounds (Fig. 3C, 3D), which correlates well with previous reports [12, 36]. Neochlorogenic and caffeic acids were the dominant marker compounds in DEE (Fig. 3A), where caffeic acid and kaempferol were the predominant markers in the ethyl acetate fraction (Fig. 3B).

H. sabdariffa L. leaves yielded 3.25% w/w mucilage (ML). HPLC analysis of the mucilage hydrolysate revealed six sugars, representing 94.12% of the total hydrolysate. Arabinose (48.27%) was the major identified sugar, followed by glucuronic acid (23.09%) and glucose (20.44%). Traces of xylose (1.91%), rhamnose (0.25%) and fructose (0.16%) were also detected.

Lymphocytes are the principal cells of the adaptive immune system [37]. Numerous plants are traditionally used to stimulate, suppress or modulate the immune system and are thereby termed "immunomodulatory" [38]. According to our results, DEE showed poor stimulation of lymphocytes at a concentration of 20 µg/ml. However, it exhibited a significant stimulation only at a concentration of 50 µg/ml by 21 %,

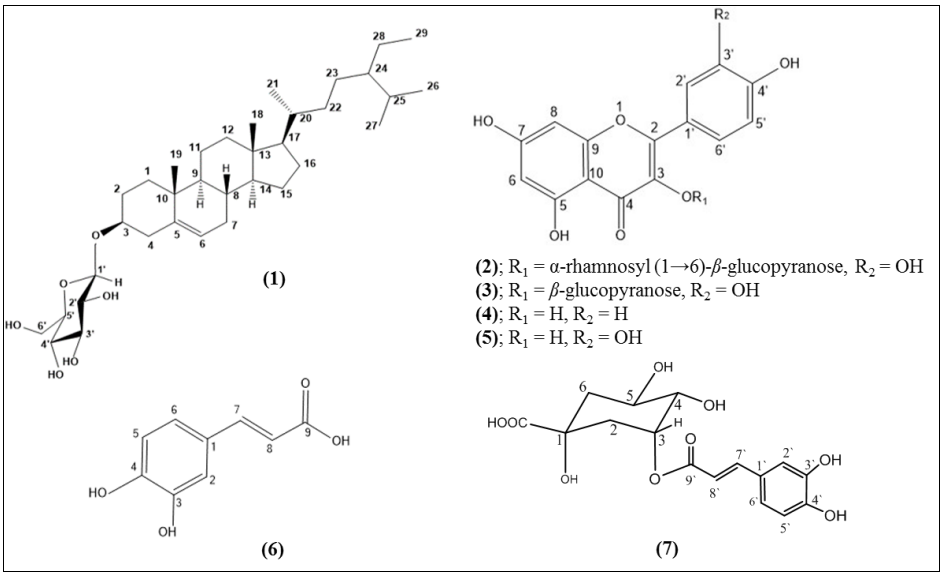

Fig 2: Structures of isolated compounds from defatted ethanolic extract of *H. sabdariffa* L. leaves (DEE)

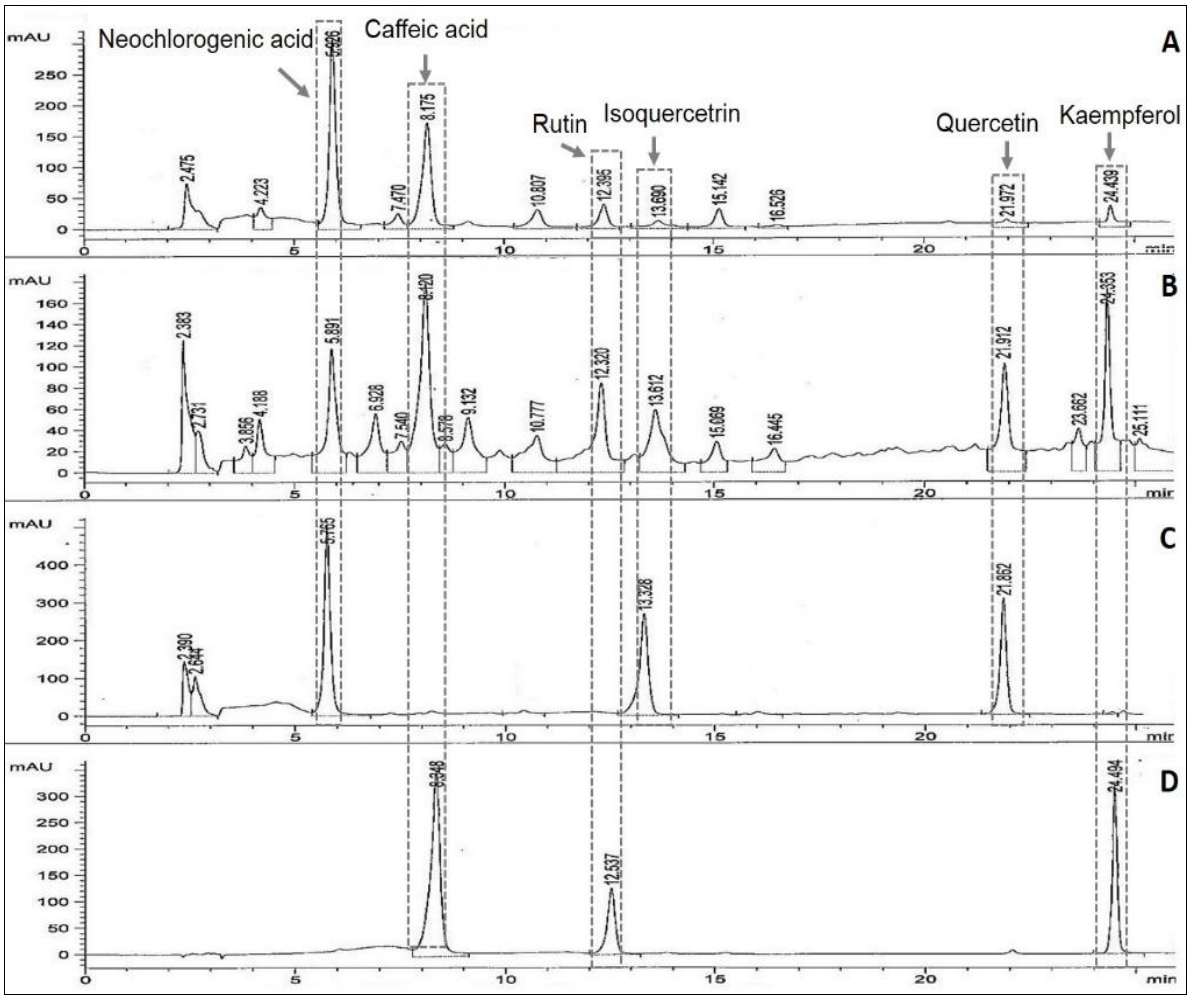

Fig 3: HPLC chromatograms of *H. sabdariffa* L. leaves defatted ethanolic extract (DEE, A) and its ethyl acetate fraction (B) versus isolated compounds (C and D)

compared to control group (p<0.05, Table 1). On the other hand, the mucilage (ML), at concentrations of 10, 20, 50 µg/ml, possessed significant inhibitory proliferative effects on lymphocytes, compared to control group (p<0.05, Table 1), with the most suppressive effect at 50 µg/ml. The immunostimulatory activity of DEE demonstrated herein might be attributed to the presence of phenolic and flavonoid compounds [2]. In this aspect, caffeic acid, a major marker in DEE, exhibited an immunomodulatory effect *via* enhancing splenocyte proliferation, and the killing activity of natural killer and cytotoxic T lymphocytes cells [39]. This is the first time for investigating the effect of DEE and ML on lymphocytes proliferation. Our results are in line with Lubega *et al.*, 2013 [14], who investigated the effect of *H. sabdariffa* L. leaves crude extract on the immune system in rats, and reported increased red blood cell production, hemagglutination titers and boosting of some phagocytes, as an indication of immunity boosting.

Table 1: Effect of defatted ethanolic extract (DEE) and mucilage (ML) of *H. sabdariffa* L. leaves on peripheral blood lymphocytes proliferation

Conc. (µg /ml)	DEE		ML	
	Optical density (Mean ± S.D.)	Proliferative %	Optical density (Mean ± S.D.)	Proliferative %
0	3.19 ± 0.21[b]	--	3.19 ± 0.21[b]	--
5	2.09 ± 0.35 [cd]	-34.48	2.99 ± 0.27 [b]	-6.27
10	2.25 ± 0.18 [cd]	-29.47	2.38 ± 0.17 [cd]	-25.39
20	3.36 ± 0.38 [b]	5.33	2.00 ± 0.49 [d]	-37.30
50	3.86 ± 0.13 [a]	21.00	1.33 ± 0.24 [e]	-58.31

Means followed by different letters within the same column are significantly different at $p < 0.05$.

Previous reports have demonstrated the inhibitory effect of standardized methanolic extracts of *H. sabdariffa* L. leaves on myeloperoxidase (MPO) activity [13] and nitric oxide synthetase [12]. Despite the rising attention towards dual COX-2/5-LOX inhibitors, nothing was traced on the influence of *H. sabdariffa* L. leaves on these enzymes. The current study showed that DEE and ML possessed anti-inflammatory activities *via* inhibition of COX and 5-LOX enzymes, compared to celecoxib and meclofenamate sodium, respectively (Table 2). The IC_{50} values of DEE and ML on COX-1 (IC_{50} = 10.24 and 8.54 µg/ml, respectively) and COX-2 (IC_{50} = 0.27 and 0.36 µg/ml, respectively) inhibition revealed higher selectivity towards COX-2, by 38 and 24 folds, respectively. Similarly, DEE exhibited more potent inhibition of 5-LOX, compared to ML (IC_{50} = 6.82 and 8.89 µg/ml, respectively). These findings are in accordance with the potential COX-2 inhibition exhibited by DEE marker compounds viz., neochlorogenic acid, caffeic acid, isoquercetrin, quercetin and kaempferol [40-44]. Nevertheless, caffeic acid, rutin, quercetin and kaempferol have also been demonstrated as 5-LOX inhibitors [45-47], whereas daucosterol has showed moderate lipoxygenase inhibitory activity [48]. In concurrence, the anti-inflammatory activity of a polysaccharide isolated from a Malvaceae plant has been previously investigated [49]. It is worth nothing that the methanol and ethyl acetate extracts of *H. sabdariffa* L. calyces exhibited higher preference towards COX-1 inhibition than COX-2, revealing tendencies of these extracts for gastric ulcers and other common side effects of NSAIDS [50]. Considering the dual COX-2/5-LOX inhibitory effects of DEE and ML demonstrated herein, the utilization of *H. sabdariffa* L. leaves in inflammatory disorders is much more recommended than its widely used calyx.

In vitro study showed that DEE exhibited more remarkable effect than ML. Therefore, the *in vivo* anti-inflammatory activity of DEE was performed, to confirm *in vitro* results, employing the carrageenan induced paw edema method, compared to indomethacin. The acute oral toxicity of *H. sabdariffa* L. leaves ethanolic extract was previously performed [51], being safe even at high doses of 2 g/kg. Consequently, two dose levels (200 and 400 mg/Kg) of DEE were selected for the current study. DEE significantly inhibited carrageenan-induced paw edema in a dose reliant manner, at different time intervals (2, 4 and 6 hr) (Table 3). However, maximum inhibition (65.71%) was achieved at a dose of 400 mg/kg, after 6 hr, being close to that of indomethacin (78.57%). This promising effect can be attributed to its phenolic [17] as well as daucosterol [52] contents.

Table 2: *In vitro* COX-1, COX-2, and 5-LOX inhibitory activities of defatted ethanolic extract (DEE) and mucilage (ML) of *H. sabdariffa* L. leaves

Tested samples	IC_{50} (µg/ml)[a]		
	COX-1	COX-2	5-LOX
DEE	10.24 ± 0.33	0.27 ± 0.18	6.82 ± 0.29
ML	8.54 ± 0.30	0.36 ± 0.13	8.89 ± 0.76
Celecoxib	15.24 ± 0.09	0.04 ± 0.02	11.48 ± 0.10
Meclofenamate sodium	nd	nd	1.78 ± 0.13

[a] Concentration of tested samples (µg/ml) producing 50% inhibition of COX-1, COX-2 and 5-LOX enzymes and expressed as M ± SD, nd: not determined.

Table 3: Acute anti-inflammatory activity of defatted ethanolic extract of *H. sabdariffa* L. leaves (DEE) in carrageenan-induced rat paw edema model

Groups	2 hr		4 hr		6 hr	
	Edema Volume (Mean ± S.D.)	% Edema Inhibition	Edema Volume (Mean ± S.D.)	% Edema Inhibition	Edema Volume (Mean ± S.D.)	% Edema Inhibition
Control (Saline)	0.59 ± 0.03	--	0.61 ±0.05	--	0.70 ± 0.08	--
Indomethacin (10 mg/kg)	0.37 ± 0.04*	37.29 %	0.31 ± 0.06*	49.18 %	0.15 ± 0.04*	78.57 %
DEE (200 mg/kg)	0.53 ± 0.02*	10.17%	0.50 ± 0.09*	18.03 %	0.41 ± 0.08*	41.43 %
DEE (400 mg/kg)	0.44 ± 0.01*	25.42 %	0.39 ± 0.02*	36.07 %	0.24 ± 0.04*	65.71 %

* Significantly different from the corresponding control group at $p < 0.05$.

4. Conclusion

This study represents the first evidence on the effect of chemically characterized defatted ethanolic extract (DEE) and mucilage (ML) of *H. sabdariffa* L. leaves on lymphocytes proliferation, using MTT assay as well as on COX and 5-LOX inhibition. DEE possessed significant stimulatory effect on lymphocytes, while ML revealed inhibitory proliferative effects, providing a new potential immunosuppressant agent for autoimmune diseases. Yet, further studies are in need to evaluate the exact mechanism of action. DEE exhibited significant anti-inflammatory activity, revealing more promising effects as dual COX-2/5-LOX inhibitor, compard to ML. One sterol glycoside (daucosterol) and six phenolic compounds (neochlorogenic acid, caffeic acid, rutin, isoquercetrin, quercetin and kaempferol) were isolated from DEE. These compounds may contribute synergistically to the aforementioned activities. These findings highlight the potential of *H. sabdariffa* L. leaves as therapeutic agent for immunomodulatory and inflammatory diseases, encouraging further investigation of this underutilized part.

5. References

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