Electronic Supplementary Material (ESI) for RSC Advances.

Table. S1. Regression statistic parameters, limit of detection and limit of quantification of the HPTLC method.

Compound	Regression equation	R^2	Linearity range (ug/spot)	LOD (ug/spot)	LOQ (ug/spot)
8-Methoxy isoscutellarein	y=1111.8x +194.91	0.990	1.69-12	0.56	1.69
Sotetsuflavone	y=1696.7x+1638.2	0.994	1.34-12	0.44	1.34
Hinokiflavone	y=383.35x+323.2	0.995	1.32-11	0.43	1.32
Cupressuflavone	y=1094.9x+7032.3	0.992	1.90-11	0.62	1.90
Isoscutellarein-7-O-β-D-xyloside	y=1120.3x+1080.6	0.997	1.13-11	0.37	1.13
Quercetrin	y=956.89x+637.3	0.995	1.37-10	0.45	1.37
Hyperoside	y=1305.8x+960.45	0.994	1.68-10	0.55	1.68

Table.S2. Intra and inter-day precision of the HPTLC method

Measurement	8-methoxy isoscutellarein		Sotetsuflavone		Hinokiflavone		Cupressuflavone		Isoscutellarein-7-*O*-*β*-D-xyloside		Quercetrin		Hyperoside	
	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RSD%)	Inter-day (RSD%)
1	1.11	2.22	1.35	2.05	2.79	3.25	2.77	3.25	2.28	3.01	2.82	3.02	2.27	3.30
2	0.80	1.56	0.60	1.16	4.23	5.01	3.88	4.52	3.72	4.15	4.42	5.21	4.40	5.35
3	0.59	0.32	3.16	4.01	3.95	4.65	1.04	2.03	4.27	5.01	3.28	4.26	1.95	2.56
4	1.03	2.25	0.34	1.12	2.91	3.88	2.49	3.51	1.10	2.32	2.26	3.24	2.75	4.01
5	1.20	2.45	0.50	2.01	1.11	2.25	1.90	2.58	1.19	2.54	2.87	4.01	0.27	1.06
6	1.26	2.65	0.59	1.56	1.95	2.98	1.71	2.17	1.23	2.91	2.42	3.54	0.45	1.25

Table.S3. Accuracy of the HPTLC method.

Model analyte	Matrix amount (ug/spot)	Added amount (ug/spot)	Expected amount (ug/spot)	Found amount (ug/spot)	% Recovery	% RSD
8-Methoxy isoscutellarein	1.39	4	5.39	5.12	94.98	4.53
				5.44	100.91	
				5.60	103.89	
	1.39	6	7.39	7.50	101.48	1.27
				7.35	99.45	
				7.49	101.35	
Sotetsuflavone	0.82	4	4.82	4.70	97.5	3.49
				4.95	102.6	
				5.02	104.31	
	0.82	6	6.82	6.70	98.24	1.92
				6.90	101.17	
				6.95	101.90	
Hinokiflavone	4.21	4	8.21	8.00	97.44	2.21
				8.30	101.09	
				7.98	97.19	
	4.21	6	6.21	6.30	101.45	1.66
				6.15	99.03	
				6.35	102.25	
Cupressuflavone	3.66	4	7.66	7.45	97.25	3.78
				7.89	103.00	
				8.01	104.56	
	3.66	6	9.66	9.52	98.55	1.68
				9.79	101.34	
				9.50	98.34	
Isoscutellarein-7-o-β-D-xyloside	6.88	4	10.88	10.65	97.88	2.11
				10.90	100.18	
				11.11	102.11	
	6.88	6	12.88	12.98	100.77	1.16
				12.75	98.99	
				12.70	98.60	
Quercetrin	8.37	4	12.37	12.01	97.08	3.07
				12.45	100.64	
				12.77	103.23	

	8.37	6	14.37	14.20	98.81	1.11
				14.45	100.55	
				14.50	100.90	
Hyperoside	7.5	4	11.5	11.11	96.60	3.36
				11.45	99.56	
				11.88	103.30	
	7.5	6	13.5	13.39	99.19	1.24
				13.66	101.19	
				13.70	101.48	

Quercetin -3-*O*-galactoside (Hyperoside); ^{1}H-NMR (400MHz, DMSO-d_6): 3.18-3.47 (6H, m, sugar protons), 5.39 (1H, d, *J*= 4.8 Hz, gal-H-1), 6.44 (1H, d, *J*= 2.1 Hz , H-6) , 6.44 (1H, d, *J*= 2.1 Hz, H-8), 6.96 (1H, dd, *J*= 8.4Hz, H-5'), 7.74 (1H, d, *J*= 2.1, 8.6 Hz, H-6'), 7.78 (1H, d, *J*= 2.1 Hz, H-2'); ^{13}C-NMR (100MHz, DMSO-d_6) δ; 61.11 (gal-C-6), 70.11 (gal-C-4), 73.60 (gal-C-2), 76.90 (gal-C-3), 77.76 (gal-C-5), 95.11 (C-8), 99.34 (C-6), 100.50 (gal-C-1), 106.15 (C-10), 116.15 (C-2'), 116.15 (C-5'), 122.29 (C-1'), 122.40 (C-6'), 136.70 (C-3), 147.77 (C-3'), 149.50 (C-4'), 156.14 (C-2), 156.14 (C-9), 160.80 (C-5), 163.20 (C-7), 176.54 (C-4). MALDI-TOF: (measured mass: 464.0954, calculated mass: 464.0963, error value: 0.0002%).

Quercetin -3-*O*-rhamnoside (Quercetrin); ^{1}H-NMR (400MHz, DMSO-d_6): 0.82 (3H, d, *J*=5.9 Hz, C$\underline{H_3}$), 3.12-3.32(4H, m, sugar protons), 5.26(1H, d, *J*=1.6Hz, rha-H-1), 6.21 (1H, d, *J*= 2.0 Hz , H-6) , 6.40(1H, d, *J*= 2.0 Hz, H-8), 7.26 (1H, dd, *J*= 2.0, 8.6Hz, H-6'), 6.87 (1H, d, *J*= 8.6 Hz, H-5'), 7.30 (1H, d, *J*= 2.0 Hz, H-2'); ^{13}C-NMR (100MHz, DMSO-d_6) δ; 17.94 (rha-C-6), 70.70 (rha-C-5), 70.90 (rha-C-2), 71.00 (rha-C-3), 71.62 (rha-C-3), 102.26 (rha-C-1), 94.10 (C-8), 99.15 (C-6), 104.5 (C-10), 115.90 (C-2'), 116.09 (C-5'), 121.17 (C-1'), 121.58 (C-6'), 134.65 (C-3), 145.64 (C-3'), 148.88 (C-4'), 156.89 (C-2), 157.76 (C-9), 161.73 (C-5), 164.64 (C-7), 178.38 (C-4). MALDI-TOF: (measured mass: 448.3755, calculated mass: 448.3769, error value: 0.0003%).

Isoscutellarein -7-*O-β*-xylopyranoside; ^{1}H-NMR (400MHz, DMSO-d_6) δ: 3.30-3.37 (m, sugar protons), 3.80 (2H,d, xyl-H-5), 4.97(1H, d, *J*=7.4Hz, xyl-H-1), 6.57 (1H,s, H-6), 6.84 (1H, s, H3), 6.96 (2H, d, *J*=8Hz, H-3',5'), 7.99 (2H, d, *J*=8.6Hz, H-2', 6'); ^{13}C-NMR (100MHz, DMSO-d_6) δ: 66.24 (xyl-C-5), 69.77 (xyl-C-4), 73.47 (xyl-C-2), 76.14 (xyl-C-3), 98.96 (C-6), 102.2 (xyl-C-1),103.13 (C-3), 106.64 (C-10), 116.46 (C-3', 5'), 121(C-1'), 127.51 (C-8), 129.03 (C-2',6'), 144.98 (C-9), 151.46 (C-7), 152.74 (C-5), 161.78 (C-4'), 164.53 (C-2), 182.82 (C-4); MALDI-TOF: (measured mass: 418.3494, calculated mass: 418.3500, error value: 0.0001%).

Cupressuflavone; ; ^{1}H-NMR (400MHz, DMSO-d_6) unity **I** and **II**; 6.44 (s, 1H, H-6), 6.74 (d, 2H, *J*= 8.8 Hz, H-3' and 5'), 6.74 (d, 1H, *J*= 8.8 Hz, H-5'), 6.78 (s, 1H, H-8), 7.49 (d, 2H, *J*=8.8, H-2', 6'); ^{13}C-NMR (100MHz, DMSO) δ; unity **I** and **II**; 99.16 (C-8), 99.25 (C-6), 103.03 (C-3), 104.06 (C-10), 116.25 (C3', 5'), 121.65 (C-1'), 128.36 (C-2', 6'), 155.24 (C-9), 161.29 (C-5), 161.49 (C-4'), 163.99 (C-2) 164.38 (C-7), 182.50 (C-4). MALDI-TOF: (measured mass: 538.0900, calculated mass: 538.0930, error value: 0.0005%).

Hinokiflavone; ^{1}H-NMR (400MHz, DMSO-d_6) unity **I**; 6.20 (1H, d, *J*= 2.1 Hz, H-6), 6.47 (1H, d, *J*= 2.1 Hz, H-8), 6.62 (1H, s, H-3), 7.03 (2H, d, *J*= 8.5, H-3', 5'), 7.99 (2H, d, *J*=8.5, H-2', 6'), unity **II**; 6.79 (1H, s, H-3), 6.82 (1H, s, H-8), 6.94 (2H, d, *J*=8.5, H-3', 5'), 7.94(2H, d, *J*=8.5, H-2', 6'); ^{13}C-NMR (100MHz, DMSO) δ; unity **I**; 94.46 (C-8), 99.42 (C-6), 102.86 (C-10), 104.16 (C-3), 115.78 (C3', 5'), 125.50 (C-1'), 128.92 (C-2', 6'), 157.70 (C-9), 160.20 (C-4'), 161.30 (C-5), 161.64 (C-7) 164.34 (C-2), 182.20 (C-4), unity **II**; 94.46 (C-8), 103.58 (C-3), 104.23 (C-10), 116.46 (C-3', 5'), 121.70 (C-1'), 124.40 (C-6), 128.74 (C-2', 6'), 153.39 (C-5), 154.49 (C-9), 157.70 (C-7), 162.66 (C-4'), 164.86 (C-2), 182.20 (C-4). MALDI-TOF: (measured mass: 538.4569, calculated mass: 538.4579, error value: 0.0002%).

Sotetsuflavone; ^{1}H-NMR (400MHz, DMSO-d_6) unity **I;** 3.76 (3H, s, OC<u>H</u>$_3$), 6.19 (1H, d, *J*= 2.0 Hz, H-6), 6.45(1H, d, *J*= 2.0 Hz, H-8), 6.83 (1H, s, H-3), 7.16(1H, d, *J*=8.6Hz, H-5'), 8.02 (1H, d, *J*=2.0 Hz, H-2'), 8.02 (1H, dd, *J*= 2.0, 8.6 Hz, H-6'), unity **II**; 6.41 (1H, s, H-6), 6.89 (1H, s, H-3), 6.93(2H, d, *J*= 8.4Hz, H-3', 5'), 7.69 (2H, d, *J*= 8.4Hz, H-2', 6'); ^{13}C-NMR (100MHz, DMSO-d_6) δ; unity **I**; 55.97 (O<u>C</u>H$_3$), 94.49 (C-8), 99.28 (C-6), 103.46 (C-3), 104.18 (C-10), 116.77 (C-5'), 120.56 (C-1'), 121.35 (C-3'), 128.25 (C-2'), 131.85 (C-6'), 157.83 (C-9), 160.20 (C-4'), 161.00 (C-5), 163.66 (C-7), 164.99 (C-2), 182.20 (C-4), unity **II**; 99.28 (C-6), 103.71 (C-3), 104.09 (C-8), 104.59 (C-10), 114.95 (C-3', 5'), 123.47 (C-1'), 128.46 (C-2', 6'), 155.02 (C-9), 161.91 (C-5), 162.66 (C-4'), 162.80 (C-7), 164.57 (C-2), 182.60 (C-4). MALDI-TOF: (measured mass: 552.4839, calculated mass: 552.4845, error value: 0.0001%).

8-Methoxy- isoscutellarein; ^{1}H-NMR (400MHz, DMSO-d_6) δ; 3.70 (3H, s, OC<u>H</u>$_3$), 6.45 (1H, s, H-6), 6.87 (1H, s, H-3), 6.96 (2H, d, *J*= 8.3Hz, H-3', 5'), 7.61 (2H, d, *J*=8.5Hz, H-2', 6'); ^{13}C-NMR (100MHz, DMSO-d_6) δ ; 56.04 (O<u>C</u>H3), 99.10 (C-6), 103.75 (C-3), 104.09 (C-10), 115.00 (C-3', 5'), 123.31 (C-1'), 127.03 (C-8), 128.5 (C-2', 6'), 155.29 (C-5), 159.44 (C-9), 161.32 (C-7), 162.66 (C-4'), 163.49 (C-2), 182.50 (C-4). MALDI-TOF: (measured mass: 300.2617, calculated mass: 300.2628, error value: 0.0003%).

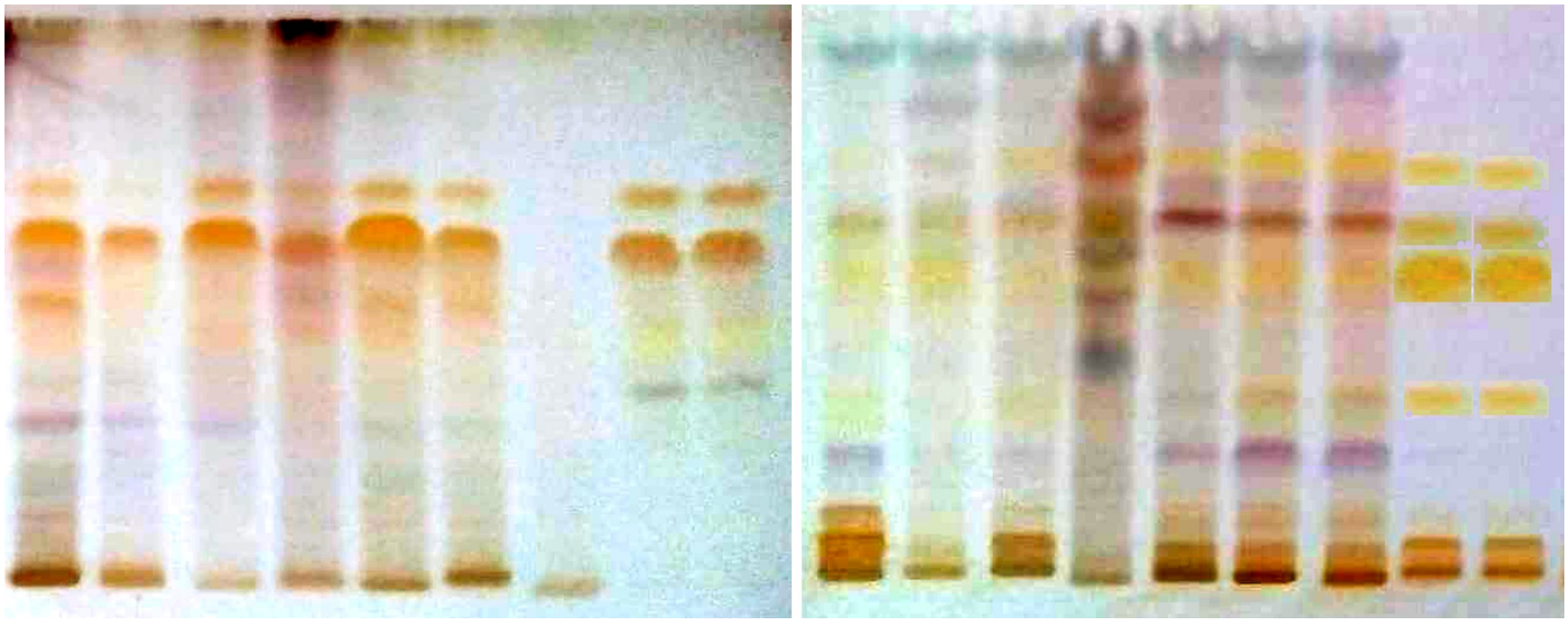

Figure S1: HPTLC chromatogram of some representative samples, plates were visualized under white light after post chromatographic derivatization using andisaldehyde/ H_2SO_4 spray reagent.

2.9.1. Method validation

The correlation coefficient of the data was detected over concentration range 2-7 ug/spot and the linearity range was determined.

Intra-day Precision (repeatability) was determined by independent repeated analysis of six different volumes of each standard, each repeated triplicate was done under the same conditions by using the same apparatus and the same analytical method in the same laboratory and on the same plate. Inter-day precision (reproducibility) was calculated by the analysis of each standard three times a day over 3 days by a different analyst. Repeatability and intermediate precision results were expressed as relative standard deviation (RSD) (%).

The limit of detection (LOD) and limit of quantification (LOQ) values expressed the sensitivity of the method and they were calculated on the basis of the residual standard deviation of a regression line and slope. Both parameters were calculated according to equations (1) and (2).

$$LOD = (STEYX/s) * 3.3 \quad (1)$$

$$LOQ = (STEYX/s) * 10 \quad (2)$$

Where STEYX is a built in formula in excel program to calculate the standard error of the predicted y-value for each x in the regression.

Accuracy of the HPTLC method was carried out by standard addition method, as two spots representing two concentration levels 4 ug and 6 ug of each model analyte were applied over two tracks containing *P. orientalis* summer leaves extract (pre-analyzed sample),

respectively. After that the plates were developed in system I for hyperoside, quercetrin, isoscutellarein-7-*O*-*β*-xyloside plate and developed in system II for cupressuflavone, hinokiflavone, sotetsuflavone and 8-methoxy isoscutellarein plate. Addition experiments were done in triplicate and the accuracy was calculated as the % of model analyte recovered. Three analyses per concentration were done and % RSD was determined.

2.10. Cytotoxicity and anti-inflammatory activity test

Isolation and cultivation of human white blood cells

In a sterile heparin tube a whole blood specimen was obtained and about 1ml blood was taken into 15 ml centrifuge tube then the tube was filled to capacity with fresh cold ammonium chloride lysing solution then inverted for ~10 minutes at room temperature until the liquid became clear red. Centrifugation of samples was done at 4^{o} C for 10 min at 2000 rpm followed by decantation of the supernatant and the tubes were allowed to drain, The pellets (WBCs) were suspended in 10 ml cold Phosphate buffer saline (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4 and 10 mM KH_2PO_4) pH 7.4, recentrifuged and pellets were resuspended in RPMI culture medium containing 10% fetal bovine and 2% L-glutamine. Assessment and counting of WBCs were done using the dye exclusion method of (Louis & Siegel, 2011). A portion of the cell suspension (e.g., 50 µl) was mixed with an equal volume of 0.5% trypan blue staining solution and loaded onto hemocytometer. Both viable unstained and nonviable stained cells were counted.

Calculation

$$\mathbf{N / ml = mean\ of\ WBCs\ counting\ x10^4 \times D}$$

N: Number of viable or nonviable cells

D: Sample dilution (1:1 with the trypan blue).

$$\mathbf{\%\ of\ cell\ viability} = \frac{\mathbf{Number\ of\ viable\ cells}}{\mathbf{Total\ number\ of\ cells}} \times \mathbf{100}$$

At least 90% of the cells must be viable in order to use the cells for assays, after that incubation of the cultured was performed in CO_2 incubator for six days. WBCs were seeded as 100,000 cells/ well (96 well cell culture plate) and incubated in CO_2 incubator (37°C, 5% CO_2, and 90% relative humidity).

Assessment of cytotoxicity of the essential oils compared to piroxicam (MTT assay)

A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. In this assay the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was used to detect metabolically active living cells by mitochondrial succinate dehydrogenase which convert the MTT into a dark purple insoluble formazan. The cells were then solubilized with an organic solvent (dimethylsulfoxide) and the quantitation of the released, solubilized formazan can done spectrophotometrically in order to check the cytotoxicity of the oils, crude extracts and the piroxicam against human WBCs by method described before by (Mosmann, 1983). Different concentrations (0, 3.125, 6.25, 12.5, 25 and 50 µg/ml) of the studied oils were plated in 96-well cell culture plate with 200 µl of cultured medium that contained 100,000 WBCs / well in RPMI medium without fetal bovine serum or standard anti-inflammatory drugs piroxicam followed by the incubation of the plate for 72 h in CO_2 incubator (37°C, 5% CO_2, and 90% relative humidity). After the incubation, addition of 20 µl of MTT solution each well was done and then plates were incubated for 3 h in CO_2 incubator to allow the MTT to be reacted. After incubation, the plates were centrifuged at 1650 rpm for 10 min and the medium was discarded. The formazan crystals (MTT byproduct) were re-suspended in 100µl DMSO and reading was measured at a wavelength of 570 nm for detecting safe dose, that cause 100% cell viability.

The % viability was calculated as follow: $(A_T-A_b/A_C-A_b) \times 100$

$\mathbf{A_T}$ = mean absorbances of cells treated with different concentration of each oil

$\mathbf{A_C}$ = mean absorbances of control untreated cells with culture medium only

$\mathbf{A_{b=}}$ mean absorbances of cells treated with vehicle of plant extract (RPMI without fetal bovine serum)

The expression of the cytotoxicity assay of the compound was as EC_{100}, which was calculated by the Graphpad Instat software using the % viability calculated from the serial dilutions of each oil.

1.1.1. Detection of the effective anti-inflammatory concentrations (EAICs) of the used treatments in lipopolysaccharides (LPS)-stimulated human WBC's culture

LPS is is a protein fragment of the gram-negative bacteria cell wall. LPS degrades into the O-antigen and Core protein which have a little immunogenic effect and Lipid-A which is highly pro-inflammatory. Lipid-A binds the CD14/TLR4/MD2 receptor on monocytes and tissue macrophages to provoke the NF-κβ protein family (Abbas & Lichtman, 2006). This messenger translocates to the cell nucleus and starts the production of pro-inflammatory cytokines through primer binding. LPS acts as common inflammatory inducer and causes

abnormal up-regulation in the proliferation of human leukocytes (Abbas & Lichtman, 2006). The abnormal increase in the cell proliferation can be used as a marker of inflammation. This assay was performed according to (Mosmann, 1983). Fifty µl of the culture medium that contained 100,000 of human WBCs was dispensed per well in a 96-well plate. The inflammation was induced by addition of 50 µl of LPS (1 mg/ ml) to the plated cells and incubated in CO_2 incubator for 24 h. After the incubation period, the plate was centrifuged at 1650 rpm for 5 min and the supernatants were discarded and then 200 µl of serial concentrations (0, 3.125, 6.25, 12.5, 25 and 50 µg/ml in culture media) of the oils or the standard drug piroxicam were added. The control cells contained cell culture medium only. Then plates were incubated for additional 72 h in CO_2 incubator. After the incubation period, cell proliferations were measured using MTT (as previously discussed). Stimulation index (SI) was used to assess the cell proliferations.

Stimulation index = (mean absorbance of LPS-stimulated cells or LPS-stimulated cells treated with different concentrations of natural product / absorbance of control untreated cells).

The effective anti-inflammatory concentration (EAICs) of each oil can be defined as the concentration that has the ability to bring back the abnormal proliferation of LPS-stimulated cells to normal proliferation of control untreated cells (SI = 1) were calculated using Instate graph pad.

Investigation of the anti-inflammatory effect of each plant extract effective dose (EAICs) in LPS-stimulated WBCs

In order to detect the anti-inflammatory properties of untreated and treated white blood cells, cell pellets and supernatant of untreated and treated cells were collected for detecting pro-inflammatory and anti-oxidant indices using biochemical assays. This assay was performed according to (Mosmann, 1983). Stimulation of about 1 x 10^6 white blood cells per well (6-well plate) LPS for 24 h was done and then treated with each oil effective dose separately or piroxicam for 72 h. After centrifugation, each supernatant was collected and used for the TNF-α, IL-1 and INF-γ and lipid peroxidation assays. Meanwhile, pellets of untreated and treated cells were collected and resuspended in phosphate buffer saline (PBS). Cell suspension was lysed in Phosphate buffer saline pH= 7.4 containing protease inhibitor in ice bath for estimating the pro-inflammatory markers and lipid peroxidation assays.

Determination of pro-oxidants or pro-inflammatory mediators

Determination of lipid peroxidation products (TBARS)

Lipid peroxidation is a sensitive marker due to the high probability of lipids to undergo oxidation, and therefore is a highly used oxidative stress marker. Thus, increased oxidative stress was primarily shown by using various markers of lipid peroxidation in plasma and serum (AbuYoussef et al., 2014). The lipid peroxidation biomarker TBARS (Thiobarbituric acid reactive substance) including lipid

hydroperoxides and aldehydes increase in concentration as a response to oxidative stress. This assay was based on the reaction of 2-thiobarbituric acid (chromogenic reagent), with malondialdehyde (MDA) at 25°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid resulting in formation of a chromophore with absorbance maximum at 532 nm. This assay was performed according to (Tappel & Zalkin, 1959) as shown below in figure

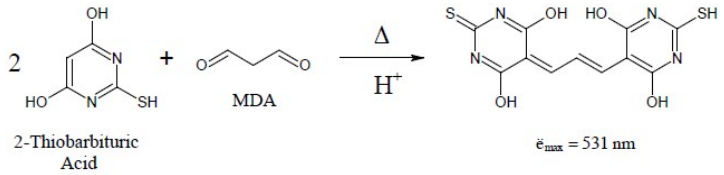

Figure Reaction between 2-thiobarbituric acid and MDA under acidic conditions

In a boiling water bath, heating of 100 µl of supernatants with 2 ml of (TBA) reagent was carried out for 20 min. After cooling, centrifugation of the solution was at 2000 rpm for 10 min and the absorbance of the supernatant was determined at 532 nm using optima spectrophotometer.

Calculation

The lipid peroxidation level in the sample was determined using standard curve of tetramethoxy propane.
Lipid peroxidation level (nmoles/ml) = $[(A_E-A_B)-I] / S$
Where:
A_E: The mean of absorbances of supernatant of treated cells or untreated cells.
A_B: The mean of absorbances of blank.
I: The intercept of the standard curve
S: The slope of the standard curve

ELISA detection of tumor necrosis factor alpha (TNF-α)

(TNF)-α kit is a solid phase sandwich enzyme linked-immuno-sorbent assay (ELISA). A human TNF-α specific monoclonal antibody has been coated onto the wells of the provided microtiter strips provided followed by pipetting of samples, standards of known human TNF-α content and cell lysates into these wells. The human TNF-α antigen binds to the immobilized (capture) antibody on one site during the first incubation. Addition of a biotinylated monoclonal antibody specific for human TNF-α was done after washing .

In the second incubation, this antibody binds to the immobilized human TNF-α which was captured during the first incubation. In order to complete the four-member sandwich, streptavidin-peroxidase was added to bind to the biotinylated antibody after removal of excess second antibody. After a third incubation and washing to remove all the unbound enzyme, a substrate solution was added, which is acted upon by the bound enzyme to produce color. The concentration of human present in the original specimen was indicated from the intensity of the color. TNF-α ELISA kit was used to perform the assay.

According to the kit's instructions, all reagents and the serial dilutions of the standard TNF-α protein were prepared. Each well of a 96 well plate was pre-coated with anti-human TNF- α. In each well a volume of 100 µl of serial dilutions of the standard or sample or RPMI medium (blank) were added. The incubation of the plate was done at room temperature for 2.5 h. The supernatants were discarded and the plate was washed 4 times with the washing solution. After washing 100 µl of the pre-prepared biotinylated anti-human TNF- α antibody (detection antibody) was added to each well. After that, the plate was incubated for a second time for1 h at room temperature. The supernatants were discarded and the washing step was repeated.

One hundred microliters of pre-prepared streptavidin solution were added to each well and incubated for 45 min at room temperature followed by discarding the supernatant and washing of the plate. One hundred microliters of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate reagent were added to each well and the plate was incubated for 30 min in the dark at room temperature. Finally, 50 µl of the stop solution were added to each well and the absorbance was measured against blank at 450 nm using ELISA reader. The concentration of TNF- α in the culture supernatants was determined using standard curve of serial dilutions (3.91- 500 pg /ml) of the standard TNF-α protein.

Calculation

The concentration of TNF-α in the culture supernatants was determined using the standard curve of the standard TNF-α protein.

TNF-α Concentration = $[(A_E-A_B)-I] / S$ Where:

A_E: The mean of absorbances of supernatant of treated cells or untreated cells.

A_B: The mean of absorbances of blank.

I: The intercept of standard curve

S: The slope of standard curve

ELISA detection of interleukin -1 beta (IL-1*β*)

(IL-1*β*) kit is a solid phase sandwich enzyme linked-immuno-sorbent assay (ELISA). The principle of the assay was the same as in case of IL-1*β* quantitative assay, please refer to **2.6.5.2.**section. IL-1*β* ELISA kit was used to perform the assay.

According to the kit's instructions, all reagents and the serial dilutions of the standard IL-1*β* protein were prepared. Each well of a 96 well plate was pre-coated with anti-human IL-1*β*. In each well a volume of 100 µl of serial dilutions of the standard or sample or RPMI

medium (blank) were added. The incubation of the plate was done at room temperature for 2.5 h. The supernatants were discarded and the plate was washed 4 times with the washing solution. After washing 100 µl of the pre-prepared biotinylated anti-human IL-1β antibody (detection antibody) was added to each well. After that, the plate was incubated for a second time for1 h at room temperature. The supernatants were discarded and the washing step was repeated.

One hundred microliters of pre-prepared streptavidin solution were added to each well and incubated for 45 min at room temperature followed by discarding the supernatant and washing of the plate. One hundred microliters of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate reagent were added to each well and the plate was incubated for 30 min in the dark at room temperature. Finally, 50 µl of the stop solution were added to each well and the absorbance was measured against blank at 450 nm using ELISA reader. The concentration of IL-1β in the culture supernatants was determined using standard curve of serial dilutions (3.91- 500 pg /ml) of the standard IL-1β protein.

Calculation

The concentration of IL-1β in the culture supernatants was determined using the standard curve of the standard IL-1β protein.

IL-1β Concentration = $[(A_E-A_B)-I] / S$ Where:

A_E: The mean of absorbances of supernatant of treated cells or untreated cells.

A_B: The mean of absorbances of blank.

I: The intercept of standard curve

S: The slope of standard curve

ELISA detection of inteferno gamma (INF-γ)

(INF-γ) kit is a solid phase sandwich enzyme linked-immuno-sorbent assay (ELISA). The principle of the assay was the same as in case of TNF-α quantitative assay, please refer to **2.6.5.2.**section. INF-γ ELISA kit was used to perform the assay.

According to the kit's instructions, all reagents and the serial dilutions of the standard INF-γ protein were prepared. Each well of a 96 well plate was pre-coated with anti-human INF-γ. In each well a volume of 100 µl of serial dilutions of the standard or sample or RPMI medium (blank) were added. The incubation of the plate was done at room temperature for 2.5 h. The supernatants were discarded and the plate was washed 4 times with the washing solution. After washing 100 µl of the pre-prepared biotinylated anti-human INF-γ antibody (detection antibody) was added to each well. After that, the plate was incubated for a second time for1 h at room temperature. The supernatants were discarded and the washing step was repeated.

One hundred microliters of pre-prepared streptavidin solution were added to each well and incubated for 45 min at room temperature followed by discarding the supernatant and washing of the plate. One hundred microliters of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate reagent were added to each well and the plate was incubated for 30 min in the dark at room temperature. Finally, 50 µl of the

stop solution were added to each well and the absorbance was measured against blank at 450 nm using ELISA reader. The concentration of INF-γ in the culture supernatants was determined using standard curve of serial dilutions (3.91- 500 pg /ml) of the standard INF-γ protein.

Calculation

The concentration of INF-γ in the culture supernatants was determined using the standard curve of the standard INF-γ protein.

INF-γ Concentration = $[(A_E-A_B)-I] / S$ Where:

A_E: The mean of absorbances of supernatant of treated cells or untreated cells.

A_B: The mean of absorbances of blank.

I: The intercept of standard curve

S: The slope of standard curve