Electronic Supplementary Material (ESI) for RSC Advances.

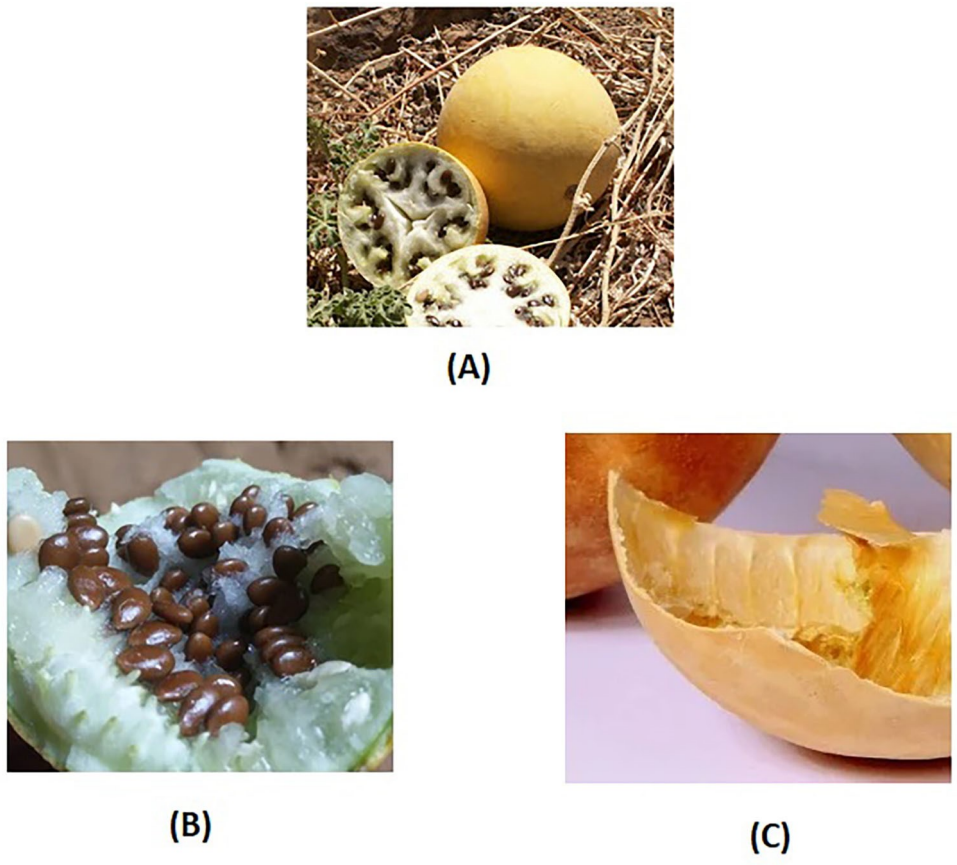

Figure S1. Colocynth fruit (A), seeds and pulp (B) and peel (C).

Standard solutions for UPLC-MS quantification

In order to investigate the variability in the chemical profile of the tested extracts and to unravel the effect of roasting on the quality and quantity of the identified metabolites, all detected metabolites were analyzed in each tested extract based on mean peak area computation using the calibration curves of corresponding standards and the result were readily presented as (mg standard equivalents/g dry extract). The stock external standard solution of *p*-hydroxybenzoic acid, quercetin, cucurbitacin E and linoleic acid (Sigma-Aldrich (St. Louis, MO, USA)) were prepared by weighing accurate weight of each standard (10 mg). Each standard was separately placed in 10-

mL volumetric flask and HPLC-grade methanol was added. After that, the solution was serially diluted to the working concentrations over the reliable range 0.0125 – 0.75 mg mL^{-1} using the same solvent. Five µL aliquots of each standard compound were injected onto the chromatographic system in duplicates for different concentration levels. The standard calibration curves were established by plotting peak areas of the standards as the analytical responses against their known concentration concentrations. Validation parameters like linearity, limit of detection (LOD) and limit of quantification (LOQ) were assessed based on FDA guideline on bioanalytical method validation (Kadian et al., 2016) (**Table S1**).

Table S1: Linearity and sensitivity parameters for *p*-hydroxybenzoic acid, quercetin, cucurbitacin E and linoleic acid

Compound	Linearity range (mg /ml)	Slope (a)	Intercept (b)	Corelation coefficientr	LOD (mg /ml)	LOQ (mg /ml)
***p*-hydroxybenzoic acid**	**0.0125-0.25**	$1.55*10^7$	$-6.91*10^3$	**0.994**	**0.009**	**0.0125**
Quercetin	**0.025-0.536**	$2.43*10^7$	$-8.34*10^4$	**0.992**	**0.011**	**0.025**
Cucurbitacin E	**0.04-0.7**	$4.43*10^7$	$-7.5*10^4$	**0.997**	**0.006**	**0.011**
Linoleic acid	**0.03-0.6**	$1.63*10^7$	$-9.2*10^4$	**0.991**	**0.013**	**0.03**

Experimental conditions as in Section 2.3.2. For each calibration curve the equation is y = ax + b, where y is the peak area, x is the concentration of the standard (mg/ml), a is

Table S2: UPLC-MS metabolite profiling data from all Colocynth samples representing the content of each variable (peak areas) calculated as (expressed as mg standard Equivalents/g dry extract)

Compound	seed unprocessed	Seed processed	pulp unprocessed	Pulp processed	Peel unprocessed	Peel processed
Methyl-heptanone **	0.051	0.117	0	0	0	0.092
Heptanoic-acid**	0.144	0	0.238	0.323	0.345	0.224
2-Ethyl-1-hexanol**	0.154	0.239	0.118	0.151	0.115	0.252
Dimethyl azelate**	0	0.088	0.035	0	0	0.065
Histidine **	0	0	0.025	0	0	0
Asparaginine **	0	0	0	0	0	0
Citrulline **	0.106	0	0.153	0	0	0
Siderin***	0	0	0.868	0	0.98	0.099
Dihydrocucurbitacin E****	0.069	0	0	0	0	0
Cucurbitacin S ****	0	0.02	0	0	0	0
Cucurbitacin L****	0	0	0	0.069	0	0

Cucurbitacin D****	0.114	0.175	0	0	0	0
Dihydrocucurbitacin C****	0.284	0.346	0	0.053	0	0
Cucurbitacin F****	0	0.067	0	0	0	0
Cucurbitacin A glucopyranoside****	0	0	0	0	0.018	0.019
Colocynthin B****	0.101	0.098	0.37	0.427	0.088	0.05
Cucurbitacin I-glucoside****	0.21	0.252	1.24	1.145	0.27	0.26
Cucurbitacin E glucoside****	0.07	0	0	0	0	0
Cucurbitacin D dehydroepirhamnoside****	0	0.065	0.56	0.116	0	0.074
Cucurbitacin B****	0	0	0	0	0.082	0
Calodendroside A***	0	0.044	0	0	0	0
Cucurbitacin E****	0.6	0.6	0.971	0.78	0.507	0.459
6’-acetyl-2-O-β-D- glucopyranosyl-cucurbitacin E ****	0.1	0.11	0.317	0.3	0.137	0.09
Cucurbitacin L-glucoside****	0.049	0.1	0.352	0.3	0.221	0.15
2-O- β -D-glucopyranosyl-16-20R-dihydroxycucurbita-1,5,23E,25(26)-tetraen-3,11,22-trione****	0	0	0.06	0	0	0
Arvenin I****	0.243	0.11	1.15	0.187	0.27	0.194
22-deoxocucurbitoside B****	0.104	0.111	0.34	0.406	0.181	0.096
Colocynthosides B****	0	0	0.036	0.227	0	0
Colocynthosides A****	0	0	0	0	0.067	0
Colocynthin A	0.111	0.028	0.75	0.768	0.323	0.167
Palmitoleicmethyl ester**	0	0.095	0	0	0.105	0.106
Linoleinc acid *	1.712	0.879	0.683	0.727	0.491	0
Heptadecanoic acid *	0	0	0	0.075	0	0
Gadoleic-acid*	0.335	0	0.222	0.261	0	0
Docosanyl-acetate *	0	0.055	0	0.101	0	0
Caprylic acid*	0.452	0.485	0.275	0.428	0.362	0.522
Caproic acid*	0	0.152	0	0	0	0
Lauric acid *	0.064	0	0	0	0	0.076
Hexanoic acid methyl ester*	0	0	0	0	0.063	0
Quercitrin***	0	0.084	0	0	0	0
Luteolin-O-hexoside ***	0	0	0.066	0	0.056	0
Kaempferol rhamnoside–hexoside***	0.083	0.077	0	0	0	0
Isovitexin***	1.853	1.499	0	0.349	0.098	0.155
Isosaponarin***	0.382	0.44	0.022	0	0	0
Isorhamnetin 3-O-rutinoside***	0	0	0.029	0	0.028	0
Isoorientin 3’-O-methyl ether ***	0	0	0	0	0	0.026
Chrysoeriol-O-hexoside ***	1.548	1.472	0.433	0.443	0.144	0.05
4-methylquinoline ***	0	0.225	0	0.057	0	0.096
Citronellol	0	0.12	0.078	0	0	0
Citral	0.072	0	0	0	0	0
Sinapic acid**	0.098	0.119	0	0	0.104	0.158
Gastrodin**	0	0	0.233	0.24	0.18	0

Cinnamyl alcohol**	0.328	0.274	0	1.143	0	0.289
Catalposide	0	0.185	0	0	0	0.148
4,5-di-O-galloylquinic acid**	0	0.193	0	0	0	0.16
3-O-Caffeoyl quinic acid**	0	0	0	0.171	0.181	0
O-feruloylquinide **	0	0.043	0	0	0	0.038
Gallic acid **	0.059	0.117	0	0	0.153	0.15
Salicylic acid-O-hexoside **	0	0	0	0.248	0.269	0
Syringic acid**	0	0	1.127	1.192	0.952	0.243
Protocatechuic acid -3-glucoside**	0	0	0.592	0	0	0
Protocatchuic acid**	0	0	0.207	0	0	0
O-Caffeoylshikimic acid**	0	0	0.127	0.257	0	0
Gallocatechin**	0.091	0.12	0.214	0	0	0
Ferulic acid **	0	0.081	0	0	0.053	0.063
Dicaffeoylshikimic acid **	0.075	0.165	0	0.038	0.117	0.139
Cinnamic acid**	0.377	0.197	0	0	0	0
Caffeic-acid**	0.062	0.088	0.193	0	0	0
Quinic acid**	0	0.123	0	0	0	0.105
Corilagin**	0.15	0.13	0	0	0	0
Stigmasta-7,25-diene-3beta-ol	0	0	0	0.13	0	0
Spinasterol	1.429	0.929	0	0.113	0	0
Bryonolic acid	0	0	0	0	0.085	0

Data are expressed as the average of three determinations (n=3).

*** Compounds are expressed as linoleic acid equivalent**

**** Compounds are expressed as p-hydroxy benzoic acid equivalent**

***** Compounds are expressed as quercetin equivalent**

****** Compounds are expressed as cucurbitacin E equivalent**

Cytotoxicity and anti-inflammatory activity test (MTT assay)

Isolation and cultivation of human white blood cells

Procedure

In a sterile heparin tube, a blood specimen was obtained then 1ml blood was taken into 15ml centrifuge tube then the tube was filled to capacity with fresh cold lysing solution. After that, the tube is inverted for about 10 minutes at room temperature until the liquid became clear red. Centrifugation of the blood specimen was carried out at 4^{o} C for 10 min at 2000 rpm followed by decantation of the supernatant and draining of the tubes was done. Suspending of the pellets (WBCs) was in 10 ml cold PBS followed by centrifugation. Then pellets were resuspended in RPMI culture medium. Dye exclusion method was used for the assessment of WBCs viability and counting (**Louis and Siegel; 2011**). Mixing fifty microliters of cell suspension with an equal volume of 0.5% trypan blue staining solution was done followed by its loading onto hemocytometer. Finally, counting of viable "unstained" and nonviable "stained" cells was carried outin each of the four corner quadrants (A, B, C, D).

Calculation

N / ml = mean of WBCs counting x10^4 × D

N: Number of viable or nonviable cells
D: Sample dilution (1:1 with the trypan blue).

% Cell viability = Number of viable cells × 100
Total number of cells

In order to use the cells for assays, at least 90% of the cells must be viable. Culturing of WBCs was in RPMI media, then they incubated in CO_2 incubator for six days. Seeding of WBC's was 100,000 cells/ well (96 well cell culture plate). The incubation conditions in CO_2 incubator were 37°C temperature, 5% CO_2, and 90% relative humidity.

Assessment of cytotoxicity of the crude extracts compared to piroxicam

Procedure

In this assay, 200 µl of cultured medium containing 100,000 WBCs / well were treated with different concentrations (0, 3.125, 6.25, 12.5, 25 and 50 µg/ml) of the crude extracts in RPMI medium without fetal bovine serum or piroxicam (standard anti-inflammatory drug. After that, plates were incubated for 72 h in CO_2 incubator and conditions were 37°C temperature, 5% CO_2, and 90% relative humidity). After incubation, 20µl of MTT solution was added to each well followed by plates incubation in order to allow MTT reaction to be performed. Then, centrifugation of plates was carried out at 1650 rpm for 10 min followed by discarding the medium. MTT byproduct (the formazan crystals) were re-suspended in 100µl DMSO. The absorbance was measured at a wavelength of 570 nm using optima spectrophotometer for detecting safe dose, which 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 plant extract
$\mathbf{A_C}$ = 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 cytotoxicity assay of the compound was expressed as EC_{100}, and was calculated by the Graphpad Instat software using the % viability calculated from the serial dilutions of each plant extract.

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

Procedure

In a 96 well plat, A volume of 50 µl of the culture medium that contained 100,000 of human WBCs was dispensed per well. The inflammation was induced by adding 50 µl of LPS to the plated cells and incubated in CO_2 incubator. After 24 h, 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 50µg/ml in culture media) of the crude extracts or the standard anti-inflammatory drugs piroxicam were added. The control cells contained cell culture medium only. The plates were incubated for additional 72 h in CO_2 incubator. After 72 h of incubation, the cell proliferations were measured using MTT (as previously illustrated). 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 extract that were able 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.

Extraction of RNA of untreated and treated LPS-stimulated human white blood cells
Procedure

In 50 µl of solution R1, cell pellets were then suspended and mixed for 30 s, then their incubation was done at room temperature for 1 min. three hundred microliters of solution R2 were added and mixed for 30 s then the centrifugation was done at 4ºC for 3-5 min. Into a spin column the supernatant was transferred and centrifuged for 30 s at 14000 rpm at 4ºC. three hundred microliters of working wash buffer were added into the spin column after discarding the flow-through was discarded and, centrifuged for 30 s and this step was repeated in twice. Centrifugation of the spin column was done for 1min at 10,000 rpm then it was transferred to a sterile 1.5 ml micro centrifuge tube. To the central of the membrane, 30µl of elution buffer were added and incubated at room temperature for 1 min followed by their centrifugation for 30 s at 14000 rpm at 4ºC. Finally, determination of the optical density (OD) of the extracted RNA was done through measuring the absorbance and purity at A260 and A260/A280 nm, respectively using spectrophotometer and kept in -80°C until real time PCR.

cDNA synthesis from RNA extracted from untreated and treated LPS-stimulated human white blood cells
Procedure

Two µg of total RNA or nuclease-free water and 1 µl of oligo dT primer were added to nuclease-free water in a total volume of 12 µl in PCR tubes, then they mixed gently. Centrifugation, incubation at 65ºC for 5 min in PCR machine was carried out then they placed back on ice immediately. The gentle mixing of 4 µl of 5X reaction buffer, 1 µl of RNase inhibitor, 2 µl of dNTPs mix and 1 µl of reverse transcriptase or 1 µl of nuclease-free water instead of reverse transcriptase for reverse transcriptase negative control with previous mixture was performed. After that, spin down and incubation for 60 min at 42ºC followed by heat inactivation at 70ºC for 5 min in PCR machine was carried out.

Determination of IL-1β, IL 6, TNF and INF-γ expression level by real time polymerase chain reaction (PCR)
Procedure

Thirteen µl of 2 X SYBR green master mix was mixed with 5µl of cDNA, 0.5 µl of 10 pmoles/ml forward primer and 0.5 µl of pmoles/ml reverse primer for each primer in PCR tubes. As for the reference tube, 0.5 µl of 10 pmoles/ml forward primer of β-actin and 0.5 µl of 10 pmoles/ml for reverse primer of β- actin were added. In order to assess for reagent contamination or primer dimers, another tube was used as a non-template control (NTC) by adding 1 µl of nuclease-free water instead of template used. After that, the gentle mixing of the tubes with 6.5 µl nuclease free water without creating bubbles was done and then spinned for few seconds. In the cycler, samples were placed and the program was started as following; initial denaturation (1 cycle of 95ºC for 10 min), followed by denaturation (40 cycles of 95ºC for 15 sec), annealing (at 60ºC for 30s) and extension (at 72ºC for 30s). the effect of LPS and extracts on gene expression was expressed as Fold change in gene expression which calculated according the following equations:
Calculation

Expressions fold levels of gene calculated by

$\Delta Ct_{normal} = Ct_{normal\ untreated\ cells} - Ct_{reference}$

$\Delta Ct_{tested\ plant\ extract} = Ct_{tested\ plant\ extract\text{-}treated\ cells} - Ct_{reference}$

$\Delta Ct_{induced} = -Ct_{LPS\text{-}exposed\ cells} - Ct_{reference}$

In case of genes:

$\Delta\Delta CT_{tested\ plant\ extract} = \Delta Ct_{tested\ plant\ extract} - \Delta Ct_{normal}$

$\Delta\Delta CT_{induced} = \Delta Ct_{induced} - \Delta Ct_{normal}$

In case of GAPDH:

$\Delta\Delta CT_{tested\ plant\ extract} = \Delta Ct_{normal} - \Delta Ct_{tested\ plant\ extract}$

$\Delta\Delta CT_{induced} = \Delta Ct_{normal} - \Delta Ct_{induced}$

Fold change in gene expression = log ($2^{-\Delta\Delta CT}$)

Where:

$Ct_{tested\ plant\ extract}$: threshold cycle value of genes of extracted mRNA of plant extract treated-LPS-stimulated WBCs which is defined as the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold.

Ct reference: threshold cycle value of GAPDH which is used for normalization.

Ct_{normal}: threshold cycle value of genes of extracted mRNA of untreated control WBCs

$Ct_{induced}$: threshold cycle value of gene of extracted mRNA of LPS-stimulated WBCs

The primers used:

TNF-α	F-CTCTTCTGCCTGCTGCACTTTG
	R- ATGGGCTACAGGCTTGTCACTC
IL-6	F, 5′-TGAACTCCTTCTCCACAAGCG-3′
	R, 5′-TCTGAAGAGGTGAGTGGCTGTC-3′
IL-1β,	F, CCACAGACCTTCCAGGAGAATG
	R, GTGCAGTTCAGTGATCGTACAGG
INF-γ	F, GAGTGTGGAGACCATCAAGGAAG
	R, TGCTTTGCGTTGGACATTCAAGTC
	R, GGAAGATGGTGATGGGATT
GAPDH	F, GGATTTGGTCGTATTGGG
	R, GGAAGATGGTGATGGGATT