NOVEL ANTI-INFLAMMATORY AND IMMUNOMODULATION EFFECTS OF ROSE ON THE ENDOTHELIUM IN NORMAL AND HYPOXIC INVITRO CONDITIONS

Mark Christopher Arokiaraj*[1], Eric Menesson[2]

1. Cardiology, Pondicherry institute of Medical Sciences, Pondicherry, India. 605014
2. Tebu Bio, France

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ABSTRACT

Aims: *The study was performed in search of a novel agent useful for inflammation modulation, and cytokine and related biomarkers levels, which would result in the treatment of cardiovascular disorders and various other clinical scenarios.*

Materials and methods: *A crushed red rose extract was prepared from the petals, and it was processed for analysis. The extract was tested on HUVEC cells at various concentrations. By microscopic examination of cells, a safe concentration was identified, and the levels below the safe limit were tested at 72 hours and seven days for selected cytokines secretion. After hypoxia treatment, the experiment was performed at various concentrations and varying degrees of hypoxia (12%, 5%, and 1% oxygen) during treatment, and the results were compared with the controls.*

Results: *The majority of the Inflammatory cytokine's secretion was reduced by the treatment of red rose extract on the endothelial cells. VEGF and angiogenic cytokine levels were reduced, but VEGF-R2 levels were maintained after the cell treatment. Below the safe concentration limit (0.5%), there were only minimal changes in the cytokine levels tested at various dilutions. IL 1, TNF α, ADAM ST13, Angiopoietin levels and other inflammatory markers were reduced. In further experiments, the rose extract also induced Fas ligand, ERB4, integrin A5, Insulin R, IGF1 R, and XIAP; and reduces Trail R1, ICAM 1, and BMPR2. LDL R receptors were elevated in the endothelial cells.*

Conclusion There is potential for a red rose extract for the reduction of in vascular inflammatory biomarkers and certain other cytokine levels. Further studies need to be performed to evaluate the benefits and pharmacokinetics.

Keywords
Inflammation; Molecular biology; Rose

INTRODUCTION

The inflammatory response to cardiovascular injury or infections is common in clinical practice. Predominantly this is a protective response in the process of healing. The inflammatory response is required in control for sepsis and the inflammation induced by autoimmune disorders. However, in many instances, this could be overwhelming, and the products of the inflammatory process initiate a negative vicious cycle that needs to be controlled. The anti-inflammatory agents are frequently toxic and induce multiorgan injuries and eventually resulting in their dysfunction. The overwhelming response to inflammation or infection could result in multiorgan malfunctions[1,2]. The current anti-inflammatory agents are steroids or non-steroidal anti-inflammatory agents (NSAIDS). Both these agents are effective, but they have potential side effects, and injudicious use of these agents could result in severe and life-threatening side effects due to these agents. Hence, a novel anti-inflammatory agent is necessary without side effects to mitigate the illness as well as maintaining safety. Also, in organ-transplant scenarios, a simple inflammatory agent without side effects is much desirable. The study

*Autor para correspondência.
Correio eletrónico: christomark@gmail.com (MC. Arokiaraj).

was performed in search of a novel anti-inflammatory and immune regulation agent, which could be used for various clinical conditions. In this study, the red rose extract was used to evaluate the beneficial effects in controlling inflammation as well as their effects on angiogenesis. Traditionally rose is known as a symbol of love, and it is also known to have positive psychological effects[3-5]. It is admired time immemorially by various artists for its aura, and it is vividly described in various literature. In some studies, it has been shown to have anti-inflammatory effects[6, 7], and antibacterial properties[8-10]. It also modulates the immune system[11]. It has mild antidiabetic properties[12], increases heart rate in Langendroff preparations[13], and it is known to inhibit angiotensin-converting enzyme[14]. It increases the levels of reproductive hormones[15], and it is also used as an ophthalmic herbal medication[16]. In this study, we evaluated the potential of the red rose extract on inflammatory and angiogenic markers. This is the first study in which red rose extract was evaluated for its vascular anti-inflammatory effects and its angiogenic potentials.

METHODS

Preparation of red rose extract

A red rose was cut from a plant, and 2091 mg of freshly collected petals were distributed in 6 Precellys CK14 lysing tubes (Bertin Technologies, ref. 03961-1-003) for homogenization of soft tissue. One ml of PBS (ThermoFisher, ref. 14190) was added into each tube. The tubes were run on Precellys 24 homogenizer at 5000 rpm for 2×30 seconds. Then, the supernatant from all tubes was gathered and spun down at 200g, 4°C for 5 min. The supernatant was spun down twice at 4°C for 5 min, the first at 2000g, and the last at 10000g. The final supernatant was 0.22 µm filtered (Sartorius, ref. 16534-K) and stored until use at -20°C.

Cell treatment

HUVEC were seeded at passage 5 in 96-well plates at 3300 cells/well in 100 µl of endothelial cell growth medium (Cell Applications, ref. 211-500). 24h after plating, the cells were treated in duplicate with 150 µl of red rose extract diluted at 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0.005% (v/v) in endothelial cell growth medium (Cell Applications, ref. 210-500) or with growth medium only as control for 72h and 7 days. One replicate cell culture medium of each different condition was collected after 72h and after seven days. The cell viability was evaluated under the microscope.

Profiling of secreted cytokines

The Human angiogenesis antibody array G-1000 is a multiplexed sandwich ELISA-based array enables to detect multiple cytokines simultaneously. It combines the advantages of the high detection sensitivity and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labelled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-conjugated Cy3 equivalent dye, using a laser scanner.

In detail, one standard glass slide is divided into eight wells of identical cytokine antibody arrays. Each antibody, together with the positive controls is arrayed in duplicate. The slide comes with an 8-well removable gasket, which allows for the process of 8 samples on one slide. By comparing signals from different arrays, the cytokine fold change between a sample and a control can be determined.

During the incubation time, the volume of medium varied differently in each well, and from the 150 µl added at the beginning of incubation, the volume collected was reduced. To normalize the profiling results, sample diluent from antibody array kit was added to the different collected media to reach 150 µl before performing the profiling assay. Then, the medium samples were tested undiluted on arrays.

The slide was scanned by using a microarray scanner (Innopsys, model InnoScan 710). The analysis of the image was done with Mapix 7.0 software (Innopsys) as follows: To determine the position of each spot, a grid containing the parameters (spot spacing, diameter, etc.) must be assigned. To assign a grid, a GAL file was used, which is a standard ATF file generated by the spotter itself. The grid was automatically positioned on the image. Once the grid is correctly positioned, the image is quantified. Each spot was framed in a gridding circle. This circle defines the border between spots and background. Background value is calculated as a local value estimated within a circular area, centered on the spot and excluding the spot itself. Photometric quantification was exported in a spreadsheet (GPR file).

Hypoxia treatment

In the next phase of the study, other inflammatory markers as targets and insulin-related biomarkers on the endothelium were studied. The red rose extract used in this study was prepared for project POMC-032018 and stored until use at -20°C. HUVEC were seeded at passage 8 in 48-well plates at 9500 cells/well (10000 cells/cm2) in 250 µl of endothelial cell growth medium (Cell Applications, ref. 211-500). 24h after plating, the cells were treated in duplicate with 250 µl of red rose extract diluted at 0.5%, 0.05% and 0.005% (v/v) in endothelial cell

growth medium (Cell Applications, ref. 211-500) or with growth medium only as control for 72h at 21%, 12%, 5% and 1% O2. The hypoxia INVIVO2 workstation (Baker Ruskinn) was used to simulate hypoxic conditions at 12%, 5%, and 1% O2. At each of these lower oxygen levels, the medium incubated on cells was previously preconditioned with the HypoxyCOOL device (Baker Ruskinn). The duplicate cell culture medium of each different condition was collected and pooled after 72h. The cell viability was evaluated under the microscope.

RESULTS

The red rose extract was evaluated on HUVEC cells at various concentrations. The cells were incubated, and the effects of the extract concentration were studied 24 hours and 48 hours after incubation under the microscope. The results are summarised in Figures 1 to 3. At concentration 0.5 percent and less, the cell lysis was minimal, and the cell morphology was well preserved. At levels >0.5 percent cell lysis was predominant. Hence, further extended duration analysis in the study, a concentration of less than 0.5 percent, i.e., 0.1 percent or less, was chosen.

The results of the cytokines secretion are summarised in table 1. Table 2 shows the fold changes between treatment vs. untreated cells for each incubation time as final results. Many inflammatory cytokines expression was reduced, and the angiogenic factors secretion was reduced to a certain extent. VEGF secretion was reduced, and VEGF receptor expression was normal. Between concentrations 0.1 to 0.005%, there were only minimal changes in the levels of cytokines observed (Table 1). Many cytokines levels were reduced at 72 hours of cell treatment (0.1 percent concentration), and the levels rise after seven days (Figure 4a and 4b). MCP 3 (CCL7) and PDGF BB levels were less than 10% of the baseline values. Based on the fold changes, i.e., compared to the baseline values, a significant shift in specific cytokines like GRO, MCP-1, IL-6, INF-gamma was noticed (Figure 5). In the next phase of the study, inflammatory markers were reduced, and adiponectin levels were mildly elevated. Anti-apoptotic XIAP was elevated, and there was also a mild increase in apoptotic markers like Fas, Fas ligand, and TRAIL R1 (Table 3). There were no significant changes in factor VII and thrombospondin levels. BMP R2 levels were reduced; Cystatin C levels were increased at 0.5% concentration, but in lower concentrations, it was reduced. Integrin A5 levels were maximally increased with 0.05% concentration treatment. ERB4 from endothelium increases during treatment with 0.005% concentration of the extract only. Osteopontin levels showed decreasing trend at lower concentrations of the extract. Insulin, Insulin R, and IGF1 R signal intensities were increased at 0.005% concentration extract treatment. Table 4 shows the values above the detection threshold wherein the detection threshold's cut-off was set at a higher value

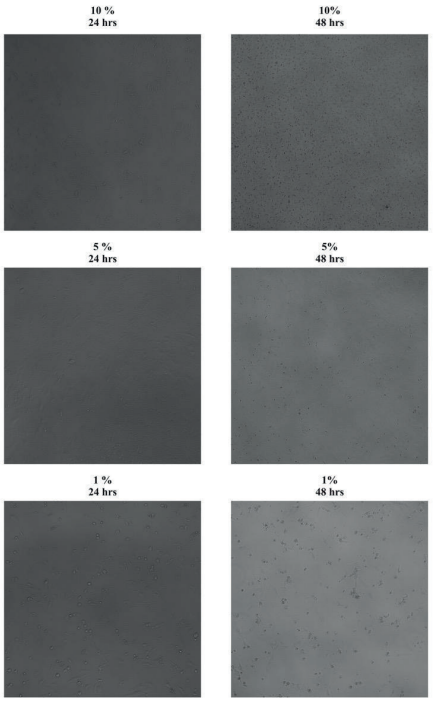

Figure 1: Microscopy of HUVEC cells incubated at concentrations 10 to 1 percent at 24 and 48 hours.

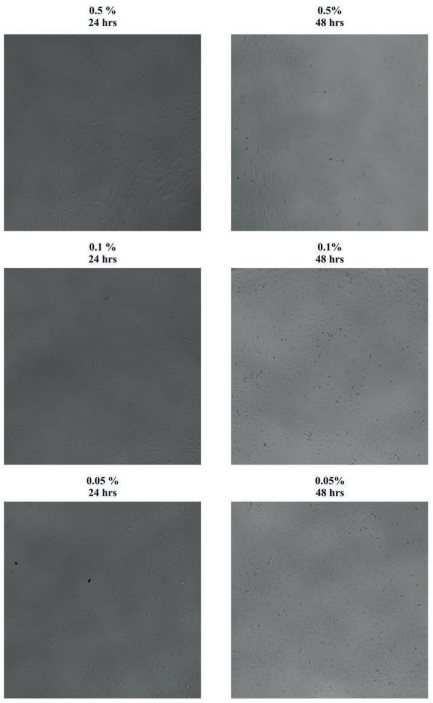

Figure 2: Microscopy of HUVEC cells incubated at concentrations 0.5 to 0.05 percent at 24 and 48 hours.

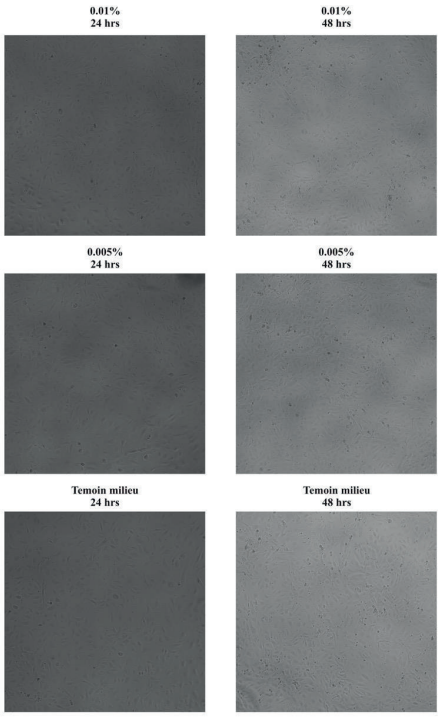

Figure 3: *Microscopy of HUVEC cells incubated at concentrations 0.01 to 0.001 percent at 24 and 48 hours.*

inclusive of 3 standard deviations. XIAP, Fas, Fas ligand, BMP R2 were not detected above the this higher threshold levels.

Phase 3 results

In the third phase of the study further investigations of the target markers were performed. The results in the phase 1 and 2 were observed in this phase also (Table 5 and 6). There was a marked reduction in the inflammatory markers IL 1A, IL-17A, TNF alpha. Among the angiogenesis markers the VEGF C was markedly reduced. VEGF A, VEGF B and Tie-2 levels were maintained in the near-baseline levels. Angiopoietin 1 and 2 levels were reduced and LDL R receptor showed an elevation with increasing concentrations of the rose concentration. ANGPLT3 levels showed an increasing trend at 0.05% concentration only. There were no significant changes in the endothelial CD 30 and CD 40 counts expressed on endothelial cells.

Hypoxia effects

Hypoxia reduced the secretion of gp130 in untreated cells, and the rose extract tended to minimize the effect of hypoxia and even increase the secretion at mid-hypoxia (12% and 5% O2) compared to 21% O2 for highest concentrations of rose extract (0.5% and 0.05%). Similarly, hypoxia reduced

Figure 4a

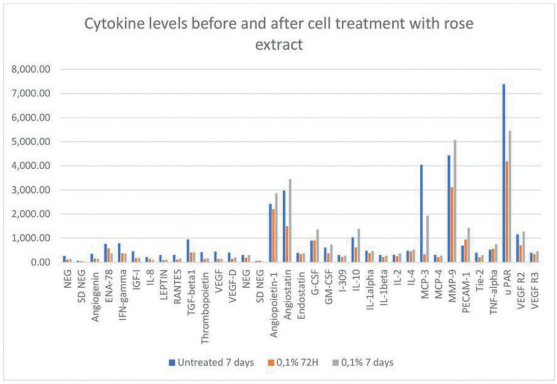

Figure 4b

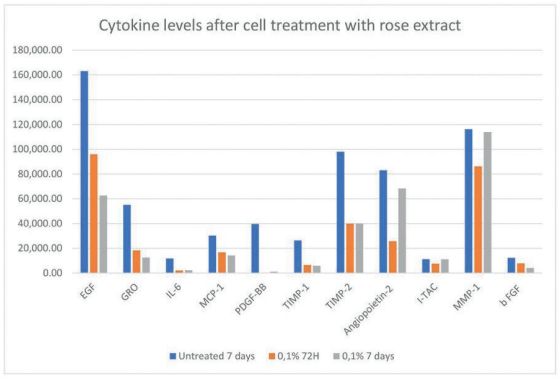

Figure 4: *Shows cytokine levels at 72 hours and at 7 days after cell treatment with red rose extract (0.1% concentration)*

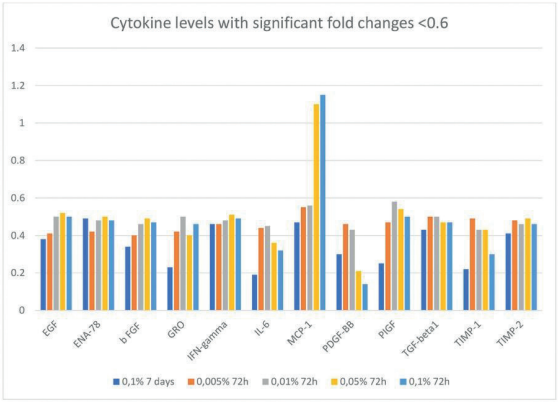

Figure 5: *Shows cytokine levels at 72 hours and at 7 days after cell treatment with red rose extract (0.1% concentration)*

the secretion of TNF R1 in untreated cells with a drop at 1% O2 but probably due to the lower cell growth at this level. The same effect was observed at each concentration of rose extract. Rose extract decreased the secretion of TNF R1, but this effect was erased at lower oxygen levels (5% and 1%).

Table 1 FLUORESCENCE INTENSITY.

Analyte	Untreated 7 days	0,1% 7 days	Untreated 72H	0,005% 72H	0,01% 72H	0,05% 72H	0,1% 72H	0,5% 72H
POS	28,262.33	28,262.33	28,262.33	28,262.33	28,262.33	28,262.33	28,262.33	28,262.33
NEG	264.22	131.79	254.12	103.04	122.93	103.77	102.90	129.70
SD NEG	55.54	27.25	100.41	38.76	69.48	40.39	44.39	49.34
Angiogenin	348.00	150.31	269.74	128.17	153.99	149.98	153.52	119.78
EGF	1,63,128.50	62,570.06	1,90,350.85	77,764.93	95,944.22	99,017.77	95,950.79	1,03,144.14
ENA-78	760.00	372.28	1,209.88	503.27	575.60	609.64	575.83	699.87
b FGF	12,319.00	4,182.92	16,494.24	6,582.28	7,537.29	8,032.14	7,757.51	8,039.48
GRO	54,975.00	12,510.80	39,647.14	16,713.04	19,763.80	15,902.77	18,326.93	2,406.53
IFN-gamma	786.50	364.32	764.84	349.40	367.60	388.66	372.85	475.28
IGF-I	457.00	189.62	474.71	195.30	193.88	201.37	175.24	245.93
IL-6	11,834.25	2,240.64	6,330.71	2,776.79	2,834.53	2,256.06	2,017.21	1,045.87
IL-8	215.50	98.92	232.83	86.63	110.84	84.64	128.52	101.46
LEPTIN	299.00	102.28	178.47	87.85	89.21	85.39	88.83	120.59
MCP-1	30,241.00	14,172.50	14,533.10	8,015.65	8,119.62	15,939.98	16,714.48	6,170.26
PDGF-BB	39,646.25	1,121.82	2,312.64	1,057.50	1,003.16	476.71	314.95	114.91
PIGF	3,924.25	999.38	1,276.92	599.54	738.33	693.75	632.08	791.12
RANTES	305.00	156.15	279.94	112.33	109.38	112.25	105.71	115.14
TGF-beta1	945.50	411.22	848.87	420.52	424.75	398.36	401.58	487.80
TIMP-1	26,422.75	5,900.23	21,687.54	10,581.41	9,220.17	9,366.65	6,461.65	5,050.77
TIMP-2	97,948.50	39,930.82	87,455.00	41,966.94	40,570.98	42,938.54	39,837.20	30,159.66
Thrombopoietin	415.00	164.61	372.34	129.17	157.69	152.12	128.08	159.31
VEGF	441.00	148.81	273.14	144.57	130.23	130.05	132.03	140.53
VEGF-D	398.25	201.20	354.22	129.61	133.48	130.26	135.76	139.95
POS	86,898.17	86,898.17	86,898.17	86,898.17	86,898.17	86,898.17	86,898.17	86,898.17
NEG	302.29	293.03	183.45	173.68	237.61	168.20	182.83	182.89
SD NEG	51.16	31.03	55.86	43.72	125.26	48.04	66.13	47.58
Angiopoietin-1	2,419.25	2,862.50	2,466.22	2,338.62	2,558.00	1,740.63	2,194.66	1,484.79
Angiopoietin-2	82,987.75	68,402.67	53,901.01	45,320.87	45,062.16	31,805.18	25,680.67	5,220.99
Angiostatin	2,971.00	3,453.88	2,959.32	2,465.14	2,598.25	2,177.06	1,483.92	524.45
Endostatin	387.50	374.27	301.03	285.65	313.54	354.75	330.46	266.01
G-CSF	897.50	1,354.90	802.07	993.97	770.33	764.69	902.98	439.50
GM-CSF	603.00	737.76	383.95	558.46	397.78	416.90	370.28	407.52
I-309	301.00	275.43	153.28	172.85	188.16	194.95	217.15	219.09
IL-10	1,030.00	1,391.20	812.78	769.26	782.68	715.61	614.79	435.33
IL-1alpha	474.50	468.18	363.82	345.34	349.13	330.64	381.33	364.01
IL-1beta	302.00	274.96	224.39	216.68	351.99	202.08	214.34	268.84
IL-2	307.75	376.14	289.02	213.83	269.18	211.93	255.56	235.55
IL-4	479.50	523.46	459.30	452.61	478.08	474.47	443.07	422.09
I-TAC	11,174.00	10,975.51	7,897.49	7,207.07	6,438.29	7,976.51	7,609.76	7,075.47
MCP-3	4,045.75	1,929.42	514.89	500.01	410.65	432.53	328.36	196.19
MCP-4	307.50	278.71	219.03	211.16	222.86	202.76	209.78	233.84
MMP-1	1,16,129.50	1,13,864.08	94,937.92	1,00,755.68	94,282.37	79,576.13	86,082.71	80,347.96
MMP-9	4,432.50	5,068.76	4,244.69	3,975.51	4,282.00	3,657.20	3,112.38	3,164.08
PECAM-1	693.75	1,423.29	636.41	794.92	1,239.83	836.18	939.47	593.31
Tie-2	391.50	293.00	257.63	272.28	288.14	202.25	206.98	222.87
TNF-alpha	517.75	752.51	483.12	570.40	531.56	577.04	555.33	426.06
u PAR	7,381.25	5,458.48	3,352.13	3,463.38	3,554.59	3,946.23	4,184.46	2,763.36
VEGF R2	1,156.50	1,275.27	700.86	769.09	675.00	654.48	706.36	527.47
VEGF R3	395.25	458.58	366.41	347.66	347.16	355.09	337.65	361.36

Table 1 shows the cytokine values and control at various concentrations and time of incubation

Table 2

Analyte	0,1% 7 days vs Untreated 7 days	0,005% 72h vs Untreated 72h	0,01% 72h vs Untreated 72h	0,05% 72h vs Untreated 72h	0,1% 72h vs Untreated 72h	0,5% 72h vs Untreated 72h
EGF	0.38	0.41	0.50	0.52	0.50	0.54
ENA-78	0.49	0.42	0.48	0.50	0.48	0.58
b FGF	0.34	0.40	0.46	0.49	0.47	0.49
GRO	0.23	0.42	0.50	0.40	0.46	0.06
IFN-gamma	0.46	0.46	0.48	0.51	0.49	0.62
IL-6	0.19	0.44	0.45	0.36	0.32	0.17
MCP-1	0.47	0.55	0.56	1.10	1.15	0.42
PDGF-BB	0.30	0.46	0.43	0.21	0.14	
PIGF	0.25	0.47	0.58	0.54	0.50	0.62
TGF-beta1	0.43	0.50	0.50	0.47	0.47	0.57
TIMP-1	0.22	0.49	0.43	0.43	0.30	0.23
TIMP-2	0.41	0.48	0.46	0.49	0.46	0.34
Angiopoietin-1	1.18	0.95	1.04	0.71	0.89	0.60
Angiopoietin-2	0.82	0.84	0.84	0.59	0.48	0.10
Angiostatin	1.16	0.83	0.88	0.74	0.50	0.18
G-CSF	1.51	1.24	0.96	0.95	1.13	0.55
GM-CSF	1.22	1.45		1.09		1.06
IL-10	1.35	0.95	0.96	0.88	0.76	0.54
IL-1alpha	0.99	0.95		0.91	1.05	1.00
IL-4	1.09	0.99		1.03	0.96	0.92
I-TAC	0.98	0.91	0.82	1.01	0.96	0.90
MCP-3	0.48	0.97		0.84		
MMP-1	0.98	1.06	0.99	0.84	0.91	0.85
MMP-9	1.14	0.94	1.01	0.86	0.73	0.75
PECAM-1	2.05	1.25	1.95	1.31	1.48	0.93
TNF-alpha	1.45	1.18		1.19	1.15	0.88
u PAR	0.74	1.03	1.06	1.18	1.25	0.82
VEGF R2	1.10	1.10	0.96	0.93	1.01	0.75
VEGF R3		0.95		0.97		0.99

Table 2 shows the cytokine levels and fold changes compared to baseline values.

DISCUSSION

Inflammation and angiogenesis

The study results show a significant reduction in the secretion of anti-inflammatory cytokines by endothelial cells treated with the red rose extract (Table 1–3). The effects of red-rose extract inhibited major inflammatory cytokines like TGF-beta, TNF-alpha, GRO, IL-6, INF-gamma, etc. These inflammatory proteins are known for their inflammatory response wherein renal, and pulmonary tissues are primarily affected, resulting in acute renal failure and acute respiratory distress syndromes, respectively. In severe conditions, the accumulation of these cytokines can lead to multiorgan dysfunction. Steroids are known for their anti-inflammatory action. The role of steroids in the adjuvant treatment of septicaemia is always under debate, and the beneficial effects against the risk involved are not yet proved decisively[17-19]. Steroids are useful in the treatment of bronchial asthma and COPD exacerbations. However, in these conditions also, if there is underlying sepsis or urinary tract infection, the infections can be exacerbated, especially in large dosages. Also, in post-transplantation scenarios, infections due to steroids are common. Non-steroidal anti-inflammatory agents are effective as an anti-inflammatory but with side effects, especially in the long term use, and most of them are nephrotoxic[20], and gastrointestinal effects are often seen[21].

In this study, VEGF levels were reduced, and it was not almost not detected in the medium under all conditions, and VEGF-R2 levels were near normal, which are markers for angiogenesis[22-24]. VEGF R3 was not well detected, and it was too close to background. This action on VEGFR2 could, however, indicate a direct effect of the red rose extract on angiogenesis, which needs to be evaluated further.

Analyte	Normoxia control	12% O2 control	0,5% rose extract normoxia	0,5% rose extract 12% O2	0,05% rose extract normoxia	0,05% rose extract 12% O2	0,005% rose extract normoxia	0,005% rose extract 12% O2	5% O2 control	1% O2 control	0,5% rose extract 5% O2	0,5% rose extract 1% O2	0,05% rose extract 5% O2	0,05% rose extract 1% O2	0,005% rose extract 5% O2	0,005% rose extract 1% O2
POS	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339
NEG	224	238	217	219	269	215	339	309	197	184	185	191	177	185	179	227
ErbB4	246	229	203	351	222	301	366	242	215	186	222	181	165	180	188	228
Integrin a5	248	194	324	204	413	424	201	256	179	257	236	233	181	211	188	220
OPN	452	270	696	358	488	585	384	381	293	326	415	332	303	326	417	351
CXCL16	4,141	2,100	386	527	3,526	2,307	4,304	2,446	1,164	583	453	498	1,250	618	1,183	652
Cystatin C	1,159	971	1,953	1,109	1,035	969	781	1,008	702	631	887	1,087	649	711	836	670
Fas	331	187	414	191	319	200	503	195	175	180	187	182	170	184	181	219
Fas Ligand	197	181	201	192	386	192	481	319	178	184	180	182	167	180	186	187
TRAIL R1	495	576	447	625	386	442	452	418	441	406	428	437	355	409	368	424
XIAP	195	182	210	297	394	299	173	186	173	180	179	177	173	177	182	181
TNF R1	1,224	491	741	349	937	563	1,067	551	486	305	469	283	588	310	551	274
gp130	3,415	3,107	2,518	3,056	2,323	2,977	3,062	2,601	2,812	1,300	3,132	1,659	3,429	1,437	3,155	1,340
Leptin R	194	183	207	255	220	198	249	192	174	179	238	179	214	227	187	178
Adiponectin	561	268	676	304	710	284	631	317	278	266	358	268	276	315	313	312
Adipsin	390	244	511	203	317	252	428	221	251	180	186	226	248	284	230	274
Insulin	444	314	358	444	487	380	544	463	385	262	361	372	348	362	366	301
Insulin R	410	472	395	533	353	379	531	362	342	369	404	368	350	334	391	375
Thrombospondin	244	183	252	244	391	278	231	194	172	186	175	183	162	220	179	189
BMP R2	299	192	708	199	225	282	177	192	177	182	177	188	169	179	169	184
CF VII	281	242	341	299	174	289	274	282	266	191	269	191	177	276	229	273
ICAM-1	352	558	401	489	323	490	410	486	369	353	371	332	349	313	453	554
IGFI R	308	239	346	301	794	255	413	236	177	228	238	276	219	282	217	225

Table 3 *The biomarkers tested and the results in normal and hypoxia milieu.*

Detection threshold: NEG + 3*SD NEG

Analyte	Normoxia control	12% O2 control	0,5% rose extract normoxia	0,5% rose extract 12% O2	0,05% rose extract normoxia	0,05% rose extract 12% O2	0,005% rose extract normoxia	0,005% rose extract 12% O2	5% O2 control	1% O2 control	0,5% rose extract 5% O2	0,5% rose extract 1% O2	0,05% rose extract 5% O2	0,05% rose extract 1% O2	0,005% rose extract 5% O2	0,005% rose extract 1% O2
POS	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339	1,08,339
NEG	247	208	236	209	272	228	236	228	175	167	181	184	175	182	177	192
SD NEG	49	46	65	24	110	60	64	47	14	19	3	11	3	3	4	18
ErbB4				351							222					
Integrin a5						424				257	236	233		211		
OPN	452		696	358		585		381	293	326	415	332	303	326	417	351
CXCL16	4,141	2,100		527	3,526	2,307	4,304	2,446	1,164	583	453	498	1,250	618	1,183	652
Cystatin C	1,159	971	1,953	1,109	1,035	969	781	1,008	702	631	887	1,087	649	711	836	670
Fas							503									
Fas Ligand							481									
TRAIL R1	495	576	447	625		442	452	418	441	406	428	437	355	409	368	424
XIAP				297												
TNF R1	1,224	491	741	349	937	563	1,067	551	486	305	469	283	588	310	551	274
gp130	3,415	3,107	2,518	3,056	2,323	2,977	3,062	2,601	2,812	1,300	3,132	1,659	3,429	1,437	3,155	1,340
Leptin R											238		214	227		
Adiponectin	561		676	304	710		631		278	266	358	268	276	315	313	312
Adipsin			511				428		251			226	248	284	230	274
Insulin	444			444			544	463	385	262	361	372	348	362	366	301
Insulin R	410	472		533			531		342	369	404	368	350	334	391	375
Thrombospondin														220		
BMP R2			708													
CF VII				299					266		269			276	229	273
ICAM-1		558		489		490		486	369	353	371	332	349	313	453	554
IGFI R				301	794					228	238	276	219	282	217	

Table 4 *The biomarkers tested and the results in normal and hypoxia milieu.*

The leptin levels, which are a marker of inflammation, was below the line of detection, and the leptin levels are associated with obesity[25]. Reduced leptin levels are observed after mammoplasty in obese patients, and it correlates with increased insulin sensitivity[26].

Atherosclerosis

Some of the cytokines tested also have effects on apoptosis[27] (TNF beta, IGF-1) and atherosclerotic processes (RANTES, MCP-1, TGF beta 1, and INF-gamma and IL-1 beta[28]. Hence, by regulating the cytokine actions, long term benefits and control of these pathways could be achieved. The significance of the marked reduction in specific cytokines like MCP-3, TIMP1, and PDGF BB needs to be evaluated by future studies.

Other inflammatory markers

In the second phase, the results were consistent with the first phase of the study i.e., a reduction in inflammatory markers. In the second phase, other inflammatory-related markers involved in immune regulation like ICAM 1 were reduced, and Integrin A5 was elevated. Integrin A5 rise may be beneficial in myocarditis[29]. ICAM modulates the neutrophil migration and adhesion to the endothelial cells[30]. Adiponectin is associated with anti-inflammatory effects, and its levels are low in obesity and diabetes or patients with insulin resistance[31,32]. Hence, a rise in adiponectin could have favourable outcomes. Adipsin was increased at 0.05% concentration only, and adipsin has been shown insulin secretion activity in the Beta cells33. There was a tendency for an increase in the leptin R receptors (table 3).

	FLUORESCENCE INTENSITY															
Analyte	Normoxia control	12% O2 control	0,5% rose extract normoxia	0,5% rose extract 12% O2	0,05% rose extract normoxia	0,05% rose extract 12% O2	0,005% rose extract normoxia Slide 747	0,005% rose extract 12% O2	5% O2 control	1% O2 control	0,5% rose extract 5% O2	0,005% rose extract normoxia Slide 748	0,05% rose extract 5% O2	0,05% rose extract 1% O2	0,005% rose extract 5% O2	0,005% rose extract 1% O2
POS	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364
NEG	213	210	213	213	209	189	199	176	284	292	238	211	241	221	202	197
VEGF A	197	174	254	260	210	185	252	232	231	327	251	190	292	264	238	232
VEGF C	2,772	2,316	252	261	1,863	1,626	2,881	2,045	1,719	1,326	297	2,148	1,544	1,057	1,572	1,077
Angiopoietin-2	3,63,209	3,37,707	21,522	56,868	1,97,353	2,58,139	2,51,867	2,68,585	2,88,402	1,64,659	43,809	2,12,202	1,86,101	77,671	1,72,783	1,42,448
Tie-2	301	340	298	293	324	334	324	278	377	336	291	307	333	355	331	355
ANGPTL3	291	304	266	254	318	300	254	224	345	323	330	284	342	305	303	308
PIGF	5,457	1,346	11,334	12,327	4,331	1,246	4,969	913	2,197	6,132	5,960	3,797	1,652	3,719	1,039	2,103
LDLR	10,926	18,543	42,247	38,395	22,366	22,233	14,315	13,155	10,519	5,573	16,694	11,244	23,342	13,779	12,604	4,885
ACE	1,113	2,245	625	3,668	2,165	2,012	2,567	627	393	273	405	750	495	268	331	297
IL-1a	984	1,099	472	554	898	672	914	732	693	581	569	1,138	680	484	449	387
IL-1Ra	302	230	219	254	248	236	246	236	291	231	239	257	242	264	199	232
IL-17A	538	414	511	538	444	423	439	387	521	533	422	386	530	453	401	391
TNF alpha	1,338	1,005	635	702	1,081	1,002	950	884	778	754	765	1,217	769	693	590	582
CD30	292	315	343	337	312	311	245	266	347	320	299	333	289	321	291	283
IGF-II	437	443	357	448	370	371	320	303	433	332	341	377	386	436	323	347
ADAMTS13	1,178	1,657	218	420	486	845	590	1,402	612	425	243	614	562	385	808	474
VEGF-B	199	177	206	273	203	190	159	155	220	213	195	229	191	270	162	207
P-selectin	666	782	711	874	732	774	737	693	718	638	648	614	674	647	608	703
Angiopoietin-1	3,587	4,374	168	322	907	1,231	2,111	3,306	4,491	2,972	330	2,734	1,981	845	4,056	2,205
SORT1	249	172	219	260	157	289	239	338	235	216	192	177	241	274	165	183
Insulin	388	509	307	345	300	357	336	474	529	337	383	456	949	437	518	325
IGF-I	476	465	412	442	471	446	384	410	438	436	444	431	467	394	394	420
Insulin R	204	179	173	169	247	157	165	152	231	221	246	201	199	261	165	203
CD40	193	225	167	183	238	233	202	229	222	218	230	178	249	261	221	239

Table 5 *The biomarkers tested in phase 3 and the results in normal and hypoxia milieu.*

Leptin R deficiency is associated with obesity and diabetes[34]. Again about leptin R, the signal is below the threshold or really close to it, so the protein is not detected for me.

A reduction in inflammatory markers and the cystatin c levels would indicate a decrease in multiorgan failures seen in various infective and inflammatory conditions[35]. Elevated cystatin c levels are an early marker of diabetes, and it is elevated in renal failures, and it is also a marker of impending renal failure in the future. Chronic reduction of these markers could result in a reduction of chronic inflammatory conditions and renal failures. Osteopontin is a well recognized vascular inflammatory marker associated with insulin resistance and diabetic complications[36], which showed a decreasing trend in the study.

BMPR2 and ERB4

BMP R2 is strongly associated with primary pulmonary hypertension, where the current treatment methods are not satisfactory[37]. BMP R2 inhibitory effects could be useful in primary pulmonary hypertension. ERB4 is reduced in neuronal tissues in psychiatric disorders like schizophrenia. The endothelium assessment of ERB4 need not reflect on the neuronal levels. ERB4 induction could reduce the incidence of atrial fibrillation and other arrhythmias[38].

Apoptosis

Fas ligand inductions were seen in lower concentrations of the extract (0.05 and 0.005%), and the Fas induction was observed at 0.05% concentration only. Fas ligand induction in experimental studies has shown benefits to regulate leukocyte extravasation and cell adhesion mechanisms[39,40]. Fas pathway apoptotic induction could be a part of normal homeostasis mechanism, and the exact effect has to be studied in detail[41]. Antiapoptotic XIAP increase, though not detected at higher thresholds, and reduction in the TRAIL R1 have favourable effects in apoptosis regulations[42,43].

Insulin related markers

The majority of inflammatory proteins and steroids result in the decrease in insulin levels or hyperglycaemia, and the impact of rose extract seems to increase insulin R levels, and IGF1 R. Increase in IGF1 R is associated with lower concentrations of the extract (0.05 and 0.005%). These would have favourable effects on insulin sensitivity, and it enhances endothelial regeneration[44]. This the first study showing the benefits of the red-rose extract on the vascular endothelial response.

Toll (IL 1) interception, LDL R, Angiopoietin levels and phase 3 results

IL1 is a major marker of inflammation and belongs to the toll receptor superfamily and participates in the early host defence [45,46]. There is marked reduction at 0.5% concentration and mild reduction in 0.05 and 0.05% concentration. When subjected to hypoxia there is marked reduction in the levels of IL1 concentration irrespective of the rose extract concentration. IL 1 RA (receptor antagonist) levels were mildly reduced. IL1Ra is a competitive inhibitor of IL 1a and it is actively involved in the regulation of inflammation[47]. IL[17] levels were also reduced by all concentrations. IL 17 is a potent mediator of inflammatory pathways[48]. TNF a is known for inflammation and immune regulation properties. Reduction in TNF a could result in reduction of inflammation mediated disorders.

Also, there was a marked reduction in the angiopoietin 1 and 2 levels compared to the untreated baseline values.

Most detected targets	FLUORESCENCE INTENSITY															
Detection threshold: NEG + C4*SD NEG																
Analyte	Normoxia control	12% O2 control	0,5% rose extract normoxia	0,5% rose extract 12% O2	0,05% rose extract normoxia	0,05% rose extract 12% O2	0,005% rose extract normoxia Slide 747	0,005% rose extract 12% O2	5% O2 control	1% O2 control	0,5% rose extract 5% O2	0,005% rose extract normoxia Slide 748	0,05% rose extract 5% O2	0,05% rose extract 1% O2	0,005% rose extract 5% O2	0,005% rose extract 1% O2
POS	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364	78,364
NEG	201	184	181	183	220	162	173	159	243	242	203	188	207	189	173	170
SD NEG	8	18	22	21	20	19	19	12	30	35	25	17	24	23	20	19
VEGF A			254	260			252	232					292	264	238	232
VEGF C	2,772	2,316	252	261	1,863	1,626	2,881	2,045	1,719	1,326	297	2,148	1,544	1,057	1,572	1,077
Angiopoietin-2	3,63,209	3,37,707	21,522	56,868	1,97,353	2,58,139	2,51,867	2,68,585	2,88,402	1,64,659	43,809	2,12,202	1,86,101	77,671	1,72,783	1,42,448
Tie-2	301	340	298	293	324	334	324	278	377		291	307	333	355	331	355
ANGPTL3	291	304	266	254	318	300	254	224	345		330	284	342	305	303	308
PIGF	5,457	1,346	11,334	12,327	4,331	1,246	4,969	913	2,197	6,132	5,960	3,797	1,652	3,719	1,039	2,103
LDLR	10,926	18,543	42,247	38,395	22,366	22,233	14,315	13,155	10,519	5,573	16,694	11,244	23,342	13,779	12,604	4,885
ACE	1,113	2,245	625	3,668	2,165	2,012	2,567	627	393		405	750	495	268	331	297
IL-1a	984	1,099	472	554	898	672	914	732	693	581	569	1,138	680	484	449	387
IL-1Ra	302			254		236	246	236				257		264		232
IL-17A	538	414	511	538	444	423	439	387	521	533	422	386	530	453	401	391
TNF alpha	1,338	1,005	635	702	1,081	1,002	950	884	778	754	765	1,217	769	693	590	582
CD30	292	315	343	337	312	311	245	266	347		299	333	289	321	291	283
IGF-II	437	443	357	448	370	371	320	303	433		341	377	386	436	323	347
ADAMTS13	1,178	1,657		420	486	845	590	1,402	612	425		614	562	385	808	474
VEGF-B				273										270		
P-selectin	666	782	711	874	732	774	737	693	718	638	648	614	674	647	608	703
Angiopoietin-1	3,587	4,374		322	907	1,231	2,111	3,306	4,491	2,972	330	2,734	1,981	845	4,056	2,205
SORT1	249			260		289	239	338						274		
Insulin	388	509	307	345	300	357	336	474	529		383	456	949	437	518	325
IGF-I	476	465	412	442	471	446	384	410	438	436	444	431	467	394	394	420
Insulin R														261		
CD40						233		229						261		239

Table 6 *The biomarkers tested in phase 3 and the results in normal and hypoxia milieu with a higher cut-off value inclusive of 3 standard deviations.*

In pre-ecclampsia the levels of VEGF and angiopoietin values are increased, whereas the placental inhibitory growth factor is reduced which can offset the balance to increase the placental growth factor and this can reduce pre-ecclampsia[50,51]. There is a tendency for a mild reduction in the levels of the PIGF when treated at lower concentrations (0.05 and 0.005%). PIGF inhibition can increase the placental growth factor levels[52].

There was a marked increase in LDL R on the endothelial cells when treated with 0.5% and 0.05% concentrations, and at 0.005 concentration treatment the LDL receptors were mildly elevated. The LDL R play a major role in the metabolic modulation of LDL[53]. Hence induction of this receptor is useful in atherosclerosis control. In analysing the diabetes related markers the IGF1 levels were not changed, whereas there was a mild reduction in the IGF2 levels. There is a tendency for SORT1 to be reduced and SORT1 is associated with calcification of the vessels[54]. The ACE levels increase at 0.5% concentration treatment and it reduces when treated with 0.005 and 0.05% levels. ADAMST13 is strongly inhibited by all concentration, and this cytokine is closely associated with microangiopathic disorders and its perpetuation including disseminated intravascular coagulation[55,56]. The CD 40 count on the endothelial cells increases with 0.05% concentration treatment, whereas in other concentrations it is normal. The CD 30 on the cells mildly increase at 0.5% treatment but in other concentrations the levels are maintained.

Limitations and future perspectives

Further studies need to be performed to analyse the effects of the extract on inflammation and angiogenesis. Also, the side effect profile and active ingredients need to be studied. The observations in this study could be of use in the potential treatment of a wide range of disorders. When the threshold levels were increased to higher by including 3 standard deviations some markers were not detected (tables 4 and 6). Hence further experiments need to be performed with a large sample volume for more validation. This is a bench study, and the observations in animal models need to be studied.

The anti-inflammatory actions of the rose extract needs to be investigated in special conditions like corona virus and Ebola virus infections, etc.

CONCLUSION

There is potential for a red rose extract for the reduction in vascular inflammatory biomarkers and related cytokine levels on endothelial cell treatment. Further studies need to be performed to evaluate the benefits and pharmacokinetics.

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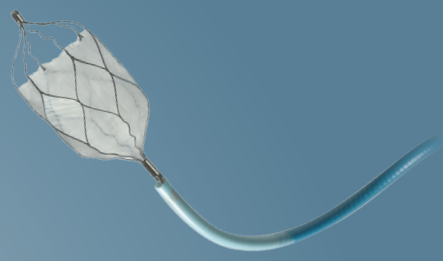

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